



Compendium of Projects in the European NanoSafety Cluster

2012 Edition

February 2012

Editors:

Michael Riediker, PD Dr.sc.nat.
Institute for Work and Health, Lausanne, Switzerland

Georgios Katalagarianakis, Ph.D.
European Commission, Directorate General for Research, Brussels, Belgium

NanoSafety Cluster 2012

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PREFACE

This is the third edition of the Nanosafety Cluster compendium. It documents the status of important projects on nanomaterial toxicity and exposure monitoring, integrated risk management, research infrastructure and coordination and support activities.

The compendium is not intended to be a guidance document for human health and environmental safety management of nanotechnologies, as such guidance documents already exist and are widely available.

Neither is the compendium intended to be a medium for the publication of scientific papers and research results, as this task is covered by scientific conferences and the peer reviewed press.

The compendium aims to bring researchers closer together and show them the potential for synergy in their work. It is a means to establish links and communication between them during the actual research phase and well before the publication of their results. It thus focuses on the communication of projects' strategic aims, extensively covers specific work objectives and the methods used in research, and documents human capacities and available laboratory infrastructure. As such, the compendium supports collaboration on common goals and the joint elaboration of future plans, whilst compromising neither the potential for scientific publication, nor intellectual property rights.

Of course this publication alone will not be able to achieve these targets. However, we hope that it will help the research community to make significant progress towards them. The compendium will continue to be a dynamic, frequently updated, web-based document available free of charge to all interested parties. A limited number of printed paperback copies of the compendium are also available.

More information about the NanoSafety Cluster can be found at <http://www.nanosafetycluster.eu>

ACKNOWLEDGMENTS

The editors thank the project managers for their contributions in the creation of this publication. This compendium would not have been possible without their help.

The reader of this compendium will find the tough work, the brilliant ideas, the frustrations, the successes, and the satisfaction of the researchers themselves. Their commitment is the foundation for this publication. The editors devote this work to them.

Projects appearing in this compendium are supported financially by the European Union and the Governments of the Framework Programme Associated States. We gratefully acknowledge their continued support.



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QUOTE

Compendium of Projects in the European NanoSafety Cluster – 2012 edition, update 2

Michael Riediker and Georgios Katalagarianakis (Eds.)

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Overview matrix: Research themes of the NanoSafety Cluster projects

Project Acronym	ENNSATOX	ENPRA	EURO-NanoTox	HINAMOX	InLiveTox	INSTANT	ITS-NANO	MARINA	ModNanoTox	Nanodetector	NANODEVICE	NanoFATE	NANOHOUSE	NanoImpactNet	NanoLyse	NanoMICEX	NANOMMUNE	NanoPolyTox	NanoReTox	NanosafePACK	NanoSustain	NanoTranskinetics	NanoValid	NEPHH	NeuroNano	NHECD	QNano	SANOWORK	Scaffold	SIIN	SMART-NANO	
Start year	2009	2009	2007	2009	2009	2012	2012	2011	2011	2012	2009	2010	2010	2008	2010	2012	2008	2010	2008	2011	2010	2011	2011	2009	2009	2008	2011	2012	2012	2011	2012	
End Year	2012	2012	open	2012	2013	2015	2013	2015	2013	2015	2013	2014	2013	2012	2013	2014	2011	2013	2012	2014	2013	2014	2014	2012	2012	2012	2015	2015	2015	2014	2014	
Characterisation & measurement	X		X	X			X	X		X	X			X			X	X			X		X							X	X	
Physico-chemical properties	X	X	X	X		X	X	X		X	X	X		X	X	X	X	X	X	X	X		X	X	X		X	X	X	X	X	
Exposure assessment for humans and the environment		X	X				X	X						X			X				X					X			X	X	X	
Develop & validate exposure measurement and modelling methods	X		X					X				X	X	X	X	X			X		X	X	X	X	X		X		X	X	X	
Human Exposure: Application of measurement and modelling methods on NM			X				X	X					X	X		X		X	X	X	X		X				X		X			
Environmental Exposure Assessment							X	X				X	X	X		X			X	X	X	X	X						X			
Interaction of NM with biological systems	X	X	X	X			X	X			X			X			X				X		X			X				X		
Interaction with physiological mechanisms	X	X	X	X				X			X	X	X	X		X		X		X	X	X	X	X	X		X	X		X		
Toxicokinetics	X	X	X	X				X			X	X	X	X		X	X		X	X	X	X	X	X	X			X	X	X		
Inter- and intraspecies variability	X		X									X	X					X			X	X	X	X	X		X			X		
Predictive models	X	X		X				X	X			X	X	X		X	X		X	X	X	X	X							X		
Long term monitoring and assessment			X	X										X									X		X				X	X		
Human Health			X	X			X	X						X							X		X			X				X		
Develop & validate testing & assessment strategy		X	X	X	X		X	X				X	X			X		X		X	X	X	X		X		X			X		
Apply testing and assessment strategy		X	X	X				X				X	X		X				X	X	X	X		X			X		X			
Coexposures / Mixture toxicology							X					X	X									X	X							X		



Project Acronym	ENNSATOX	ENPRA	EURO-NanoTox	HINAMOX	InLiveTox	INSTANT	ITS-NANO	MARINA	ModNanoTox	Nanodetector	NANODEVICE	NanoFATE	NANOHOUSE	NanoImpactNet	NanoLyse	NanoMICEX	NANOMMUNE	NanoPolyTox	NanoReTox	NanosafePACK	NanoSustain	NanoTranskinetics	NanoValid	NEPHH	NeuroNano	NHECD	QNano	SANOWORK	Scaffold	SIIN	SMART-NANO
Ecotoxicology	X						X	X						X								X			X					X	
Develop testing and assessment strategy	X						X	X				X	X	X		X				X	X		X	X			X			X	X
Apply testing and assessment strategy	X							X				X		X							X		X	X						X	X
Control measures at workplace			X					X						X								X								X	
Develop & validate methods to evaluate control measures at workplaces			X					X		X				X							X		X				X		X	X	
Apply methods to evaluate control measures at workplaces			X					X		X				X		X				X	X		X		X		X	X	X	X	
Control banding approach								X						X		X				X		X					X	X	X		
Preliminary handling guidelines	X		X					X						X							X		X							X	
Collect available and ongoing approaches	X		X				X	X						X					X		X		X	X					X	X	
Evaluation and further development	X		X				X	X	X					X							X		X	X				X	X	X	
Information transfer	X		X								X			X								X			X				X	X	
Database generation	X	X	X			X	X	X	X		X			X			X				X	X	X	X	X		X	X	X	X	X
Public dialogue	X	X		X		X	X	X			X	X	X	X					X				X	X		X	X		X	X	
Information to and training of workers, business and employers				X		X						X	X	X		X				X	X		X	X		X	X	X	X		
National and international collaboration	X		X	X				X			X			X							X		X			X				X	X
Development	X	X	X	X		X	X	X		X	X	X	X	X	X		X				X		X	X	X		X	X	X	X	X
Testing	X		X	X		X	X	X		X	X	X		X	X	X	X			X	X		X	X	X		X	X		X	X
Validation	X		X				X	X			X	X		X	X		X				X	X	X		X		X		X	X	
Standardisation			X	X		X	X	X			X	X	X	X	X							X	X		X		X		X	X	X
Assessment activities		X					X	X				X	X	X		X	X			X	X	X	X			X	X	X	X	X	X





Foreword

Nanotechnological research gives us a better understanding and control of physical, chemical and biological processes at the nano-scale, which is at the interface between the molecular and supramolecular level. The control of structures in this scale results in new material properties, a more economical use of precious raw materials, smarter medical devices and pharmaceutical agents, and products with increased energy efficiency. Nanotechnology is of benefit to almost every domain of our daily life. Safety, health and environmental research are means to ensure that the enormous investments into the development of nanotechnology result in long-term benefits and sustainable, smart and healthy materials and products. It identifies the key factors to guide engineers and investors so that they can address these issues in an early phase of material development and product design when the flexibility is highest. The vision is a future where most of the risks are recognized and addressed well before products reach the market.

The European Commission encourages and supports safety, health and environmental research on nanomaterials and technology as an important part of its Framework Programmes. It has recognized already at the beginning of the seventh FP the need for coordination and collaboration between researchers by funding activities such as NanoImpactNet (see p. 119 ff). FP projects are limited in time. To ensure long-term coordination beyond the individual projects' lifetimes, the NanoSafety Cluster was created as a super-structure that ensures long-term coordination, facilitates collaboration between research teams, transfers knowledge between ending and newly starting projects and makes information about scientific insight and developed methods available to the scientific community and disseminates the core messages to industry, regulators, and other relevant stakeholders.

The Nanosafety Cluster is structured into highly interlinked working groups that cover the domains Materials, Hazards, Exposure, Databases, Risks, Modelling, and Dissemination. The end of 2011 marked an important step forward in the NanoSafety Cluster with the first elections of the NanoSafety Cluster management team consisting of a chair and seven working group chairs. Also first joint research activities between Cluster projects started by different project teams working towards joint summary reports, training schools and workshops.

In addition, the European nanosafety research community, represented through EU FP7 funded projects, the Nanosafety Cluster, has initiated a new action that aims at providing an insight on the future research needs in Europe. This initiative includes the production of a "Strategic Research Agenda for Nanosafety Research 2020". The document aims to support the Commission in shaping its views on future needs on nanosafety research funded under the EU 8th Framework Programme for Research and Innovation - Horizon 2020. Hence, if successful, this action may have an impact on the contents of the future nanosafety research funding within the European Union.

The European nanosafety research community is a key-player also in the global scene in assuring the safety of engineered nanomaterials and nanotechnologies. A new task for the Cluster and all the Cluster projects is the collaboration with the US nanosafety research community, as exemplified by the new series of EU-US Nanosafety Workshops. The main aim of this collaboration, in which the Cluster and the Cluster projects play an important role, is to promote global collaboration of nanosafety research to ensure the safe use of engineered nanomaterials and their technological applications.

This compendium provides an update of the status of already running EU FP7 funded nanosafety projects, and summarizes the achievements of the ended project NANOMMUNE. It is also a pleasure to include in this compendium the description of several upcoming projects that are planned to start in early 2012, namely INSTANT, ITS-NANO, Nanodetector, NanoMICEX, SANOWORK and Scaffold.

Michael Riediker and Kai Savolainen
Dissemination WG chair NanoSafety Cluster chair





ENNSATOX

Engineered Nanoparticle Impact on Aquatic Environments: Structure, Activity and Toxicology



Contract Agreement: NMP4-SL-2009-229244

Website: <http://www.ennatox.eu>

Scientific Coordinator: Professor Andrew Nelson, Centre for Molecular Nanoscience (CMNS), School of Chemistry, University of Leeds, UK

Project Manager: Dr Karen Steenson, Faculty of Engineering, University of Leeds, UK

No.	Beneficiary name	Short name	Country
1	University of Leeds (Coordinator)	UNIVLEEDS	UK
2	Wageningen University	WU	Netherlands
3	University of Antwerp	UA	Belgium
4	Stazione Zoologica Anton Dohrn	SZN	Italy
5	Lleida University	UdL	Spain
6	Marine Biological Association of UK	MBA	UK
7	Society of Environmental Toxicology And Chemistry (SETAC) – Europe	SETAC	Belgium

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1 Summary

Project Duration: 1 July 2009 – 30 June 2012

Project Funding: €3,655,316 Total Budget; €2,816,500 EC Contribution

The use of engineered nanoparticles (NP) in cosmetics, pharmaceuticals, sensors and many other commercial applications has been growing exponentially over the past decade. EU and Member States' research into the environmental impact of these materials, particularly in aquatic systems, is at an early stage. There is a large uncertainty into the environmental risk posed by these new materials. ENNSATOX addresses this deficit through a comprehensive investigation relating the structure and functionality of well characterised engineered nanoparticles to their biological activity in environmental aquatic systems. ENNSATOX takes account of the impact of nanoparticles on environmental systems from the initial discharge to the uptake by organisms. Accordingly an integrated approach will assess the

activity of the particles in a series of biological models of increasing complexity. Parallel environmental studies will take place on the behaviour of the nanoparticles in natural waters and how they modify the particles' chemical reactivity, physical form and biological activity. A comprehensive theoretical model will be developed describing the environmental system as a series of biological compartments where particles transport between a) compartments by advection-diffusion and b) between phases by a transfer function. Following optimisation of the transfer functions a generic predictive model will be derived for the environmental impact of each class of nanoparticle in aqueous systems. The project will include the use of unique biological membrane models not only to understand better the interaction of nanoparticles with cell membranes from an organism health point of view but also to develop suitable nanoparticle screening procedures which can substitute for the more lengthy *in vivo* tests. ENNSATOX will generate: 1) Exploitable IP of screening devices and simulation software; 2) Set of standard protocols; 3) Global dissemination of



results; 4) Creation of an EU laboratory service; 5) Tools and data to inform EU Regulation; 6) Risk assessment procedures.

2 Background – The ENNSATOX Project

2.1 Introduction, Scientific / Industry Needs & Problem Addressed

Nanomaterials are becoming increasingly important in their applications and uses in many industries, consumer products and healthcare (*The Nanotech Report, 6th Edition, Lux Inc, New York, 2008*). Current worldwide sales of products incorporating nanomaterials are €1.1 trillion and are expected to rise to €4.1 trillion by 2015. Engineered nanoparticles represent a major part of this growth. However an understanding of their toxicological properties has not kept pace with the exponential rate of increase of research into their synthesis, characterisation and applications. Research into their behaviour, impact and fate in aquatic environments is at a very early stage. Out of 14 funded FP5/FP6 nanotoxicology projects only one is dedicated fully to this area (*“EU Nanotechnology R&D in Health and Environmental Impact of Nanoparticles”* <http://cordis.europa.eu/nanotechnology/home.html> report, Jan 2008). This report details the member states with the largest number of nanotoxicity research projects as follows: UK (46), Switzerland (24) and Denmark (12) of which the numbers dedicated to the fate of nanoparticles and their impact in the aquatic environment are UK (6), Switzerland (2) and Denmark (1) respectively. The majority of risk studies are concentrated on airborne particulates. A similar situation is also seen in the recently updated US National Nanotechnology Initiative (NNI) *Strategy for Nanotech-related Research for the Environment, Health and Safety Research, Feb 2008* http://www.nano.gov/NNI_EHS_Research_Strategy.pdf where the focus for aquatic environmental research is into environmental transport mechanisms and standardisation of nanoparticles rather than their ecotoxicological effects.

The toxic effects of nanomaterials are poorly understood and their effects on aquatic wildlife are largely unknown. In the absence of such basic toxicological information, it is difficult to set environmental quality standards or perform risk assessments for these materials. As a result two EU Member States have recently recommended a voluntary moratorium on the release of engineered nanoparticles into the environment backed by a voluntary reporting system. These Member States are Germany (*Nanotechnology: Health and Environmental Risk of Nanoparticles, Joint Working Party Report, Aug 2006*, <http://www.baua.de/nn49456/en/Topics-from-A-to-Z/Hazardous-Substances/Nanotechnology/pdf/draft-research-strategy.pdf>) and the UK (*Council for Science & Technology Review of Government Progress of its Action Plan for Nanoscience and Technology, March 2007*, <http://www.baua.de/nn49456/en/Topics-from-A-to-Z/Hazardous-Substances/Nanotechnology/pdf/draft-research-strategy.pdf>), to be administered by the Department of Environment, Food and Rural Affairs (DEFRA). On 17th January 2008 the UK's Soil Association, the national organic food certification body, issued a complete moratorium on the use of engineered nanoparticles for organic food production (<http://www.soilassociation.org>). More recently, Poland et al. (20th

May 2008), described important findings relating the dimensional characteristics of carbon nanotube and inorganic fibres to the inability of macrophages to prevent mesothelioma risks in rat lungs. <http://www.nature.com/nnano/journal/v3/n7/abs/nnano.2008.111.html>.

ENNSATOX addresses this crucial uncertainty by seeking to relate the structure and functionality of a well known class of nanoparticles of varying morphology to its biological activity at successive levels of molecular, cellular and organism organisation. Its research will focus in particular on the impact of nanoparticles on these biological systems in aqueous environments with relevance to the interpretation of their effects on ecosystems. The work programmes will examine the importance of the biological membrane in the toxicology and bioaccumulation of nanoparticles in aquatic organisms. The study will thus operate at a series of levels and will take into account not only the responses of the individual organism to the specific agent but also relate this to the mechanism of activity of the agent. This goal will be achieved by engaging in a multidisciplinary approach and integrating the results in a multi component model. In so doing it will fill an important knowledge gap and inform the EU's *code of conduct for responsible nanosciences and nanotechnologies research*, ftp://ftp.cordis.europa.eu/pub/nanotechnology/docs/nanocode-recommendation-pe0894c08424_en.pdf for the purpose of future regulation by the EU (REACH Directive) and Member states.

The underlying concept of the proposed research is to address the current uncertainty of nanoparticle toxicity and environmental impact using an integrated multidisciplinary approach

The philosophy of ENNSATOX's work plan is to initially produce and thoroughly characterise different morphologies and sizes of a model nanoparticle, such as zinc oxide (ZnO), using the most advanced state-of-the-art methods in physical chemistry and microscopy. This will be extended to additional classes of nanoparticles in particular silicon dioxide (SiO₂) and titanium dioxide (TiO₂). At the same time the programme will look at the nanoparticles' activity towards a series of biological models of increasing complexity and organisation. Next, the behaviour of the nanoparticles in environmentally relevant aquatic systems will be examined see whether the environment alters the chemical and/or structural nature of these particles. Throughout the study an integrative model will be used to plan the activities and at the end of the programme, a predictive mathematical model will be developed incorporating all of the elucidated parameters.

The hypothesis is:

The biological activity and environmental impact of nanoparticles is directly dependent on their structure and functionality. By evaluating these relationships we can develop predictive models which can be deployed for statutory controls of nanoparticle use.

Toxicity assays will be performed using *in vitro* models of cell and tissue culture and *in vivo* models of several different aquatic species of key indicator organisms. As part of this proposal, all the procedures for toxicity testing will be selectively developed and optimised for nanoparticles. This means that streamlined protocols for nanoparticle toxicity testing will be formulated which can later be exploited as routine tests for nanomaterials.



The biological membrane and its dependent mechanisms play important roles in nanoparticle toxicity for two reasons. Firstly the biological membrane forms the boundary of the living cell which nanoparticles will need to cross and, secondly, the biological membrane hosts many of the physiological processes such as respiration and nerve conduction and any disruption in its structure will lead to a disruption in the function of the incumbent processes. The effect of nanoparticles on biological membrane structure is entirely unknown as is the permeability of nanoparticles in cell membranes. This study therefore allocates considerable resources to look at the interaction of nanoparticles with biological membranes by using highly novel supported membrane models of successive complexity. These model membranes represent the most basic model for nanoparticle interaction and will deliver important preliminary structure-activity relationships which are used to guide the more complex *in vitro* and *in vivo* studies. Already one of the model membrane tests being deployed in this study is in the process of being patented¹ as a generalised toxicity testing procedure which can be applied to investigate the activity of nanoparticles. We see a major outcome of this study as the delivery of calibrated and accredited toxicity testing protocols for nanoparticle biological activity. A very recent SETAC World Congress in Sydney (August 2008) had an extensive session on nanomaterials and it was apparent that there were many issues to be addressed concerning how the materials should be tested for biological activity and the mechanism of toxicity. ENNSATOX therefore has a great opportunity to make advances which could be a significant asset commercially.

2.2 Scope & Objectives

- 1) To source and comprehensively characterise a representative group of nanoparticles: initially ZnO and later SiO₂ and TiO₂ and other metal oxides of varying morphology and dimension. *In-house* synthesis is limited to special nanoparticles not obtainable commercially or from other projects. In these cases, the production methods are well defined. This objective will be continued as an iterative process throughout the programme of work. The success of this objective is directly measurable by the standardised particles which it delivers.
- 2) To characterise the interaction of the nanoparticles with the following biological models: supported phospholipid membranes of increasing complexity, *in vitro* models of cell and tissue culture, *in vivo* models of several different species of key indicator organisms. A feature of this objective is the direct comparison of the effects in the different groups which leads to the configuration of generalisations of nanoparticle biological activity.
- 3) To formulate direct and predictive structure-activity relationships between nanoparticle form and nanoparticle biological activity. Success in this objective will be achieved following results from objectives 1 and 2 and is a central feature of ENNSATOX.

- 4) To analyse the behaviour and fate of nanoparticles and their impact on models of biota in environmental aquatic systems. This advances on the initial structural-activity relationships by testing their application in the environmental aquatic situation.
- 5) To configure a mathematical model for the behaviour of nanoparticles in aquatic environments taking account of their interactions with biota of increasing complexity. This objective quantifies the interactions and will serve as a means of verifying and measuring previous objectives 1-4.
- 6) To draw up standard procedures for the exploitation and dissemination of the results for statutory planning and accredited use.

In order to accomplish the challenge ENNSATOX has assembled a group of RTD performers of unprecedented excellence from across Europe. The ENNSATOX Consortium has outstanding capabilities and achievements in:

- Nanoparticle manipulation, synthesis and characterisation (Leeds, Wageningen);
- Supported model membrane technology (Leeds, Naples, Wageningen);
- Environmental and molecular mathematical modelling (Lleida, Wageningen, Leeds, Antwerp);
- *In vitro* and *in vivo* biological models (Naples, Leeds, Antwerp, Wageningen, MBA);
- Surface and colloid chemistry (Leeds, Wageningen, Naples);
- Environmental impact assessment (Wageningen, Antwerp, MBA); and,
- Dissemination of best practice worldwide (MBA, SETAC).

The objectives directly address, in an integrated manner, the impact of the nanoparticles on the environment. Implicit in this is the approach towards understanding the environmental and biological fate, transport, and transformation of nanoparticles in various biological compartments in aquatic systems. It is clear that the above objectives incorporate investigations into the toxicokinetics, cellular and molecular mechanisms, behaviour and fate, bio-persistence and biokinetics of nanoparticles. This enables a fundamental understanding of the exposure, behaviour, mechanisms, consequences and potential effects to various endpoints of nanoparticle-biological entities interactions.

Contained within the objectives the following important questions will be addressed:

- What is the dispersion and solubility of nanoparticles in water?
- What are the most likely routes of exposure for environmentally relevant species?
- Can nanoparticles interfere with critical physiological mechanisms in aquatic organisms?
- Can nanoparticles bioaccumulate in aquatic organisms?
- Can nanoparticles be metabolised to less toxic forms?
- What biomarkers are relevant for determining nanoparticle exposure levels?
- What end-points are significant for determining risk of nanoparticles?

¹ Inventors Nelson, A. and Coldrick, Z. UK Patent No 0714866.1, filed 31/07/07.

- What are the mechanisms of toxicity of nanoparticles in environmentally relevant systems?
- Does the presence of nanoparticles in the environment affect the toxicity of other compounds and vice versa?

are being related to their activity towards the model membrane systems. Anton Dohrn is examining the effects of nanoparticles at the level of single channels (HERG K⁺ channels). The principle is to

2.3 Technical Approach, Work Description & Achievements To-Date

The scientific (RTD) activities are conducted within seven work packages (WP1-7), with two other work packages being specifically concerned with exploitation/IPR and pre-validation (WP7), and dissemination (WP8):

WP1: Synthesis and characterisation of a selected group of nanoparticles. To keep the study focused three groups of nanoparticles are being examined: silicon dioxide (SiO₂), zinc oxide (ZnO) and titanium dioxide (TiO₂), of different morphology and dimension. Although Leeds is responsible for the synthesis, sourcing and processing of the nanoparticles, their characterisation is being cross calibrated with Wageningen. Nanoparticle characterisation in the *in vitro*, *in vivo* and aquatic systems is being carried out throughout the programme as and when appropriate (WPs 2, 3, 4 and 5) in order to follow their behaviour and fate in the respective systems. *Figure 1* shows a characteristic image from this study of ZnO nanoparticles which have been synthesised at Leeds and have very small constant particle size. These particles are representative of the many samples which have been distributed round the Consortium for testing.

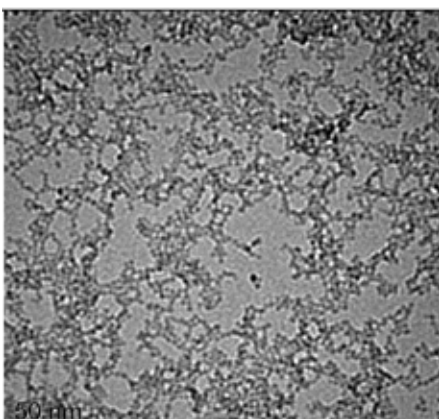


Figure 1. TEM image of in-house synthesised ZnO nanoparticles dried from fresh water suspension

WP2: Interactions of different classes of nanoparticles with model membrane systems. Leeds and Wageningen possess a whole suite of experimental model biological membrane systems of increasing levels of complexity (*Figures 2 & 3*). Wageningen have considerable expertise in surface and colloid chemistry and extensive expertise modelling membrane interactions, and are responsible therefore for correlating the model membrane-nanoparticle interactions with theoretical mathematical models using self consistent mean field theory². The form, structure and functionality of the particles

² Leermakers, F.A.M. & Kleijn, J.M., in Physicochemical kinetics and transport at biointerfaces. Series: IUPAC Series on analytical and physical chemistry of environmental systems. Published online: 2004. Editors: van Leeuwen, H.P. & Köster, W.

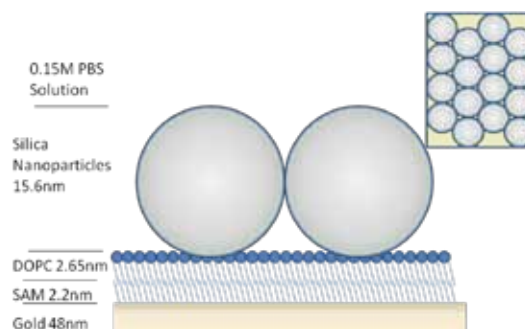


Figure 2: Shows schematic of nanoparticle adsorption on layers of lipid (DOPC), self-assembled monolayer (octadecanethiol) and gold surface. Inset: top-down view showing theoretical close-packed nanoparticle arrangement, with 0.6046 layer volume.

understand how nanoparticles affect the organisation and fluidity of the biological membrane, how they influence the functioning of ion channels and enzymes located in the membrane environment and whether the nanoparticles are themselves permeable in the membrane structure. *Figure 4* shows an image of SiO₂ nanoparticles adsorbed on to a cyanobacterium membrane. To

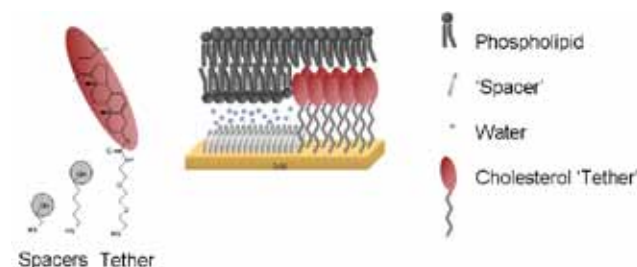


Figure 3: Shows examples of supported phospholipid membranes used for toxicity assay of nanoparticles dispersions.

test the biomembrane activity of all nanoparticles a custom built high throughput sensor has been developed. This device can assess the biomembrane activity of a nanoparticle dispersion in 10 minutes. Interesting results have been found using this sensor. For instance, SiO₂ nanoparticles have been shown to adsorb on the surface of phospholipid membranes, as seen in SEM images of silica nanoparticles on phospholipid monolayers on electrode surfaces. The extent of contact of the SiO₂ particles' surface with the phospholipid membrane surface determines the effect on the membrane's properties and is dependent on the particle size. The interaction of ZnO particles is also very significant. Unlike amorphous silica, ZnO particles have a strong tendency to aggregate. The nanosensor shows that in the first few minutes after aqueous dispersions are formed, ZnO particles have a high permeability in the membrane. The home made ZnO particles with smaller primary particle size (6 nm) interact strongly with the membrane surface. The electrochemical nanosensor readily

distinguishes between the interaction of particulate and soluble Zn with the membrane surface.

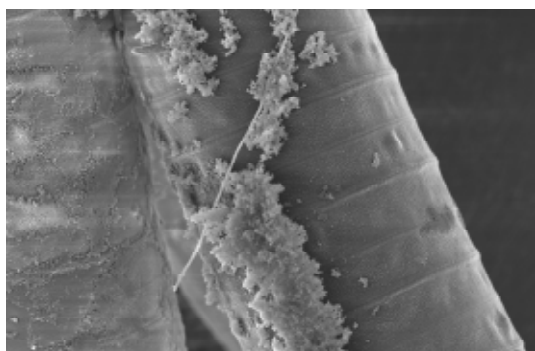


Figure 4: Shows *Oscillatoria princeps* incubated with SiO_2 . Cells separated by septa (arrowed). Right filament partially covered by SiO_2 , left filament completely covered.

A simple geometric model (see Figure 5) has been developed to describe the adsorption of particles on to a supported membrane. The model is directly transposed from the experimental data of the packing of silica nanoparticles on to a supported membrane. The model has one adjustable parameter which is the maximum distance between the particle surface and the DOPC layer where there is an interaction between the particle and the DOPC.

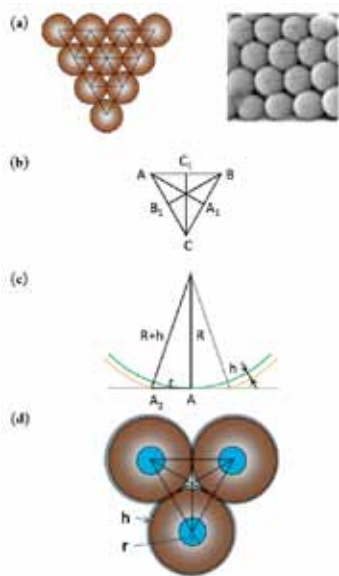


Figure 5.

(a) Model (top view) of silica nanoparticles binding to DOPC is presented in this picture.

(b) Triangle ABC, where $AB=AC=BC=2R$ and A, B and C are the centres of three nanoparticles abutting each other in (a).

(c) Vertical section through (b) showing nanoparticle touching the DOPC surface at point A, h is 'interfacial layer thickness'.

(d) Model (horizontal sectional view) of nanoparticles bound to DOPC showing radius, r , of 'interfacial contact area'.

WP3: Interactions with *in vitro* models. These studies are directed to nanoparticle interactions at both the cellular level and the tissue level. The test systems will be established on *in vitro* models. The cellular level will include test systems ranging from tissues and cultured cells to DNA. The tissue level includes nerve axons from the squid consisting of a single axon and glia, and ascidian embryos (rapidly developing chordate embryos to 12 hrs). The principle is to understand how the nanoparticles affect the structure and function of these systems using both real time assays and electron microscopy. The *in vitro* work is led by Anton Dohrn and is spread

between Anton Dohrn and Leeds (WP 3). Anton Dohrn has extensive facilities in electron microscopy and biophysical and molecular biological techniques and considerable world expertise in electrophysiology.

Some of the most exciting recent work carried out by WP3 has been on the effect of ZnO particles on membrane proteins. NPs provided by WP2 were tested directly on HEK cells that heterologously express the hERG K^+ channel. This gave us the opportunity of assessing the impact of the NPs on defined membrane proteins directly. The range of concentrations used was $0.1\text{-}10 \text{ mg ml}^{-1}$ for both SiO_2 (dialyzed and non -dialyzed) and ZnO. Cells were held at -70 mV under voltage clamp and hERG K^+ channels were activated by patterns of voltage steps which produced outward ionic currents which were subject to biophysical analysis (Figure 6). The channel activity was stable for at least an hour without run-down although experiments were normally carried out in the first 20 minutes. Examination of the hERG current kinetics (activation / inactivation and peak currents revealed no effect of SiO_2 up to 10 mg ml^{-1} but a notable selective effect of ZnO on channel kinetics (Figure 6). To establish if this effect was due to release of Zn^{2+} ions from the NPs, we carried out experiments where increasing concentrations of ZnCl_2 were added and the peak currents measured. As can be seen in Figure 7, increasing the concentration of Zn^{2+} begins to block the channel only in the mM range. The effect of the NPs in Figure 6 is the opposite to this, i.e. they increase the current. Therefore the NP effect cannot be due to residual Zn^{2+} .

ZnO significantly alters steady state current of hERG.

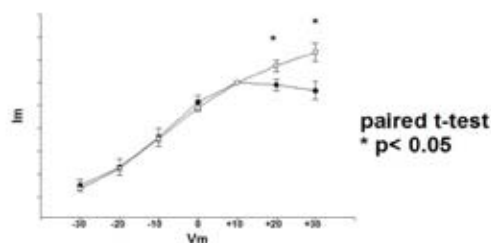


Figure 6. ZnO removes the fast inactivation of hERG and is so doing increases the amplitude of the current at positive voltages. The graph shows the extracted values for current voltage relations of the steady state K^+ current under different voltage steps.

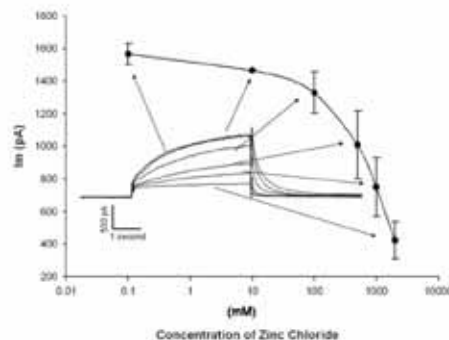


Figure 7. Dose response of hERG peak currents in various concentrations of Zn^{2+} . The EC_{50} of Zn^{2+} on hERG is estimated to be of the order of 1 mM . The results represent the mean and standard deviation of results from at least five experiments.



WP4: Interactions with *in vivo* models. *In vivo* testing is being performed on at least eight different species to allow the construction of species sensitivity distributions for the selected nanoparticles. This also includes three standard toxicity species of which the acute and chronic toxicity is well documented and characterised for a variety of toxicants (e.g. *Chlorella*, *Daphnia*, and *Danio*). The *in vivo* experiments address three main issues: namely bioavailability, accumulation and toxicity. A series of chronic experiments are being performed in which effects on growth and reproduction are being determined. This work is led by Antwerp with an input from Anton Dohrn. Antwerp is one of the world leaders in molecular, cellular and whole-organism toxicology, and both experimental and predictive modelling. In the last year studies have concentrated on looking at the chronic toxicity and internalisation routes of nanoparticles. In a chronic exposure scenario over 21 days, the ZnO nanodispersion (Alpha Aesar NanoTek) is toxic to *Daphnia magna* at low concentrations (around 0.02 mg Zn/L), whereby especially the reproduction was affected. As it has already been observed in studies carried out in **WP3**, toxicity of the ZnO nanodispersion cannot be solely due to the Zn or Zn²⁺ toxicity (see Figure 8).

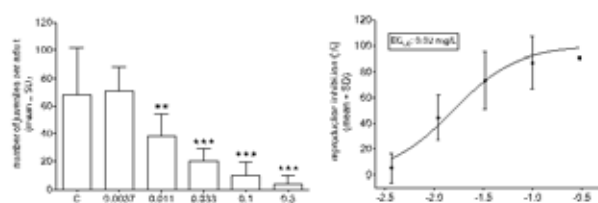


Figure 8: Chronic toxicity of ZnO nanodispersion on the reproduction of *Daphnia magna* (mean ± SD) after 21 days of exposure. Number of juveniles per adult *Daphnia*; one-way ANOVA with Dunnet's post test, significant differences $p < 0.001-0.01$ (left) and sigmoidal dose-response curve on the inhibition of reproduction (right).

Results also showed the uptake and internalisation of the ZnO nanoparticles in *Ciona intestinalis* (see Figure 9). However, under the tested conditions, algae and daphnids did not take up the particles.

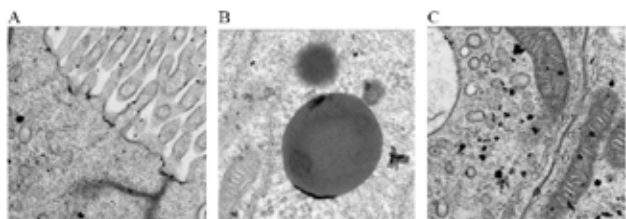


Figure 9: Feeding the animals with ZnO nanoparticles results in an accumulation of the particles in the stomach tissue. TEM analysis of this organ indicates that nanoparticles, found at the plasma membrane of the cells facing the lumen, pass into the cytoplasm and through the junctions (A). Clusters of ZnO nanoparticles can be observed at the edge of zymogen granules (B), in the mitochondria and scattered in the cytoplasm (C). Scale bars 0.5 μm .

WP5: Nanoparticle environmental impact. The biophysicochemical behaviour of nanoparticles, and their ensuing bioavailability and toxicity characteristics, strongly depends on the nature and the extent of molecular interactions with organic and inorganic materials in the environment. Wageningen together with an input from Antwerp are responsible for analysing the influence of chemical conditions and binding of particular species on the biointeraction and bioaccumulation of nanoparticles. Wageningen have extensive experience in the relationship between the chemical speciation of dissolved and particulate material in 'natural' waters and its bioavailability. They are studying how the nanoparticles and the nanoparticle-water interface is modified when they enter a typical aqueous environmental system such as river, estuarine and sea water and how this affects their biological activity. Experiments are being carried out in laboratory controlled and relevant microcosms. The rate of the actual transfer of oxide nanoparticles across the cell membrane of a few selected aquatic organisms (microorganisms, invertebrates and fish); in relation to their local speciation and the physicochemical conditions at the outer side of the biointerface will also be investigated. The alteration of the nanoparticles during the *in vivo* experiments described in (**WP4**) is being investigated in this section and related to their effects. In the first half of the programme, studies have mainly concentrated on the surface chemistry of SiO₂ particles and their interaction with the soluble heavy metal ion Pb²⁺.

WP6: Integrated Modelling. No environmental toxicological study is complete unless the various parts are integrated together in a theoretical model. This is essential not only for planning the study but also for assessing the final transfer parameters. Such a process is continuously iterative throughout the investigation until towards the end of the study, when the parameters are completely optimised, and predictions as to the impact of the nanoparticles on the aquatic environment can be made. The model is being developed along the lines of previous environmental ecological models which predicted the transport and fate of soluble contaminants. A compartmental model is being used where the compartments are represented by the cell membrane, cell organelles, total cell, tissue and model aquatic organisms. The model based on *ECoS3* (developed by Plymouth Marine Laboratory) allows the set-up and integration of sets of advection-diffusion equations representing multiple constituents interacting in a spatial context³. **WP6** has developed working models of the solubility of oxide NPs and the validity of these models has been tested experimentally. The first step in the development of reliable structure-activity toxicological predictive relationships has been carried out and the modelling of the amount of NP that effectively reaches an organism and is, subsequently, internalized by it, prior to the toxic effect itself is taken care of using this model (see Figure 10). The main advantage of this model is that it provides a prediction of the amount of NP attached (or absorbed) and internalized by a microorganism as a function of time, based on a simple physicochemical mechanism.

³ Harris, J.R.W. & Gorley, R.N. 2005. *ECoS*, a framework for modelling hierarchical spatial systems. *Sci. Tot. Env.* 314 –316:625–635.

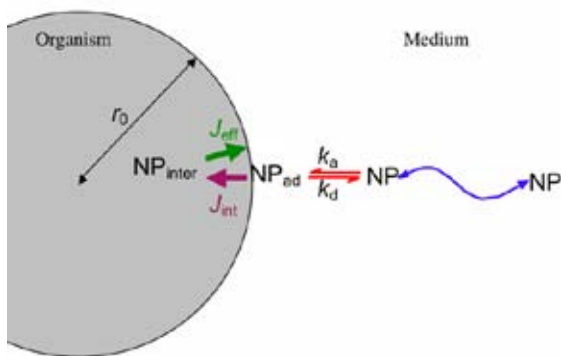


Figure 10. Schematic representation of the diffusion-adsorption-internalization model. Uptake is described through the following sequential steps: a) diffusion of NPs from bulk solution (blue); b) reversible adsorption/desorption of NPs on the biomembrane following a Langmuir-like kinetics (red); c) internalization of NPs through endocytosis or similar mechanisms (purple); and finally d) efflux of NPs out of the organism (green).

WP7: Exploitation and pre-validation. The Marine Biological Association of the UK a leading environmental charitable organisation is leading this activity. MBA has a track record of coordinating EU contracts and of carrying out bioassays for developing environmental quality objectives, with expertise in transferring analytical technology and significant regulatory experience. This includes considering all the above issues as well as developing accredited toxicity tests and assays for NPs in the aquatic situation. An important output will be aiding environmental legislation on these materials. Another important outcome is guidance on as to an effective means of calibrating and accrediting the toxicity testing procedures being developed. The results of a point of contact high throughput test using marine algae which has been developed for cross-checking all other toxicity tests in the programme.

The MBA this year has been consolidating all tests around the Consortium and evaluating their results with their own. The majority of *in vivo* and *in vitro* tests within the ENNSATOX would tend to indicate that ZnO nanoparticles are more likely to cause deleterious effects than those composed of SiO₂. However there are different requirements for Zn across taxonomic groups which consequently result in a range of sensitivities to excess metal. *In vivo* ZnO toxicity to the alga *Isochrysis galbana* was not markedly different across size ranges of NP tested here and were slightly less toxic than ionic Zn (Figure 11). Toxicity of the NP decreased in the sequence: nanotech suspension > Metals Basis undialysed > Metals Basis dialysed > powder (Sigma Aldrich). This implies that, over the studied size range, toxicity was highest for the larger Zn particles, though it is not clear if these differences were a function of some other characteristic (including actual Zn content of the stock suspensions rather than particle size *per se*).

WP8: Dissemination. Although this work package concerns dissemination it also feeds into WP7: Exploitation & Pre-validation. It identifies opportunities to publicise the achievements and

Effect of ZnO nanoparticles and ionic Zn on growth of *Isochrysis galbana*

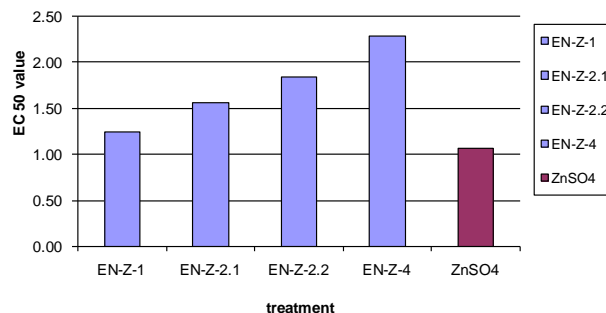


Figure 11.

capabilities developed under the auspices of ENNSATOX, engaging with potential end-users in industry, regulatory authorities, NGOs, academia, as well as the wider European and International public. The work package therefore also serves to aid *Exploitation* and will also have general marketing benefits for both the participating members of the ENNSATOX Consortium and the overall FP7 program.

WP9: Scientific Coordination & Project Management. This work package establishes effective coordination and decision structures that address the scientific and business needs of the project. It ensures that all project beneficiaries participate in decision-making and that the project is run efficiently on a day-to-day basis. It also maintains Quality Assurance (QA) on all procedures run by the Consortium. Finally it ensures the participation and representation of the ENNSATOX Consortium in the NanoSafety Cluster.

In Figure 12 the above objectives and activities are set within an integrated strategic environmental framework. The figure shows the environmental discharge and behaviour of the nanoparticles in the left hand compartment (a) and, the impact of nanoparticles on the biological barriers in between the two compartments (b) and on the aquatic organisms in the right hand compartment (c). Activities WP1 and WP5 will focus on understanding processes in compartment (a). Activity WP2 focuses on interaction and transport mechanisms at the interface between the compartments (b). Activities WP3 and WP4 focus on interaction and bioaccumulation mechanisms in compartment (c). Activity WP6 will integrate and model all processes represented in the figure summarising the RTD activities in this project.

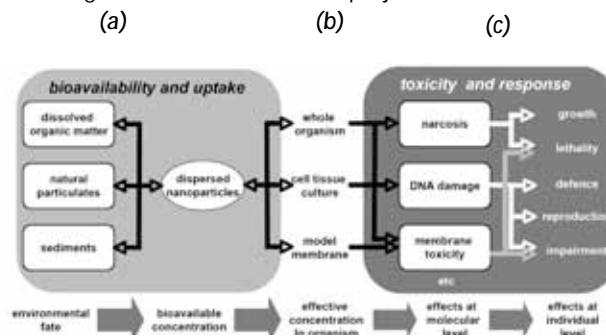


Figure 12



A list of deliverables arising out of these activities can be summarised as:

- Fundamental insight into nanoparticle interactions and transport in the aquatic environment and in living cells and organisms.
- Relation between structure and functionality and activity of nanoparticles and modified nanoparticles at all levels of biological organisation.
- Integrated model to assess and predict the fate and risks of nanoparticles in the environment.
- Protocols for screening nanoparticle activity to be accredited for statutory use.

The following has been achieved in the second year of the project:

- Synthesis of constant particle size >10 nm ZnO particles.
- Comprehensive characterisation of biomembrane activity of SiO₂ and ZnO particles of wide range of particle size.
- Chemical characterisation and modelling of solubility of ZnO particles.
- Cross calibration of results throughout the Consortium.
- Chronic toxicity and bioavailability of indicator organisms to nanoparticles.
- Development of theoretical models simulating interaction of NP with phospholipid membranes, the transport and fate of nanoparticles in environmental aqueous systems and the uptake of nanoparticles by unicellular organisms.
- Interaction of ZnO with ion channel proteins.

2.4 Conclusion To-Date & Plans for the Future

Initial conclusions from the first half of the work programme associated with relevant work package are outlined below:

WP1:

- Water solubility of ZnO nanoparticles of varying size and functionality has been determined.
- Work has sourced and synthesised stable dispersions of ZnO nanoparticles, and provided a set of well characterised single class of ZnO nanoparticles to the Consortium.
- Characterisation of NPs after exposure to biological media and characterisation of NPs internalized by cells and organisms.

WP1/WP2:

- Full comprehensive relationship between the structure and functionality of SiO₂ and ZnO particles and their biomembrane activity and their *in vitro* and *in vivo* biological activity established.

WP2:

- Toxicity screens consolidated and inter-calibrated.
- Mechanisms of SiO₂ and ZnO particle interaction with biomembrane model determined using electrochemical

methods of impedance.

WP3:

- Detailed assessment and comprehensive feedback of *in vitro* test results to WPs 2 and 4.
- EMs of NPs in cells and tissues.
- *On-line in vitro* assays for NP toxicity developed.

WP4:

- NP effects on *Ciona intestinalis* growth determined, correlation with specific/non specific response.
- Structure Activity (SA) relationships established with WPs 2 and 3.

WP5:

- Determination of the surface charge density and zeta-potential of the NP dispersions in the presence of other ions and organic substances fulvic and humic acid present in the aqueous environment.
- Analysis of the heterogeneity of metal ion binding by silica NPs, and comparison with heterogeneities observed in the metal ion binding by macroscopic silica surfaces.
- Verification of the lability of Pb²⁺/SiO₂ nanoparticulate complexes by Eigen type reconstruction of the kinetic steps involved in the surface complex formation Surface characterisation of ZnO NPs.

WP6:

- Advection/diffusion of NPs in aquatic environment studied.
- Results from WPs 2 and 5 integrated into model.

WP7:

- Intercalibrative testing of ZnO on MBA indicator organisms.

The scientific work plan for the last part of the project includes:

WP1:

- Extensive characterisation of sourced samples of TiO₂ and distribution round Consortium. Correlation of surface chemical with physical characterisation.

WP1/WP2:

- Full comprehensive relationship between the structure and functionality of SiO₂, ZnO and TiO₂ particles and their biomembrane activity and their *in vitro* and *in vivo* biological activity to be established.

WP2:

- Comprehensive studies on TiO₂ biomembrane activity.

WP3:

- *In vitro* studies of TiO₂ biological activity.

WP4:

- TiO₂ effects on *Ciona intestinalis* growth determined, correlation with specific/non specific response.
- Structure Activity (SA) relationships established with WPs 2 and 3.



WP5:

- Permeability of NPs in hydrogels/model cell walls. Physiochemical conditions on permeability rate established.
- Form, permeation of SiO₂ and TiO₂ determined. Rigorous kinetic analysis of permeation.

WP6:

- Advection/diffusion of NPs in aquatic environment consolidated.
- Results from WPs 2 and 5 integrated into model.
- Integrated model developed as protocol.

WP7:

- Organisation of procedures for inter-laboratory toxicity trials for nanomaterials using standard and novel testing procedures.



3 Directory

Table 1 Directory of people involved in ENNSATOX.

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**ENPRA****RISK ASSESSMENT OF ENGINEERED NANOPARTICLES**

Contract Agreement: NMP4-SL-2009-228789 Website: <http://www.enpra.eu>
 Coordinator: Lang Tran, Institute of Occupational Medicine, Edinburgh (UK)

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4	Université Paris Diderot - Paris 7	UPD	FR
5	Commissariat à l' Énergie Atomique	CEA	FR
6	Université Catholique de Louvain	UCL	BE
7	Katholieke Universiteit Leuven	KULeuven	BE
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9	Helmholtz Zentrum München German Research Center for Environmental Health	HMGU	GE
10	Institut für umweltmedizinische Forschung	IUF	GE
11	University of Copenhagen	UCPH	DK
12	National Research Centre for the Working Environment	NRCWE	DK
13	Universita Ca' Foscari di Venezia	UniVE	IT
14	Joint Research Centre	JRC	Pan-EU
15	Rijksinstituut voor Volksgezondheid en Milieu	RIVM	NL
US PARTICIPANTS			
16	University of Rochester	Rochester	US
17	Duke University	Duke	US
US PARTICIPANTS			
The following partners are linked to the project by a Memorandum of Understanding signed by their legal representatives			
18	US Environment Protection Agency	EPA	US
19	National Institute of Occupational Safety & Health	NIOSH	US
20	National Institutes of Health - National Institute of Environmental Health Sciences	NIH-NIEHS	US
21	The Woodrow Wilson Center	WWC	US

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1 Summary

Engineered Nanoparticles (ENP) are increasingly produced for use in a wide range of industrial and consumer products. Yet it is known that exposure to some types of particles can cause severe health effects. Therefore it is essential to ascertain whether exposure to ENP can lead to possible health risks for workers and consumers. We have formed a consortium of well-known scientists from European Universities and Research Institutes, with over 100 publications in the field of Nanotoxicology. Our aim is to develop an approach for the Risk Assessment of ENP (ENPRA). Our objectives are:

- to obtain a bank of commercial ENP with contrasting physico-chemical characteristics and measure them;
- to investigate the toxic effects of ENP on 5 (pulmonary, hepatic, renal, cardiovascular and developmental) target systems and 5 endpoints (oxidative stress, inflammation; immuno-toxicity; fibrogenicity; genotoxicity) using in vitro animal/human models;
- to validate the in vitro findings with a small set of carefully chosen in vivo animal experiments;
- to construct mathematical models to extrapolate the exposure-dose-response relationship from in vitro to in vivo and to humans;
- to use QSAR like models to identify the key ENP characteristics driving the adverse effects;
- to implement a risk assessment of ENP using the Weight-of-Evidence approach;
- to disseminate our findings to potential stakeholders. To harmonize the research activities between our EU group and the US, we have established links with scientists from US Universities (Duke, Rochester) and Government Agencies (NIH/NIEHS, NIOSH and EPA) with on-going research in Nanotoxicology.

Our objectives here are

- to share information and agree on experimental protocols;
- to avoid duplication of work.
- to further validate the findings of this proposed study.

2 Concept

Nanotechnology is one of the key industries in Europe¹. The estimated economic impact of nanoparticles in industrial, consumer, and medical products will be US\$ 292 billion by 2010 and US \$1 trillion by 2015. The prosperity of our continent depends on the safe and sustainable development of this emerging technology². Every new technology brings with it new risks and for nanotechnology, the potential health risks to

workers and consumers are paramount. They can arise from exposure to nanomaterials either at work or through consumer products. These risks, if not assessed and managed properly, can prevent economic growth and deprive us of a much needed competitive edge, but more importantly could have grave potential consequences for human and environmental health^{2;3}. Being aware of the health issues concerning engineered nanomaterials, in 2006, some of the ENPRA partners have written an article, published in Nature⁴, outlining the grand challenges for the safe handling of nanotechnology. It is clear that the production of safe nanomaterials is essential to establish and sustain the confidence of end users. This confidence is the ultimate guarantor for nanotechnology growth. It is therefore essential to develop an effective approach for improving the assessment and management of potential health risks from exposure to engineered nanoparticles (ENP)⁵. This is the overall aim of ENPRA.

2.1 Aim and Objectives

The principal aim of ENPRA is to develop and implement a novel integrated approach for ENP Risk Assessment (ENPRA). This approach is based on the Exposure-Dose-Response Paradigm for ENP (Figure 1). This paradigm states that exposure to ENP of different physico-chemical characteristics via inhalation, ingestion or dermal exposure is likely to lead to their distribution, beyond the portal-of-entry organ to other body systems. The cumulative dose in a target organ will eventually lead to an adverse response in a dose-response manner. Our approach will adapt the traditional Risk Assessment approach to ENP and will cover: Hazard Identification; Dose-Response Assessment; Exposure Assessment and Risk Assessment, Management.

The specific objectives of ENPRA are: (i) for Hazard Identification: To characterize a panel of commercially available ENP carefully chosen to address the relevant hazards, properties and potential mechanisms¹; (ii) for Dose-response Assessment: To assess the hazards of these ENP by means of in vitro toxicology tests based on five body systems: (1) pulmonary; (2) hepatic; (3) renal; (4) cardio-vascular and (5) developmental, and five endpoints: (a) oxidative stress; (b) inflammation and immune-responses; (c) genotoxicity; (d) fibrogenicity and (e) developmental toxicity; (iii) To verify the in

¹ The ENP selected represent a subset from a panel of ENP chosen as reference materials for testing in a UK government (DEFRA) funded project and is very likely to be fed into the OECD plan for reference materials testing. The samples were chosen with contrasting properties on size/surface area (TiO₂), charge (silica), shape (MWCNT), surface chemistry (silver, iron).



vitro findings with in vivo models; (iv) for Exposure and Risk Assessment: To use data from this project and other sources (including US data) to: (1) model exposure and the exposure-dose-response relationships by means of mathematical modelling such as PBPK and QSAR-like methods, and extend these deterministic models into probabilistic models (2) to conduct the risk assessment with uncertainty analysis; (v) for Risk Management: To develop and implement a strategy for dissemination to maximize the anticipated high impact of our findings.

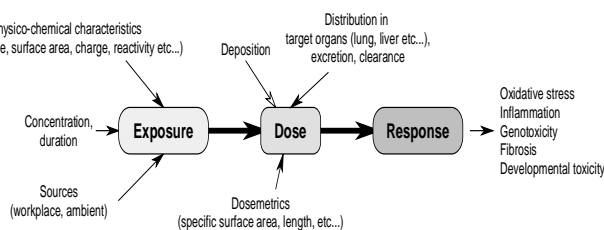


Fig 1. The Exposure-Dose-Response paradigm

The main deliverables of ENPRA are:

A novel risk assessment approach – with uncertainty analysis - specific to ENP;

In vitro and in silico models of exposure-dose-response relationships for 5 target organs and 5 endpoints to be used for the hazard assessment of ENP and considered for high-throughput screening tests;

In vivo models for the hazard assessment of ENP to complement the REACH and OECD guidelines.

The rationale of the ENPRA project is to generate essential data on ENP characteristics and toxicity to be used with data from other sources for a risk assessment of ENP.

Using the traditional Risk Assessment approach as starting point, our approach consists of:

Hazard Identification: We will implement (i) a comprehensive set of measurements of the physico-chemical characteristics of ENP, both in bulk samples and in body tissues; (ii) common protocols for ENP characterization and preliminary validation of measurement techniques; (iii) relationship between particle characteristics and hazards.

Dose-Response Assessment: We will implement a development of in vitro testing systems using models representing the most important target systems affected by ENP.

These in vitro tests need to be verified with in vivo models, (carefully designed to minimize the numbers of animals used and/or their inconvenience).

The verified tests will be validated by a round robin process between the ENPRA partners.

The selected in vitro tests could then be integrated as part of a low-cost, high-throughput screening test system, as a cost effective way of testing a large number of ENP expected to enter the EU market in the near future.

The in vitro data will be used to develop a QSAR model linking ENP characteristics with the adverse effects.

The in vivo models will also be considered as additions to OECD guidelines for regulatory toxicology tests of ENP.

The design of our in vitro and in vivo studies takes into account the need to promote the principles of 3R.

Exposure Assessment: We will review existing exposure models in the public domain; Collect exposure information from existing EU and National Project and from our US partner; Construct a model of ENP exposure in occupational settings; Extend the traditional risk assessment approach by quantifying the uncertainty in ENP exposure.

Risk Assessment: We will extend the current risk assessment approach to ENP by building mathematical models of exposure-dose-response, including uncertainty analysis, to be used in estimating the DNEL and make comparison to the values obtained in Exposure Assessment.

Risk Management: We will implement a communication strategy to bring the ENPRA results to stakeholders including government agencies and Nanotechnology industry.

The approach proposed by ENPRA is in line with the grand challenges described in our article in Nature4. The rationale of ENPRA is summarised graphically in Figure 2.

The ENPRA Consortium To implement the ENPRA plan, we have assembled a consortium of 21 partners (15 Europeans and 6 Americans) with an excellent academic record measured in hundreds of publications on Nanotoxicology (and three relevant articles in Nature and Nature Nanotechnology). Our partners also include prominent members of government bodies, participating in the regulatory process, on both sides of the Atlantic (e.g. JRC and US EPA, NIOSH). Most importantly, different groups within the ENPRA consortium have experience in working together in FP projects as well as other national projects and will be able to share their extensive experience on working with ENP in achieving the objectives laid out in ENPRA.

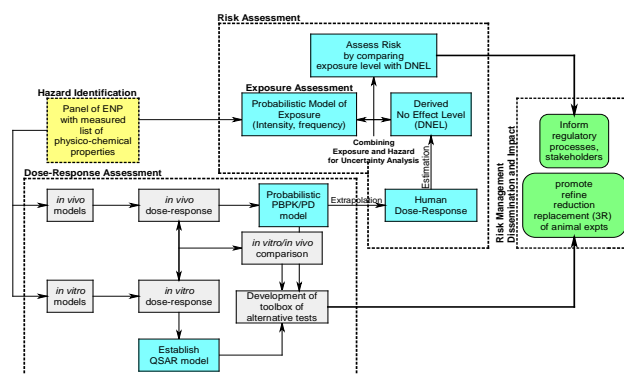


Fig 2. The rationale of the ENPRA approach



2.1 Overall view of the Workplan

ENPRA consists of 7 complementary Work Packages (WP), summarised in Table 1. In the text below, we will present each WP in details. In each WP, we will give the objectives; the WP leader (in bold) and team members; the Hypotheses and Methods; the Deliverables; and linkage to other Work Packages.

Timescale: We envisage that ENPRA will require the total of 3.5 years (42 months) to complete.

Table 1: Work Packages of ENPRA

WP1	Project Coordination and Management
WP2	EU-US Collaboration
WP3	Hazard Identification: Characterisation of the Physico-chemical Properties of ENP
WP4	Dose-Response Assessment I: Development of in vitro Models for Assessing the Potential Hazards of ENP
WP5	Dose-Response Assessment II: Using in vivo models for a kinetics study and verification of in vitro results
WP6	Risk Assessment: Models of Exposure-Dose-Response & QSAR-Like Model Development. Risk Analysis: Combining Hazard and Exposure
WP7	Risk Management: Dissemination Strategy to Maximize Impact

Figure 3 describes the interrelationships between the Work Packages and the link between ENPRA and other EU and US activities.

The project has reached the mid-term (18 month) milestone. The work performed by each WP and the main results are summarised below:

WP2 EU-US Collaboration

The progress of WP2 in the first reporting period is according to the original workplan and we have achieved the current milestones. The notable achievements of WP2 so far are: (i) the implementation and updating of the project website; (ii) the maintenance of the EU-US relation. Specifically, the transfer of ENP materials and protocols to our US partners. Some of the Tasks in WP2 are continual and will be reported accordingly.

The most notable results so far for WP2 are:

1. The ENPRA website which is available for internal and external use
2. The continuing collaboration between EU and US partners in the supply of ENP samples and protocols.

In the second reporting period, we have implemented the following activities:

1. Partner 17 performs complementary ICP-analysis of the catalast particles in MWCNT to cross-compare with data from ENPRA
2. The first in vitro set of results from Partner 19 with MWCNT has been sent to Partner 1 to incorporate in the ENPRA database for use in WP6.
3. Partner 18 and Partner 1 are designing the data templates for transfer the TOXCAST data over to ENPRA for WP6.
4. Perform the comparison on size distributions and stabilities of dispersions made at Partner 19 with the results obtained in ENPRA.
5. Presence of impurities in CNT will be investigated also by Partner 19 for comparison.

WP3 Hazard identification through characterization of the Physico-chemical properties of ENP

The progress of WP3 for the first reporting period is according to the ENPRA workplan. Specifically, a probe-sonication particle dispersion protocol based on 2% w/v serum-water has been developed and adopted by the consortium. Validation of the protocol is in progress in EU-US collaboration. Primary physico-chemical characterization data has been submitted as part of D3.1 with minor analytical results remaining. Developments of protocols for analysing particles in biological matrices is in progress

The most notable results so far for WP3 are:

1. Test materials have been identified or produced and distributed.
2. The 2%serum-water-based batch dispersion protocol (\pm use of 0.5 vol% EtOH) has been developed and adopted within ENPRA and beyond.
3. Size distributions of batch dispersions have been collated and distributed informally to ENPRA.
4. Primary physicochemical characteristics have been obtained and a report is currently being prepared

For the second reporting period,

1. all primary physicochemical characterization has been completed and analyses of organic coatings have been completed.
2. Extensive supporting analysis for the toxicity testing has been completed.
3. A complete database on physical and chemical characterization of ENP has been distributed for incorporation in the ENPRA database.

We continue to measure the hydrochemical reactivity and biodurability of ENP. We will also validate the developed



protocols with real biological samples from WP and perform a **final** intra-vial and vial-vial homogeneity study will be done on the TiO₂ samples. Finally, we prepare publications on the ENPRA dispersion protocol and co-authoring papers lead by researchers outside the WP

WP4 – Dose-response assessment I: Development of in vitro models for assessing the potential hazards of ENP

The progress of WP4 in the first reporting period is according to the ENPRA workplan. Specifically, we have achieved the following:

1. For the kick off meeting all tasks were detailed via a spreadsheet, dividing each task into target organ/cell types and the relevant endpoints for each target.
2. Using the spreadsheet, each partner identified which target and endpoints they were to be responsible for in order to identify areas for collaboration and potential gaps. This was discussed via teleconference in order to allow coordination of collaboration. No gap was identified.
3. Key users of protocols that spanned multiple targets were identified and these groups generated standard operating procedures for these protocols. These protocols have been shared amongst partners via the ENPRA website. In addition they have been provided to NanoImpactNet for conversion into NIN protocol format.
4. A panel of 10 ENP were distributed to all partners via Mercator.
5. Using the agreed protocols, each group tested all of the particles for cytotoxicity in the relevant target cell types in order to determine LC50 values.
6. LC50 values have been assembled into a summary spreadsheet to enable future strategic decision making for WP4 and WP5. This spreadsheet has allowed identification of relatively high toxicity materials (ZnO and Ag) and relatively low toxicity materials (MWCNT and TiO₂), but it has also allowed identification of cell type - particle type interactions which are relatively sensitive (e.g. macrophages and long MWCNT). This information, combined with the information from the characterisation WP, will allow prioritisation of particles for mechanistic studies and in vivo studies.

The most notable results so far for WP4 are:

1. The clear consistency between all partners that the ZnO and Ag particles are relatively toxic;
2. all other ENP are generally of no significant toxicity at the doses tested.

For the second reporting period, we continue with the following:

1. Each group has tested all of the particles in the relevant target cell types in order to determine LC50 values.
2. LC50 values have been assembled into a summary spreadsheet to enable future strategy decision making for WP4 and WP5.

3. All Cytotoxicity data has been submitted for data analysis and database generation in other WPs.
4. Much of the cytokine data has been submitted for data analysis and database generation in other WPs.
5. Mechanistic studies now focus on a minimum of the 2 MWCNT, the Ag, uncoated ZnO and one TiO₂.
6. One publication is already in press for the genotoxicology studies and a number of other manuscripts are in advanced stages of preparation.

WP5 – Dose-response assessment II: Using in vivo models for a kinetics study and verification of in vitro results

The progress of WP5 in the first reporting period is according to the ENPRA workplan. Specifically, we have achieved the following:

1. The major part of the study has been conducted
2. The dose-response relationships of the full panel of ENP after acute exposure have been executed. The study has been performed using the finalised dispersion protocol to make the particle suspensions and the finalised instillation protocol. The data are being collected and currently analyzed.

The most notable results so far for WP5 are:

1. Results of kinetic study using TiO₂ have been reported. ENP taken up into the lungs cross the lung membrane and reach the blood stream. This leads to an accumulation in organs in a size dependent manner: the smaller the ENP, the higher the accumulation in the organs.
2. The intratracheal instillation has been performed for the acute dose-response study. Preliminary results show a very low acute inflammatory potential, except for ZnO.
3. In contrast to the in vitro results, nano-silver does not evoke an acute inflammatory response.
4. The MWCNT seem to be less acutely toxic compared to other MWCNT studies described in literature, but a detailed comparison in particle characteristics needs to be done to give an idea why that is.
5. Data from the in vitro studies and the preliminary results from the acute in vivo study have lead to a critical re-evaluation of the study set-up for the in vivo studies in mice with a pre-existing risk factor.

For the second reporting period we have continue with the following

1. Bio-kinetics inhalation studies with Gold and Silver ENP have been performed and the data analysed. The gold ENP undergo the same metabolic and biokinetics when comparing inhalation versus instillation. Rather unexpectedly the entire NP translocation across the air-blood-barrier did not differ significantly and the retained fractions in prominent secondary organs like liver and spleen were also not significantly different. This result is insofar important as it suggests that the rather inexpensive method of instillation provides a rather simple application for NP delivery to the lungs



including comparable translocation towards secondary organs compared to the application by inhalation – the gold standard of lung toxicology research.

2. In vivo studies to determine toxic effects after acute (24hrs) exposure for the full panel of ENPs have been conducted. The data have been analysed and dose-response relationships have been established for a range of endpoints. The intratracheal instillation of ZnO NM-110 and NM-111 will be repeated for 2 reasons. 1. Due to technical difficulties during blood drawing after administration of NM-110, no blood analysis was possible while effects are seen in blood parameters with ZnO NM-111. 2. Due to advances with ZnO toxicity testing in other projects, there could be indication for early changes in lung tissue. In the current protocol the lung tissue was used for protein and RNA analysis and not prepared for histopathological analysis.
3. Administration of nanomaterial in ApoE mice has finished and analysis of all parameters is underway. The studies in a rapid ageing mouse model have also started.
4. Oxidative stress and inflammation studies in wildtype mice by visualizing Matrix MetalloProteinase (MMP) and Cathepsin have also commenced.

WP6 – Risk Assessment: Models of exposure-dose-response & structure-activity model development. Risk Analysis: combining hazard and exposure

The progress of WP6 in the first reporting period is according to the ENPRA workplan. Specifically, we have achieved the following:

1. The ENPRA database templates have been produced for data collection from WP3, 4 and 5.
2. The ground work for Exposure modelling, QSAR, PBPK and PD modelling is ongoing. When data will be available, in the first quarter of 2011, results will be generated.

The most notable results so far for WP6 are:

1. on preparatory work for modelling, including uncertainty analysis using Monte Carlo simulation. This is done in collaboration with US partner 19. Period 1 work has concentrated on data generation, with much of the WP6 work so far being preparatory. In Period 2, significant and important results from the Risk Assessment will be available.

In the second reporting period, the data available from other WP are now increasingly available. The notable progresses in WP6 are:

1. The collection of data from WP3, 4 and 5 and later also from the US partners into the ENPRA database for QSAR analysis.
2. Partner 1 and Partner 14 have met to discuss the transfer the data for analysis

3. Bio-kinetics data from WP5 have been used for PBPK modelling
4. In vitro and in vivo endpoints commensurate for comparison have been identified
5. Model of exposure of ENP are being constructed using data from Partner 19.

WP7 – Dissemination Strategy to maximise impact

The progress of WP7 in the first reporting period is according to the ENPRA workplan. Specifically, we have achieved the following:

1. The main contributors (P14, P5 and P1) of this work package met face-to-face during the kick-off meeting (where a general agreement on the initial 6 months work plan and working procedures was achieved) and several times virtually by teleconference to agree organise further steps. Also the annual management meeting and the expert panel meeting offered opportunities to meet.
2. The dissemination strategy was implemented as foreseen by:
 - holding the first annual workshop with participation of the main stakeholders (EU CAs, Industry, NGOs, COM Services, OECD, other FP6/7 projects) and disseminating its outcome
 - organising the three experts' panel meeting and producing and disseminating the two EONS reports
 - participating in a number of workshops/conferences both in the EU and the US
 - participating in one OECD experts' meeting

The most notable results for WP7 so far are the regular and high-impact events to disseminate the idea and results of ENPRA. Overall, the work package is well on track and the foreseen milestones and deliverables have been reached without major problems. This seems to be the case as well for the next milestones and deliverables.

- 3.1.1. Expected final results and their potential impact and use (including the socio-economic impact and the wider societal implications of the project so far),

ENPRA has reached the Mid-Term Milestones. As demonstrated above, the major achievement of ENPRA so far are the results from the in vitro (WP4) and in vivo tests (WP5) together with the measurement of the ENP physico-chemical characteristics for Hazard Identification. So far, there is remarkable concordance between in vitro and vivo results (with the exception of nano silver). The ENPRA team is currently analysing and formulating new hypotheses regarding the low toxicity of the ENP samples in contrast to the published results in the public domain.

In the second reporting period we continue with the following:

1. The 2nd Annual ENPRA Workshop and Stakeholders Panel Meeting took place in Somma Lombardo (Italy) from 10 to 12 May 2011 organised by the JRC-IHCP. This time the workshop theme was "Challenges of Regulation and Risk Assessment of Nanomaterials"



and it was organised in the frame of the JRC Enlargement and Integration Action, that allowed to support the participation of scientists from EU and Associate Candidate Countries. Attendance of members of the Competent Authorities for REACH and Classification and Labelling Sub-Group on Nanomaterials (CASG Nano) and the European Chemicals Agency (ECHA) was supported. Also other strategic stakeholders as OECD, NGO's (representing environment and workers' protection organisations), the contractors for the RIP-oNs projects and chemicals and nanomaterials industry and EFSA were involved. Some related projects under FP7 Research Program participated too. It involved about 80 participants. Generalist and specialised press were also present. During the workshop, 34 experts from 26 different organisations informed the participants on the latest scientific progress in the field of nanoparticles risk assessment produced within the ENPRA and other related projects and presented and discussed recent developments concerning legislation in the EU and beyond. A full report of the event is currently in the final steps for publication and distribution. Information can be found in the JRC site http://ihcp.jrc.ec.europa.eu/events_workshops/joint-jrc-nano-enpra-2011.

The results of the 2nd workshop will be published on-line on the JRC site (presentations already on-line, full report by mid November) and widely distributed among targeted stakeholder.

2. The 4th EONS report (state of the art report summarising the discussion of the 4th ENPRA expert panel meeting held in Brussels on March 31st) was

published on June 2011 (D7.2 – M24) The report (10 experts' article commentaries) was then disseminated.

The 5th ENPRA expert panel meeting will be held in Paris on October 13, 2011. The event will gather 13 participants including ENPRA partners (P1, P5, P6, P8, and P14) and French OMNT experts (5).

In the next period, ENPRA will

1. Analyse the body of experimental data generated by the WP4 and 5 as well as data contributed by the US partners in order to Identify the reason(s) behind the relative low toxicity of the ENP tested. Specifically, the mechanisms of toxicity of ENP selected in ENPRA;
2. relate these facts to the physico-chemical characteristics of ENP in WP3 by means of modelling.
3. Assess the potential health risks with regards to the tested ENP

With our regular dissemination events, we will be able to keep the stakeholder community informed. The impacts on industry strategy for the production of ENP as well as the assurance for nanotechnology workers and consumers on the relative safety of these ENP is expected to be significant. A further impact on the use of ENP in general for nanomedicine is also to be expected.

In summary, the second term of ENPRA will bring together many significant results and advance the state of the art knowledge on nanotoxicology further.

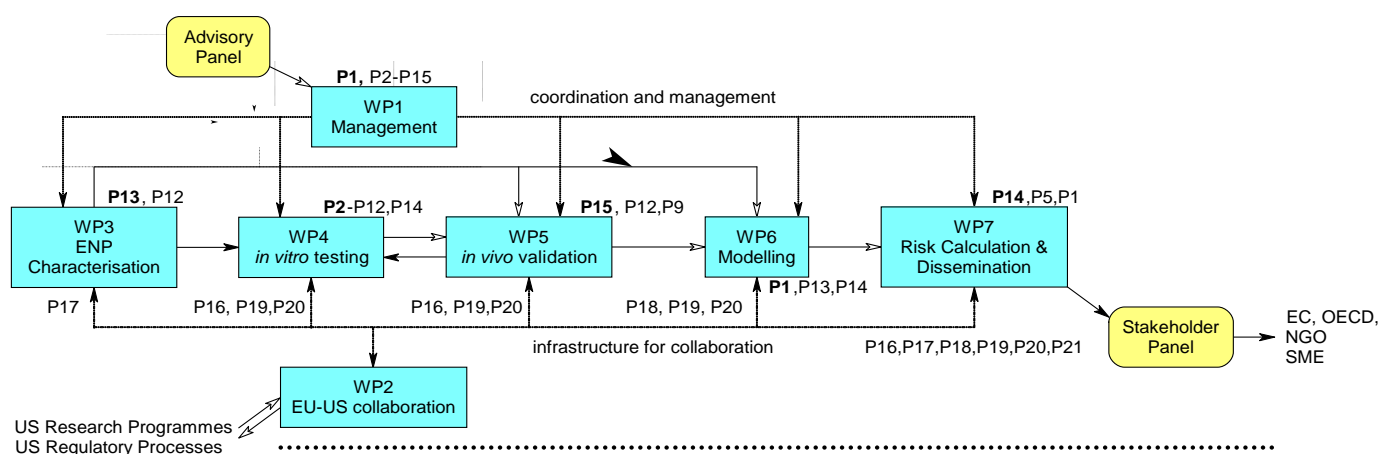


Fig 3. Flow chart describing the information flow between different WP of ENPRA (solid lines) and the process of coordination, management and collaboration (dotted lines)



3 Directory

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2	Institute of Pharmaceutical Sciences/ Pharmaceutical Technology, Karl-Franzens University of Graz	IPW	Austria
3	Medical University Graz, Center for Medical Research	MUG	Austria
4	JOANNEUM RESEARCH, HEALTH – Institute for Biomedicine and Health Sciences	JR-HTH	Austria
5	BioMed-zet-Life Sciences GmbH, Linz	BioMed-zet	Austria
6	University of Vienna, Faculty of Life Sciences, Department of Pharmaceutical Technology and Biopharmaceutics	IPB	Austria
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1 Introduction

Nanotechnology is, along with biotechnology and information technology, a key technology of the 21st Century that has far-reaching implications for science, industrial development and the creation of new products. Therefore, it is considered highly important for successful economic development over the coming decades. The subject of nanotechnology is a collective term that relates to different techniques in the nanometer range, the production, study and in the application of structures. Molecular materials, internal interfaces and surfaces with critical dimensions or production tolerances ranging from a few to about a hundred nanometers are studied structural factors. In the most important industries, it is increasingly recognized that the control of structural and functional properties of novel materials - so-called "Advanced Materials" - on the nanometer scale is the key to technological advances and new products that will conquer emerging markets [1]. Besides the use of nanotechnology in material science, there are great impacts of nanotechnology that are expected to alter medicine. Nano-based techniques in the fields of diagnostics (e.g. imaging, biosensors) and therapy (e.g. drug delivery, drug targeting or regenerative medicine) are creating new possibilities in medicine [2, 3]. Cancer therapy along with the treatment of viral and a number of degenerative diseases has shown significant progress with nano-based techniques [4-8].

However, despite the obvious benefits of such advanced materials, there are potential adverse effects on the environment and people due to the fact that humans are exposed to nanoparticles through various routes: inhalation via the respiratory tract, dermal absorption/penetration through hair follicles, ingestion by the gastrointestinal tract, and injection. Nevertheless, the toxicology of these materials has been investigated insufficiently.

Regarding the degradation processes of advanced materials (e.g. waste deposit, air, and groundwater), nanostructured materials are being distributed in the environment. Until now, it has not been possible to show whether nanoparticles that are ingested or inhaled from the environment are systemically absorbed on a larger magnitude and if it is possible to calculate their long-term effects [9, 10].

In addition to the desired physio-chemical changes, a modification of toxicological behaviour is observed due to the structuring of the materials on the nanometer scale. Systematic studies exist regarding the effects of environmental nanoparticles (ultrafine particles) in relation to a reported increase in the incidence of cardiovascular disease and propensity for asthmatic disease [11-15]. The change of the toxicological potential, which is due to the reduction of the material in the nanometer range, was negated a few years ago. In many cases, the importance has only been recognized in recent years. People and the environment are permanently exposed to nanostructured materials as a result of an ever-widening use and also from their release from within their life cycle; these effects are not negligible. Therefore, a profound knowledge of the toxicological potential of nanostructured materials, breakdown products, penetration of and metabolism in the human body, and their emission is of enormous importance.

The knowledge of toxicology, the possibility of critical assessment of the potential danger of using standardized testing procedures and the systematic studies carried out on nanomaterials are hereby determined by public acceptance. Public acceptance is a prerequisite for the sustainable and successful development of nanotechnology. A poor acceptance (e.g. caused by a lack of awareness in the field of toxicology) could probably lead to a negative trend in perception similar to that of genetic engineering.

Due to the fact that nanomaterials are increasingly present in our environment, international experts have a growing interest regarding this issue. It is increasingly clear, however, that there is a tremendous need for standardization. A portion of data published to date, which has been used, contains insufficiently characterized nanostructured materials. Moreover, many of the in vivo studies carried out with mice or rats have used overly high doses of the investigated nanostructured materials. This demonstrates that the results are not conclusive and that a classification of the key parameters for assessing the toxicity of nanostructured materials is urgently needed.

2 Background

In the field of toxicology in recent years, a paradigm shift towards a proactive risk assessment has been identified. The public reporting has increased significantly, making the need for the objective communication of risks paramount. Public opinion and acceptance of nanotechnology contribute to four main areas and therefore must be given special consideration: (a) public attitudes, (b) public perception, (c) the role of the media, and (d) trust from those who communicate the risk in Public behaviour and attitudes [16]. This development is also taken into account on an international scientific level and is recognized by The European Commission. Janez Potocnik, a member of the Commission for Science and Research until November 2009, cited this: "*Nanotechnology is a key area where Europe leads the way and we must ensure that this remains so. The potential of nanotechnology for European industry and society is enormous so we need to research a clear strategy and effective measures in this area. At the same time we must consider eventual health, safety and environmental risks and address them as early as possible.*" [17]. The bidding of the 7th Framework Program of the European Union reflects this trend by making a specific call for clarifying research in the field of toxicology.

Another initiative in this field sets the Organization for Economic Co-Operation and Development (OECD) with the "Sponsorship Program for the Testing of Manufactured Nanomaterials" This program pools expertise and funds the safety testing methods of specific manufactured nanomaterials (NMs). The priority list includes 14 NMs for testing based on materials which are in, or close to, commerce: Fullerenes (C60), single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs),



silver nanoparticles, iron nanoparticles, carbon black, titanium dioxide, aluminium oxide, cerium oxide, zinc oxide, silicon dioxide, polystyrene, dendrimers and nanoclays [18].

In 2004, Donaldson and colleagues claimed, '*We suggest that a discipline of nanotoxicology be built up to address the new potential threats that widespread use of new nanoparticles could bring in support of the growth of a safe and sustainable nanotechnology industry*' [20]. The term 'nanotoxicology' was introduced into the literature and Austria became involved with various activities right from the beginning.

2.1 From international to Austrian needs in the field of nanotoxicology

The network NanoNet Styria was the first in Austria to touch upon the topic of nanotechnology and has dealt with nanotoxicology from the beginning. Initiated through these regional activities in nanoresearch, the Austrian Nanoinitiative was founded at the federal level. The group, which has been working within NanoNet Styria with bionanotechnology, has evolved into BioNanoNet Forschungsgesellschaft mbH. In 2004, the Austrian Nanoinitiative announced the program "National cooperative research and technological development in collaborative projects." BioNanoNet worked as an administrative coordinator for the proposal of the joint project "Nano-HEALTH - Nano-structured materials for drug targeting, release and imaging" (www.nano-HEALTH.at). This project deals with nanostructured materials and integrated the toxicological concerns as a sub-project. International experts have critically evaluated the submitted research project. They have positively reviewed the project twice. The budget for "Nano-HEALTH" was allotted at 8 million Euros for the time period of 2005 to 2012. The toxicological work under this project was the basis for the establishment of the European Center for Nanotoxicology.

In 2007, giving active support to decision makers to implement an Austrian strategy on nanotoxicology, Helge Torgersen and Frank Sinner laid down the basis for the joint recommendation to the Austrian Federal Ministry for Transport, Innovation and Technology (BMVIT) regarding the questions of risk and societal issues in nanotechnology. These recommendations summarized the state-of-the-art in nanotechnologies and risk assessment as well as the possible effects on human health. Additionally, they outlined the knowledge gaps that need closing and the methods that will ensure safe and sustainable development of the entire field of nanotechnologies. The authors also proposed a set of strategies to implement these recommendations on a short, medium and long-term basis. Some aspects of these recommendations were implemented by the funding of the project Nano-Trust which is managed by the Austrian Academy of Science.

The core of Nano-Trust is to provide a point of contact for issues dealing with the potential health and environmental risks of nanotechnology for citizens, government and politicians. Furthermore, a multidisciplinary team has established an annotated literature database that covers different aspects of nanotechnologies including the effects on human health, ecotoxicity, and governance. The team consists of the Austrian Academy of Science, Environmental Agency, BioNanoNet

Forschungsgesellschaft mbH and the Austrian Agency for Health and Food Ltd (AGES).

2.2 Interdisciplinary Network

The key to the development of safer nanomaterials (including the factors mentioned above) is the establishment of interdisciplinary networks of nanomedicine and the transference of existing knowledge in Austria. In order to focus on this necessary expertise in Austria, the European Center for Nanotoxicology (EURO-NanoTox) from the BioNanoNet Forschungsgesellschaft mbH was founded in 2007, funded by the Austrian Federal Ministry of Science and Research (BMWF).

The European Center for Nanotoxicology (EURO-NanoTox) is the Austrian hub for scientific knowledge in the field of human nanotoxicology. The Center's science and networking industry contributes significantly to improving safety in the workplace when dealing with nanostructured materials.

EURO-NanoTox is designed to address all aspects of nanotoxicology and is a national contact point with international visibility for researchers and industries. The EURO-NanoTox is managed by the BioNanoNet Forschungsgesellschaft mbH, a non-profit network company active in the field of pharmaceutical development. The partners of the EURO-NanoTox are Joanneum Research, the Medical University of Graz, the Karl-Franzens-University of Graz, Seibersdorf Laboratories GmbH, BioMed-zet Life Sciences GmbH, the University of Salzburg, Mondi Uncoated Fine & Kraft Papers GmbH – Department Research & Development, and the University of Vienna. The variety of the scientific backgrounds and the techniques offered by the partners allows the Center to describe biological actions of nanoparticles from different perspectives (see standard-method-catalogue on www.EURO-NanoTox.at). The EURO-NanoTox is in collaboration with national and international working parties. EURO-NanoTox is an open network that is accessible to all Austrian groups active in or interested in the field of nanotoxicology. Furthermore, EURO-NanoTox establishes a strong cooperation to key institutions on European level. EURO-NanoTox is involved in all major activities in the field and is a data contributor in the OECD Working Party on manufactured nanomaterials (WPMN) on the international level.

3 Core-Activities of EURO-NanoTox

The Center is active in the following areas:

1. Development and structuring of the field of nanotoxicology in Austria.
2. The Development, establishment and implementation of standardized in vitro and in vivo toxicological methods for nanostructured material
3. The development of national and international research projects on nanotoxicology
4. It provides industry with a tool kit of methods for the in-vitro and in-vivo measurement of the toxicological potential of nanostructured materials as well as carrying out and interpreting these tests
5. The active establishment of international contacts with key players in the area of nanotoxicology



6. The active monitoring of relevant literature and the providing of an information point for interested scientists and industry partners
7. Participation in and organization of comparative studies including ring studies.

The core function of the Center, however, is to develop and implement standardized in vitro and in vivo tests for the determination of the toxicity of nanostructured materials. This is an absolute necessary basis for the systematic investigation of toxicological effects as well as for toxicological mechanisms. Hence, the EURO-NanoTox was conceived as a vehicle that will bring all these aspects together. Through the application of standardized methods in a quality assured environment, expensive failures in product development and/or potential hazards occurring upon product release can be avoided.

The toxicological profile of a given nanostructured material is determined by multiple parameters, including, but are not limited to: size, payload, composition and geometrical structure. Thus, it is essential to develop, in each case, an individual toxicological strategy tailored to each unique nanostructured material. The strategy should reflect current literature-based knowledge and enable an approach that is both cost-effective and well structured (see figure 1).

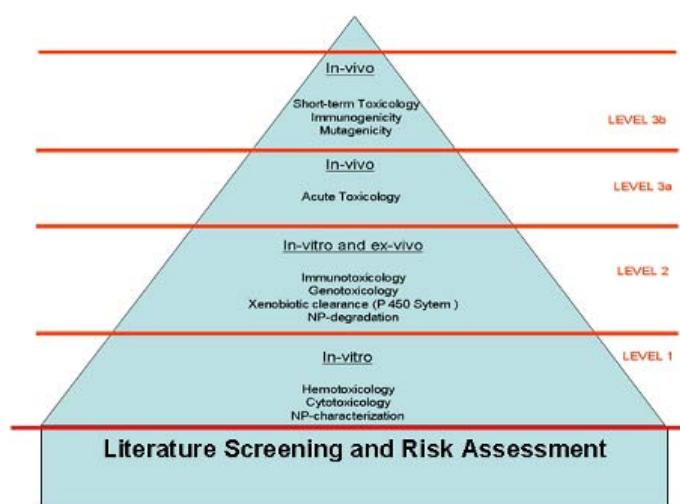


Figure 1: Risk assessment and development of a strategy for the determination of a nanotoxicological profile.

EURO-NanoTox prepares such testing strategies, accompanied by an overview of the relevant published information. The risk assessment and the development of a strategy for the determination of the nanotoxicological profile should constitute the first step in the toxicological testing of each novel nanostructured material.

Additionally, when gaps in the portfolio of available methods become visible, they will be filled by the development of new methods within the context of national or international research projects.

Starting with the formulation of testing strategies for nanostructured materials and with the preparation of a review to evaluate the state of the art literature, nanostructured materials are characterized in different (biological) media according to their

size, size distribution, surface, agglomeration and zeta-potential. These are significant factors influencing the standardization of a method. Standardized protocols addressing nanoparticle-specific interferences by the inclusion of additional controls are used for these assays.

The systematic in-vitro toxicology is based on cytotoxicology and hemotoxicology concerning the effect of the port of entry into the human body (pulmonary, dermal, nasal, buccal, oral, and endothelial) and the effect onto specific organs (liver, kidneys, spleen). Additionally, a 3D liver model can be used for testing metabolic activity, cell viability, cell toxicity, biochemical assessment of ROS generation (oxidative stress), CYP450 activity (xenobiotic metabolism), stress and genotoxic as well as inflammatory responses. Genotoxic effects are identified by the assessment of changes in the structure of chromosomes and DNA. Evaluations of the in-vivo effect of nanoparticles include blood count and clinical chemistry (serum parameters for liver damage, kidney function, inflammation, and immune response), histopathology and immunohistochemistry, all of which address specific questions (proliferation, inflammation, oxidative stress etc.).

An improved understanding of tissue specific toxicology of nanoparticles is critically dependent on the development of procedures that are able to sample the tissue microenvironment in a manner that enables continuous sampling, i.e. without taking biopsies. Open Flow Microperfusion [OFM] enables such an approach to be realized in a highly effective and elegant manner given that it: (i) is a minimal invasive procedure, (ii) allows continuous sampling and (iii) enables the full spectrum of analytes to be harvested from the surrounding milieu, i.e. ranging from small molecules to nanoparticles (micro dialysis in contrast employs a catheter containing a semi-permeable membrane).

The latter features allow a broad spectrum for analysis of all potential nanoparticles and substances (electrolytes, small molecules, peptides or proteins) to be performed. All these expertises are collected in the "Assessment of Toxicological Effects by in-vitro and in-vivo Assays and open flow microperfusion"-folder available on the EURO-NanoTox Homepage (www.EURO-NanoTox.at).

4 Organisation of EURO-NanoTox

The pooling of the scientific expertise of all partners involved and the formation of a link with the structured network of BioNanoNet Forschungsgesellschaft mbH has facilitated the creation of a broad base for a toxicology Center. The embedding of this know-how in international research and development landscape in collaboration with regulatory bodies and authorities will lead to the extension and further development of EURO-NanoTox as an international hub. EURO-NanoTox is also eager to pursue strategic collaborations with other European nanotoxicology centers which will lead to the establishment of a European nanotoxicology network.



The core functions of the Center, however, are: (i) to serve as the Austrian junction point at which industry and science can submit their nanostructured materials for investigation regarding human toxicity and (ii) to develop and implement standardized in-vitro and in-vivo methods for the determination of the toxicity of nanostructured materials (including workplace safety). This is absolutely necessary because without this basis of determining toxicity, no systematic investigation of the toxicological effects will be possible.

Therefore, EURO-NanoTox was conceived as a vehicle by which the coordination of these aspects is possible. Through the application of standardized methods in a quality assured environment, costly failures product development or potential hazards due to product release can be avoided. Besides the applied aspects of nanotechnologies for scientific expertise, EURO-NanoTox builds in the area of workplace safety. Furthermore, the Center elaborates upon the requirements for a European information platform in order to ensure that the workers and decision makers, who are responsible for the safety of the employees, have access to important emerging knowledge in the field of nanotechnology.

Aspects of converging technologies have the capacity to be viewed in a negative way by the public. The development of scientific expertises, provisions for the availability of information and management for public expectation will be important parts for the acceptance of this innovative technology.

5 EURO-NanoTox Expertise-Portfolio

During the first two years of the project, EURO-NanoTox created a methods-catalogue, and in 2010 already revised and expanded this document.

The assessment of toxicological effects induced by conventional drugs, nanoparticles or medical devices includes a series of in vitro and in vivo tests. The EURO-NanoTox partners offer a first assessment of toxicological effects for producers of chemical substances and especially of nanoparticles.

The document is available online:

http://www.euro-nanotox.at/images/stories/folder_euronanotox_webversion.pdf

6 EURO-NanoTox-Letters – ONLINE-Journal

EURO-NanoTox-Letters is a new journal in the biomedical field that fills the gap between material science orientated and medical journals. The main aim of EURO-NanoTox-Letters is to increase the knowledge in the field of nanotoxicology and to help to pave the way from the present case-to-case to a holistic approach. This journal should help to ensure a sustainable development of the entire field of nanotechnology. The journal will publish in vitro, ex vivo and in vivo studies elucidating NMs behavior in physiological environment. It will describe absorption, distribution, metabolism

and elimination of NMs in order to find out to which extent toxicity testing guidelines for drug products can be used for the toxicological assessment of these materials.

The following top-level category structure is proposed for EURO-NanoTox-Letters:

- Interaction of nanoparticles with cells
- Changes of nanoparticles by interaction with physiological fluids
- Absorption, distribution, metabolization and elimination of nanoparticles
- Physico-chemical characterization of nanoparticles
- Bio-persistence of nanoparticles
- Interference with test systems

The journal publishes original articles on all aspects of nanotoxicology as well as on toxicological issues in nanomedicine; reviews- these inform readers of the latest advances in nanotoxicology and short papers- these feature exciting research breakthroughs in the field are available resources.

Neither the authors nor their institutions will be charged for publication processing fees.

The editors are looking forward to receiving high quality papers from experts in the field of nanotoxicology and nanomedicine in order to make this journal a leading publication in the field.

6.1 Aim of EURO-NanoTox-Letters

The aim of *EURO-NanoTox-Letters* is to increase knowledge on interactions of nanoparticles in the physiological context by investigating adsorption, distribution, metabolism and elimination of nanoparticles in order to find out to which extent toxicity testing guidelines of drug products for nanoparticles can be used for the assessment of nanoparticles' toxicity.

6.2 Background of EURO-NanoTox-Letters

Research on the toxic effects of nanoparticles was started by reports that nano-sized combustion products may cause health problems. The potential toxic effects on workers exposed to nanoparticles, which are generated either as by-products during the production or that consist of the final product itself, are another important topic for nanotoxicological research. Increasingly, nanoparticles are also designed for drug-delivery, medical devices and imaging in medicine. Although products, which are used in medical treatment, are subjected to strict guidelines, these guidelines may not apply for nanoparticle-based therapeutics.

7 SUMMARY and OUTLOOK

EURO-NanoTox pursues the main goal to condense and structure all available scientific expertise in Austria and to develop standardised methods for toxicology assessment of nano-structured materials. The catalogue of Austrian nanotoxicology expertise, which is available online (http://www.euro-nanotox.eu/images/stories/folder_euronanotox_webversion.pdf)



has been updated, new methods have been, and continuously will be included.

EURO-NanoTox is the AUSTRIAN hub for nanotoxicology and serves as the port for all scientific driven aspects of nanotoxicology and human health.

In future, EURO-NanoTox will additionally serve as a scientific foundation for regulatory aspects as for example worker safety/workplace safety. To enhance safety for workers EURO-NanoTox actively tested workplaces and assessed the risk and safety aspects at these workplaces. The EURO-NanoTox partners additionally provide valid scientific data and perform validated scientific experiments to address the potential toxic profile of nano-structured materials. Extract of the publications from EURO-NanoTox and its partners:

Fröhlich E. and Roblegg E. (2011) Risk of oral exposure to nanoparticles in consumer products. Toxicology, 2011, in press.

Fröhlich, E; Meindl, C; Pieber, TR. (2010) Important issues in the cytotoxicity screening of nano-sized materials. ENT-Letters 2(2010) pp.1-8.

Roblegg, E.; Falk, A.; Fröhlich, E.; Zimmer, A.; Sinner F. (2011) European Centre for Nanotoxicology: A Proactive Risk-assessment Nanotechnology Initiative. ASM Science Journal, Volume 5(1), pp.43-52.

Roblegg, E; Fröhlich, E; Meindl, C; Teubl, B; Zaversky, M; Zimmer, A (2011) Evaluation of a physiological in vitro system to study the transport of nanomaterials through the buccal mucosa. Nanotoxicology, May 18. [Epub ahead of print]

Fröhlich, E.; Meindl, C.; Roblegg, E; Griesbacher, A; Pieber, TR (2011) Cytotoxicity of nanoparticles is influenced by size, proliferation and embryonal origin of the cells used for testing. Nanotoxicology, May 31. [Epub ahead of print]

Furthermore, EURO-NanoTox is setting up a European-wide network of national hubs for nanotoxicology in collaboration with already existing platforms. This network will help to interchange recent developments and developed methods between different European countries and promote the development of European standards to help ensuring the successful development of nanotechnologies as a key for European growth. EURO-NanoTox actively contributes to European projects dealing with regulatory aspects of nanotechnology (e.g. NANOFORCE) and plans to enlarge its initiative in this field in future projects.



8 Directory

Table 1 Directory of people involved in this project.

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HINAMOX

Health Impact of Engineered Metal and Metal Oxide Nanoparticles: Response, Bioimaging and Distribution at Cellular and Body Level



Contract Agreement: NMP4-SL-2009-228825 - HINAMOX Website: <http://www.hinamox.eu>
Coordinator: Sergio E. Moya, Centro de Investigación Cooperativa en Biomateriales – CIC biomaGUNE

Consortium:

No.	Beneficiary name	Short name	Country
1	Centro de Investigación Cooperativa en Biomateriales	CIC biomaGUNE	Spain
2	Universidad de Vigo	UVIGO	Spain
3	University of Leipzig	ULEI	Germany
4	Centro de Investigación en Química Aplicada	CIQA	Mexico
5	Zhejiang University	ZJU	China
6	PlasmaCHEM	PLASMACHEM	Germany
7	National Research Centre for the Working Environment	NRCWE	Denmark
8	Finish Institute of Occupational Health	FIOH	Finland

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1 Summary

Project number: 228825

Project Duration: 1 October 2009 – 30 September 2012

Project Funding: EUR 2,297,337.26

HINAMOX is a 7th Framework project dedicated to the study of metal and metal oxide nanoparticles (NPs) such as TiO₂, ZnO, Al₂O₃, CeO₂, as potentially hazardous to biological organisms

HINAMOX will accomplish an advanced work in the field of in vivo and in vitro studies of NPs. The importance of this work lays on the fact that will set the basis for proper dose relation quantifications and distribution studies both at cellular and body level. This represents indeed a milestone for the future definition of nanosafety regulations, standards definitions and for the assessment of the health effects of NPs on humans.

Research in HINAMOX focus on the correlation of materials properties to their toxicological endpoints.

HINAMOX will provide knowledge on the biodistribution and biological fate of metal oxide NPs. To achieve this goal procedures for NP radiolabelling are being developed to allow for the application of PET and SPECT techniques.

In parallel to the in vivo studies the work in HINAMOX will be paramount in establishing quantitative data and practical procedures to determine the concentration and distribution of NPs at cellular level applying Ion Beam Microscopy, Confocal Raman Microscopy and Transmission Electron Microscopy techniques.

The consortium generates new knowledge on the cytological and pathological response to NPs, targeting innovative work of the inflammatory response of the alveoli as a possible vehicle for the introduction of NPs in the body. Detailed analysis of NP leaching and dissolution for the assessment of NP biodegradability and residence times in tissue and lung-lining fluids will be developed.

All together, these studies will make an important contribution to a deeper understanding of NP toxicology and will be fundamental on defining regulations where dose effect relation are required both at the cellular and body level.

2 Project Background

Among the immense variety of industrial and medically important NP), the HINAMOX proposal will focus on metal and metal oxide NPs as potentially dangerous to biological organisms. Metal oxide and metal NPs are widely used in various industrial processes and common products.

Metal oxide and metal NPs may be dangerous for humans because of two reasons: their special catalytic activity coming from the properties of their nanointerface may interfere with numerous intracellular biochemical processes and the decomposition of NPs and subsequent ion leakage could heavily interfere with the intracellular free metal ion homeostasis, which is essential for cell metabolism.

A very specific problem dealing with metal oxide NPs is the difficult of localizing and quantifying them in cells and organs. Obtaining dose effect relationships for these NPs is not simple, because of the unknown amount of material present in affected cells.

Previous research and hypothesis suggest that the particle size, shape, chemical composition and the chemistry of the capping agent determine the catalytic properties and surface activity of NPs as well as the materials where the NPs are incorporated. These properties are important for the applications of the NPs and must be studied in the context of their effects on human health. The substantiated evaluation of the health risk, associated with the exposure to metal oxide and metal NPs, requires a concerted action. Our approach is a close collaboration in a highly interdisciplinary consortium with expertise in synthetic chemistry, production technology, particle physics and characterization, biochemistry, toxicology, immunology and occupational hygiene.

3 What is HINAMOX

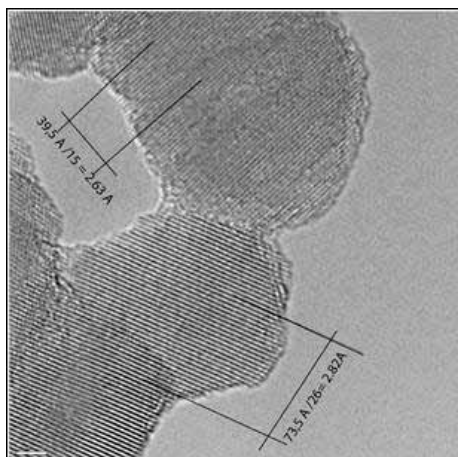
3.1 Project description

The integrated study of NP health effects in our project involves the following steps:

- 1) **Characterization of commercially available NPs, and the fabrication and characterization of NPs with specific properties and with either fluorescence or radioactive labelling.**

The company PlasmaChem, which is a member of our consortium, provides us with metal oxide NPs. CeO₂ and ZnO NPs have also been purchased from Evonik, Germany. At least one example of each of the metal oxide NPs with the most relevant applications or potential, according to the Nanomaterial Roadmap 2015 of the 6th Framework programme, is being considered for study. The consortium is also working in the design of NPs endowed with either fluorescence or radio labels. An important aspect of this project is indeed the fabrication of NPs with proper labelling to trace the fate of the NPs both in vitro and in vivo. This task implies the development of proper routes of labelling both NP core and capping agent. Also, it is important to learn to what extent the labelling affects properties of the NPs such as aggregation, size, charge, morphology and crystallinity, which can have a direct impact on the interaction of the NPs with cells and organs and in their toxicity.

Characterization of the structural properties of the commercial NPs and those fabricated by the consortium is a key aspect in this project. Surface and structural properties of NPs will be related to their toxicological effect and a strong effort will be made in understanding and relating to the characteristics of the materials the differences in toxicity, uptake or distribution among NPs of the same materials but from different sources.



High Resolution Electron Microscopy image of ZnO particles

2) Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET) for the analysis of the uptake, distribution and release of NPs *in vivo*.

At the organism level, we propose the novel use of methodologies such as Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET). Both techniques allow for the three dimensional mapping or imaging of organs and functional processes in the body through the detection of radioactive species. SPECT and PET will be used to directly follow the uptake, distribution and release of the particles in animal models by different ingestion ways. To perform this task, special NPs have to be designed with tracers of gamma radiation (SPECT) or positron emitters (PET). This complicated task requires the fabrication and stabilization of these particles under conditions of hot chemistry, taking into account the limited decay time. Whole-body analysis using direct imaging techniques of potentially toxic NP distribution and kinetics has been accomplished.

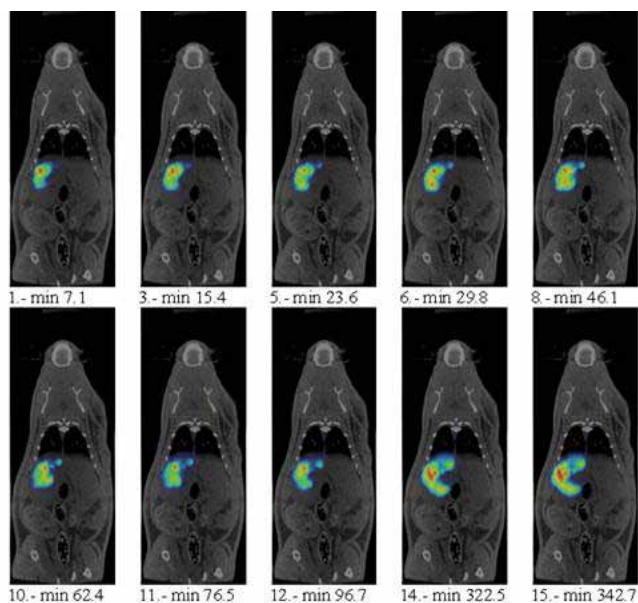
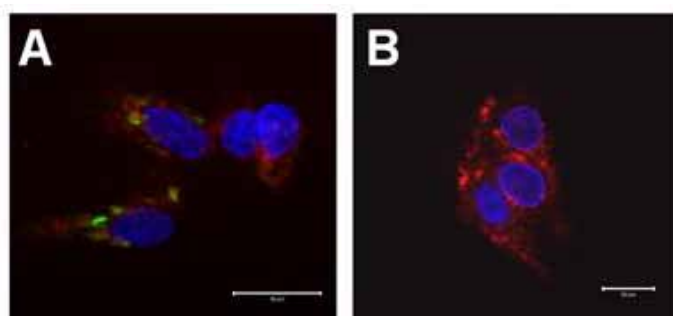


Image sequence of PET scanning for g-Al₂1803 NPs by IV route

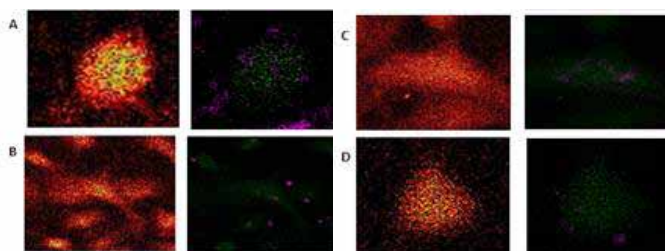
3) Quantification and distribution studies at cellular level, by Ion Beam Microscopy (IBM), Electron Microscopy (EM) and Confocal Laser Scanning (CLSM).

There is a profound lack of knowledge concerning the amount of NPs present in a cell for an applied NP dose. In other words, a quantitative relation between dose and uptake of NPs at both organ and cellular level is missing. The particle uptake depends on the activity of the cells, as well as the size, shape and physico-chemical properties of the nanomaterial. Therefore, the absence of dose-effect relationships represents a serious drawback for proper risk evaluation of special intracellular developed effects. At cellular level, the localization and quantification of metal and metal oxide NPs will be performed by Ion Beam Microscopy (IBM), Electron Microscopy (EM) and Confocal Laser Scanning Microscopy (CLSM or LSCM).



A) Confocal laser scanning microscopy image of HepG2 cells after being incubated with CeO₂ NPs for 24hrs. (B) HepG2 cells without exposure to NPs as control.

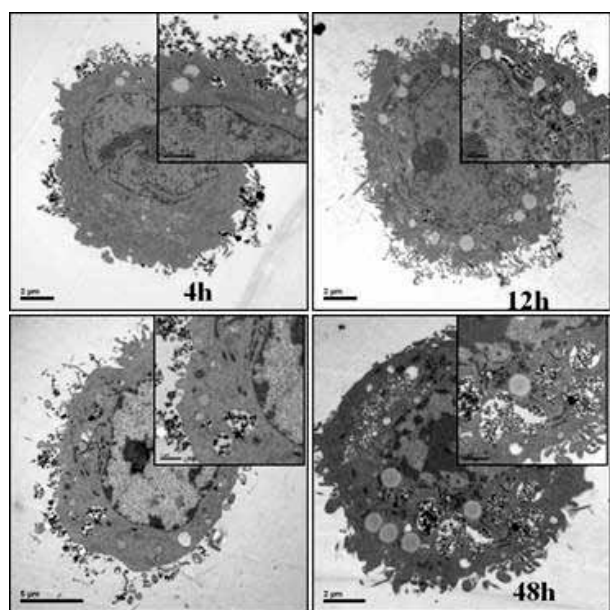
IBM is a unique and very powerful technique capable of localizing and quantifying these particles as well as performing elemental map distributions inside cells. It does not require particle labelling and relatively thick specimens can be investigated. The IBM technique is based on the targeting of a sample with high energetic ions (with approximately 2-3 MeV energy), which penetrate the targeted sample interacting with the electrons and nuclei present. This leads to an excitation of electron shells, which rearrange themselves under emission of electromagnetic radiation (X-rays and light).



PIXE elemental mapping of A549 cells treated during 72 h with various NPs: A - ZnO; B - CeO₂; C - TiO_x; D - FeO_x. Left images demonstrate the P distribution (yellow is maximum, black is minimum) and right images - overlapping of two elements in the cells: S (green) and NPs metal (Zn, Ce, Ti, and Fe) (purple).



Since the interaction processes depend on the encountered atoms, on the structure of the sample and on the sort and energy of the ions, the detection of secondary products of the interactions allows the determination of the elemental content and distribution in a sample. IBM has been used successfully to study the permeation of titanium dioxide NPs after topical application (Menzel et al., 2004). Since the technique is time consuming, EM and CLSM serve as supporting techniques. CLSM requires sophisticated labelling of the NPs ensuring strong fluorescence, but trying to avoid the use of conjugated labels, which would interfere with the cellular uptake and response.



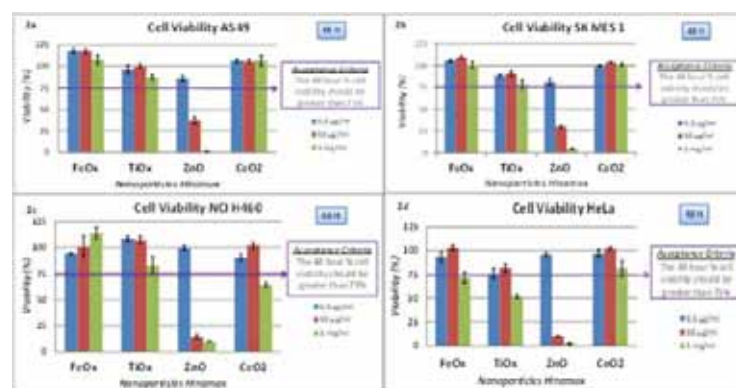
TEM images of H460 cells incubated with TiO₂ NPs taken after 4, 12, 24, and 48 h incubation

4) Understanding the interaction of NPs with cellular and extra-cellular components.

For assessment of the fate and interaction of NPs in the organism, investigations of NP-protein interaction and stability of NPs in different biological compartments will be carried out by biochemical methods focusing on measuring complementary agents and by means of binding studies with Fluorescence Correlation Spectroscopy (FCS). In FCS fluctuations in the fluorescence intensity from a confocal volume in a sample, which are caused by diffusional and rotational processes are measured and correlated temporally (Haustein et al., 2003). These results can be related to aggregation, association, polymer dynamics and, most importantly, in the proposed research, binding reactions. The technique has been successfully applied to measure binding constants and association of biomolecules. It has the advantage of only requiring very few fluorescent molecules in the confocal volume, and this can be applied in combination with CLSM to measure binding and association within cell compartments. The stability and corrosion of NPs is being investigated by biodissolution tests in an environmentally controlled, stirred batch reactor.

5) Determination of physiological effects of NPs *in vitro*.

It is a well-accepted hypothesis that reactive oxygen species may play an important role in particle-induced toxicity (Limbach et al., 2007; Xia et al. 2006; Sayes et al., 2006). Great differences in toxicity have been found between different oxide NPs, and *in vitro* studies suggest that the levels of toxicity may correlate to the reactive oxygen species (ROS) formation capacity of the NPs (Limbach et al., 2007; Jeng & Swanson, 2006, W. Lin et al. 2008), and also the release of ions. Comparatively more work has been completed on quantum dots, fullerenes and carbon nanotubes (CNTs) (Cui et al, 2004; Monteiro-Riviere et al, 2005; Lam et al, 2004; Maynard et al, 2004; Derfus et al, 2004; Kirchner et al, 2005; Hoshino et al, 2004; Schrand et al., 2007). Immune competent cells are specialized in the recognition of external factors in the skin, mucosa, blood, digestive and lung tissue, etc. They are also responsible for the subsequent production of signal molecules (cytokines), which activate other mechanisms of the immune defence system such as antibody production, macrophage activation, lymphocyte activation and proliferation. In addition, humoral factors such as complement, or acute phase proteins, participate actively in the inflammatory process and in the destruction of foreign elements. The subsequent changes in cell physiology induced by the presence of the NPs will have a tremendous impact on the induction and course of the immune reactions as a whole. For example, NPs can modify endogenous protein, inducing allergy processes, or induce macrophage activation in lungs, leading to a chronic inflammation with fibrosis (Lam et al., Crit Rev Toxicol. 2006). ATII cells are by far the most frequent cell in the alveolar lining. They are, among other functions, responsible for secretion and recycling of lung surfactant and a number of defence proteins.



Effect of the different NPs in cell viability. Graphs showing the effect on cell viability of the different Nps, by Quick cell colorimetric assay, at 48 h, in different cell types: A549 (2a), SK MES1 (2b) NCI H460 (2c), and HeLa (2d). Nanoparticles were considered non-toxic if at 48h cell viability reached at least 75%. All the assays were performed twice for each cell line, and in triplicate for each nanoparticle concentration

6) Risk of exposure and toxicological effects of metal and metal oxide NPs.

The knowledge generated by the different workpackages of HINAMOX will be used to make an assessment of the risks associated with these kinds of NPs following European standards suggested by the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2007). The data gathered in this project utilizing *in vitro* and *in vivo* models will be used to support



integrated risk assessment. Integrated Risk Assessment Framework (IRA), as identified in the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH EC/1907/2006), will be utilized in the sense that exposure scenarios from experimental setting will be designed, and, in toxicity studies, predicted no-effect levels (PNEL) on predicted minimum-effective levels (PMELs) will be determined and compared with the predicted exposure levels e.g. in the work environment.

3.2 Partners

HINAMOX project fully adheres to the European Recommendation of 07/02/2008 on a code of Conduct for Responsible Nanosciences and Nanotechnology Research. Furthermore, HINAMOX strongly identifies with the objectives of sustainability expressed by the Code of conduct that states that research activities in Nanotechnology and Nanosciences (N&N) must be safe, ethical and contribute to sustainable development. N&N research activities should not harm or create a biological, physical or moral threat to people, animals, plants or the environment at present or in the future. Therefore, HINAMOX will strive for the generation of a culture of responsibility and precaution to protect not only the researchers taking part in the project but also professionals, consumers, citizens and the environment that may get involved in the activities to be developed in the course of the research activities of HINAMOX.

The HINAMOX consortium is formed by eight different academic institutions and companies located in Europe, Asia and Latin America. The consortium blends a wide range of expertise ranging from the synthetic skills and the physical characterization to the bioimaging, including molecular biology, immunology and microscopy.

CIC biomaGUNE is a non-profit research organization created to promote scientific research and technological innovation at the highest levels in Spain, in order to help strengthen and further develop the new business sector based on biosciences in the country. CIC biomaGUNE blends a unique mixture of expertise. The institute combines synthetic chemistry, material science, physical and biophysical characterization, with *in vivo* and *in vitro* imaging. CIC biomaGUNE is endowed with a cyclotron, radiochemistry labs for hot chemistry and animal PET, SPECT and MRI cameras. State of the art techniques for material characterization are presented in the institute such as TEM, SEM, NMR, Raman, FITR, light scattering, etc. Among the different research lines in the institute there is a compromise to develop nanomedicine tools and to become a leading institution in *in vivo* studies concerning nanotechnology. CIC biomaGUNE is the coordinating partner in the project, its role is the characterization of commercial NPs, the synthesis and characterization of radio and fluorescently labelled NPs and "*in vivo*" studies with animal models.

The **University of Vigo** (UVIGO) is a recent University (1990) located in Galicia (Northwest Spain), covering many educational disciplines including Biology, Chemistry, Engineering, Law, Economics, etc. The Immunology Area was set up on 1996 in the Department of Biochemistry, Genetic and Immunology, and brought in researchers with experience in Medical Immunology and Biology. Since then, UVIGO has developed and trained scientists with experience in basic and applied Immunology, development of monoclonal antibodies and immune responses to vaccines. The role of the University of Vigo in HINAMOX is to study the cytotoxicity of NPs in different human cell lines (lymphoid, lung

origin), interaction of nanomaterials with complement and serum proteins, effect of sterility and immunogenicity of nanomaterials *in vitro* and *in vivo*.

The University of Leipzig is one of the largest and oldest universities in Germany, covering all educational disciplines. The proposed research work will be conducted in close collaboration with two different faculties (Physics and Medicine), and in three different departments or Institutes.

- 1) Institute of Medical Physics and Biophysics: The institute has its focus on membrane and cell biophysics for medical applications.
- 2) Institute for Experimental Physics II, Division of Nuclear Solid State Physics: The focus of the research of the accelerator laboratory, using the high energy ion nanoprobe LIPSION, is on spatially resolved quantitative trace element analysis in neuroscience, cell biology and in elemental analysis of natural and artificial micro- and nano-structures and ion beam modification of materials with sub-micrometer resolution.
- 3) Medical Hospital, Department of Pneumology: The department is responsible for the in-patient treatment of severe pulmonary disorders such as asthma, pneumonia or lung carcinoma. In parallel, clinical research is carried out to improve the treatment of pneumological disorders.

The role of the **University of Leipzig** in the project is the quantification of NPs in cells by means of IBM techniques and FCS, and studies of the lung function in presence of NPs, uptake and immunological response of lung cell lines under different breathing regimes.

PlasmaChem GmbH is an SME with research facilities dedicated to the development, production and sales of medical devices, analytical equipment and nanomaterials and their formulations. PlasmaChem GmbH was founded at 1993 in Mainz. In 2005 the company moved to Berlin. The main area of the company concerns nano-materials, detonation-, vacuum-, plasma- and ultra-thin film technologies and their biomedical and technical applications. The main technology focus of PlasmaChem concerns the development of processes, induced by low temperature plasma on different surfaces, in atomically flat, inorganic solids and in liquid interfaces. The important business line of PlasmaChem GmbH is production and sale of new industrial products - Nanopowders (NanoDiamonds, NanoCeramics, NanoMetals and composite Nanoparticles - Nano-capsules). In 2005 PlasmaChem launched the world's first General Catalogue on Nanomaterials and related products. PlasmaChem performs chemical and low temperature plasma modification of nanopowders, with the purpose of functionalizing the nano-particle's surface with assistance from a new plasma chemical method developed by PlasmaChem for ultra-dispersed materials. Along with new nano-particles, PlasmaChem develops new, ready-to-use industrial nano-products like nano-abrasives, additives to engine oils and composite nano-suspensions for electroplating and electroless-plating of metals. The role of PlasmaChem in HINAMOX is the design, fabrication and scale up of NPs.

National Research Centre for the Working Environment (NRCWE) is a Danish governmental research institute in the field of occupational health and safety under the Ministry of Employment. NRCWE's goal is to generate and disseminate knowledge contributing to a safe, healthy and developing work environment in accordance with the technical and social development of the Danish society. NRCWE contributes to securing the coordination of



Danish work environment research and monitors national and international work environment development and research. The knowledge is disseminated via NRCWE's Working Environment Information Centre. Health risks from occupational exposure to NPs is one of NRCWE's seven strategic research areas. The role of NRCWE in HINAMOX is the study of biodurability of NPs, genotoxicity, exposure of NPs and risk assessment.

Centro de Investigaciones Químicas Aplicadas (CIQA) is one of 27 Mexican public research institutions covering the major fields of scientific and technological knowledge funded by the Consejo Nacional de Ciencia y Tecnología (CONACYT). CIQA's focus is on research and development in polymers and advanced materials and has a full time faculty staff of about 45, 70 technicians and 120 PhD and MSc students. CIQA has broad expertise in new materials synthesis and characterization including nanoscale structures and metamaterials, and in polymer synthesis, processing and engineering. State-of-the-art techniques for material characterization at CIQA include TEM, SEM, SQUID magnetometry, magnetoelectric capability and ISO-9000 facilities.

The role of CIQA in the project is the support in the design routes for NPs to be suitable of being labelled and the application of High Resolution TEM for characterization.

Zhejiang University is located in Hangzhou, Zhejiang Province, China. It was initially founded in 1897, and is the third oldest University in China. It has 5 campuses and occupies a total area of 518 hectares. It is a key comprehensive university whose fields of study cover eleven branches of learning, namely philosophy, literature, history, education, science, economics, law, management, engineering, agriculture and medicine. The university now has 112 specialties for undergraduate studies, and it is entitled to confer Masters' degrees in 317 programs and Doctoral degrees in 283 programs. Under its administration, there are 14 Key National Laboratories, 2 National Engineering Research Centres and 3 National Engineering Technology Centres.

The role of Zhejiang University in the project is the study of the cellular uptake and distribution of NPs by means of TEM and CLSM.

The **Finish Institute of Occupational Health (FIOH)** is a governmental research institute whose main emphasis is on a safe work environment and workers' health and well-being. FIOH operates under the Ministry of Social Affairs and Health, is directed by a Board representing employers, employees and the government, and has about 800 employees. FIOH is responsible for most research and development in the field of occupational safety and health in Finland, and its main general focus areas are work environment development, promotion of workers' health, healthy work organizations, safe working conditions, and dissemination and implementation of knowledge in these areas. The role of FIOH is the integration of the knowledge generated in HINAMOX in a final risk assessment.

3.3 Dissemination activities

HINAMOX has participated in several international events related to its research topic, mainly workshops and conferences. Concerning publications, a list of journals, magazines and newspapers pertaining to the nanotoxicology subject has been identified and over a dozen publications have already been published. Both events and publications are an effective way to widely disseminate the project results among the appropriate communities.

In particular, the following conferences are of special interest:

- At **NanoBio&Med 2011**, several of the HINAMOX partners had the opportunity of presenting their work: University of Vigo, University of Leipzig and CICbiomaGUNE. HINAMOX organized seminar targeted to industrial stakeholders who are now disseminating on scientific and legal issues related to the toxicity of nanomaterials.

- The **Safety Issues of Nanomaterials along their Life Cycle** conference in Barcelona was co-organised with other two consortia, NANOPOLYTOX and NEPHH.

HINAMOX participates actively in the **EU NanoSafety Cluster** and the HINAMOX project contributes to the joint newsletter by sharing its findings. All events organised by HINAMOX are communicated to EU NanoSafety Cluster who kindly help with their dissemination. HINAMOX contributes this way to create an environment to foster discussion, exchange ideas and common activities together with other groups and European projects in the field of nanotoxicology.

In addition, work of HINAMOX has already been published in scientific journals.

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4 Directory

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InLiveTox

Intestinal, Liver and Endothelial Nanoparticle Toxicity - development and evaluation of a novel tool for high-throughput data generation



Contract Agreement: CP-FP 228625-2 Website: <http://www.inlivetox.eu>
 Coordinator: Martha Liley, CSEM SA, Jaquet-Droz 1, 2002 Neuchâtel, Switzerland

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5	Helmholtz Zentrum München - Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH)	HMGU	Germany
6	Kirkstall Ltd	Kirkstall	United Kingdom
7	University of Rochester	URMC	USA
8	ALMA Consulting Group SAS	ALMA	France

* Edinburgh Napier University has now been replaced by Heriot-Watt University, HWU, United Kingdom

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1 Summary

InLiveTox consists of an interdisciplinary consortium at the European level, together with a key American research group brought together to develop an improved *in vitro* model for the study of nanoparticle (NP) uptake, transport and cellular interaction, thus advancing our understanding of NP toxicity.

Rather than repeat what has, or is being done in the field of aerosol NP and lung toxicology, InLiveTox is focused on the impact of NP exposure via ingestion, in the healthy and diseased gastrointestinal (GI) tract, vascular endothelium and liver. The key questions in this study are: (i) How do these tissues individually respond to NPs? (ii) How do the interactions between the different tissues modulate their responses? (iii)

How does inflammation affect the toxicity of NPs and their ability to cross the intestinal barrier? (iv) Which physico-chemical characteristics of NPs influence their uptake by intestinal epithelial cells and their subsequent interactions with endothelial and liver cells?

The objective of InLiveTox is to develop a novel modular microfluidics-based *in vitro* test system modelling the response of cells and tissues to the ingestion of NPs. Cell culture models of target tissues such as the GI tract, the liver and the endothelium will be connected via a microfluidics system so that knock-on and cross talk effects between organs and tissues can be studied. The InLiveTox system will be validated by an *in vivo* study of NP toxicity in rats carried out in parallel.



A major innovative aspect of the InLiveTox project is the implementation of biological tissue models in a microfabricated compartmental cell culture system that allows multiple cell types to be addressed and investigated in combination. This system will be much easier, more convenient and ethically less questionable than animal testing, as well as more relevant than the *in vitro* single cell /co-culture models currently used. For this study, applications of the model will focus on NP toxicology, but the system could also be widely used in various applications of toxicology and pharmacology.

2 Introduction

Context

Nanotechnology is defined as the ability to create and use materials, devices and systems with unique properties at the scale of approximately 1 to 100 nanometres. The use of nanotechnology in consumer and industrial sectors is expected to increase significantly in the future. Nanotechnology offers society the promise of major benefits, but also raises questions of potential adverse effects. The challenge for health (and environmental) protection is to ensure that as nanomaterials¹ are developed and used, any unintended consequences of exposure to humans are prevented or minimised.

In Europe and in the USA, governments, non-governmental organisations, and others have expressed concern that the number of consumer products incorporating nanomaterials is increasing dramatically, that, in many cases, the safety of these materials has not been demonstrated and that there are still a large number of unanswered questions. For example, little is known about the relationship between the physicochemical characteristics of nanoparticles (NPs) and their ability to cross cell-barriers and to enter the general circulation, their fate within the body (toxicokinetics), their subsequent toxic impact, or the ability of our bodies to defend against such toxic impact.

In order to understand such behaviour and responses, and to manage the resulting risks, it is essential to investigate the hazard (toxicology) of the large number of engineered NPs in different formulations and at different points in their life cycle (from production to disposal), in relation to different routes of exposure and different target organs and tissues. The number of experiments required to address all of these issues is enormous and so it is essential to develop rapid and reliable non-animal models to assess NP hazards.

The InLiveTox project has formed an interdisciplinary consortium at the European level, together with an American key research group to develop an improved *in vitro* model for

NP uptake and the impact of the NP on different cell types, thereby advancing our understanding of NP toxicity.

Rather than repeat what has been done in the field of aerosol NPs and lung toxicology, InLiveTox focuses on the impact of NP exposure via ingestion, in the healthy and diseased (susceptible) gastrointestinal tract, and the subsequent impact on the endothelium and liver parenchymal cells (hepatocytes). Exposure via ingestion is particularly relevant due to the inclusion of NPs in food, food packaging and in oral medicines. The key questions pertaining to this research are: (i) How do these tissues individually respond to NP? (ii) How do the interactions between the different organs modulate their individual responses? (iii) How does inflammation affect the toxicity of NP and their ability cross the intestinal barrier? (iv) Which physico-chemical characteristics of NP influence their uptake by intestinal epithelial cells and their subsequent interactions with the vascular endothelium and liver cells?

Concepts

The origin of the InLiveTox project is the idea of developing a novel modular microfluidics-based *in vitro* test system modelling the response of cells and tissues to the ingestion of NPs. Models of target tissues such as the gastrointestinal tract, the liver and the endothelium will be connected to each other via a microfluidics system, so that knock-on and cross-talk effects between organs and tissues can be closely monitored.

The innovative aspect of InLiveTox project pertains to the implementation of biological tissue models in a microfabricated compartmental cell culture system which allows multiple cell types to be addressed and interrogated in a single device, the InLiveTox system. This system will be much more convenient and ethically less questionable than animal testing, as well as more relevant than the single /co-culture cell *in vitro* models currently used. For this study, the model will focus on NP toxicology, but the InLiveTox system can also be more widely used in various applications of toxicology and pharmacology.

Currently, the study of the interaction between organs and tissues during NP exposure via ingestion is complex and laborious *in vivo*, and has not been attempted *in vitro* except by InLiveTox partner groups. *In vitro* test models for nano- or any other type of toxicology, are either based on one cell type, crude mixes of different cell types, or transfer of conditioned medium between different cell types.

The InLiveTox system will be based on the technologies and tools developed by the different project partners to implement model biological barriers and tissues in a microfluidics system. Together, these bring the *in vitro* system much closer to *in vivo* reality and will provide the means to study NP effects in a healthy or diseased model of ingestion.

3 Objectives

The objectives of the project are

¹ The term nanomaterials refers to engineered nanomaterials and particles.



- to develop and validate a novel model for assaying ingested NP toxicity, the InLiveTox system
- to gain new insights into NP toxicity.

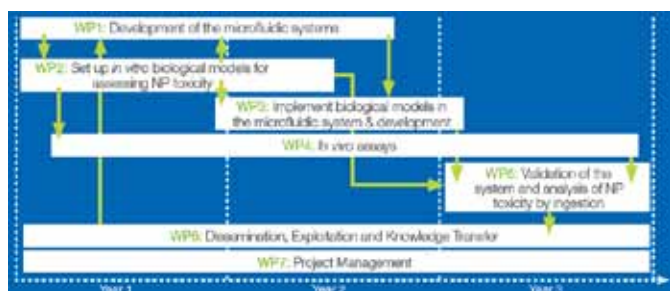
These objectives will be achieved by bringing together microfluidics technologies with cell culture models of human tissues to produce an *in vitro* test system that is more physiologically relevant. The microfluidics system will be flexible and modular so that the complexity of the system can be increased stepwise to include additional cell types, more complex 3D models of tissues, and more sophisticated tests of cellular responses to the presence of nanoparticles. Thus, while the main focus of the project concentrates on cell culture models of healthy tissues, there will also be work on a more complex model of the 'susceptible' or inflamed intestinal epithelium.

The InLiveTox system will be validated using *in vivo* assays of biokinetics and toxic response using a rat model. In contrast, the cell culture models in the InLiveTox system use established human cell lines. The use of human cell lines ensures more reproducible results and a more stable culture system. Great care is being taken to obtain well-characterised and reproducible NP preparations for both *in vivo* and *in vitro* experiments, so that relevant and useful comparisons can be made between them.

The consortium has chosen to validate and demonstrate the InLiveTox system by studying a relevant but largely neglected route of entry of NPs into the body: ingestion. The cell lines to be cultured in the InLiveTox system have been chosen as models for organs and tissues of particular relevance for ingestion: the intestinal epithelium, the vascular endothelium and the liver. Similarly, validation assays will focus on NP toxicity by gavage. In this way, new insights will be generated into NP toxicity on ingestion, based on both *in vivo* and *in vitro* data.

4 Scientific/technical methodology and work plan

The organisation of the project into different work packages is shown below.



PERT diagram showing the structure of the project in work packages and the flow of results and information between the different work packages

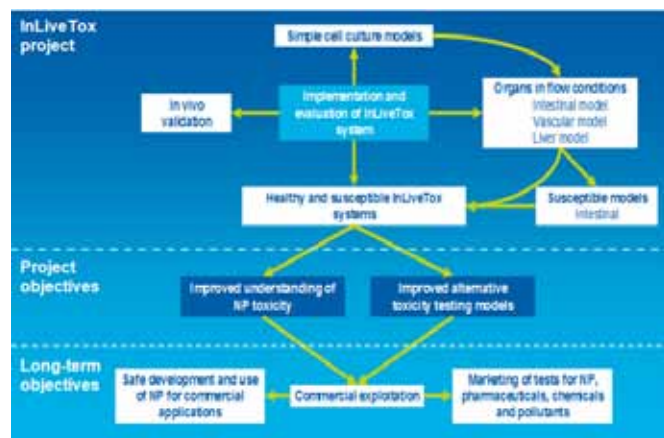
In a first phase of the project, in work package 1, a microfluidics device will be designed and fabricated for the simultaneous culture of different cell lines representing the intestinal epithelium, the vascular endothelium and the liver. In a second phase, feedback from the other work packages will be used to produce an improved microfluidics device. In parallel, development work will be carried out on the cell lines, the NPs and the viability and toxicity assays in work package 2. The chosen cell lines will be optimised for co-culture of all cell lines together under flow conditions. The NPs will be characterised in detail. Assays for cell viability and cytotoxic response will be tested and protocols and endpoints will be defined.

In work package 3 the different cell lines from WP2 and the microfluidics device from WP1 will be brought together. Simultaneous co-culture of all the cell lines in the microfluidics device will be established and the whole system will be tested.

Work package 4, *in vivo* testing of NP fate and of the toxic effects of the NPs in rats, will run for almost the entire duration of the project. The NP preparations optimised in WP2 will be tested. The rats will be exposed by gavage (ingestion) and also by injection.

In work package 5 the InLiveTox system will be used to characterise the fate of the NPs and the toxicological response they induce *in vitro*. Finally, the *in vivo* data will be compared with results obtained from the *in vitro* system.

Work package 6, dissemination, exploitation and knowledge transfer, as well as work package 7, project management, will run for the whole duration of the project.



The InLiveTox project, project objectives and long-term objectives

As shown in the figure above, the long-term objectives of the project are:

- to ensure the safe development and use of NPs for commercial applications



- to commercialise a test system to screen NPs for their toxicity.

These two objectives are linked and related to the foreseen impacts of the project. The commercialisation of the InLiveTox system will make it available to the whole toxicology community.

5 Major R&D deliverables foreseen in the project

1. A microfluidics "InLiveTox system" to test the potential consequences of NP uptake via ingestion.
2. A protocol manual to construct, maintain and use the InLiveTox system to assess the toxicity of NP
3. Uptake and toxicity data pertaining to the ingestion of TiO₂ and silver nanoparticles generated by:
 - a. Individual cell types *in vitro*
 - b. Multiple interacting cell types cultured within the InLiveTox system
 - c. *In vivo* rodent models
4. A statistical comparison between the individual cell types, the *in vivo* model and the InLiveTox system in order to assess the relevance and appropriateness of the new microfluidics system as an alternative to animal testing and as an improvement of mono-culture systems.

6 Progress

In the 1st reporting period, the project has produced a modular fluidics system for the connected culture of 3 different *in vitro* tissue models (the InLiveTox system). An essential part of this system is the newly-developed ILT2 bioreactor. A protocol manual for the setting-up and use of the InLiveTox system is now being written.

Much progress has also been made in the cell culture models to be studied using the InLiveTox system. Common culture conditions have been defined that allow all 3 tissue models to be maintained under flow conditions and in a common culture medium. In addition, protocols for the assay of the viability and functionality of the tissue models within the fluidics system have been established. First results are now coming in on a 2 tissue model (vascular endothelium and liver) and the maintenance of

a model intestinal epithelium within the ILT2 bioreactor has been demonstrated.

Novel data about NP ingestion has also been obtained in *in vivo* biokinetics studies on the effects of exposure to gold NPs of different sizes by both ingestion and injection. These studies in rats have been completed by investigations of the hepatobiliary excretion of gold NPs. Studies of gene expression on NP ingestion and injection in selected tissues and for different NPs have revealed strong patterns of changes in gene expression.

Characterisation of the three tissue model (intestinal epithelium, vascular endothelium and liver) in the InLiveTox system is now underway. This will be followed by first studies of the effects of NPs on the model.

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INSTANT

Innovative Sensor for the fast Analysis of Nanoparticles in Selected Target Products

Contract Agreement: NMP4-SE-2012-280550

Website:

Coordinator: Günther gauglitz, Institute of Physical and Theoretical Chemistry, Tübingen, Germany

No.	Beneficiary name	Short name	Country
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4	University of Vienna	UNIVIE	Austria
5	University of Cordoba, Department of Analytical Chemistry	UCO	Spain
6	Sitex45	SITEX	Romania
7	Sociedad de Investigación en Nanoestructuras S.L	SIANTEC	Spain
8	BAM Federal Institute for Materials Research and Testing	BAM	Germany
9	Corpus Datamining	CD	Sweden
10	Nanordic	NANO	Finland

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1 Summary

Project Duration: 1 March 2012 – 31 August 2015

Project Funding: 3,772 Mio. EUR

INSTANT will face the challenge of the detection, identification and quantification of engineered nanoparticles (ENPs) in complex matrices such as cosmetic products and engineered food and drinks. Therefore, new detection methods and technologies are mandatory. This is completely in line with the Call FP7-NMP.2011.1.3-1 which deals especially with innovative, practically implementable and cost effective measurement approaches for ENPs in complex matrices. Recently emerging ENPs include Ag, SiO₂, TiO₂, ZnO, and organic NPs. The "Opinion of the Scientific Committee on the Potential Risks Arising from Nanoscience and Nanotechnologies on Food and Feed Safety" released by the European Food Safety Authority (EFSA) (2009) also highlights the urgent need for such a tool. Accordingly, the interdisciplinary project INSTANT will develop an innovative and integrated technology for monitoring the exposure of consumers to ENPs

using a label free opto-electrochemical sensor array in combination with novel recognition elements.

The SME driven INSTANT will develop an innovative, cost effective, and easy to use analytical tool to extract, detect and identify ENPs typically used in cosmetic products (e.g. sunscreen, toothpaste, deodorant, ...) and engineered food (e.g. instant soups, ketchup, ice cream, ...) and drinks (e.g. fruit juice, energy drinks, bottled water, ...). A crucial point of measuring in these complex matrices is the sample preparation and extraction. Therefore, INSTANT will develop and integrate tailored extraction methods. Especially the size distribution of ENPs in the sample and the influence of the matrix on chemical and physical properties of the ENPs have to be taken into account. The INSTANT device will be designed to be used as a cost effective monitoring tool which is suitable for characterization and classification of ENPs for the future implementation of quantitative structure-activity relationship studies.



2 Background

In recent years, nanotechnology has been a hot topic in the scientific community due to the specific properties in the nanoscale and has become an enabling technology for numerous applications. Especially engineered nanoparticles (ENPs) have shown various beneficial properties. In many fields of application, these ENPs have left the scientific laboratories and made their way to consumer products¹. Beside their advantages, ENPs are under discussion in the scientific community due to possible unforeseen hazards and an unknown disposition in living organisms and the environment. Nanoparticles (NPs) have drawn vast public attention due to their application in many consumer products (e.g. cosmetics, food and food packaging, drinks). Following the "Opinion of the Scientific Committee on the Potential Risks Arising from Nanoscience and Nanotechnologies on Food and Feed Safety" released by the European Food Safety Authority (EFSA) in 2009², the European Commission tackles this arising matter of public concern within the current FP7 NMP theme.

One of the key challenges is the detection, identification and quantification of engineered nanoparticles in complex matrices, such as products, food and the environment. However, currently none of the existing techniques allows for a holistic approach which is able to analyze all ENPs' properties in a single step.

3 What is INSTANT

INSTANT will face this challenge by developing a fully integrated tool for the extraction of ENPs from complex matrices and their subsequent detection and identification. The device will be tailored to be used as a cost-effective monitoring tool, allowing for analytics of food and cosmetics close to the point of need (Point-of-Product Testing POPT and Point-of-Food Testing POFT).

Accordingly, the project INSTANT is organized in a workflow using complementary expertise of well-known partners in their fields from all over Europe.

The detection and identification of ENPs in cosmetic products, food and/or drinks require an efficient sample preparation and extraction of ENPs from these complex matrices. Especially the size distribution of ENPs in the sample and the influence of the matrix on chemical and physical properties of the ENPs have to be taken into account. INSTANT will develop a generalized extraction protocol to isolate and pre-concentrate ENPs from food and cosmetic samples. An extraction protocol, as generalized as possible, will be developed for the extraction of ENPs from complex matrices. This generalized protocol allows for an extension in the future to a wider range of samples (e.g. for environmental monitoring).

¹ Tran, Lang; Chaudhry, Qasim, RSC Nanoscience & Nanotechnology, (2010), 14, 120-133

² Scientific Opinion of the Scientific Committee on a request from the European Commission on the Potential Risks Arising from Nanoscience and Nanotechnologies on Food and Feed Safety. The EFSA Journal (2009) 958, 1-39.

After extraction and pre-concentration of ENPs, an innovative, cheap and robust sensor device is used for their detection. This sensor will be developed within INSTANT. For the detection of ENPs, recognition structures with a high affinity to ENPs are mandatory. INSTANT will use two different types of recognition elements (REs) with different selectivity combined on an array. On the one hand, technologies will be applied to for generating REs to distinguish between size, shape and material. On the other hand, REs will be chemically modified in order to generate a material for selective sorption of targeted ENP species.

Also, the sensor will combine two complementary transduction principles, an optical and an electrochemical one. Electrochemical sensing is sensitive to ENPs' speciation including conductivity, surface properties and chemical composition. Optical transduction will provide information on ENP size, size distribution and refractive index. Both transduction principles will be adapted to a sensor array, which allows for simultaneous detection of various ENPs.

By combining two transduction principles with two types of recognition elements on a single array, a huge amount of data will be produced. In order to reduce redundancies, to separate noise from signal and to extract relevant information, strong chemometric techniques for the detection, identification and quantification of these ENPs will be needed.

3.1 Summary of INSTANT's key strengths

- Develop a simple and fully integrated sensing system, together with a suitable number of sensor elements for the detection and identification of ENPs in one device.
- Combine two complementary transduction principles to create a robust sensor system providing high-content information about ENPs.
- Implement innovative recognition elements (REs).
- Improve and modify sampling and separation techniques in regard to the complex matrices.
- Develop advanced chemometrics to extract information from the complex data sets.
- Provide characterized and standardized ENPs for the comparison of their properties as pure material used as product and food additives as well as during storage and processing.

4 Organization of INSTANT

INSTANT merges high ranked European partners with remarkable knowledge in each area of the proposed work. Abundant complementary expertise is provided by an interdisciplinary working group of researchers, whose contribution is essential for a successful outcome of the project. The combined resources mobilized completely fulfill all the requirements of the Programme in terms of facilities, equipment, personnel and resources. On a national level it would not have been possible to gather a consortium of this high quality and complementarity.

SMEs and research institutions are brought together to cover the various tasks by distributed expertise and to carry out



complementary research which will lead to a highly innovative technology. SMEs are involved in all parts of the project that are interesting for future exploitation and will benefit from joint research activities of academia and industry.

Table 1 Workpackages (WP) of NanoImpactNet

WP	Title	Topic
1	Sensor development	In WP 1, the sensor array for the INSTANT device is developed. For this purpose, design and specific assembling of both transduction methods are elaborated.
2	Sample preparation and standard materials	WP 2 has two main objectives. Firstly, WP 2 synthesizes and characterizes reference ENPs. Secondly, WP 2 develops a generalized extraction protocol to isolate and pre-concentrate ENPs from food and cosmetic samples.
3	Recognition elements	WP 3 is the main platform for designing selective layers for the multivariate sensor platforms. The goal is to achieve as high "chemical" selectivity (i.e. directly on the measuring device) as possible.
4	Chemometrics and experimental design	WP 4 deals with data mining for extracting minute signals from the complex and probably noisy measurement data obtained by the sensor system(s).
5	System integration	The aim of WP 5 is to setup a fully integrated analytical tool based on the components provided by WP 1 (Sensor development), WP 2 (Sample preparation and standard materials), and WP 4 (Chemometrics and experimental design).
6	Management	This work package coordinates the overall financial, administrative, legal and contractual management of INSTANT.
7	Dissemination and exploitation of results	Dissemination activities beyond the consortium to the scientific community and towards a wider international audience through specialized events like conferences, fairs and exhibitions.

5 INSTANT Events and Reports

The project INSTANT targets three key issues of ongoing debate about nanomaterials, their safety, monitoring, and risk assessment:

- INSTANT will provide a powerful tool to researchers dealing with NP detection and characterization.
- With help of the INSTANT device, it will be possible to ensure that the consumer will feel safe that the product he or she is buying is either free of nanoparticles (if desired) or contains amounts and speciation of nanoparticles that are considered as safe by the EU.
- With the development of the INSTANT device, the consortium delivers a tailor-made instrument to the legislative organs of the EU. This will be the device of choice to gather enough data about nanoparticles in different products and exposure of the consumer to these nanoparticles to help the decision-making process within the EU.

In addition to these three points, the developed device will enable the participating SMEs to take a leading role in the production and

distribution of the next generation device for the detection of nanoparticles in all fields of applications.

Beside this technological goal several events are planned in order to strengthen European knowledge and cooperation within the field of nanotechnology.

5.1 Events

In order to strengthen European knowledge and cooperation within the field of nanotechnology, INSTANT will join forces with other EC funded projects like SMART-NANO and QNANO. Besides an ongoing exchange of samples and knowledge joint events as workshops and a public midterm seminar are planned.

5.1.1 Workshops

- INSTANT will conduct workshops where recent development in the detection of NPs will be presented to a wider audience. Representatives from EU projects will



be invited as well in order to share knowledge and experiences with other EC funded projects dealing with the preparation of reference materials and the detection of nanoparticles (e.g. SMART-NANO, QNano). This will draw the attention from customers to the INSTANT technology and their possibilities.

5.1.2 Public midterm seminar

The midterm seminar will disseminate the results of the INSTANT project to an enlarged number of scientists in the field of sensor technology, nanotechnology and separation sciences, including representatives from other EU projects.

6 Directory

Table 2 Directory of people involved in this project.

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ITS-NANO

Intelligent testing strategies for engineered nanomaterials

Project Number: 290589 Website: <http://www.its-nano.eu>
 Coordinator: Vicky Stone, Heriot-Watt University, Edinburgh, United Kingdom

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3	Aarhus University	AU	Denmark
4	Fondazione Istituto Italiano di Tecnologia	IIT	Italy
5	Centro Ricerche FIAT SCPA	CRF	Italy
6	Fraunhofer-Gesellschaft zur Foerderung der Angewandten Forshung E.V	IME	Germany
7	Institute of Occupational Medicine	IOM	United Kingdom
8	Det Nationale Forskningscenter Forarbejdsmljo	NRCWE	Denmark
9	JRC – Joint Research Centre – European Commission	JRC	Transnational (EU)
10	European Research Services gmbh	ERS	Germany

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1 Summary

The background, concept and objectives of ITS-NANO are straightforward. The volume of information on hazard characterisation of ENM is increasing fast. In parallel with the scientific development, regulation orientated initiatives are also taking place to identify needs.

The ITS-NANO concept is 1: Gather scientific evidence by communication with leading scientists. 2: Develop an initial assessment (document) of the available knowledge, focussed on an intelligent approach to grouping ENMs based on their properties and on their subsequent biological impacts in order to intelligently design next-generation nanosafety evaluation and risk assessment strategies. 3: Assemble stakeholders for a dialog on how this relates to their aims/needs and how to make a consent-driven strategy . 4: Revise the initial assessment document with the input from the stakeholder sent around for commenting,

presenting the next draft for a smaller group for final commenting.
5: Publish it

The work focuses on the following areas: Human toxicology, ecotoxicology and risk assessment. The project will last fifteen months, and organize two events to discuss the development of the testing strategy with the researchers and the stakeholders dealing with the issues. After these workshops, and after online consultation with the widest community, a roll-out event will be organized to present project results to all key stakeholders, including industry, legislators, and funding agencies, and to raise awareness in the media.



2 Background

The volume of information on hazard characterisation of ENM is increasing fast. In parallel with the scientific development in toxicology and eco-toxicology, regulation orientated initiatives are also taking place at the international as well as European and nation state levels. The main initiatives are the OECD Sponsorship Programme for the testing of Nanomaterials, the ISO classification of nanomaterials, and the REACH guidance on regulatory safety testing for chemicals, which is to be updated for ENM. Together, all these sources of information form the state-of-the-art landscape of the safety testing of ENM. The ITS-NANO concept is to gather scientific evidence and assemble representative stakeholders for the consent on a research strategy for rational grouping of ENM and a risk assessment approach for ENM.

Moreover, the framework for future research aiming at grouping ENM according to (i) their physico-characteristics and (ii) specific endpoints will be based on the nanosafety landscape above and specifically on the knowledge gaps that need to be bridged in order for such rational grouping to be made. However, as expected, there will be a considerable knowledge gap. Therefore it is essential to narrow this gap. Of course, with all the time and funding available, this could be eventually be achieved. However, time is critical and funding is restricted, so a rationale is needed for future research. This rationale will reflect the need for different types of evidence by industry, regulators (e.g. OECD WPMN, REACH) and scientists to prioritise the process for information generation (i.e. (eco)-toxicology tests) accordingly. This is why it is an 'Intelligent Testing Strategy'. Our approach is also 'intelligent' at the testing level because we will identify:

- a battery of in vitro (eco)-toxicology tests with predictive power
- in vivo tests only where it is essential (in respect of the 3R principle).
- A risk assessment strategy is only effective if the key stakeholders (e.g. food industry, nano-material manufacturers, pharma- and health-related industry) approve and adopt it. This will be achieved by integrating, engaging them in the very shaping and development of the strategy.

3 What is ITS-NANO

The ITS-NANO consortium consists of 11 partners from 4 European Countries (Italy, United Kingdom, Denmark and Germany) and the Joint Research Centre of the European Commission; it includes industries, renowned universities, research centres of excellence, and a consultancy firm to provide a management structure in line with the needs of the European Commission.

The ITS-NANO consortium consists of experts in nanotechnology, nanotoxicology, nanoecotoxicology, and risk assessment, from academia and industries with considerable experiences in collaborative research in FP6 and 7 NMP programme. Some of the collaborators are also members of the OECD Working Party on Manufactured Nanomaterials (WPMN), ISO-TC229, and are contributors to the review and extension of REACH for nanomaterials as well as other National initiatives. Project partners

has a direct access to state-of-the-art knowledge, being involved in several initiatives already ongoing and about to start, including the two large NMP research projects MARINA and NanoValid. Finally, sustainable industrial implementation themes are also represented, due to the presence of industry, technology transfer experts but also occupational health research institutes.

ITS-NANO will receive further inputs from the integration of the most relevant stakeholders, regulatory bodies, policy makers, and industries, being the latter both the most significant actors of nanotechnology R&D at the moment, but also the end-users. Integrating such competences in the project, and also networking and interacting with the wide scientific community represented by the NanoSafetyCluster and by selected experts in the United States, will provide the consortium with a clear understanding of which are the priorities to be addressed in the future, according both to stakeholders' needs, but also identifying the direction in which nanotechnology development and transfer is moving towards.

ITS-NANO first objective is to create a scientific basis for ensuring the safe and responsible development of manufactured nanoparticles and nanotechnology-based materials and products, and to support the determination of regulatory measures and implementation of legislation in Europe. Current (eco)-toxicological approaches to assessing nano-material hazard are based either on classical toxicology approaches or on novel multiplexed assays. These approaches do not provide a comprehensive assessment due to the many unique aspects of nanomaterials, such as the transport mechanisms (in the body and within cells) and, in particular, the relationship between the physico-chemical properties of the nanoparticles with:

1. the biological identity *in situ* (i.e. in various culture media);
2. the fate and behaviour (uptake, translocation, localisation);
3. the functional impacts at system and cellular level

Thus, new (eco)-toxicology approaches, which consider and exploit these unique aspects of engineered nanoparticles, are urgently needed. Specifically, an Intelligent Testing Strategy for Engineered Nano-Materials is required. This strategy is 'intelligent' both at the *strategic level* by identifying and setting a priority research agenda to reduce the research gaps according to the need of the stakeholders (industry, regulators) and at the *tactical level*, to be economical and ethical. This is the aim of the ITS-NANO proposal.

Specifically, our objectives are to develop:

- A framework for future research aiming at rational grouping, through well standardized methods, of engineered nanomaterials (ENM) according to their i) physical, ii) chemical, iii) biological characteristics.
- A framework for future research aiming at specific grouping of ENM according to the specific health risk they present towards the immunological, respiratory, reproductive, circulatory, etc... systems.
- A strategy to increase the integration among stakeholders (food industry, nano-material manufacturers, pharma- and health-related industry) for a shared, agreed-upon risk assessment strategy and



approach to conveying the appropriate, evidence-based information to the public.

3.1 Summary of ITS-NANO key expected impacts

- An integrated and agreed upon research strategy to address priority needs of key stakeholders.
- The integration of key stakeholders into the decision making process towards the definition of the Intelligent Testing Strategy.
- The definition of a reliable methodology for exposure assessment, hazard identification and risk assessment applicable in future research, speeding up the reliable generation of new knowledge
- The promotion of innovative methodologies and concepts, to help in the clarification of molecular mechanisms involved in interactions between nanomaterials and biological systems

environmental impact and risk assessment, from various countries of Europe (Italy, United Kingdom, Denmark, and Germany), and to take advantage of the existing broad European cultural wealth and variety to find best solutions for an integrated testing strategy to promote sustainable development of this increasingly growing economic sector.

ITS-NANO has a strong commitment to communication and openness, and will actively operate to invite all researchers and stakeholders to participate in the project activities. For this purpose, different layers of participation are foreseen: a small group of experts (the project partners) will identify key areas and knowledge gaps to be addressed by the Intelligent Testing Strategy, and will organise workshops with a core group of invited stakeholders (the Core Stakeholder Panel) to develop the initial strategy, which will be eventually evaluated and integrated by the widest scientific community of the NanoSafetyCluster, and other interest groups who wish to collaborate with the project.

The ITS-NANO work plan is broken down into four inter-related and interconnected work packages (WPs, see table below). Interaction and communication between the WPs is guaranteed by a network of inter-relationships, as outcomes of each WP are inputs for the others. In all work packages, existing data is taken into consideration and every attempt is made to include results and contributions from other ongoing projects.

4 Organisation of ITS-NANO

The overall goal of ITS-NANO is to bring together top specialists in nanoscience and technology, nanotoxicology, ecotoxicology,

Table 1 Workpackages (WP) of ITS-NANO

WP	Title	Topic
1	Project Management	This workpackage deals with the technical, financial, and administrative management. It supports the timely and efficient implementation of the work plan
2	Goal-setting for Toxicology, Eco-Toxicology and Risk Assessment of ENM	This workpackage will identify and set the strategic goals for (eco)-toxicology testing and Risk Assessment of ENM. The experts represented in the consortium will use their knowledge and other information coming from other projects and reports, including the European NanoSafetyVision for 2015-2020, to identify which are the goals that need to be achieved, also integrating them with the foreseen of the potential development both in nanotechnology (to identify future changes/trends that will impact upon the types of human and environmental exposures that are likely to occur), and in the developments in biological and environmental sciences that can impact on how nano(eco)tox research would be performed.
3	Intelligent testing strategy design	This workpackage aims to develop a structured research framework. The integrated test strategy will be developed starting from the work performed in WP2. Emphasis will be put on ensuring the inputs coming from the stakeholders contacted in the development of the testing strategy. The strategy will: i) be tailored towards the rational grouping of nanomaterials according to physic-chemical characteristics and common biological descriptors; ii) prioritize future research according to the necessity of the most important stakeholders, namely regulatory bodies and industries, and to foster the acceleration of the sustainable transfer of nanotechnology innovation from research to market; iii) propose the adoption of novel concepts and methods into nanoecotoxicology research, in particular fostering the adoption of innovative technologies and approaches into (eco)toxicological testing and risk assessment.
4	Integration of Stakeholders and Dissemination	This workpackage aims to maximise the communication and the integration of all the stakeholders, by: i) establishing a Core Stakeholder Panel, which will work in close collaboration with the project partners to define research priorities; ii) organising a series of workshop, international conference, and roll-out event, to promote the dissemination of project results, and to stimulate the wider scientific communities to provide a constructive feedback to project activities; iii) setting up an online consultation to promote the transfer of opinions, suggestions, and feedback from the widest range of stakeholders; iv) effectively disseminating project results and achievements, publishing newsletters, reports, articles on scientific journals.



5 Activities of ITS-NANO

5.1 *Intelligent approaches to grouping of nanomaterials based on their properties and their subsequent biological impacts in order to intelligently design next-generation nanosafety evaluation and risk assessment strategies*

While much of the function of nanomaterials is due to their core structure, the surface properties defines much of their bioactivity, as it influences their interaction with the environment or the host organism. Several parameters may be used to describe the physicochemical characteristics of nanomaterials, some of them being conventional descriptors already used for chemicals (chemistry, size, size distribution, surface charge, water/octanol partition coefficient, etc.), whilst other becoming more important when considering engineered nanomaterials, like surface area, presence of nano-pores, absorption on different molecules (including biomolecules) on the surface, etc.

These factors influence strongly the interactions of the particles with the environment and exposed organisms, as they are responsible for events such as chemical reactions that might occur on the surface, for the compartmentalisation in different environmental matrices, for the altered binding of different biological molecules, and for the possibility to cross biological barriers. Such events that may, indeed, cause unwanted toxic effects. Only a few studies have been performed to quantitatively correlate such characteristics with possible biological activities (Burello and Worth, 2010), the reasons being the lack of standard *in vitro* methods that univocally assign a precise toxicity value to the tested nanomaterials, the deficient knowledge regarding the mechanisms of action of these materials and the difficulty to mathematically describe a system which is too large as well as too complex for accurate calculations. The Delphi approach that ITS-NANO is going to develop will provide a first mean to elucidate a selection of physicochemical characteristics for which there is relatively clear evidence of impacts upon a series of exposure, toxicology and ecotoxicology endpoints. The first outcome of this Delphi consultation will be the creation of functional groups of particles according to their physicochemical characteristics that can eventually lead to given (eco)toxicological endpoints, but also to move in the opposite direction, clearly associating groups of endpoints to physicochemical characteristics.

Furthermore, this rational grouping will have a direct impact to the definition of the techniques used in laboratory research. It is known (See OECD ENV/JM/MONO(2010)46, 01.12.2010) that several nanomaterials can interact with reagents used to assess their effect during laboratory assays; defining group of particles according their physicochemical characteristics, and associating such characteristics also with the experimental bias scientist may incur in when using tests that have been shown to be inappropriate for given groups, will avoid the production of biased results.

5.2 *Quick screening and identification of high risk materials, and implementation of strategies to counter these risks*

Rational grouping of nanoparticles and nanomaterials can be itself a way of screening high risk materials. In fact, functional groups of physicochemical characteristics may be associated to strong toxic behaviours, or to extreme likelihood of exposure in given circumstances. When these associations will become evident through our state-of-the-art analysis, the intelligent testing strategy representing the core output of ITS-NANO will be developed in order to contain a optimised set of tests to be used in rapid screening of toxic potential.

Through this strategy, it will also be possible to reduce risks associated to physic-chemical characteristics of nanomaterials. This strategy, in fact, can be transferred from the identification of risks to the design phase, suggesting to avoid to develop of nanomaterials with characteristics that can give them potential risks in term of release in the environment, human and ecotoxicity, or promoting the incorporation of chemical features that have been shown to be safer.

5.3 *Increased integration and advancement of EU policy on nanosafety evaluation and communication*

Research performed so far, based on a basic research approach, performed on pure commercial nanoparticles, often poorly characterised, has generated a significant amount of data, but this results obtained are now difficult to compare due to the absence on information of physicochemical characteristics. Furthermore, data achieved to date, do not represent a significant mean of predicting the actual behaviour of nanomaterials in the environment and when they will come in contact with humans, as in several cases they lack of the consideration and the assessment of which modifications occur during the whole life cycle of the product. These uncertainties are reflected in the general absence of a specific regulation for the use of nanomaterials in general applications. In fact, on November 20th, 2009, the European Union (EU) Council approved an updated cosmetics regulation that would require cosmetic products that contain nanoscale ingredients to be labeled as such. Concerning food applications of nanomaterials, the European Commission has explicitly stated that specific methods to assess the risk are developed, no food with nanomaterials should be put on the EU market, and is committed to only approve the marketing of food containing nanomaterials for which the food safety has been established. ITS-NANO will address this critical issue in nanotechnology risk assessment proposing an intelligent testing strategy that will try to respond to such relevant points. The first point that will be developed of this project is in fact the integration of the most relevant stakeholders, regulatory bodies, policy makers, and industries, being the latter both the most significant actors of nanotechnology R&D at the moment, but also the end-users. Integrating such competences in the project will provide scientists with a clear understanding of which are the priorities to be addressed in the future, according both to stakeholders' needs, but also identifying the direction in which nanotechnology development and transfer is moving towards. To understand this point, it is crucial to involve industries



in the discussion. The integration of scientific excellence, industrial and regulators' needs, within the ITS-NANO consortium will set the backbone to design an prioritised research strategy, to be implemented in future research projects, but also in the EU projects already started and in which project partners are already actively participating. This research strategy will necessarily highlight which are the key issues to be addressed as a first priority, to help industries in designing safer particles for their application, to provide the regulatory bodies the mean to protect workers and consumers, but also to address the fears, justified or not, of the society, since we have already witnessed how a strong contrary reaction of the public opinion can block the possible development of promising technologies. Indeed, facilitating the understanding of the relevant toxicity mechanisms of nanomaterials will also benefit the possibility to respond to the safety concerns of the general public, as providing scientific dissemination agencies significant scientific data, achieved according to strategies and protocols agreed upon the independent scientific community.

5.4 Meetings, consultations and workshop

Firstly, the consortium will draft a concise set of goals for the strategy, based on the current state-of-the-art. This will be a conceptual draft, and the consortium will rely on its inherent expertise.

After 6 months this first draft will be discussed with a small panel of top-level experts (the Core Stakeholders Panel, to be defined as

the project starts) in a focused workshop. This workshop aims to significant improvement of the first draft, of which an evolved version will be published for an open online consultation, starting month 9. This draft will include approaches to testing strategies, already.

Using an online consultation ITS-NANO will reach out to a much wider audience. Further, this way it will be possible collect opinions of certain stakeholders, including experts on the public perception to innovative technologies. While these positions may not add much in way of scientific facts, they are very important to make the researchers aware of present anxieties and fears, that need to be dealt with. Where possible the feedback from the online consultation will be classified using the scheme developed by Klimisch et al., which is used widely in OECD initiatives.

In month 12 ITS-NANO will organise a major international conference, to discuss a second draft, which includes conclusions of the online consultation. This event is the key element of our consultation procedure. The expert-panel members of our 6-month Workshop will chair sessions of the conference. In addition there will be high-level co-chairs representing the various stakeholder groups. This will in particular serve to integrate industry, legislators, and funding bodies strongly. The proceedings of this event will lay the foundation for an ongoing dialogue, and we will seek opportunities to secure an ongoing funding for such conference, after the project.

3 months after this conference, results will be presented to all key stakeholders: scientific community, industry, legislators, and funding agencies, in a roll-out event.

6 Directory

Table 2 Directory of people involved in this project.

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MARINA

MANAGING RISKS of NANOMATERIALS

Contract Agreement: Under Negotiation Website: <http://www.marina-fp7.eu>
Coordinator: Dr Lang Tran, Institute of Occupational Medicine, Edinburgh (UK)

no.	Participant Legal Name	Country	Organisation Type
1	Institute of Occupational Medicine (IOM)	United Kingdom	SME
2	European Research Services GmbH (ERS)	Germany	Industry
3	Aarhus University (AU)	Denmark	University
4	BASF(BASF)	Germany	Industry
5	Commissariat à l'énergie atomique (CEA)	France	Research Organisation
6	Das Institut für Energie- und Umwelttechnik (IUTA e.V.)	Germany	Research Organisation
7	Swiss Federal Laboratories for Materials Testing and Research (EMPA)	Switzerland	Research Organisation
8	Finish Institute for Occupational Health (FIOH)	Finland	Research Organisation
9	Fraunhofer-Institut für Molekularbiologie und Angewandte Oekologie IME-AE (IME)	Germany	Research Organisation
10	Freie Universität Berlin (FUB)	Germany	University
11	Gothenburg University (UGOT)	Sweden	University
12	Health & Safety Laboratory (HSL)	United Kingdom	Research Organisation
13	Institut National de l'Environnement Industriel et des Risques (INERIS)	France	Research Organisation
14	Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)	Spain	Research Organisation
15	Joint Research Centre of the European Commission (JRC)	Italy	Research Organisation
16	Max-Planck-Institute for Molecular Genetics (MPI)	Germany	Research Organisation
17	Nanotechnology Industries Association (NIA)	Belgium	SME
18	National Physical Laboratory (NPL)	United Kingdom	Research Organisation
19	Stichting Dienst Landbouwkundig Onderzoek (DLO)	The Netherlands	Research Organisation
20	National Institute for Public Health and the Environment (RIVM)	The Netherlands	Research Organisation
21	Netherlands Organisation for Applied Scientific Research (TNO)	The Netherlands	Research Organisation
22	Universität Salzburg (PLUS)	Austria	University
23	University College Dublin (UCD)	Ireland	University
24	University of Leeds	UK	University
25	University of Wien (UVIE)	Austria	University
26	VTT Technical Research Centre of Finland (VTT)	Finland	Research Organisation
27	Westfälische Wilhelms-Universität Münster (WWU)	Germany	University



28	Technical University of Denmark (DTU)	Denmark	University
29	ACCIONA (ACC)	Spain	Industry
30	Venice Research Consortium (CVR)	Italy	Research Organisation
31	The REACH Centre Ltd (TRC)	United Kingdom	SME
32	Karolinska Institute (KTH)	Sweden	University
33	National Center for Nanoscience and Technology, Chinese Academy of Sciences (NCNT)	China	Research Organisation
34	Institute of Biochemistry, Russian Academy of Sciences (INBI)	Russia	University
35	University of Parma (UP)	Italy	University
36	Tor Vergata University Roma 2 (TVUR)	Italy	University
37	Edinburgh Napier University (Napier)	United Kingdom	University
38	Ludwig-Maximilians-Universität München (LMU)	Germany	University
39	University of Plymouth (UoP)	United Kingdom	University
40	The Food and Environment Research Agency (FERA)	United Kingdom	Research Organisation
41	University of Birmingham (BHAM)	United Kingdom	University
42	University of Tübingen	Germany	University
43	Nofer Institute of Occupational Medicine (NIOM)	Poland	Research Organisation
44	Institut universitaire romand de Santé au Travail (IST)	Switzerland	Research Organisation
45	NANOCYL sa (Ncyl)	Belgium	SME
46	National Institute for Materials Science (NIMS)	Japan	Research Organisation
47	Heriot Watt University (HWU)	UK	University

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1 Concept

Nanotechnology is recognised as one of the most important new technologies of the 21st century. The global investment in nanotechnology from all public sources for 2008 exceeds \$7 billion¹.

The market size for nanotechnology is expected to grow to over \$3 trillion by 2015² and nanotechnology promises new materials for industrial applications by having new or enhanced physico-chemical properties that are different in comparison to their micron-sized counterparts. However, as in all industrial applications, the potential exposure of humans and the

environment to these materials is inevitable. As these new materials go through their life-cycle – from development, to manufacture, to consumer usage, to final disposal – different human groups (workers, bystanders, users) environmental compartments, (air, soil, sediment, water), and species (e.g. worm, fish or human through secondary exposure), will be exposed to them. Emerging data show a range of toxic (hazardous) effects from engineered nanoparticles, suggesting that any exposure will result in a risk to human health or the environment, risk being the product of exposure and hazard... While standard methods exist



for risk analysis, these tools need to be applied, modified and verified for nanomaterials. Previously used standard approaches to risk management, control and reduction need to be proven for the novel paradigm presented by nanomaterials. Thus, the development of nanotechnology-based products needs to be complemented with appropriate validated methods to assess, monitor and reduce the potential risks of engineered nanomaterials (ENM) to human health and the environment. Public mistrust of any new technology is often high, and demonstrating 'safe' products of nanotechnology will enhance the confidence of consumers, workers and other stakeholders. Furthermore, these measures must be validated and integrated in an overarching, coherent strategy for regulators and industry to adapt them. Thus, a safe and environmentally responsible nanotechnology will safeguard current and future global investments and will be the key to the sustainability of this industry.

While there are standard procedures for product life cycle analysis, exposure, hazard, and risk assessment for traditional chemicals, is not yet clear how these procedures need to be modified to address all the novel properties of nanomaterials. There is a need to develop specific reference methods for all the main steps in managing the potential risk of ENM. The aim of MARINA is to develop such methods. **MARINA will address the four central themes in the risk management paradigm for ENM: *Materials, Exposure, Hazard and Risk*. The methods developed by MARINA will be (i) based on beyond-state-of-the-art understanding of the properties, interaction and fate of ENM in relation to human health and the quality of the environment and will either (ii) be newly developed or adapted from existing ones but ultimately, they will be compared/validated and harmonised/standardised as reference methods for managing the risk of ENM. MARINA will also develop a strategy for Risk Management including monitoring systems and measures for minimising massive exposure via explosion or environmental spillage**

2 Objectives

The specific objectives of MARINA are:

1. For *Materials*, to obtain reference nanomaterials for testing; to develop validated methods for characterising the physico-chemical properties of ENM as pristine materials, in biological matrices, in environmental samples and field detection; to isotope-label ENM for their use in bio-distribution studies
2. For *Exposure*, to conduct exposure assessment in the workplace throughout the life-cycle of a ENM, developing different exposure scenarios. To assess the fate and behaviour of ENM in soil/sediment/water. To characterize the actually released ENM (aged ENM) and compare them to the pristine ENM. To evaluate, as part of a performance assessment, different approaches to conduct exposure assessment for use in the MARINA integrated risk assessment.
3. For *Hazard*, to address the knowledge gap, especially in areas of non-genomic toxic mechanisms, toxicogenomics, proteomics

and metabolomics by developing new test systems; to develop reference methods for in vitro toxicology tests (including and fully incorporating those developed in other FP projects) by means of a scientific validation strategy; to implement in vivo dose-response models of healthy and susceptible subjects exposed through repeated dosing to ENM via inhalation, ingestion, intravenous injection and dermal exposure; to develop and scientifically validate in vitro and in vivo tests for soil/sediment/aquatic toxicity and secondary poisoning.

4. For *Risk*, to combine phase (1), (2) and (3) in developing reference methods for assessing the health and environmental risk posed by ENM; to develop a strategy for Risk Management including monitoring systems and measures for minimising massive exposure via explosion or environmental spillage.

MARINA is to achieve the objectives described above in **48 months**.

3 The MARINA approach

The European Commission, to date, has funded some 15 projects relevant to health and safety issues regarding ENM. This commitment is set to continue in the future. At the national level, there are other similar efforts^{3,4}. However, to date, the valuable results generated from these projects have in the main been unable to generate concepts, methodology and data which have been practically used for risk assessment and management.. Thus, there is clearly a need to use the most up-to-date available information and methodology for guidance on health and safety risk management to industry and regulators. To respond to this need, in MARINA, we have created a consortium consisting of first class scientists and organisations with a track records for research in Health and Safety Issues of ENM. Most importantly,

- we have representatives from more than ten FP projects¹. Our aim is to take the beyond the state-of-the-art results from these projects and use them for creating validated reference tools for Risk Assessment and Management
- we recognise the relevance of our results to industry therefore we have involved the direct participation of the Nanotech Industries Association² and industrial key partners such as BASF and Nanocyl.
- we also recognised the geopolitical and economical

¹ The FP projects are: **FP6** PARTICLE_RISK, NANOSH, NANOINTERACT, NANOSAFE2, **FP7** NANOMMUNE, NANOTEST, ENPRA, NEURONANO, NANODEVICE, NANOLYSE, NANOIMPACTNET, NANEX, ENNSATOX, NANOFATE, NANOHOUSE and ObservatoryNANO.

² NIA is also involved in another proposal on the same call (NMP.2010.1.3-1: NanoREFORM); this involvement allows the establishment of added-value components to both projects and benefits the nanotechnology research and industries community..



importance of Third Countries such as China, Russia and Japan. The inclusion of the prestigious Academies of Sciences from China, Russia (for Toxicology) and the Japanese National Institute of Materials Science (for ENM synthesis, characterisation and Toxicology) as well as our existing US partners through current FP7 projects goes beyond the scientific excellence and will enable MARINA to reflect a true global effort in addressing this important issue and to promote our strategy for risk management of ENM globally.

- we will interconnect all MARINA activities with other relevant ongoing activities such as the forthcoming EC European Technology Platform(s), Nanofutures, Infrastructure and cluster activities of FP7 projects, the ERAnet, the OECD WPMN sponsorship programme as well as National Research programmes, such as NanoCare2 and NanoNature.

Although the database that supports risk assessment and management continues to expand, the general approaches have not changed significantly. Risk assessment and management must be based on the best available science, which is continually progressing. These changes appearing in the nature and the interpretation of data prompt the MARINA approach: Specifically:

1. The likelihood of increasing restrictions and public acceptance of the use of animals for testing purposes in the EU drive MARINA to go for **integrated test systems (ITS)** targeting modules of hazard endpoints, fate and exposure, and monitoring.
2. The availability of data from new/rapidly advancing methodologies is fully acknowledged in MARINA - systems biology and early marker detection are used for **integrated assessment schemes (IAS)** for occupational and environmental exposure assessment and monitoring schemes.
3. Advances in mode of action research and in the understanding of effects/disease mechanistic processes in MARINA lead to addressing hazard more specifically and develop **interconnecting module systems (IMS)** for risk assessment and risk management as methodology for supporting decision making.

In summary, MARINA stands for **integrated testing, integrated assessment and modular interconnection of knowledge and information for science-based risk management methods**. The approach is to translate scientific advancements and methodology in contribution to shifting from toxicological studies of specific individual nanomaterials towards a more systematic health and environmental safety assessment and management that handle the overall risks for types or classes of ENM based on their intrinsic, e.g. physico-chemical properties.

4 Progress beyond the state-of-the-art

In the following sections, the scientific state-of-the-art is summarised and the many aspects of MARINA which go beyond the state-of-the-art to achieve the objectives listed above will be clearly described.

4.1 The state-of-the-art

Currently, the EC has funded many projects through their 6th and 7th Framework programmes. These projects generally cover the state-of-the-art landscape of health and safety issues related to ENM:

(a) *for Materials*, characterising the physico-chemical properties of ENM in bulk materials is generally accepted as essential to all toxicology studies. The detection and characterisation of ENM in biological matrices is being investigated in FP7 NANOLYSE. To date there is no attempt in harmonising and standardizing characterisation methods, although this has been widely debated.. Furthermore, the lack of reference materials to be used, means that there is difficulty in extrapolating the results between studies. Also, almost no study has characterized the ENM that are actually released into the environment. The new FP7 NANOHOUSE is going to do this but only for ENM released from paints.

(b) *for Toxicology*, it is well known that exposure to particles is likely to lead to adverse effects. Following exposure, some ENM have been shown to translocate beyond the portal of entry organ⁵, the extent of translocation is dependent on the ability of ENM to cross biological barriers (blood-brain, blood-air, placental, and so on) which is a function of ENM size, surface properties, (e.g. surface charge⁶) or the formation of the protein or lipid corona⁷ on the ENM surface. Inhaled ENM could be translocated to the brain via the olfactory bulb⁸. These ENM can also reach the terminal bronchial region and for high-aspect ratio ENM, they are likely to be further translocated to the pleura⁹. A small ENM fraction can reach the blood system where they can travel to secondary organs⁵ like the heart, liver, kidney and beyond. The bio-distribution of internalised ENM follows a different pattern depending on the route of exposure: inhalation, ingestion or dermal¹⁰. For dermal exposure, there is some evidence that ENM can penetrate beyond the stratum corneum¹¹. For oral exposure, TiO₂ ENM have been shown to induce DNA damage and genetic instability in mice¹². The paradigm for the adverse response is currently oxidative stress leading to inflammation¹³. Chronic inflammation could lead further to fibrosis, DNA damage and cancer¹⁴. ENM can also be genotoxic by direct by a totally new mechanism¹⁵. ENM driven oxidative stress also plays an important role in the immune response and apoptosis¹⁶. Cardio-vascular effects have been demonstrated in susceptible animal models exposed to ENM¹⁷ although it is not clear whether these are caused by systemic inflammation or direct ENM interaction with cardio-vascular plaques causing plaque disruption. The ability of some ENM to cause a pro-thrombotic effect like platelet aggregation should also be noted¹⁸. Data on the adverse responses are generated from many FP projects. For example, the protein corona issue is being investigated in the FP6 NANOINTERACT project, the central nervous response is investigated in FP7 NEURONANO, the pulmonary and cardiovascular effects were investigated in FP6 NANOSH, PARTICLE_RISK and more recently in FP7 ENPRA, NANOTEST. Other target organ effects are also studied in ENPRA and NANOTEST and immunotoxicity is the research topic for FP7 NANOMMUNE. Taken together, a coherent toxicity profile of ENM begins to emerge. However, there are still many short-comings to be addressed. These include the proper validation of the test protocols developed in these FP projects, and the comparison of in vitro and in vivo results to reduce animal testing as part of the 3R strategy for toxicology testing.

(c) *for Eco-Toxicology*, relatively little is known about the



environmental ENM toxicity, but toxicity has been reported from the molecular to the population level¹⁹, including food chain effects. ENM uptake and tissue distribution within species are mostly unknown, except for in a few larger species²⁰. It is however clear that ENM uptake mechanisms are different those for conventional chemicals (see *toxicology*). One of the early examples of ENM effects in an environmental species was the study by Hund-Rinke et al²¹ who showed oxidative stress in algae and daphnids following TiO₂ exposure. Since then data are emerging at increasing numbers every year. Almost all published ecotoxicological studies with ENM³³ have focused on the aquatic environment with little or no attention to the soil and sediment compartments, the latter even tested in aqueous suspensions or on filter paper soaked with the test suspensions²². Within the aquatic environment freshwater species, mostly pelagic have been tested²³. The effects reported often differ depending on the method used to prepare the ENM for testing e.g. stirred ENM versus solvent carried ENM²⁴. There is currently no guidance or guidelines for toxicological assessment of ENM, or guidance on how to adapt the guidelines used for conventional chemicals. This is probably one of the reasons for the diverse range of test conditions/protocols currently used and for the reported differences in effect levels. There is no clear pattern as to which ENM characteristics are important for toxicity, although surface area may be a candidate for certain ENM²⁵. The lack of convincing patterns could be because the ENM characteristics reported are at best derived for primary particles and for ENM suspended in the exposure media, where ionic strength, pH and others changes to the primary ENM take place (e.g. agglomeration, aggregation and surface chemistry) i.e. media-specific factors that modulate effective exposure and hence toxicity. (d) *for Human Exposure*, the workplace is still the most likely space where human beings will be exposed to ENM, although exposure is likely high throughout the entire life-cycle of the ENM from production to disposal²⁶. At each stage of the life-cycle, there is potential for exposure to different groups of workers. Methods for measuring the ENM aerosol concentration in the workplace are currently being developed (e.g. FP7 NANODEVICE³⁹), with measurements of mass, particle size and size distribution²⁷. Currently, there is no method for measuring the surface physico-chemical characteristics of the collected samples²⁸. Also, dermal exposure from airborne ENM and secondary exposure to ENM from the environment are also not considered²⁹. It has been recognised that models of ENM exposure will be important to counter the paucity of data. Workplace exposure assessment has been conducted in many national and European projects^{30,31}. Of particular importance, is FP7 NANEX³² which will establish exposure scenarios for the workplace throughout the ENM life-cycle. (e) *for Environmental Exposure*, ENM are likely to end up in the environment, although uncertain estimates ranging from ng to mg per kg levels in various compartments³³. Some ENM may be persistent while others rapidly dissolve. Soils, sediments and surface waters are complex matrices with many possible different exposure and interaction scenarios³⁴. The environmental behaviour of ENM can be particularly complex with a high propensity for aggregation, agglomeration and deposition, along with dis-agglomeration and re-partitioning into the solution phase³². Formation of aggregates or agglomerates can take place between ENM or with natural organic and/or inorganic colloids^{33, 34}, and is influenced by environmental factors like pH and ionic strength³⁵⁻³⁹; These factors combined with inherent the physical-chemical properties, structure and concentration/dose, contributes to the complexity of

quantifying environmentally relevant and bio-available concentrations^{40,41}. The physico-chemical distribution of ENM between dissolved, colloidal and particulate phases is largely unknown⁴²⁻⁴⁶, and remains a key unknown in regard to exposure of organisms (NANOFATE). The problems are also currently being addressed in FP7 ENNSATOX which is specifically concerned with the environmental aqueous behaviour of ENM in relation to their toxicity. This underlines the need for detailed experimental work on the environmental fate of ENM in a coherent manner only possible in an integrated project. (f) *for Risk*, there are two important elements: the assessment and management of risk. To assess risk is to compare the measured or predicted exposure level (PEC) from evidence in (d) and (e) with the derived-no-effect-level (DNEL) level from toxicology data or the predicted-no-effect-concentration (PNEC) in the case of the environment. To predict or estimate risk and consequently to manage risk is to implement procedures for the purpose risk mitigation. This includes, inter alia, establishing exposure control limit, controlling and monitoring exposure including accidental explosive or massive release of ENM into the environment, identifying risk scenarios, i.e. groups for health surveillance or geographical areas for health protection, communicating to key stakeholders and training about risk, including developing protective standard operating procedures and informing the regulatory process such as REACH. FP7 ENPRA is developing methods and tools for Risk Assessment. There are currently many Risk Management approaches, such as the HACCP (Hazard Analysis and Critical Control Point) for food safety control, but an integrated Risk Management Strategy specific to ENM is still to be developed

4.2 Beyond the state-of-the-art

It is clear from the summary above that there is still a considerable large knowledge gap in all the four major themes relevant to the risk management of ENM. MARINA will be able to go far beyond the state-of-the-art on all of the points above, because it represents the most comprehensive consortium on Nanosafety issues, with 46 partners merging knowledge into MARINA from numerous EU and large national projects.

To develop reference methods for risk management of ENM, we need to go beyond the state-of-the-art. The specific areas to be included in MARINA are (i) the development of reference materials; (ii) exposure assessment in human and environment settings; (iii) identification of key ENM parameters e.g. size, charge or coating important for describing dosimetry (iv) validation of existing (eco)-toxicology tests and development of new, relevant ones; (v) implementation of in vivo dose-response models of healthy and susceptible individuals exposed to ENM through repeated dosing via inhalation, ingestion, iv injection and dermal routes; (vi) combination of (iv) and (v) into an Intelligent Testing Strategy (vii) implementation of all relevant evidence generated by MARINA and from other projects into a rigorous Risk Assessment for ENM; (viii) development of an overarching Risk Management Strategy for ENM including exposure monitoring schemes and the management of rare events of massive exposure due to explosion or spillage. Most importantly, in all the themes stated above, we will emphasise the production of reference methods applicable



ultimately in the Risk Management of ENM. In the text below, the specific beyond the state-of-the-art research in MARINA for each important Themes are described in more detail.

Materials

We will establish a panel of representative ENM of high volume production and of high economic importance (e.g. TiO₂ – in different size, shape and surface charge, SiO₂, Ceria Oxide, ZnO, nanoAg, Multi-Wall Carbon Nanotubes (MWCNT) – in different lengths) as Reference Nanomaterials (RNM) for use in MARINA. Commercially relevant, fully characterised and quality-controlled ENM will be sourced from both industry partners (via NIA) and the JRC's repository for reference nanomaterials, which is already sub-sampling and distributing several commercially relevant ENM for other nanotoxicology projects, including the OECD Sponsorship programme. These RNM will be characterised, assessed for homogeneity, stability and described shelf-life according to the OECD WPMN SG3⁶⁷ endpoints and criteria. We will use these RNM to validate the metrology methods for measuring key physico-chemical ENM characteristics, which are suggested to drive the adverse effects. Important inputs to these activities will come from the nanometrology community (e.g. FP7 co-Nanomet and ISO TC229). We will harmonise and standardise these methods for the qualification/certification of these reference materials according to ISO Guide 30-35 and OECD Guide 34 as well as ongoing work at the OECD Sponsorship Programme for both risk assessment and nanometrology purposes. To date, there is no consistent method for labelling ENM, although knowing the target organ/cell dose is essential in understanding the nature of the dose-response relationship. In MARINA we will develop and validate methods for labelling ENM for studying the bio-distribution of ENM in body tissues. We will also develop and validate methods for characterising ENM in biological matrices and environmental samples from air/soil/sediment/water for field detection. MARINA will also characterise ENM released from products and aged under environmental conditions, as these are the ENM that the organisms are exposed to. Comparisons to the pristine ENM will be made.

Exposure

i. For Occupational and Consumer Exposure, In collaboration with the relevant industries, we will identify the relevant current and future occupational exposure scenarios and review available occupational/consumer exposure information and conduct exposure surveys to complement the occupational and consumer exposure data. We will review and revise models for predicting exposure to ENM in the workplace and from consumer products and implement these in an advanced control banding tool. We will also develop and implement a strategy for occupational and consumer exposure monitoring including the characterisation of workplace and consumer product samples; these strategies will be verified through industrial case studies, using both real-case exposure scenarios.

ii. For Environmental Exposure, we will review available environmental, identify and formulate the current and future environmental exposure scenarios, validated by monitoring. We will develop adapt and validated experimental guidelines for the

fate and behaviour assessment of ENM in soil, sediment and water. This will be based on analysis ENM binding to and partitioning from natural components, including importance of agglomeration; besides distribution, availability and stability of ENM under standardised and real environmental conditions will be assessed. The data generated will allow parameterisation of the fate processes scientifically and permit the implementation of regulatory exposure assessment frameworks.

iii. For both spillage and explosion, critical parameters controlling risk, like concentration of agglomerates, the explosion severity, the minimal ignition energy and many others, will be identified experimentally, new reference evaluation methods of such parameters will be developed and quantified. using the unique expertise in pulse/intermittent exposure in our consortium. Moreover, for accidental release models, industry case-studies will also be used in support of the development of experimental models for massive accidental exposure from explosion.

Hazard

i. For Toxicology, we will develop new in vitro toxicology test methods on the following target systems: the immune, central nervous, cardio-vascular, pulmonary, hepatic, renal, reproductive, developmental and dermal systems. The adverse endpoints are target specific as well as oxidative stress, inflammation, genotoxicity, fibrosis. We will also investigate the ability of ENM to translocate across biological barriers such as the blood-brain, blood-air, endothelial and placental barriers and determine the ENM physico-chemical properties which facilitate this dynamics. Moreover, the interaction between ENM surface physico-chemical characteristics and body proteins and lipids is fundamental in how cells react to the presence of foreign entities. Thus, we will investigate this phenomenon in relation to the potential toxicity and translocability of ENM Most importantly, we will implement animal experiments dose-response and bio-distribution ADME models of healthy, pregnant and susceptible (to cardio-vascular problems) individuals exposed through repeated dosing to ENM via inhalation, ingestion, iv injection and dermal routes.

ii. For Eco-Toxicology, we will adapt and if develop in vitro and in vivo tests for soil, sediment and aquatic toxicity including secondary poisoning. Current test will be modified or if needed developed then standardised and validated for use with ENM. Key effect endpoints and dosimetry parameters directly specific to ENM will be identified, this will be done across all media benefiting on the size of the consortium. Data will be complemented by mechanistic information (see iii).

iii. For both Toxicology and Eco-Toxicology, we will develop methods for toxicological profiling using toxicogenomics, proteomics and metabolomics including some unique arrays that are being adapted for ENM available to the consortium and therefore identifying ENM specific Modes of Actions (MoA). We will adapt existing in vitro tests nominated by current FP projects, and harmonise toxicology and eco-toxicology endpoints into one unified framework for hazard assessment. The tests will be validated by reliability assessment and inter-laboratory round robin comparative tests and the selected ones will be implemented in High-Throughput Systems (HTS). Ultimately, we will integrate the validated tests into an intelligent Testing Strategy (ITS) and



propose it as a Method Validation Framework for use by ECVAM in compliance with the 3R principles and we will update the OECD test guidelines with this ITS.

Risk

i. Assessment, we will implement a database for storing MARINA data and available data from other FP and national projects by using the existing NAPIRAhub database; We will implement and harmonise *in silico* models of exposure-dose-response (PBPK/PD) and QSAR models for both toxicology and eco-toxicology and to use them as tools for Risk Assessment (RA) (we will work with other successful projects in FP7 NMP-2010.1.3-2). Key differences from the present RA will be identified and ENM specific issues will be clarified. Based on the weight-of-evidence generated in MARINA and from other projects, we will implement a RA strategy for the humans and the environment and integrate both strategies into an Integrated Risk Assessment (iRA) Strategy for ENM.

ii. Management based on the results of the iRA, and in close collaboration with industries (i.e. via case-study verification), we will develop a Risk Reduction Strategy (RRS) in the form of a toolboxes for (a) the management massive release risk, (b) the assessment of monitoring systems for the control of occupational/consumer/environmental exposure, and (c) identification of susceptible groups (humans and other species) for future health surveillance. We will develop guidance manuals and SOPs and communicate them to all relevant stakeholders (e.g. research labs, industrial manufacturing, processing and research labs). For both (i) and (ii), we will contribute the iRA and RRS as part of the development of the REACH process.

iii. *Other issues* relevant to MARINA

MARINA will implement a strategy for (i) training of the next generation of researchers and relevant industry stakeholders through a series training schools and workshops and (ii) dissemination of MARINA approach and results targeted at policy-informing and -making bodies (e.g. OECD, EC Scientific Committees, EC regulatory working groups, etc.), national public authorities, nanotechnology industries, and the wider nanotechnology research community and citizens by means of public forums, website and newsletter. Therefore enhancing the public awareness about the developments of sustainable nanotechnology through emphasis among the participants and encouragement of transparent and direct communication to the public. Most importantly, MARINA will collaborate with lead institutes of Nanosciences, the forthcoming INFRASTRUCTURE FP project, the existing nanosafety cluster activities promoted by the EC and also the very successful FP NANOIMPACTNET project for an effective dissemination effort. Through direct participation of Industry Associations and dedicated industrial partners, dissemination and uptake of RRS to key industry in different sectors including chemical industry, cosmetics and consumer products will be guaranteed. **MARINA strives for integrated testing, integrated assessment and modular interconnection of knowledge and information for science-based risk management methods.** The approach is to translate scientific advancements and methodology in contribution to shifting from toxicological studies of specific individual nanomaterials towards more holistic health

and environmental safety assessment and management that manages overall risks. Finally, we are aware that for this large consortium to function efficiently, a rigorous management system must be implemented. For this reason, the management of MARINA is divided into two fundamental areas: The administrative and scientific management. We endeavour to manage MARINA using the latest techniques in project management and the expertise and experience of coordinating FP projects from the core MARINA members.

We present an overview of the project structure (see also Fig 2), with references to the WP that are summarised in the table below. The text here, together with the summarised WP, describes the MARINA project. The workplan covers both human health and environment and is comprised of the four main themes Materials, Exposure, Hazard and Risk.

For **materials**, WP3 and WP4 will obtain a panel of ENM including - TiO₂, ZnO, SiO₂, CeO₂, nanoAg, MWCNT – and characterise by measuring the physico-chemical properties of these ENM suspected of driving the adverse human and eco-effects. WP3 will also assemble an Industrial Case Study, consisting of the physico-chemical properties (in the pristine state and in different media) and the (eco)-toxicity profile, for each of the materials considered.

For **exposure**, WP5 address release scenarios, WP6 will develop a tiered human exposure assessment approach (Occupational/Consumer exposure scenarios) while WP7 and WP8 will do the same for the environment.

For **hazard**, WP9 and WP10 are specifically for the human and eco-toxicity of ENM. WP11 is for omics-based system toxicology approaches relevant to both humans and the environment.

For **risk**, the assessment of human and environmental risks is implemented in WP12 and 13. WP14 is dedicated to the management of accidental risk while WP15 is to develop and implement monitoring systems. WP16 is to develop a strategy for risk reduction which include the derivation of control limits, control banding and the exploration of new ENM synthesis which be used for substitution.

Finally, as a FLAGSHIP programme, we will devote two WP (17 and 18) for training and dissemination targeted at specific relevant stakeholders of NANOSAFETY. We are committed to providing long-lasting impact in the area of nanosafety and risk assessment, in Europe and at the international level.

The relation between the WP is illustrated in Figure 1.

5 Impact

MARINA is expected to make a significant and long-lasting impact on the European objectives for the safe, integrated and responsible approach to the development of Nanotechnology. Specifically, for *the development of comprehensive understanding of the properties, interaction and fate of ENM in relation to human health and environment*, MARINA is a multidisciplinary consortium of 46 organisations at the leading edge of European and world-wide research on ENM risk issues or industrial commercialisation of ENM and their products. As inputs we will incorporate state-of-the-art scientific findings, including those of over ten FP projects in the



field and accessible national and international programmes and including the OECD WPMN sponsorship programme and transatlantic co-operations. Building on these inputs we will integrate into a web-based, comprehensive, searchable IT platform to create an integrated resource to bridge the gaps caused by confidentiality, delays between findings and data access and limitations by search engines. Results generated within MARINA will serve to narrow the critical knowledge gaps in all of the principal areas in the risk management paradigm for ENM linking Materials, Exposure, Hazard and Risk, in order to provide an overarching understanding of the interaction of ENM with humans and the environment towards a sound, scientifically based, coherent approach to assessing and managing the potential risk of ENM. MARINA is dedicated towards the development of validated reference methods for managing the risks of ENM and is the first project to also address monitoring of exposure of ENM and its contribution to RA and RMM. An integral part of the MARINA project is also to make available the findings and the methods to other users in an accessible format in order to maximize the benefit of the project. For **support to policy and decision making concerning nanotechnology in respect to various stakeholders**, MARINA will provide a unique resource of information and methodology. It will support shifting from case-by-case evaluations to holistic health and environmental safety assessment and management that addresses overall risks. By using frames and modules of integrated testing and integrated assessment, conclusions are set in a science-policy perspective. All information is provided web-accessible in real time. Decision makers will find information directly in a format, which allows analysis across endpoints, across material types or preparation forms. Industry depends on evidence-based safety assessment to safeguard market potential and sustainability of their products. MARINA will provide the industry with important data and tools to take decisions about products, processes, risk management measures and safety assessment. This includes validation, weight of evidence approaches, expressions of uncertainties, quantitative expression of risks and setting the risk in appropriate context in e.g. risk-sustainability or risk-benefit analysis. MARINA has a clear strategy for engaging the European and wider International Community. The design of the MARINA consortium and our dissemination strategy reflect our commitment to bring the MARINA results to the global community in general and to the international regulatory bodies and interested stakeholder groups and NGO in particular. For **contribution to the future definition of appropriate measures, where needed**, MARINA objectives are to develop standard, reference methods covering a wide range of themes, from Materials to Risk. These reference methods will contribute actively to the much needed global efforts in standardisation and harmonisation of methods and measures. The implementation of reliable reference methods will generate public confidence in the sustainable development of nanotechnologies. For **support to good governance in nanotechnology** MARINA will develop an overarching strategy for risk management and reduction and enabling the EU regulatory bodies, agencies and authorities to make informed decisions and policies to safeguard consumers while taking full advantage of the advancements that nanotechnology will bring to the economy and competitiveness of

EU industry. MARINA will facilitate dialogue in the field via the advisory board and a dissemination plan adapted to international stakeholders in governance. We expect this to support European policy, specifically horizontal standardisation, harmonisation, as well as worker and consumer protection. For **Support to pre and co-normative activities, such as with reference to the implementation of REACH**, MARINA is clearly committed to the REACH process through our integrated activities and ITS. MARINA will closely work together with the Commission services involved in development of adaptations of REACH guidance documents concerning nanomaterials. In particular, our definition developments, adequacy and supplementation of reference methods are of direct value for REACH, especially the focus on reliable methods and combined use of data including from modelling and monitoring. Noteworthy are new in silico approaches for cross-reading (QSAR-like) implemented in MARINA and the acceptance of approaches warranted by a communication strategy directed at relevant bodies including European Commission, European Food safety authority (EFSA), European Chemicals Agency (ECHA), ISO, CEN and OECD. For **Support to the safe, integrated and responsible approach as laid down in "Nanosciences and Nanotechnologies: An action plan for Europe"**, the risk management recommendations will be developed in cooperation between the scientists and industrial stakeholders. It will identify conditions, challenges, and provisions to account for the impact on a responsible and flourishing industrial development of nanotechnologies within Europe. Decision-making by stakeholders will be supported in MARINA to enable risk issues to be addressed on the earliest possible level in order to improve assessment methodology and subsequently safe and responsible use of ENP. Contributions to validated reference methods for risk management will contribute to improve favourable conditions for innovation. MARINA will contribute to reinforcement of the international dimension of European research and collaboration between industry, researchers, NGO, authorities (at Member State and European level) and international standardisation bodies, such as OECD WPMN, WPN, WNT, ISO TC 229 and TC 24, CEN TC 352, and IUPAC.

MARINA has started on the 1st November 2011. The Kick-Off meeting was held in Rome on the 16th to 18th November 2011 jointly with the project NANOVALD. The key activities implemented at the start of the project were:

1. To synchronise MARINA and NANOVALID activities;
2. To choose and procure ENM for a quick start of the MARINA experimental programme.
3. To train scientists on Nanosafety through the Nanosafety Autumn School in Venice.

A joint working group for MARINA/NANOVALID, consisting of key researchers from both projects, has been formed. The specific objective is to synchronise the research activities between the two projects. This group will meet every 6 months and will have a teleconference every three months.

Much effort has been made to choose the relevant ENM for testing. We have identified TiO₂-NM101, ZnO-NM110, Ag-NM300K



and SiO₂-NM200 and NM203, and a SiO₂ from NANOVALID. This batch of ENM will be followed by MWCNT. The ENM will be delivered to the MARINA partner in early 2012.

Following the Kick-Off meeting several WP meeting were held (e.g. WP9 in Stockholm) to initiate their studies.

MARINA has also made use of the NANOSAFETY Autumn School in Venice to initiate their training programme. The course was covered the topics of Materials, Exposure, Hazard and Risk. It was attended by staff and students working in MARINA and NANOVALID as well as students from outside Europe like Iran.

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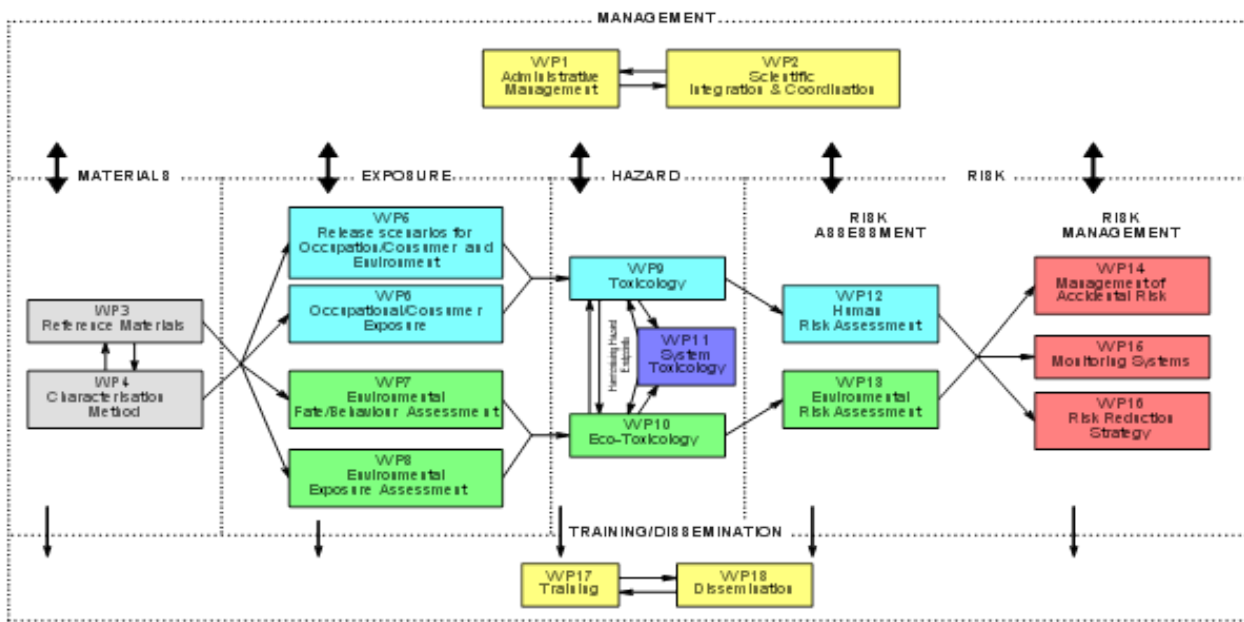


Figure 1 Flow chart of MARINA

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ModNanoTox

Modelling nanoparticle toxicity: principles, methods, novel approaches



Contract Agreement: NMP4-SL-2011-266712

Website: TBA

Coordinator: Professor Eugenia Valsami-Jones, University of Birmingham, Birmingham, UK

No.	Beneficiary name	Short name	Country
1	University of Birmingham	UB	United Kingdom
2	Roskilde Universitetscenter	RU	Denmark
3	Eidgenössische Anstalt für Wasserversorgung, Abwasserreinigung und Gewässerschutz	EAWAG	Switzerland
4	Eidgenössische Materialprüfungs- und Forschungsanstalt	EMPA	Switzerland
5	In-silico toxicology gmbh	IST	Switzerland
6	University of Nebraska Lincoln	UNL	USA

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Project Duration: 1 November 2011 – 31 October 2013

Project Funding: 1 Mio. EUR

1 Summary

ModNanoTox will develop a number of well-documented and technically advanced models describing the behaviour of engineered nanoparticles in organisms and the environment. Background to these models will be a thoroughly documented database, constructed based on: (1) an advanced evaluation of physicochemical properties of nanoparticles and *in silico* modelling of their reactivity; and (2) assessment of the characterisation methodologies as well as toxicity protocols used to develop biological responses in toxicological studies. At the next level whole datasets will be evaluated for internal consistency and then compared with other relevant sets. The evaluation stage will be followed by development of toxicity models based at the individual organism level, using statistical and mechanistic models, in parallel with models predicting environmental fate. The toxicity and fate models will be integrated in mechanistic models to predict the long term risks of engineered nanoparticles for populations under realistic environmental conditions. The risk assessment models will be developed in close collaboration with appropriate stakeholders and end users to ensure their suitability for practical use in relevant legislative contexts.

2 Background

The physicochemical properties of nano-sized particles are distinct from the properties of equivalent bulk substances and are also often unpredictable. As the use of nanomaterials increases, so must the research into any potential adverse effects on the environment or health. ModNanoTox was inspired by the idea that a number of projects are currently generating large datasets of experimental results. Global nanosafety research would benefit greatly by harmonizing, rationalizing and converging these datasets and then use as the basis for robust large scale models of toxicity. Such models can best be developed by teams with experience in collecting the data, who also have a deep understanding of their limitations and relative quality and can influence the progress of ongoing experimental work.

ModNanoTox will be focusing on *in silico* methodology, to complement and support research on and regulation of the environmental and human implications of exposure to engineered metal nanoparticles. There has been a relative explosion of interest in nanotoxicology and growing concerns that the assessments of the risks of nanotechnology is not keeping pace with developments of the technology. As a result, in recent years, a number of nanotoxicology projects have been funded, notably under the FP7 programme of the European Commission, which are currently in progress and generating nanosafety data. A significant research effort is also under way in the US. ModNanoTox will aim to evaluate and synthesise the best available datasets from these sources, and fit them into new models. Such models, whether statistical or mechanistic need to take into consideration the novel



properties of nanomaterials (NMs) and their potentially unpredictable behaviour, and thus models need to acquire a level of sophistication to accommodate that. Both statistical and mechanistic models are needed. Statistical models are necessary when mechanistic understanding is lacking and to capture uncertainties in relationships between nanoparticle properties and their behaviour. However, mechanistic models are more appropriate for extrapolating beyond existing data sets and exploring different scenarios.

3 What is ModNanoTox

ModNanoTox is a small project, designed to develop a number of well-documented integrated and technically advanced models describing the behaviour of engineered nanoparticles in an environmental or biological context to comprehensively address the following key hypotheses:

1. Toxicity of nanoparticles is the result of physicochemical properties and this has been documented reliably in completed/ongoing studies. Properties found to be relevant include size, surface area, structure and composition (WP1, 2)
2. Nanoparticle reactivity can be modelled computationally and can be linked to toxicity (WP1).
3. Toxic responses from cell culture studies and whole organisms can be correlated and rationalised and can be translated into tools useful for model development (WP3).
4. Bioaccumulation into cells or whole organisms can be characterized and modelled using biodynamic principles (i.e. by characterizing uptake rate constants from food and water as well as loss rate constants) (WP3).
5. Toxic responses from cell culture studies and whole organisms can be modelled reliably by QSAR type approaches (WP4).
6. Exposure concentrations can be assessed reliably and incorporated in appropriate models (WP5).
7. Mechanistic effect models can be developed by extrapolation from ecological and (eco)toxicological observations and can be built into risk assessment models (WP6).

4 Organisation of ModNanoTox

ModNanoTox consists of six RTD and one management workpackage. The workpackages and their interdependence are shown schematically in Figure 1. The specific objectives of each workpackage are as follows:

WP1: Physicochemical properties assessment

- 1) To identify and select suitable physicochemical properties (size, shape, phase, concentration, composition, surface modification, method of synthesis) so that groups of structurally similar particles can be identified for toxicity models.
- 2) To carry out atomistic simulations of surface reactivity of a reference set of particles (Ag NPs) to support this selection.

- 3) To develop mechanistic understanding of nanoparticle reactivity based on molecular models.

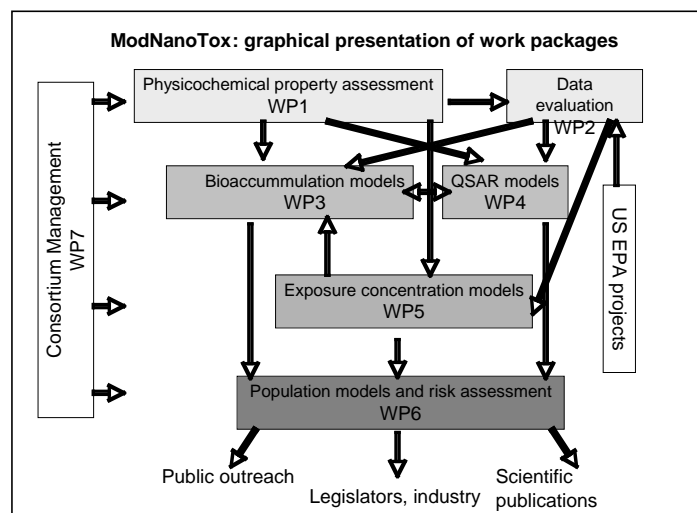


Figure 1. Workpackage structure of ModNanoTox.

WP2: Data evaluation

- 1) To characterise and classify existing toxicity data (data mining), including evaluation of datasets from completed and on-going projects, in order to prioritise data for modelling in other workpackages.
- 2) To evaluate differences in parameters due to contributing factors such as synthesis, storage, and through to testing.
- 3) To recognise data quality limitations.
- 4) To evaluate characterisation and classification techniques.

WP3: Bioaccumulation models

- 1) To model toxicity at the individual organism level using bioaccumulation based toxicokinetic- toxicodynamic models.
- 2) To generate models, based on individual organisms, capable of incorporating experimental nanoparticle uptake and toxicity data and identify links to the mechanism of toxic action.

WP4: QSAR models

- 1) To model toxicity at individual organism level using QSAR models, based on data mining and machine learning algorithms.
- 2) To adapt existing models and generate new models capable of linking toxicity to nanoparticle properties.

WP5: Exposure concentration models

- 1) To evaluate models to estimate environmental exposure, including the REACH procedures.
- 2) To parameterize existing models for nanoparticles by extracting data from the literature and by collecting results from ongoing FP7 projects.



3) To model environmental exposure concentrations of nanoparticles in water, air, soils and sediments from the local to the continental scale.

4) To validate the results by comparison with analytical results from published research.

WP6: Population models and risk assessment

1) To model effects on ecological systems (i.e. populations) and generate risk assessment models.

2) To assimilate the data from the previous objectives to scale up effects to ecologically relevant entities (i.e. populations) and to produce complete risk assessment models developed appropriately for end users.

WP7: Project management

1) To manage the consortium and its stipulated agreement between partners & EU.

2) To coordinate the flow of scientific knowledge, the decision making structures, the control of milestones and deliverables, the promotion of interchange and linkage between project components and partners, and the control and monitoring of administrative and financial issues.

3) To harmonise actions, to keep the various activities of the project convergent with the central aim of final integration.

4) To review and assess project progress.

5 ModNanoTox: progress to date

The project started officially on November 1st 2011 and had a successful kick-off meeting at the Royal Society in London on December 1st and 2nd, 2011. The kick-off was joint with another modelling project NanoTransKinetics and was attended by representatives from all project partners from both projects, as well as a joint advisory committee the members of which are Rafi Korenstein (University of Tel Aviv), Francesc Giralt (Universitat Rovira i Virgili) and Tomasz Puzyn (University of Gdansk), the projects' PTA, Cedric Notredame, and PO, Nicolas Segebarth. Highlights from the kick-off include a joint decision of the two projects to share databases and resources (for example to continue joint meetings throughout their duration, share web space) and to work closely together to liaise with other existing large databasing initiatives in biological sciences (e.g. ELIXIR) to learn from and implement similar approaches.

6 Directory

Table 1 Directory of people involved in this project.

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Nanodetector

European Network on the Health and Environmental Impact of Nanomaterials

Contract Agreement: in negotiation Website: <http://www.nanodetector.eu.eu>
Coordinator: Vladimir Mirsky, Lausitz University of Applied Sciences, Senftenberg, Germany

No.	Beneficiary name	Short name	Country
1	Lausitz University of Applied Sciences - Hochschule Lausitz	LUAS	Germany
(2)	Leibniz-Institut für Analytische Wissenschaften – ISAS- e. V.	ISAS	Germany
3	Imperial College London	ICL	UK
4	Fraunhofer-Institute for Biomedical Engineering	IBMT	Germany
5	Hybrid Integrated Technologies (HIT) Ltd.	HIT	Czech. Rep.
6	Phasis	PHASIS	Switzerland
7	Mivitec GmbH	MIVITEC	Germany
8	Optolita	OPTOLITA	Lithuania
9	Upperton Ltd	UPPERTON	UK
10	MBN Nanomaterialia S.p.A.	MBN	Italy

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1 Summary

Project Duration: 1 May 2012 – 31 September 2015

Project Funding: 2.968 Mio. EUR

The project is based on the new experimental phenomenon discovered recently by a project partner: single subwavelength-size objects give rise to optical signals in surface plasmon resonance microscopy. This provides a unique possibility for ultrasensitive on-line detection of engineered nanoparticles.

Within the project a development of the device for detection of nanoparticles and its application for a number of practically important tasks will be performed. The work includes the development of theoretical description of the new effect, optimization of main components of plasmonic microscope, development of sophisticated software for effective image analyses and isolation of nanoparticle signals from background optical signals and noise.

Preliminary experiments demonstrated a possibility to use surface modification to distinguish different types of nanoparticles. This approach will be used in the project to identify of nanoparticles

and to achieve this, an array with different receptor groups will be formed, and pattern recognition algorithms will be applied.

Measurements will be performed in aqueous media as well as in air. Inorganic, plastic and protein nanoparticles will be examined. At the final step of the project monitoring of nanoparticles in simple and complicated probes as well as for monitoring of workplaces and waste during production of inorganic and protein nanoparticles and during aging of nanostructured materials will be performed.

2 Background

2.1. Plasmonic detection of single nanoparticles

A recent experimental discovery made by a project partner forms the basis for the novel approach to nanoparticle diagnostics proposed here. The proposed detector relies on exploiting an until



now unnoticed plasmonic effect that allows the detection and identification of single nanoparticles near metallic surfaces, and the determination of nanoparticle concentration in a sample.

The phenomenon of Surface Plasmon Resonance (SPR) determines the known effect of the attenuated total reflection of light from metal surfaces. In the well studied Kretschmann geometry for ATR experiments, the light travels through a prism which is bounded by a thin metal surface layer (typically gold), and is then reflected back from that layer (Fig. 1, left). Near to a certain resonance angle (for a given wavelength of light) the collective Eigen-modes, the surface waves of electron gas density in the film known as surface plasmons, are excited. This gives rise to a sharp minimum in the reflection curve (Fig. 1, right). The position of this minimum is sensitive to changes in the refractive indices of the materials of the prism, the external medium, and the metal layer. Species binding on the outer-side of the metal layer cause a change in the local dielectric properties of the system and hence a shift of the resonance position, this shift can be used for their detection and characterization. This effect is exploited in the well known SPR microscopy, frequently used in bioanalysis, e.g. for DNA/DNA, DNA/protein, protein/protein reactions. SPR microscopy applies imaging of the sensor surface onto a 2D detector (usually - CCD camera), allowing the examination of up to several thousands surface processes simultaneously.

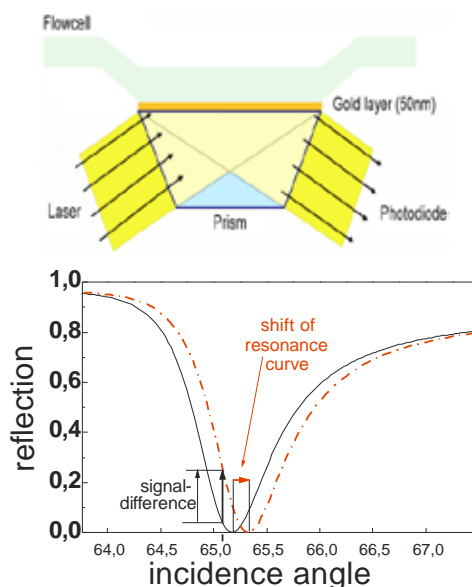


Fig. 1 The classical Kretschmann configuration mostly applied for SPR measurements in bioanalytical applications.

Limitations in the lateral resolution of SPR microscopy have been discussed since work in this area began. The large length of plasmon propagation is usually seen as an obstacle, limiting optical resolution to approximately 5 - 20 μm , preventing the observation of individual nanoparticles by this technique. However, it has been recently demonstrated by the project participant 2 that the plasmon penetration length in fact does not play the limiting role in detection of small particles bound to the surface. By precisely optimized SPR microscopy with digital subtraction of background it was demonstrated 1 that also particles of the size of few tens of nanometers can be observed. Nanoparticles are detected by SPR microscopy using CCD-camera as bright spots with a size much smaller than the plasmon propagation length (Fig. 2). A precise physical model for this effect forms part of the current proposal,

preliminary theoretical estimates suggest that these spots are caused by radiation of secondary plasmon waves around the particles. The spots are a few μm in size this is much larger than the particles that cause them. Illumination with a wavelength of 680 nm, allows dielectric particles with sizes ranging between 40 and 100nm to be observed.

The suggested physical principle can be illustrated by the analogy shown in Fig. 3: a hindrance (a rock in the sea) interacting with the surface waves results in formation of secondary circular waves. Subtraction of the background (surface waves) would reveal an image with concentric waves similar the images obtained from nanoparticles absorbed to the metal layer given in Fig. 2.

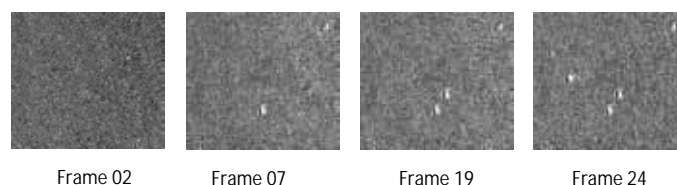


Fig. 2 Detection of 200-nm nanoparticles performed by SPR-microscopy.



Fig. 3. Illustration of the new method for detection of nanoparticles. The stone interacting with surface waves leads to the formation of secondary concentric waves. These waves can be detected with a spatial resolution which may be not enough to observe the stone itself.

This effect offers the potential new opportunities for measuring extremely low concentrations of nanoparticles. The feasibility and limitations of this method will be studied during this project. In particular, the project will focus on optimizing this new principle of visualization and characterization of single nanoparticles of different materials. Coating the gold surface with corresponding receptors will allow for selective recognition of nanoparticles.

The selectivity can be improved by deposition of receptor spots with different coatings; this principle, well known in artificial noses, provides a possibility to make selective measurements using even poorly selective (but different) individual receptors.

2.2 Quantification of nanoparticles

The new method has already been validated by measurements made using 200 nm polystyrene nanoparticles and protein particles. The particles were negatively charged; while the gold surface was coated by positively charged aluminium compound. The suspension was pumped continuously through the flow cell. The number of bound particles was counted during the

measurement interval. Measurements with the blank solution exhibited a negligible number of counts. The obtained dependence is shown in Fig. 4. The solid line is the linear fit of the data measured for 200 nm PS-nanoparticles. One can see that the slope of the line in the double-logarithmic scale is close to unity and the measured concentration dependence is well fitted by linear function over at least three orders of magnitude of particles concentration. At constant viscosity and flow the counting rate depends only on the particles size. This offers the opportunity for concentration measurements. This principle will be validated within the project and applied to quantification of nanoparticles in different samples.

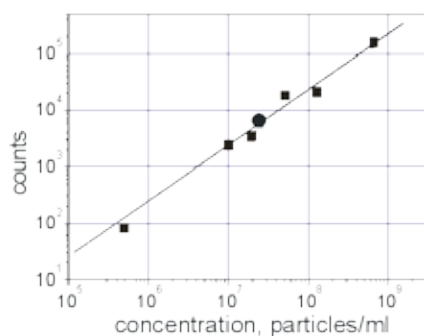


Fig. 4 Dependence of counts of nanoparticle signals on concentration of nanoparticles in the sample obtained for 200 nm polystyrene nanoparticles (squares) and for protein particles (circle).

2.3 Identification of nanoparticles

Identification of nanoparticles will be based on two principles: quantitative analysis of images from individual nanoparticles and modulation of nanoparticle-surface interaction.

Preliminary results demonstrate that the first approach provides information on size and effective refractive index of the material of nanoparticles. The second approach will be achieved by modification of the affinity properties of the receptor surface. Preliminary results have already demonstrated the potential of this approach for selective detection of biotinylated nanoparticles by streptavidin coated gold layer. Selectivity of the second approach can also be improved further by the application of sensor array and by use of pattern recognition algorithms currently applied in artificial noses and tongues.

3 Objectives of the project

The main objectives of the project are (1) the development and validation of technologies for the detection and analysis of ultra-low concentrations of both engineered nanoparticles (ENP) and nanoparticles of biological origin in different environments and (2) the construction of a laboratory prototype of the device based on this technology.

The scope of possible applications for this new technology spans the entire spectrum of the nanotechnology industry. To confirm this broad utility, the current project will test for the selective detection of a broad range of different engineered nanoparticles, including plastic, metallic, and oxide nanoparticles as well as

nanoparticles of biological origin. The technology will be tested in both liquid and gaseous environment.

Ultimately, the technology will be applied by the end user for monitoring of nanoparticles in the work place to monitor the production waste in the immediate environment surrounding production facilities. This should provide a real time detection of nanoparticles and issuing of warnings in the event of a release of nanoparticles into a wider environment.

The table below summarizes the main current methods for detection of nanoparticles. The methods are broadly divided into two categories according to the detection principle.

	Method	Drawbacks
integral methods	static or dynamic light scattering	low sensitivity; poorly compatible with qualitative analysis
	optical detection of plasmonic bands	low sensitivity; applicable only for plasmonic (gold or silver) nanoparticles
	using of labels (for example, radioactive, fluorescent)	applicable only for labelled nanoparticles
	surface enhanced Raman spectroscopy	requires a presence of Raman active moieties, applicable only for plasmonic nanoparticles
	photoacoustic imaging	low sensitivity, time consuming technique
	optical coherence tomography	low sensitivity
methods based on detection of single nanoparticles	electron microscopy	time consuming technique, requires probe preparation, cannot be realized as a portable device for field applications
	scanning probe microscopy (AFM, STM, etc)	time-consuming, sophisticated and expensive techniques, cannot be realized as a portable device for field applications
	using of labels	applicable only for labelled nanoparticles
	spectroscopy of single nanoparticles using localized plasmon resonance	applicable only for plasmonic nanoparticles (silver or gold), time consuming and complicated, cannot be realized for field applications
	light scattering microscopy	non selective methods, size measuring requires sophisticated signal processing
	chemosensitive transistors based on nanowires	sophisticated technique, very small sensor area
	waveguide based detection	small sensor area, low concentration sensitivity

The new technology proposed combines a number of unique features which cannot be provided by any other detection technology (see table below). During the project these possibilities will be developed and characterized more exactly.

Expected feature	Method of its realization
Extremely high levels of sensitivity, due to large sensor area, up to tens mm ²	The method is based on detection of single nanoparticles bound to the sensor surface
Quantitative information on nanoparticle concentrations	Large number of nanoparticles can be detected, which enables a statistical analysis of the frequency of binding
Information on the size of nanoparticles (if material is known)	Preliminary results demonstrate that it can be obtained from the intensity of image
Information on the refractive index of the material (if the size is known)	Preliminary tests demonstrate that the image intensity depends on the refractive index of the material
Information on chemical content of the surface of nanoparticles	Will be done using chemical receptors and/or variations of chemical groups on the surface. Selectivity will be enhanced by using of an array with different coatings.
Possibility of on-line detection in native surrounding	On-line detection is possible with appropriate software.
Continuous or (quasi)continuous operation mode	Even in the case of non-reversible adsorption, nanoparticles occupy only a very small part of the surface, therefore continuous operation can be realized by simple subtraction of the background.



4 Workplan

The realization of the project concept is divided into 11 work packages (WP). The first work package (WP1) is focused on the project management while the last (WP11) describes dissemination and exploitation of the project results.

The goal of WP2 is the development of theory of the recently observed phenomenon which will form a scientific base of the present proposal and formulation of ideas for new experiments. The most reliable theory will be a direct solution of Maxwell's equations of electrodynamics with a spherical particle located at the interface of the thin-metal film and semi-infinite dielectric in the classic Kretschmann ATR geometry. Once developed, this theory will be applicable to any material of the nanoparticle, described through its frequency dependent dielectric permittivity $\epsilon(\omega)$. Systematic investigations will focus on: (i) the effect of the material of nanoparticles (incorporated through its complex permittivity), their size and shape; (ii) the effect of the surface concentration of nanoparticles; (iii) the effect of the structure of the nanoparticles layer (lattice symmetry, clustering and such). Additionally the thickness of the metal resonant layer and an influence of electrochemical conditions for the experiments in electrolytes will be tested.

The WP3 and WPs 5 - 9 form a block of work that will be focused on the development of the initial and subsequently the final laboratory prototype of the device for ultrasensitive detection, quantification and identification of nanoparticles. The completion of these WP's will result in the fabrication of 8 pieces of the final laboratory prototype device.

The WP3 includes optimization of optics, resonant layers and measurement approach. The concept of bimetallic layers, suggested few years ago by one project partner, will also be tested. The resonance quality depends on the exactness the layer thickness as well as on roughness and homogeneity of the gold layer. This concept will be improved by optimization of metal deposition conditions. The results obtained from the theoretical analysis will be used to further improve the measurement technique. It can also be expected that analysis of angle or wavelength dependencies will enable the uncoupling of the influence of the size and refractive index on the image of nanoparticles.

The WP4 focuses on the development of coatings with affinity to defined types of nanoparticles. The coating will be based on self-assembly of θ -modified alkylthiols on the gold surface and on the use of photografting. These θ -groups will operate as receptors for nanoparticles. As an alternative, immobilization of carboxy-modified alkylthiols and subsequent immobilization of receptor groups on the carboxy-groups will also be used.

If a stronger binding is required, rough surfaces formed by immobilization of polymers on the gold will be considered; the receptor groups will be subsequently immobilized on this polymer or pre-synthesized polymers with corresponding receptors will be applied.

An improvement of selectivity will be reached by combination of sensors into array and by application of algorithms for pattern recognition (WP7). It is expected that an application of sensor array would give an effective mechanism for chemical analysis of

main classes of inorganic nanoparticles. Selective detection of protein-based nanoparticles will be performed using corresponding antibodies as receptors.

The system optimized within WP3 as initial laboratory prototype will be further developed to form the "final laboratory prototype". This will be done within WP5 (optomechanical components), WP6 (electronics, fluidics and controlling software) and WP9 (integration). The final laboratory prototype will be designed, optimized and produced as 8 devices.

The final laboratory prototype should be close to the next version of the device – it will be a prototype of the commercial device, however the development of a commercial device will demand significant resources and its development is not included in the project goals.

An improvement of measurement technology requires the development of image analysis software and this will be done within WP7. Different approaches for signal filtration and for recognition of images of nanoparticles will be tested. In addition to a simple statistical approach, background subtraction and filtrations, mathematical approaches with the aim of isolation of defined diffraction images (calculated in the WP2) in the stream of information from CCD- (or CMOS) camera will also be used. The software will also be used to count the adsorbed nanoparticles and for quantitative characterization of their images. This data can subsequently be used to generate information on the nanoparticle size and refractive index. Another goal of the WP7 is the development of software for identification of nanoparticles by sensor arrays. It will be performed using known approaches for pattern recognition, such as principal component analysis or neuronal networks.

The detection performance depends strongly on the quality of resonance layer. The goal of the WP8 is to develop resonant layers providing minimal rest signal in resonance, possible sharp resonance and minimal surface roughness. Another task of this WP is a development of inexpensive polymer prisms combined with the resonance layer. Success in WP8 will result in the simplification of the detection technique, making it easier for the operator to handle and reducing the requirement for operator training. Success in WP8 will be also important for the ability of the detector to detect/measure dangerous nanomaterials (eg highly toxic, radioactive, viruses, etc.) that will require disposable components within the measurement probes.

Although one can foresee a vast number of applications of the new technology, its complete potential is yet to be explored. The utility of the technology will be evaluated within WP10. Measurements will be performed using final laboratory prototypes in the laboratories and production facilities of the end users. The work will include a validation of the system performance and its application for detection and analysis of nanoparticles in drinking water and in non-colloidal drinks, for investigation of aging of nanostructured materials, for monitoring of work places during production of nanoparticles, for analysis and quality control of produced nanoparticles.

5 Directory

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NANODEVICE

Novel Concepts, Methods, and Technologies for the Production of Portable, Easy-to-Use Devices for the Measurement and Analysis of Airborne Engineered Nanoparticles in Workplace Air



Contract Agreement: Website: <http://www.nano-device.eu>

Coordinator: Professor Kai Savolainen, Finnish Institute of Occupational Health

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3	HEALTH AND SAFETY EXECUTIVE	HSE.HSL	UK
4	NEDERLANDSE ORGANISATIE VOOR TOEGEPAST NATUURWETENSCHAPPELIJK ONDERZOEK - TNO	TNO	Netherlands
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6	INSTITUT FÜR ENERGIE UND UMWELTTECHNIK EV - IUTA	IUTA	Germany
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13	NANEUM LIMITED	NANEUM	UK
14	TSI GmbH	TSI	Germany
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16	BUNDESANSTALT FUER ARBEITSSCHUTZ UND ARBEITSMEDIZIN	BAuA	Germany
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19	UNIVERSITY OF EASTERN FINLAND	UEF	Finland



20	DANMARKS TEKNISKE UNIVERSITET	DTU	Denmark
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1 Introduction

The motive of the NANODEVICE project is based on the lack of knowledge of the health effects of the widely used engineered nanoparticles (ENP) and on the shortage of field-worthy, cost-effective ways - especially in real time - for reliable assessment of exposure levels to ENP in workplace air.

2 Project summary

Due to their unique properties, engineered nanoparticles (ENP) are now used for a myriad of novel applications with great economic and technological importance. However, some of these properties, especially their surface reactivity, have raised health concerns, which have prompted scientists, regulators, and industry to seek consensus protocols for their safe production and use of the different forms of ENP.

There is currently a shortage of field-worthy, cost-effective ways - especially in real time - for reliable assessment of exposure levels to ENP in workplace air. In addition to the problems with the size distribution, a major uncertainty in the safety assessment of airborne ENP arises from the lack of knowledge of their physical and chemical properties, and their levels of exposure. A special challenge of ENP monitoring is to separate ubiquitous background nanoparticles from different sources from the ENP.

Here the main project goal is to develop innovative concepts and reliable methods for characterizing ENP in workplace air

with novel, portable and easy-to-use devices suitable for workplaces.

Additional research objectives are:

- 1) identification of relevant physico-chemical properties and metrics of airborne ENP, establishment of reference materials
- 2) exploring the association between physico-chemical and toxicological properties of ENP
- 3) analyzing industrial processes as a source of ENP in workplace air
- 4) developing methods for calibration and testing of the novel devices in real and simulated exposure situations
- 5) dissemination of the research results to promote the safe use of ENP through guidance, standards and education, implementing of safety objectives in ENP production and handling, and promotion of safety related collaborations through an international nanosafety forum.

3 Scientific and technological objectives of the NANODEVICE project

Engineered nanoparticles (ENP), defined as having at least one dimension ≤ 100 nm, have attracted a great deal of interest during recent years, due to their many technologically interesting properties. The unique properties of ENP and their applications have given birth to immense technological and economic expectations for industries using ENP. However, some of these properties have given rise to concern that they may be harmful to humans. This has prompted scientists, regulators, and the industrial representatives to investigate the features of



ENP in order to be sure of their safe use in nanotechnologies (NT), i.e. technologies utilizing ENP. The European Commission has also explored in-depth the characteristics of ENP and issued a document on ways to assure the safety of ENP.

Overall objectives of the research: New and innovative concepts and methods for measuring and characterizing airborne ENP with novel, portable and easy-to-use device(s) for workplaces.

4 Summary preliminary results

4.1 Devices

17 new devices in 4 device families and 3 feasibility studies will be developed in the project. The 4 families are:

1. Total or size specific N-S-M concentration in real time
2. Material specific monitors in real-time or quasi-real-time
3. Samplers for off-line particle analysis
4. Preseparator modules for size fractions relevant to the human respiratory tract (modular components)

4.1.1 Total or size specific N-S-M concentration in real time

Specimens of the devices in the first family are presented in figures. 1-3.

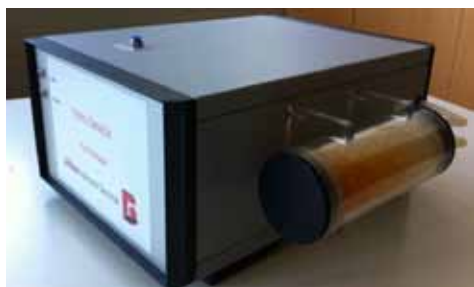


Figure 1. Electrical and optical (pre-)prototype to measure particle size distribution, total number concentration, surface and volume distribution, total surface and total mass fractions. Developed by GRIMM



Figure 2. Pre-prototype instrument to measure particle size developed by DEKATI and TUT



Figure 3. Standard instrument for measuring particle size distributions developed by IUTA

4.1.2 Material specific monitors in real-time or quasi-real-time

Specimens of the devices in the second family are presented in Figure 4 and Figure 5.



Figure 4. CNT detector developed by NANEUM

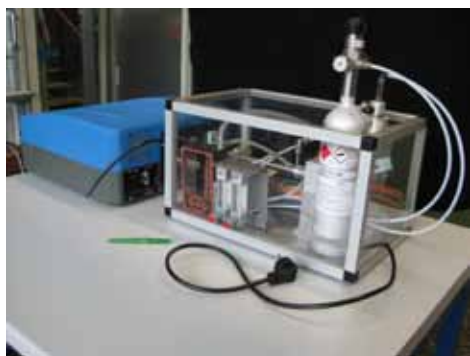


Figure 5. The Catalytic Activity Aerosol Monitor (CAAM) developed by Karlsruhe Institute of Technology

4.1.3 Samplers for off-line particle analysis

Specimens of devices in the third family are presented in Figure 6 and Figure 7.



Figure 6. Gas-exchange region (GE modular) pre-separator developed by University of Lund



Figure 7. Extra-thoracic region (ETI) modular pre-separator developed by University of Lund

4.1.4 Preseparator modules for size fractions relevant to the human respiratory tract (modular components)

Specimen of device in the fourth family is presented in Figure 8.



Figure 8. Pre-prototype CNT specific sampler by Fraunhofer IPA.

4.2 Identification of relevant physico-chemical properties and metrics of airborne ENP, establishment of reference materials

SWCNT floating catalyst synthesis reactor has been designed, built and used to generate fresh SWCNT aerosols for the project's needs (Figure 9)

The metal oxide flame reactor for nanoparticle generation was finalized and now operational, the generated powder is collected and the particles are being analysed.

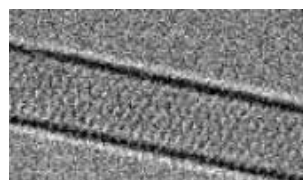


Figure 9. TEM image of a CNT tube sample

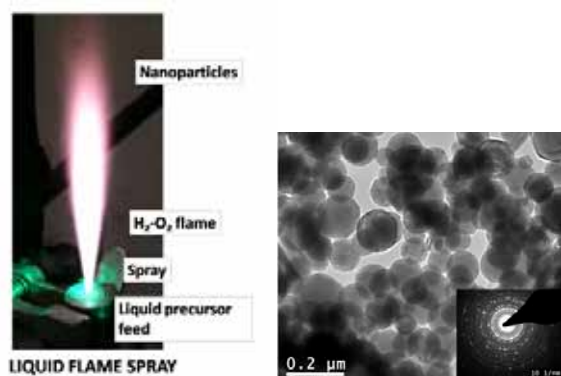


Figure 10. The basics of the metal oxide flame reactor (left) and TEM image of generated metal oxide particles (right)

4.3 Exploring the association between physico-chemical and toxicological properties of ENP

The Cytotoxicity (cell death, ATP-level, Cell cycle delay and oxidative stress), genotoxicity (DNA damage, micronuclei), and immunotoxicity (Pro-inflammatory reactions in cell models) of engineered nanoparticles have been studied and a databank is created for the results and it is available. The results have been published in journals and in scientific meetings. Images of some samples are presented in Figure 11.

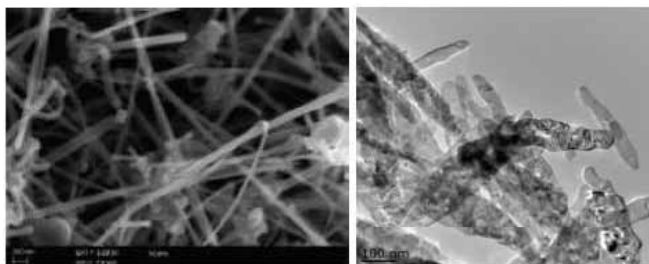


Figure 11. SEM image of a sample of Mitsui MWCNT (left) and TEM image of a sample of Fe₂O₃-fiber (right)

4.4 Analyzing industrial processes as a source of ENP in workplace air

Experimental studies of source characterization, agglomeration and ventilation have been conducted and a data analysis on them has been completed. Computational Fluid Dynamic (CFD) modelling has been achieved with scenario building completed and simulation runs conducted.

4.5 Developing methods for calibration and testing of the novel devices in real and simulated exposure situations

A calibration tool "Characterization of Aerosol Instrumentation devoted for Measuring Aerosols of Nanoparticles (CAIMAN)" has been extensively used by the device developers in the project.

A Nanotest facility has been validated with established aerosol measurement technology concerning ranges of particle sizes and concentrations as well as mixtures of particles. The first prototypes have been tested with defined aerosols. This was done in the scope of a second, larger round robin test ("stress test") under real conditions compared to those expected in the further course of the NANODEVICE project. The Nanotest facility has been extensively used by device developers and a third, and final; round robin tests are yet expected. A pilot study shall be provided for the device developers on the test results.

4.6 Dissemination of the results

The project's website <http://www.nano-device.eu> is a communication channel to communities. A brochure introducing the project in a nutshell has been carefully developed and distributed (Figure 12). Multiple poster presentations have been held in several conferences and scientific meetings on the results of the project (Figure 13). The

NANODEVICE project peaks at organizing the SENN2012 Congress Oct. 28-31, 2012 in Helsinki, Finland (Figure 14).



Figure 12. Image of the project website (left) and the cover of the brochure (right)

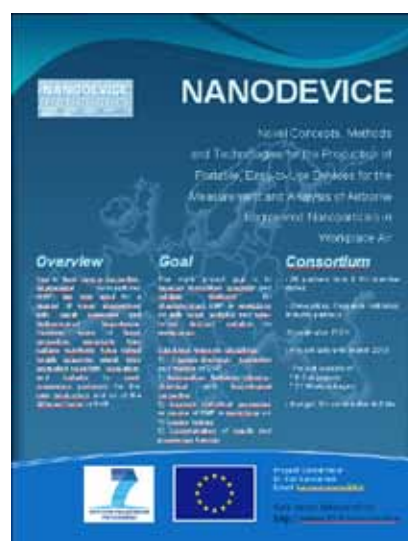


Figure 13. The project poster



Figure 14. International Congress on Safety of Engineered Nanoparticles and Nanotechnologies



5 Conclusions

NANODEVICE will provide new information on the physico-chemical properties of engineered nanoparticles (ENPs) and information about their toxicology. Also a novel measuring device will be developed to assess the exposure to ENPs from workplace air. The purpose of the project is also to promote the safe use of ENPs through guidance, standards and education, implementing of safety objectives in ENP production and

handling, and promotion of safety related collaborations through an international nanosafety forum.

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NanoFATE

Nanoparticle Fate Assessment and Toxicity in the Environment



Contract Agreement: NMP4-SL-2010-247739 Website: <http://www.nanofate.eu>
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5	Faust & Backhaus	F+B	Germany
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1 Summary

Concept: NanoFATE has been conceived to fill knowledge and methodological gaps currently impeding sound assessment of environmental risks posed by engineered nanoparticles (ENPs). Our vision is to assess environmental ENP fate and risk in for example high-volume products for which recycling is not an option, namely; fuel additives, polishing agents, personal care products and antibacterial products. To represent these products two commercial ENPs of CeO₂, ZnO and Ag (of varying size, surface and core chemistries) will be followed through their post-production life cycles, i.e. from environmental entry as "spent product", through waste treatment to their final fates and potential toxic effects. This will test the applicability of current fate and risk assessment methods and identify

improvements required for assessment of ENPs at an early stage.

Objectives: Delivery of a systematic study of the environmental fate and toxicity of selected ENPs will entail addressing nine S&T objectives:

- Design, tagging and manufacture of ENPs
- Analysis of ENP interactions with abiotic and biotic entities
- Generating predictive models for ENP exposure in waters and sludge-amended soils



- Studying the fate and behaviour of ENPs through wastewater treatment
- Determining acute and chronic ecotoxicity
- Assessing effects of physico-chemical properties on ENP bioavailability
- Defining mechanisms of uptake, internal trafficking, and toxicity
- Developing spatial RA model(s)
- Improving understanding of ENP risks

Methodology: The work plan is designed to deliver research and progress beyond the state-of-the-art. While some objectives are focused in single WPs, others are cross-cutting, so ensuring the integration of the work plan to support delivery of novel ENP risk quantification methods.

Impact: NanoFATE will provide robust tools, techniques and knowledge needed by stakeholders to understand and communicate risks associated with ENPs of different physical or chemical properties, including their environmental interactions and toxicity.

Keywords: Nano, fate, exposure, bioavailability, uptake, toxicity, risk, environmental.

2 The NanoFATE aim and focus

NanoFATE focuses on developing a systematic understanding of fate and mechanisms of effects in a core set of ENPs and addressing how these may affect the application of current tools for ecological risk assessment. The fact that the ENPs we will study are associated with commonly and widely used products provides environmental and economic relevance to our work. Furthermore, the selected ENPs will each have different core and surface chemistry and physical properties. This will allow us to elaborate on current understanding of how ENP properties influence fate and behaviour in the environment, and their potential toxicity. This will be achieved by systematically studying aspects that are related to fate and toxicity, and seeking to refine risk assessment practices for use with ENPs, leading to the nine NanoFATE objectives detailed below (3.2)

3 How NanoFATE will improve the State-of-the-art for environmental fate and effects of ENPs

3.1 Background

The potential human health effects of ENPs are of obvious importance and a review of European research and national programs indicates that a number of ongoing projects are already addressing this issue (e.g. NANOTOX, CELLNANOTOX, IMPART, NANOSH, NanoReTox). In distinct contrast, there are as yet few studies that have focused on developing and refining methods to assess the fate of ENPs in ecosystems (e.g. soils and natural waters) and any resulting ecotoxicological effects. For this reason NanoFATE intends to focus on these neglected aspects and their integration.

To support the responsible development of the nanotechnology sector, it must be recognised that the development of environmental risk assessment methods should not lag too far behind those for human health. Past experiences highlight a number of other environmental issues such as organochlorine pesticide usage (Newton and Wyllie 1992; Newton et al. 1999; Sibly et al. 2000), endocrine disruptions (Jobling et al. 1998; Tyler et al. 1998), secondary effects of pharmaceuticals on wildlife (Oaks et al. 2004), and genetic modification (Houghton et al. 2003; Heard et al. 2003), where environmental impacts rather than direct effects on human health, emerged as the major area of concern. In each of these cases, the unexpected nature of these effects had a profound affect on public confidence in new technologies. This required that rapid regulatory action was put in place to control and mitigate risks. By ignoring effects on the environment, nanotechnology runs the risk that similar damaging and costly effects could occur.

Because of the initial and wholly understandable focus on direct risk to human health, knowledge of fundamental aspects of the environmental risks associated with ENPs is low in several key areas. These include:

- the post-production fate of ENPs from entry into the environment to final residence;
- how ENP-ENP and environmental interactions affect the biotic availability of ENPs and how different ENP properties (size, surface) affect exposure/uptake;
- how crucial ENP properties such as size distribution, surface chemistry, shape and optical properties influence toxicity;
- chronic aspects of ecotoxicity, which to date has mainly been assessed at environmentally unrealistic concentrations or in inconclusive studies where it was uncertain whether the co-solvent used for dispersal, impurities, or the ENP itself resulted in the observed toxic effect;
- the mechanisms of toxicity of ENPs when compared to the bulk chemical or free metal ion and how observed effects of ENPs on the expression of genes



or proteins associated with particular pathways (e.g. such as oxidative stress in cell lines) relate to higher level *in vivo* effects;

- the fitness for purpose of existing risk assessment approaches designed for standard chemicals for use with ENPs and the modifications needed to allow existing frameworks and policies to be used in future for the risk assessments of nanotechnology products.

By studying the fate and behaviour of the selected ENPs and their effects on biota, NanoFATE will go beyond the superficial initial assessments that have been possible so far, thereby enabling a scientifically rigorous analysis in relation to each of the above aspects. The data gained in meeting each of the nine NanoFATE objectives will allow us to go beyond the current state-of-the-art as set out in the section below.

3.2 Current baseline of knowledge and points where NanoFATE will progress beyond the state-of-the-art in meeting project objectives.

Obj.1: Design and manufacture of tagged ENPs for tracking in fate and toxicity studies.

Baseline. Differentiation of ENPs from the natural background has been a critical problem in understanding their fate in complex environmental systems. Even though some of the ENP core metals have low concentrations in the environment (Ce and to an extent Ag), approaches beyond simple elemental analysis using ICP-MS based methods are needed to study the partition process that determine the final destiny of ENPs. Furthermore, some types of labelled nano-sized particles (e.g. fluorescent silica NP) that have been used to track fate in the environment often lack the physical characteristics of production ENPs and so can not be expected to behave in a similar way to commercial ENPs. As a result, specifically designed ENPs that can mimic commercial particles, are needed to support the fate and effects work conducted in WP 2, WP3, WP 4 and WP 5.

NanoFATE progression beyond the “state-of-the-art”. To undertake realistic real world fate studies, NanoFATE will design and fabricate ENPs “tagged” with selected ions that are detectable in bulk samples that will offer real advantages over the current state-of-the-art. ENPs tagged with ions of low background in the environment can under ideal conditions be detected by elemental analysis. Further, using cathodoluminescence spectroscopy it will be possible to detect the nanoparticles in small samples and investigate their degree of aggregation. Since the tagged ions will be inside the particles, they do not affect their behaviour and are also protected from chemical attack in the environment, hence preserving the tag:ENP ratios.

To provide tagged particles for use in NanoFATE, partners, IHPP, UOXF.DJ and UGOT will work together to identify any available uniquely identifiable ENPs suitable for off the shelf use that are relevant to the three product groups and incorporate particle types considered in NanoFATE. Where suitable tagged ENPs are

not available, these will be synthesised by IHPP with input from UOXF.DJ. These two partners have particular experience in ENP design, production and characterisation. Acquisition or production of the tagged ENPs will be done with consideration to match the properties of the two variant ENPs of each type selected for NanoFATE. Studies will be conducted to validate the ability to track designed tagged ENPs within sewage treatment systems, environmental media and organisms. The resulting information will help the design of the targeted studies in WP 2 and WP 5 that will address these issues in detail. The detailed work to be conducted to meet this objective is set-out below.

1. NT and AXME will allow access to existing ENPs that are currently used commercially in our target product types (diesel additives, cosmetics, antimicrobial surfaces and products). These partners will also provide information on particle properties and characteristics to support detailed experimentation, to establish how closely tagged particles generated in our project match these commercially available ENPs.
2. IHPP will use their solvothermal process, in which a mixture of chemicals soluble in a water-ethanol mixture is enclosed in a pressure vessel and heated using microwaves to nearly supercritical conditions, to produce rare earth metal-tagged nanoparticles in volumes that can be supplied to all partners (Lojkowski, 2008; Cabanas et al., 2007). This manufacturing method allows ENPs of different core chemistries, sizes and coatings to be produced, with none of the disadvantages (poor ion concentration control, particle aggregation) associated with gas phase or wet chemical synthesis. Initial product particle characterisation (surface bonds, zeta potential, surface charge and particle size) will be undertaken.
3. UOXF.DJ will lead particle characterisation, measuring surface bonds, zeta potential and light scattering of ENPs will be determined by combinations of X-ray diffraction, electron microscopy, infra-red and Raman spectroscopy and dynamic light scattering to provide measurements of surface charge and particle size. When studies include work focusing on properties in environmental media, UOXF.DJ and UGOT will collaborate.
4. UGOT will refine Flow Field-Flow Fractionation with high resolution ICP-MS (FLFFF-HR-ICP-MS), and if needed other *in situ* trace techniques (Stolpe and Hassellöv 2007), for detecting the interactions of the selected sets of tagged and untagged particles with environmental colloids in order to establish the methods for later detailed work targeted in Obj 3 that will be conducted in WP 2.

Obj.2: Generate models for predicting the likely levels and states of ENPs in receiving waters and soils.

Baseline. Current publicly available databases provide information on the use of ENPs within nanotechnology products (e.g. Project on Emerging Nanotechnologies) and this in turn provides information on the magnitude and nature of potential sources of ENP released into the environment. This identification of sources within consumer products has allowed

initial risk assessments to be conducted to predict the potential levels of ENPs that may occur in environmental media at assumed levels of marker penetration. Combining the data with existing effects data has allowed initial estimates of potential risk to be conducted (Boxall et al 2007). So far, however, work to validate a number of the assumptions within these model predictions have yet to be tested and validated. These include the extent of potential market penetration of nanotechnology products, release rates of ENPs from products, how patterns of seasonal usage will influence concentrations reaching the environment under different scenarios, and the potential impact of the heterogeneous distribution of sources on realised environmental concentrations.

NanoFATE progression beyond the “state-of-the-art”. To improve the current state of spatial and temporal exposure assessments, NanoFATE will, as a first step, compile source inventories and from this data derive plausible future scenarios of release (including median and extreme predictions) for the selected nanotechnology products and associated ENPs. This will be done through a stakeholder consultation led by F+B and involving NanoFATE’s nanotechnology sector partners NT and AXME and other amenable companies. Additionally, information on the development of the nanotechnology field provided by other EU projects, within publications highlighted in the ICPCNANONET EU funded database and through the Inventory of Nanotechnology-Based Consumer Products Currently on the Market (<http://www.nanotechproject.org/inventories/consumer/>) will also be utilised.

In addition to acquiring usage information, industrial information on ENP usage rates in products and ENP properties associated with our focus products and information on release rates and states will be collated. This information will include data on particle sizes of CeO₂ associated with diesel exhaust fumes, ZnO concentrations and release from sunscreens, Ag loss from impregnated material during washing etc. This data will be used to support release scenario development. Initially, environmental concentrations of all the ENPs will be modelled with the current standard multi-media model, EUSES, based on the relevant release pathways addressed. This is important as it will allow linkage of the project’s results with ongoing work on how ENPs can be adequately addressed within the REACH framework.

The developed release scenarios will provide a starting point for further modelling of the potential fate of ENPs in the environment using state-of-the-art approaches. This will allow a refinement of calculation of environmental concentrations and states of ENPs reaching particular environmental compartments. For modelling wastewater release for assessment of the fate of ENP, the process of disposal is visualised according to the schematic shown in Fig. 1. Modelling of deposition to soil will be the focus for CeO₂. Initial predictions will be generated based on worst case conditions.

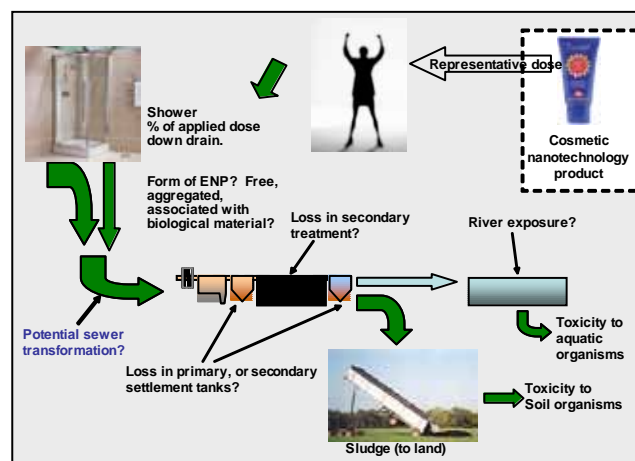


Fig. 1. Schematic illustrating key issues concerning the disposal, fate and environmental release pathways of an example “down the drain” nanotechnology product (e.g. ZnO ENP containing sunscreen).

This includes for example, assumptions of complete release from products, no removal during waste treatment, long persistence of ENP as free particles, and high traffic volumes. Since these are clearly unrealistic, predicted environmental concentrations will be iteratively refined to include information on fate available in the literature and also from model system studies, such as those on ENP removal efficiency in sewage treatment works and fate in WP 2. This takes us beyond what has been done to date either with “unit world” type fugacity models, or with simple dilution factor models for ENPs supporting prediction of multimedia fate and exposure (Hollander et al., 2006; Sumpter et al., 2006; Hollander et al., 2007). For modelling of environmental concentrations in different compartments for our set of six ENPs under different usage scenarios, simulation approaches relevant to each release pathway will be used.

1. For CeO₂ the assessment will focus on direct deposition of particles to soil. This work will be conducted using air dispersion modelling tools available within the Cambridge Environmental Research Consultants ADMS modelling suite. During model derivation, the ADMS model will be used to provide geospatial predictions of CeO₂ concentrations in air and deposition to soil surface in relation to rates of traffic flow. Information for air will be useful for human health assessment and so will be made available to human health focused projects. Within NanoFATE, the information on deposition will be used to calculate concentrations in soil based on simple assumptions regarding distribution through only the top 5 cm of the receiving soil surface. This is based on well established knowledge of metal deposition and distribution in soils subject to particulate metal deposition from smelter stacks (e.g. Martin et al 1983) (NERC, F+B).
2. For both ZnO and Ag ENPs the major route of release to the environment is likely to be through the wastewater stream. A simple wastewater process model for each ENP will be developed to predict quantities going to effluent, or



sludge. Information on rates of sludge application to soils across Europe will be used to estimate concentrations reached via this route. For that which partitions into effluent, realistic water levels will be modelled using the GIS water quality model LF2000-WQX Wales (Williams et al., 2009). Predicted environmental concentrations (PECs) will be generated for a representative set of river catchments in the Thames, Midland and Anglia regions of the UK, which are known to have the least dilution of sewage effluent across the UK. These catchment scenarios will be compared with catchments across Europe in the GREAT-ER model. The model will be driven by consumption and discharge values together with wastewater fate. With its underlying database of wastewater treatment plants (location, size and flow) together with river hydrological data (all discharges, abstractions and natural flow), the LF2000-WQX model provides unparalleled ability to predict concentrations that may reach real environments (NERC, F+B).

The predicted environmental concentrations in different compartments derived from the modelling work for our selected ENPs under different usage scenarios will be used in the project both to inform the design of toxicity studies in WP 3, WP 4 and WP 5 and as input into spatially explicit risk assessment models in WP 6.

Obj.3: Analyse ENP interactions with environmental and biological entities using advanced microscope and physical analysis.

Baseline. NanoSafe II (FP6 - which involved NanoFATE partners) has defined the current state-of-the-art for characterising and measuring ENP interactions with each other and with different biological model environments. The project used industrially supplied ENPs in model systems (e.g. cells) to determine their toxicities and demonstrated that understanding the shape and composition of ENPs and how they behave in different media is critical to understanding their potential toxicity (NanoSAFEII, 2008). Currently a major barrier to extending this work to more complex environments is the ability to differentiate ENPs from naturally occurring NPs or clusters.

NanoFATE progression beyond the “state-of-the-art”. The NanoFATE consortium will address ENP interactions with environmental and biological systems by specifically mobilising the expertise of researchers with extensive experience of preparing real environmental and biotic samples for analysis of the interactions of ENPs with, for example, natural colloids and bacterial cells in wastewater and soil pore water. A range of the advanced techniques suitable for detection of the commercially available and bespoke manufactured and doped ENPs will be used for the specific studies in NanoFATE. These will allow NanoFATE researchers to track the interaction of particle with colloidal and particulate matter, since these interactions are important determinants of particle bioavailability. Methods that also allow determination of the uptake and localisation of ENPs within prokaryotic and eukaryotic organisms will also be utilised. The major techniques that will be used in the studies in NanoFATE are as follows:

1. Raman microscopy for the detection of ENP behaviour both in waste water systems and in biological entities including the internalisation of particles in prokaryotic and eukaryotic organisms (Huang et al., 2004; Singer et al., 2005) (UOXF.DJ, NERC);
2. Light, X-ray and neutron scattering spectroscopy for detection of ENP-ENP and ENP-colloidal interactions in waters and assessing the role played by colloids in facilitating particle aggregation in waste and surface waters (Jarvie and King, 2007) (NERC);
3. Electron microscopy techniques such as scanning Electron Microscopy (coupled with Energy-Dispersive X-ray analysis (ESEM-EDX) and Transmission Electron Microscopy (TEM-EDX) and Energy Dispersive X-ray analysis for visualisation of ENP interactions with environmental media, aquatic colloids and biological entities in support of assessment of ENP bioavailability in soil and water systems and the detection and localisation of internalised ENP in organisms (CU, UOXF.DJ)
4. Matrix Assisted Laser Desorption/Ionization (MALDI)-Imaging mass spectrometry for detection of surface interactions of ENP with particulate matter and possibly also image of tissues for metal ENPs inclusions (UOXF.DJ, UNIPMN).
5. Flow Field-Flow Fractionation with high resolution ICP-MS (FLFFF-HR-ICP-MS) including use of a new detection mode. This detection method, called single particle ICPMS, built on an ultra fast (<1ms) scanning of the elemental signal for a single element of interest. For most of the time there is no signal during the short acquisitions but when there is a nanoparticle which homogeneously consists of the element of interest then there is a high signal spike. For dilute samples this method enables detection of single nanoparticles, and quantification of the number of nanoparticles by counting the number of spikes. The method has been successfully used as a stand-alone screening method for filtered samples and as a detection mode after FFF to derive number based size distributions. This method has been used for detection of metal ENPs in Gothenburg wastewater treatment plant effluent (UGOT).

The use of fluorescence labelling and detection by fluorescence microscopy is not at the present time a feasible option for ENPs relevant to the types that NanoFATE will focus upon. Work outside NanoFATE, using approaches such as incorporating a rhodamine dye in the silica shell of certain ENPs may provide new approaches for fluorescence detection and subsequently valuable information in due course. Such developments will be monitored by the NanoFATE consortium and exploited should they provide new methods that are an improvement over the developments made within NanoFATE.

Meeting this objective will allow us to study interactions through the post production life cycle of ENPs, and simultaneously assess how the properties of ENPs may change over their environmental lifecycle. The data obtained in these studies will be used to inform the design of studies that are



intended to track ENP fate during wastewater treatment process or following the deposition of diffuse ENP directly to soil ecosystems in WP 2.

Obj.4: Study ENP fate and behaviour through wastewater treatment processes and in soils.

Baseline. Published studies on the environmental fate of oxide NPs have focused mainly on transport through porous media (groundwater/soils) and will be useful to an extent in NanoFATE. Despite the fact that wastewater discharges provide a major route for emissions of oxide NPs in cosmetic/personal care products to the environment, there has been very little attention focused on their fate during wastewater treatment (Chang et al., 2007). Clearly such studies are vital to frame environmental hazard and risk.

NanoFATE progression beyond the “state-of-the-art”. NanoFATE will improve current understanding in relation to ENP behaviour during wastewater treatment by providing the following information relating to ENPs post release fate that will support predictions of ENP concentrations delivered to waters via discharges and to soil via sludge disposal.

1. Examination of the colloidal behaviour of ENPs in *real* wastewater matrices using small angle neutron scattering to directly quantify, in real time, ENP partitioning during primary (settlement) treatment, between (i) non-settleable constituents which continue through the effluent stream to secondary treatment, and (ii) sewage sludge which settles out within typical residence times of approximately 2 – 6 hours in primary settlement tanks (NERC, UGOT).
2. Distribution of tagged ENPs in flow-through test reactors installed at a UK sewage works and using real activated sludge feed. Analysis of the aqueous and solid phases for the tagged ENP would be done by ICP-MS and fluorescence or SQUID magnetometry (IHPP, NERC).
3. Use of scanning and transmission electron microscopy and dynamic light scattering techniques to measure changes in aggregate size, shape and fractal dimension of ENPs to characterise the nature and mechanisms of ENP flocculation during wastewater treatment (UOXF.DJ). Also IHPP has excellent field emission scanning microscope Leo1530 that could be employed here.
4. Use of scanning and transmission electron microscopy and nanoparticle visualisation techniques (e.g. NanoSight) to measure changes in ENP size and aggregation in different soil pore water and wastewater extracts to provide estimates of ENP dissolution rates (UOXF.DJ, UGOT).

The data derived from the studies conducted above will be used to refine the estimates of exposure conducted in the risk assessment phase of the project. Additionally, the data on dissolution rates will be used to support later detailed measurements of ENP bioavailability as particles or as free, colloidal bound forms during ecotoxicity testing in studies conducted in different environmental media in WP 4.

Obj.5: Determine the chronic toxicity of ENPs of different properties, including co-exposures with other stressors (e.g. UV and combustion derived pollutants).

Baseline. To date, published data concerning the effects of ENP *in vivo* are principally restricted to acute toxicity tests (Handy et al 2008; Luoma 2008). Chronic toxicity data are mostly lacking. Furthermore, since the available studies each used a different ENP with different characteristic, it is difficult to compare these data directly. Another issue that is often highlighted (Royal Commission on Environmental Pollution, 2008; Luoma 2008), but to date remains poorly investigated is that of co-exposure of ENP with other pollutants and/or environmental stressors. Both have the potential to lead to greater than additive effects through processes, such as facilitating pollutant transport by ENPs (AKA piggybacking) and ROS generation (Baun et al. 2008).

NanoFATE progression beyond the “state-of-the-art”. The knowledge gaps concerning ENP effects highlighted above indicate the pressing need to provide more detailed information on aspects of ENP toxicity. These include issues such as the relative sensitivities of species, acute-to-chronic ratios, the effects of ENP properties on toxicity, and the interactive effects of ENP with other co-stressors. NanoFATE will deliver such information by the following studies.

1. Literature review of data on ENP ecotoxicity for aquatic and terrestrial species. This will include information of the characteristics of the particles used for testing, the physicochemical properties of the test medium and the nature of the dose response relationship for different endpoints. The data set will be enhanced by our own studies of chronic toxicity on our selected set of ENPs in species from both aquatic (microorganisms as biofilm communities, algae, *Daphnia*, mussel) and terrestrial (nematode, springtail, earthworm, woodlouse) organisms (NERC, VUA, UAVR).
2. Establishing whether UV co-exposure affects toxicity in selected species *in vivo* for ZnO ENPs in *Daphnia*. This will build on work that has established that the cytotoxicity of some UV absorbing ENPs is mediated through radical oxygen species generation and is enhanced in the presence of UV light in mammalian cells (Sayes et al., 2006) and bacteria (Adams et al., 2006) (UAVR).
3. Assessing whether the ability of ENPs to bind and transport other molecules into biological systems modifies the toxicity of co-occurring pollutants, as shown previously for polycyclic aromatic hydrocarbon in the presence of sucrose polyester ENPs (Moore et al., 1997). While relevant to all the selected ENPs it is especially of concern for CeO₂ ENPs, which may serve to co-transport other combustion pollutants into biota. This will be addressed by taking a multiple exposure approach and analysing if the combinations of CeO₂ ENP with associated PAHs lead to higher uptake and effects than should be observed from the two components in isolation (UNIPMN, VUA, NERC).



The exposures to be conducted will utilize a range of environmentally relevant species in different exposure media and will measure a range of endpoints, thereby improving the current state-of-the-art. Variables such as aggregation and dissolution of ENPs will be monitored in the test media using qualitative and quantitative methods. Our experiences will also allow us to recommend refinements to existing ecotoxicity test protocols for ENP studies and will provide information that can be used to investigate approaches for calculating predicted no-effect concentrations in WP 6.

Obj.6: Establish and model how environmental physico-chemical properties in wastewater, natural waters and soil govern ENP parameters such as stability, soil-solution partitioning, downward transport and transformation (e.g. dissolution) that each may ultimately affect bioavailability to organisms.

Baseline. The properties of the selected ENPs will be characterised in detail (in WP 1); however, the consequences of these properties for behaviour of the ENPs in the natural environment (e.g. aggregation/dispersion, association with natural organic matter, binding to suspended sediments and soils, dissolution rates) have so far not been studied. Although knowledge of the behaviour of natural metal oxides suggests that chemical factors (e.g. dissolved organic matter, pH, ionic strength) should influence the stability of metal oxide ENP, the bioavailability of ENPs to organisms has only been studied in simple or environmentally unrealistic systems, and it is unknown how these factors affect ENP uptake and toxicity. Work has been published showing that both pH and the presence of naturally occurring macromolecules can influence the dissolution and aggregation of ENPs and it is likely that these affects may change bioavailability (Baalousha, et al. 2008; Diegoli, et al. 2008).

NanoFATE progression beyond the “state-of-the-art”. In NanoFATE we will address the role of water and soil physicochemical properties and particle characteristics by determining the magnitude of ENP effects for key organisms exposed to different particle types and under different environmental conditions. Specifically we will adopt the following approach.

1. Conduct tests to measure the toxicity of a selected set of ENPs in a set of soils and waters of known physicochemical properties (VUA, UAVR, CU).
2. Account for the role of dissolved metal in toxicity, by linking information on dissolution rates to predictions of free metal ion concentration using the Windermere Humic Acid Model (WHAM) (Tipping 1984) or empirical relationships with either the free ion activity model (Morel 1993), free ion effective dose model (Lofts et al. 2005, 2006) or biotic ligand model, as a prediction of available exposure and associated effect (DiToro et al. 2001) (NERC, VUA, UAVR).
3. Quantify additional toxicity (if any) beyond that predicted to be caused by the free metal ion.

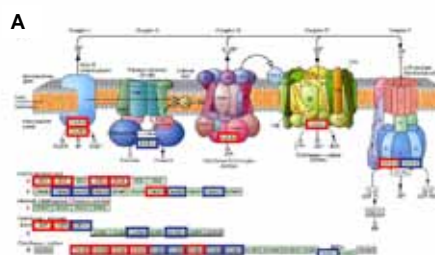
4. Use multivariate statistical methods such as principal component analysis and partial least squares regression to investigate the relationships between ENP derived toxicity and soil and water chemistry (VUA, NERC, UAVR).
5. Investigate the use of rate transfer constants as a means to account for dissolution and the subsequent transfer of the causation of toxicity from ENP to free metal ion forms (VUA, NERC).

Meeting this objective will require integrative working among ecotoxicologists and environmental and physical chemists. We will need to quantify how physical properties of ENPs change with time in diverse chemical environments and how this affects ENP exposure. The information derived from these studies will allow us to modify assessments of risk in receiving waters and soils made in WP 6.

Obj.7: Establish the mechanisms of uptake, internal trafficking and toxicity of ENPs.

Baseline. To date, information on the toxicokinetics of ENPs is very sparse. Very little is known of their uptake, internal trafficking and distribution and the effects of ENP properties on these parameters. This is despite the fact that these aspects are important to understand mechanisms of action and long-term effects of ENPs.

In relation to mechanisms of toxicity, some observations do indicate that nanoscale materials used in biomedical and pharmaceutical research may modulate the expression of cancer genes (Omidi et al., 2003), and genes involved in cell signalling (Regnstrom et al., 2006). For ENPs, recent studies have indicated genotoxicity and cytotoxicity in cultured human cells and generation of pulmonary fibrosis and lung tumours in rats (Wang et al., 2007). Such effects have, however, only recently been studied in aquatic organisms (see review of Moore, 2006; also Klaper et al. 2009; Shinohara et al. 2009) and we know of no published genotoxic studies in terrestrial invertebrates (although NERC have submitted a paper on ENP immunotoxicity in earthworms) and only a single molecular toxicity study for terrestrial plants (Lee et al. 2009).



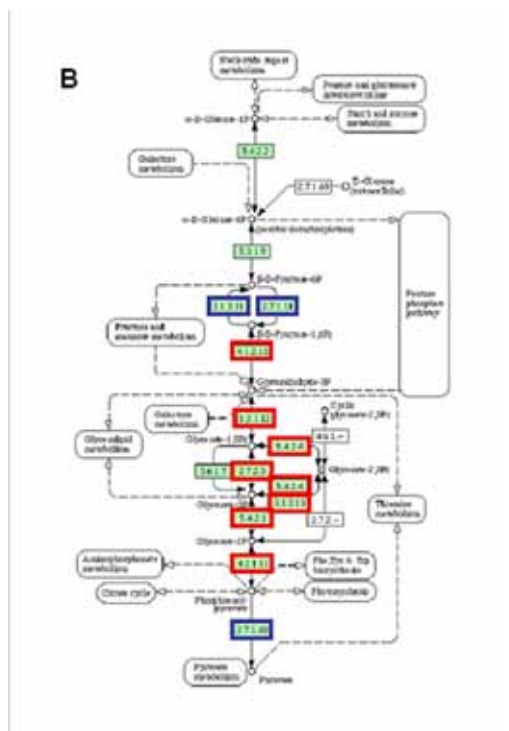


Fig. 2. Example analysis of the impact of ionic copper on oxidative phosphorylation (A) and on glycolysis/gluconeogenesis (B) of earthworms in a pathway based visualisation of the mechanism of toxicity. Transcripts outlined in bold are represented on the utilised microarray, those with < 2 fold change following copper exposure are outlined in blue.

NanoFATE progression beyond the “state-of-the-art”. Since extensive studies on tissue and cellular localization and the mechanisms of action of ENPs remain lacking in aquatic and terrestrial species, NanoFATE will progress these aspects using a number of techniques that have been developed and used previously for conventional chemical assessment. To assess uptake and elimination, methods to both directly measure and also infer toxicokinetic parameters will be applied (see WP5.1 for details). Mechanisms of action will be investigated using a systems toxicology approach, which has proved valuable for the unbiased characterisation of the molecular basis of the toxicity of PM10 / UFPs (Karoly et al., 2007) and ENPs in macrophages (Long et al., 2007; Xiao et al., 2003). This systems toxicology approach has never been applied for ENPs in organisms exposed to chronic ENP concentrations *in vivo*, although consortium members have applied the approach to assessing metal ion toxicity in a range of species (see Fig. 2 for example), which has the potential to reveal novel insights on the nature of chronic effects. Specific studies will comprise:

1. Time series studies of effects of ENPs on lifecycle parameters of species where full lifecycle data can be obtained (e.g. *Daphnia*, nematodes, springtails). This data will be used to parameterise the physiologically based model DEBtox (Kooijmann and Bedaux, 1996, Jager et al. 2003) to predict parameters relating to energy dynamics and ENP toxicokinetics (VUA, NERC, UAVR).

2. Electron microscopy of cryo-sectioned preparations from time series exposures to identify major uptake routes and gross tissue distributions of ENPs in earthworms using energy dispersive x-ray analysis (Cotter-Howells et al., 2005). This will provide information on the internal distribution of ENP in major organs (CU, UOXF.DJ).
3. The use of Raman spectroscopy to chart signatures of the interaction between ENPs in unicellular organisms (Huang et al., 2004; Singer et al., 2005) and also in the cells in body fluid samples from larger organisms (earthworms and/or mussel) (UOXF.DJ).
4. Measurement of biomarkers relevant to known modes of action of ENPs (e.g. genotoxicity, immune function and ROS production assays) (Long et al., 2006; Nel et al., 2006; Xia et al., 2006) to evaluate the cellular, organelle and molecular effects of ENPs in earthworms (Svendsen and Weeks, 1997; Svendsen et al., 1998) and mussels (Dagnino et al. 2007) (UNIPMN, CU).
5. Transcriptomics studies to directly compare gene expression responses following exposure to bulk material/free metal ion and a variant ENP. Established microarray technologies for *Caenorhabditis elegans* (Reichert and Menzel 2005; Menzel et al. 2007) and *Folsomia candida* (Nota et al. 2008), along with a full genome earthworm (*Lumbricus rubellus*) microarray and extended feature *Mytilus* microarray developed, based on results of an ongoing sequencing programs will be used (Dondero et al., 2006; Owen et al., 2008; Svendsen et al., 2008; Viarengo and Dondero, 2006). Pyrosequencing initiatives currently in progress at CU will also allow the use of a digital transcriptomic approach using Solexa-based tag sequencing technology to probe the transcriptome more deeply to identify changes in expression of low abundance genes. Bioinformatic support given within these existing sequencing programs will assist in identifying the pathways associated with ENP toxicity and will also allow inter-species comparisons through web-accessible integrated systems developed by UNIPMN in EU FP6 IP NoMIRACLE for the storage, meta-analysis, and retrieval of toxicogenomics datasets (CU, UNIPMN, VUA).

Obj.8: Develop risk assessment model(s) that integrate ENP fate, availability, accumulation and toxicity over the full post production lifecycle including provision of data for use in full lifecycle assessment.

Baseline. The current state-of-the-art approach to risk assessment relies on the use of generic data to derive predicted environmental concentrations (PECs) and on the use of toxicity data from standard tests at best within a species sensitivity distribution (Posthuma et al. 2001) or otherwise merely in combination with uncertainty factors of between 10 and 1000, to derive predicted no-effect concentrations (PNECs). While possibly suitable for predicting generic risks, this approach is rather simple, deterministic and provides no information on the spatial distribution of risk.



NanoFATE progression beyond the “state-of-the-art”. To develop and refine approaches for the risk assessment of ENPs that potentially may allow a more robust and detailed assessment, in NanoFATE we will evaluate the applicability of advanced risk assessment tools for use with ENPs. These include models for predicting no effect concentrations based on the species sensitivity approach; bioavailability models that develop the biotic ligand model to also incorporate ligand binding associated surface charge of ENPs to account for ENP mediate toxic effects; a GIS-based model such as the Air Dispersion Modelling Systems; and EUSES and LF2000-WQX hydrological model for visualising ENP risk in receiving ecosystems including river catchments.

1. For assessing risk, both generically and in a spatial context, we will first predict concentrations of the ENPs in different environmental compartments. As outlined previously these will be derived using two spatial based modelling approaches. ADMS and LF2000-WQX are two well established models that can be used to study the distribution of chemicals in air and surface water respectively. ADMS is an industry standard air pollution model that is well suited for modelling pollutant dispersion from road vehicle sources. EUSES and LF2000-WQX are chemical fate models, with EUSES being the current industry standard and LF2000-WQX an advance coupled hydrological and chemical discharge model that can be used to predict the spatial concentrations of chemicals in river systems. Each of these models has the potential to become established tools for predicting environmental concentrations of ENPs in air and water. Assessment for our selected ENPs with our different usage scenarios will start with a worst case assessment. We will progressively update PEC and PNEC values to the risk assessment model as we gain more data and understanding of ENP fate from the tracking studies conducted using particles synthesised and characterised in WP 1 and tracked within real systems in WP 2 (NERC).
2. To derive a suitable PNEC, we will examine the issues surrounding the application of the species sensitivity distribution approach for ENPs. Given that ENPs may have an infinite variety of physical properties it is not immediately clear that SSDs can be applied to ENPs even if only particles of the same core type are considered. Further it is not clear what exposure metric should be used (concentration, surface area, reactivity etc.). To establish the potential for applying SSDs and also to provide guidance on the selection of the exposure metric, we will analyse the collated data on ENP toxicity to identify patterns and trends within the data. Data can be retrieved from studies collated and available within the NAPIRAHub set of publicly available data resources. This will include studying correlations between ENP properties and toxicity, environmental properties and toxicity, and the influence of species-relevant traits including phylogeny and ecological traits (such as feeding mode, soft vs. hard bodied organisms). On the basis of this analysis, we will seek to establish best practice for ENP PNEC generation, including identifying the most suitable dose metric. We will also define the operational limits of the SSD approach (NERC).
3. We will examine the relationship between PECs for receiving soils and an indicative PNEC derived from available toxicity data. From our studies of fate in soils (e.g. dissolution rates) in WP 2 and WP 4, information on the bioavailability and the relative toxicity and effect of CeO₂ in dissolved and nanoparticle form will be used to address issues relating to the relative contribution of ENP forms to toxicity. Information on bioavailability will be built using models developed in WP 4 that will build on the biotic ligand model and also information on particle properties including surface charge and dissolution. Such information will be of fundamental importance to the development of the concept of ecologically responsible design of nanotechnology products and is a key project outcome.
4. To visualise spatial risks for ZnO and Ag ENPs, usage scenario data, hydrological data, relevant literature information and experimental results on exposure and toxicity will be used to parameterise catchment based spatial risk assessments for a selection of UK river catchment and three indicative European catchments. The approach developed builds on that for endocrine disrupting chemicals to support the spatial assessment of risk (see Sumpter et al., 2006 and Fig. 3 for specific examples). Spatially explicitly risk maps for a range of catchments under normal and extreme flow conditions will be developed for a range of usage scenarios. If suitable insight is gained from studies of ENP physicochemistry, bioavailability and uptake mechanisms, the model will be updated to consider the effects of water chemistry on particle fate and on exposure and effects in organisms.
5. NanoFATE specifically addresses the fate, effects and associated risk of ENPs during their use phase. However, the consortium also recognises that the collected data is also highly relevant to studies that seek more comprehensive and high level lifecycle assessments for nanotechnology products. To allow researchers in the LCA community to utilise NanoFATE data, applicable project data will be collected within data holdings in a manner compatible for use in lifecycle analysis as set out in the International Life Cycle Data System (ILCD) Handbook. To support exchange of data with the LCA community, NanoFATE has included experts in LCA within the project advisory board. Prof. Sverker Molander from Chalmers Institute of Technology in Göteborg is a LCA expert who has been working in the area of nanotechnology LCA, with a particular focus on metal and metal oxide ENPs. Prof. Molander has been approached (and has agreed) to provide input into the development of LCAs based on NanoFATE data holdings and also to work within NanoFATE to ensure the compatibility of NanoFATE studies with national and international LCA guidelines and projects.

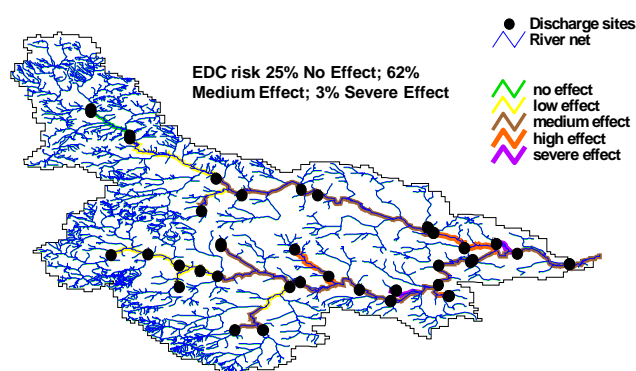


Fig 3. Catchment risk map of predicted endocrine disruption of fish from effects of oestrogenic chemicals for the Aire and Calder rivers, Yorkshire, UK.

Obj.9: Improve stakeholder understanding of ENP risks.

Baseline. Due to current uncertainties, public perception of the risks from nanotechnology could represent a barrier to the safe and sustainable development of the sector, even if ultimately the nature of such risks actually turned out to be rather limited. One thing that is missing from the nanotechnology debate is scientifically robust case studies that can be utilised as tools to communicate the real risk of potential adverse effects. Such studies can provide both a means to facilitate understanding within the regulatory community and also if correctly presented, effective platforms for discussion of actual risks for real world situations.

NanoFATE progression beyond the “state-of-the-art”. By conducting a comprehensive scientific assessment of the fitness for purpose of existing risk assessment approaches and techniques for estimating ENP risks in real environments, NanoFATE will establish the state-of-the-art for evidence-based ENP risk assessment. Developed tools for assessment will be communicated to national and EU based responsible authorities and stakeholders to encourage adoption and exploitation through conference presentations, user-friendly reports and information (on WWW), webinars, and formal scientific outputs. A project newsletter will be produced biannually. For the regulatory and policy maker audience, we will prepare project briefing notes and offer presentations given by the Coordinator or appropriate selected partners to key international and national agencies. This material will be developed in collaboration with Advisory Board members from the regulatory community (National Environment Agencies) and also the Commission (as appropriate). Further the NanoFATE team will play a full and active part within the newly inaugurated NANOSAFETY cluster that has been developed at the EU level to establish a network of experts that are involved in (EU-) projects focused on the health and safety aspects of Nanotechnology. This will ensure NanoFATE is able to work with other EU projects to meet NANOSAFETY cluster objectives regarding consensus, effective communication and discussion, and avoidance of overlap in ENP studies.

To provide industrial stakeholders and the general public with appropriate knowledge on the risks of ENPs and nanomaterials

for human health and the environment, we will also submit articles to the industrial press. Provision of information to the public in an easily understandable form will be an important part of the communication process. Because we will have data from specifically designed and systematically conducted studies, we will be in a strong position to provide coherent information to the public on this debate. This will open up understanding not only of the nanotechnology area, but also of the risk assessment approaches, their inherent assumptions and their precautionary nature. Again, links with the NANOSAFETY cluster will ensure that consistent messages regarding these aspects are delivered to regulators, industry and the wider public.

3.3 Overall project structure

The NanoFATE PERT diagram (Fig. 4) below shows the relation of the nine work packages, each of which is embedded into the three main project components.

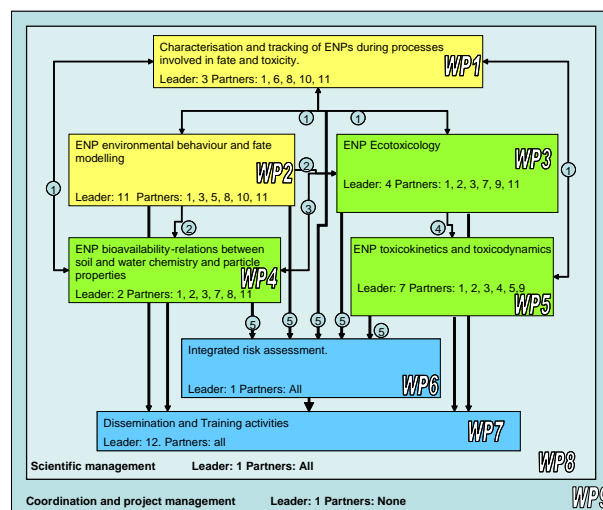


Fig 4. NanoFATE PERT diagram showing flow of data and products between the seven workpackages within particle chemistry and fate component (yellow), ecotoxicology and bioavailability component (green) and the risk assessment and communication component (blue). The flow of knowledge, technology and data are illustrated with the numbered arrows; 1) The material sciences technology and analytical skills WP1 are used for sample and ENP characterisation during studies in WP2, 3, 4 & 5. 2) Fate modelling in WP2 delivers PECs to WP3, 4 & 5 to allow design of realistic bioavailability and ecotoxicity studies. 3) Linking the exposure and effect data from WP3 & 4 will inform and validate improved bioavailability models 4) Samples from toxicity studies in WP3 will form a tissue archive for use in WP5 for identification of species-specific and generic mechanisms concerning the comparative toxicity of ENPs to the studied taxa 5) Data and knowledge from all theoretical and experimental activities of WP1-5 will feed into WP6 to form the basis for improving the integrated risk assessment.



4 Description of the work performed since the beginning of the project and the main results achieved

While work has begun on all nine of the main NanoFATE S&T objectives (see above), obviously some are targeted for early delivery (i.e. Obj. 1 and 2) while full delivery for the others will not come till the end of the project. For more details than the summary below and for access to completed public deliverables, updates and subscription to newsletters, please use the website www.nanofate.eu.

The working objectives addressed in the first 18 months of NanoFATE to ensure timely progress towards the overall objectives can be summarised as follows:

- I. Source, produce and fully characterise the commercial ENPs and match the tagged version of ZnO as closely as possible. (Delivering Main Obj. 1)
- II. Establish particle behaviour in the pure ecotox media to be used and at higher than environmental concentrations (to enable hazard assessment studies). (Working towards Main Obj. 3, 4 & 5)
- III. Establish acute and chronic toxicity (to enable selection of relevant doses for the progression into the work in environmentally relevant media conditions. (Working towards Main Obj. 5, 6, 7 & 8)
- IV. Develop initial simple assumption based fate models and estimate worst case environmental concentrations (Working towards Main Obj. 2 & 8)
- V. Train all staff cross discipline, and disseminate our early findings and planned directions to other EU projects and stakeholders (Main Obj. 9)

WP 1. Characterisation and tracking of ENPs during processes involved in fate and toxicity.

In the first 18 months the major deliverables here related to provision of high quality well characterised particles for the remaining project partners. A larger than planned range of commercial ENPs were characterised and assessed so that supply was consistent and without significant batch to batch variation issues. Consequently the final set of NanoFATE commercial particles are:

- The main ZnO particles are 30nm Nanosun from micronisers in Australia, with matching tagged ZnO ENP by IHPP, with some work on BASF z-cote and z-cote HP1 ZnO
- Amepox 3-8nm Ag ENP and a 50nm Ag NP from NanoTrade
- CeO₂ will be the Envirox or Antaria fuel additive and most likely a polishing agent from Umicore.

WP 2. ENP environmental behaviour and fate modelling

Have identified and prioritised specific properties that need principal consideration during the development, adaptation and validation of environmental fate models for nanoparticles (D2.1). They have, based on this, developed the initial basic fate models

(with WP6) and supplied the initial worst case environmental concentration estimates serving to inform decision making and exposure design in WP 3 & 4 (M 2.1 and D 2.2).

WP 3 ENP Ecotoxicology

Developed improved standard ecotox exposure protocols, principally adjusting properties of test media, media renewal frequencies and soil and food spiking methodologies, to ensure relevant and homogenous presentation of nanoparticles during toxicity testing. Employing these improved protocols most of the exposures needed for the hazard assessment has been completed (except for CeO₂). This information has allowed progress into the chronic testing phase and also some work to deliver the data for WP4 on bioavailability drivers.

WP 4 ENP bioavailability - relations between soil and water chemistry and particle properties

Collected and databased all available information from literature, conferences and other projects and conducted a critical review of this available data and its quality. This identified which environmental factors have the greatest proven effect on the bioavailability and toxicity of nanoparticles to organisms living in soil and water. Based on this initial bioavailability trials testing for pH, organic matter and cation effects have been designed and run within WP3, plus additional long-term (12 months) exposures addressing ageing have been set up.

WP 5 ENP toxicokinetics and toxicodynamics

The WP5 workshop session in Portugal (Jan 2011) developed a practical and workable sample handling and preservation system to enable the success of NanoFATE tissue banking. A well thought through tired approach to the tracking of ENPs in tissues was also developed, allowing us to make the most of our technical abilities (and tissue samples) by ensuring that the high-end expensive low throughput techniques only to be applied to samples where we have good evidence ENPs are present. Samples have been run looking at biological markers of ENP and dissolved metal effects to develop the knowledge of signatures of possible ENP tissue damage. An agreed data structure has been developed for this systematic data to allow later cross species comparison, and the format has been kept flexible to enable adaptation to the Cluster database when agreed. A few samples have been run on the UK synchrotron at Diamond Light Source to provide a highly characterised test bed for development of other more accessible techniques.

WP 6 Integrated risk assessment

The initial milestone for WP 6 was to assess ENP production and product incorporation estimation engaging industry directly through a survey. With over 560 companies and associations involved in ENP production being contacted this represented an important attempt to gain intelligence on market size and growth for ENPs in Europe. However, a devastating lack of industry willingness to interact was suffered, as was the case for a similar effort by the NanoSustain project. Therefore the report was based upon a review of the peer-reviewed as well as grey literature (reports from R&D projects, reports to governmental



authorities, etc.) on production volumes of the three ENPs and reported (predicted) environmental concentrations in surface water, STP effluents, soils and sediments. The compilation of usage scenarios were thus completed without direct data from industry and initial fate and distribution models (local to EU region scale) developed. The resulting predicted environmental concentration maps are very informative albeit obviously conservative (high estimates) due to the assumptions currently surrounding production size and distribution.

WP 7 Dissemination and Training

The NanoFATE web site and the interlinking of this with the e-based Newsletters has proven very successful in terms of bringing our work to the attention of the wider stakeholder community (see www.nanofate.eu and subscribe to the newsletters).

In terms of direct engagement with stakeholders NanoFATE organized with the fellow NanoSafety Cluster projects NanoRetox and Ennsatox an EU Cluster meeting in London aiming to link Environmental fate and Ecotoxicology aspects within EU NanoSafety Cluster projects. Representatives from many EU projects, industry and regulatory bodies. Additionally, there have of course been numerous presentations of the NanoFATE work to international conferences and workshops, and the first peer reviewed papers are starting to come out.

In terms of training NanoFATE has an inherent large training and capacity building element in that we directly have created 14 PhD or Post Doctoral positions across Europe. There are further indirectly associated students and fellows working closely with the NanoFATE partners. For the wider community the 1st NanoFATE open PhD training workshop was held in Jan. 2011 at UAVR (Aveiro, Portugal), brought together 60 participants (24 from NanoFATE) and representing 14 nationalities.

Impact: NanoFATE will provide robust tools, techniques and knowledge needed by stakeholders to understand and communicate risks associated with ENPs of different physical or chemical properties, including their environmental interactions and toxicity. Dissemination is done through a wealth of channels and activities, but centred around interactive e-Newsletters and the project website www.nanofate.eu.

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NanoHouse

Cycle of Nanoparticle-based Products used in House Coating



Contract Agreement: NMP-2009- 247810

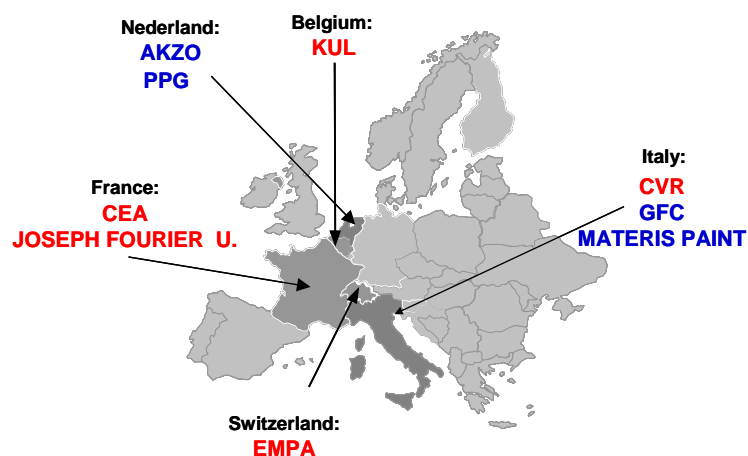
Website: <http://www-nanohouse.cea.fr>

Coordinator: François Tardif Commissariat à l'Énergie Atomique et aux Énergies Alternatives, Grenoble, France

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6	MATERIS PAINTS ITALIA	MATERIS	IT
7	GFC CHIMICA	GFC	IT
8	AKZO NOBEL COATINGS S.A.	AKZO	NL
9	PPG Europe BV	PPG	NL

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1 Summary

NanoHouse project in some words:

NanoHouse collaborative project is founded by the European Commission in the frame of FP7 programs: NMP-2009-1.3-1 & ENV2009.3.1.3.2 "Activities towards the development of appropriate solutions for the use, recycling and/or final treatment of nanotechnology-based products.

This project started January 2010 for a duration of 42 months (until 06/2013) and a total budget of 3.1 M€.

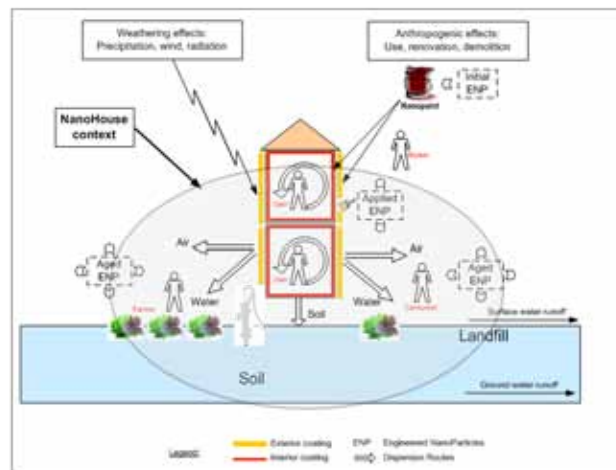
The current and projected applications of **Engineered NanoParticles (ENPs)** span a very wide range of industrial and consumer sectors such as : biomedicine, pharmaceuticals, cosmetics, new sources of energy, environmental analysis and remediation, material science . At the same time, the potential impact of these new materials their production and their **life-cycle implications** on the places where people live on Environmental Health and Safety (EHS) is a key issue regarding the future **acceptability and sustainability of nanoproducts**. In this perspective, buildings and individual houses are critical in that they constitute the major surrounding of people in developed countries.

The NanoHOUSE project concentrate on this issue and aims at promoting a responsible and sustainable development of

nanomaterials in building industry through a Life Cycle Thinking approach.

The NanoHOUSE project focuses on the most commonly used ENPs in construction materials : nano-Ag , nano-TiO₂ and nano-SiO₂ comprised in large amounts in paints and coatings for indoor and outdoor applications.

The goal of this project is to gather and to **generate**, when missing, **reliable scientific information** and analysis, using appropriate methodologies to understand the potential **EHS impacts of nanoproducts** used in building (coatings and paints).



2 Background

Nanosciences and Nanotechnologies (N&N) provide many opportunities to significantly improve materials properties and sustainability. The current and projected applications of **Engineered NanoParticles (ENPs)** span a very wide range of industrial and consumer sectors such as: biomedicine, pharmaceuticals, cosmetics, new sources of energy, environmental analysis and remediation, material sciences

At the same time, the potential impact of these new materials their production and their **life-cycle implications** on the places where people live on **human health and the environment** is viewed with apprehension by citizens. A growing body of scientific evidence indicates that exposure to some ENPs can lead to harmful effects. In the Nanotechnology Action Plan 2004 (COM(2004) 338), the European Commission highlighted that *"R&D need to take into account the impacts of nanotechnology throughout the whole of their life-cycle"*. In the "Nanosciences and nanotechnologies: An action plan for Europe 2005-2009 (2005)" it is emphasized as well: *"Health, safety and environmental risks that may be associated with products and applications of N&N need to be addressed upfront and throughout their life cycle"*. Therefore, the environmental and health consequences of these materials, their production and

the **life-cycle implications** of the products deserve attention now, during the early stages of development. This is a key issue regarding the future **acceptability and sustainability of nanoproducts**.

As far as human chronic exposure is concerned, addressing the issue of safety, and consequently of acceptability of nanoproducts calls for a focus on the places where people live. In this perspective, buildings and individual houses are critical in that they constitute the major surrounding of people in developed countries. The NanoHOUSE project will concentrate on this issue.

Therefore, the NanoHOUSE project covers not only a group of a specific population, but all the population and addresses a complementary issue of FP6-Nanosafe2 project that was focused on the human exposure at the working place.

Indeed, through the use of many different types of ENPs such as silica (hardener, antireflection effect), zinc oxide, **titanium dioxide**, cerium oxide (anti-UV) and **silver** (biocide), nanotechnologies have been introduced in construction materials: concrete, glass window, coatings for metallic pieces, anti-scratch floor coatings, concrete or wooden façade



coatings, decorative paints, and anti-microbial coatings and plastics in hospital

In the context of the trend to increase energy efficiency of buildings by thermal insulation, the demand for protecting outside façades with functional façade paints could increase. Façade paints products containing ENPs could for example be an alternative solution for façade paints containing hazardous biocides. Nanotechnologies also are expected to hold potential for example for antibacterial or air-purifying inside paints containing ENPs.

The NanoHOUSE project focuses on the most commonly used ENPs in construction materials nano-Ag, nano-TiO₂ and nano-SiO₂ comprised in large amounts in paints and coatings for indoor and outdoor applications.

The scope of the project is circumscribed to the release of ENPs during the post-production stages in the life cycle of both indoor and outdoor paints and coatings for housing

3 What is NanoHouse

The goal of this project is to gather and to generate, when missing, **reliable scientific information** and analysis, using appropriate methodologies to understand the potential **EHS impacts of nanoproducts** used in building (coatings and paints).

A life cycle approach prospectively gathers information about the EHS aspects throughout all the life cycle stages of these products and identifies the data gaps and drives the precise needs of experimental work. Firstly, experimental work focuses on the **quantification of the actual sources of ENPs** during the use and ageing of indoor and outdoor coatings, during renovation and demolition operations and during their final disposal.

The main innovative aspects of the NanoHOUSE project are: (i) to consider the **whole product life cycle** in regard to EHS and (ii) to study the environmental behaviour and the toxicological effects of the **actually released ENPs ("aged" ENPs)**, and to compare them with the pristine ENPs

As an important component of the environmental and ecological system, NanoHOUSE aims at quantifying the uptake of released ENPs by plants and determining the impact of ENPs on those organisms.

NanoHOUSE aims at identifying and quantifying **the effects on human health** along the pathways of exposure to human in urban or residential environment. The major goal is to gain insight into the influence of **ENPs transformations ("aged" vs pristine)**, routes of intake, duration of exposure on the **biokinetics** throughout the entire organism (*in vivo* tests) and **the mechanisms of toxicity** at the cellular level (*in vitro* tests) and to develop a Physiologically Based Pharmacokinetic model (PBPK) with a pulmonary dispersion model to integrate different parts of human health effect measurement.

Finally, NanoHOUSE **will improve end of life treatments** regarding ENPs release in the environment, and will participate to **the development of sustainable and competitive nanoproducts** by decreasing their potential to release ENPs. NanoHOUSE project will thus contribute to the development of

appropriate solutions for the use of safe, sustainable and competitive nanoproducts in housing through their whole life cycle.

3.1 Summary of NanoHouse's key strengths

The main outcomes of the project are:

- evaluate the risks associated with the use of ENPs in materials for housing,
- improve the **sustainability of ENPs** containing paints and coatings for housing and other applications by decreasing their release-ability,
- propose a **generic risk assessment** methodology tested for a selected group of nanoproducts that takes into account the specificity of actually released ENPs,
- support the **regulation concerning risk assessment** by contributing recommendations specific to nanoproducts considering the whole life cycle of these products and elaborating a first attempt of LCA,
- participate to the normalisation of release tests for **certification of nanoproducts** in construction and other applications,
- improve the current technical solutions for **end of life treatments of nanoproducts**,
- propose a **decision-making tool for sustainable and competitive innovation** and for nanorisk management addressed to manufacturers,
- promote nanoproducts **social acceptability**.

4 Organisation of NanoHouse

The NanoHOUSE project is structured around five scientific work packages (WP1-WP5) whose the previous aims and the interdependency are described hereafter and in the Table 1.

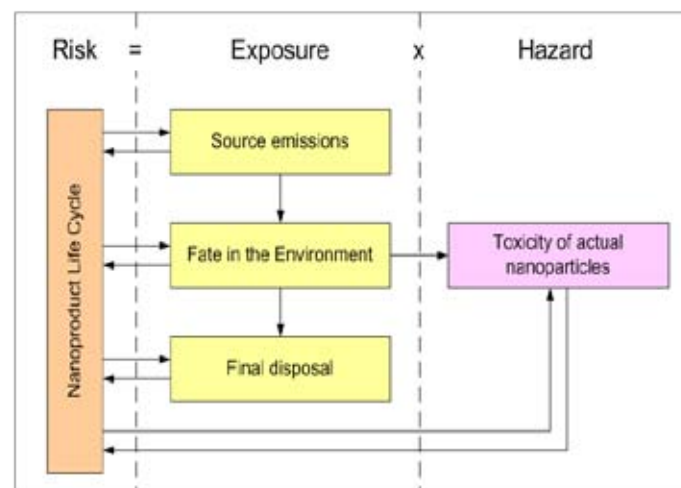




Table 1 Workpackages (WP) of NanoHouse

WP	Title	Topic
1	Life Cycle Thinking	This workpackage aims at investigating the potential health, safety and environmental (EHS) impacts during all life cycle stages of façade coating products containing ENP. A first attempt of a comparative Life Cycle Assessment (LCA) study in accordance with the ISO 14'040 standards shall be established; supporting the comprehensive assessment of the EHS impacts along the complete life cycle and allowing a comparison with traditional façade coatings, not containing ENPs. This WP is designed to systematically combine the quantitative risk assessment data on toxicology (WP4) and environmental fate (WP3) with other relevant knowledge on EHS impacts during the life cycle stages of façade coatings such as exposure situations (WP2) during the use phase among others. The combination of Life Cycle Thinking with the current knowledge on risk assessment may provide a basis for informed decision making by the industry and regulators.
2	Source Identification	WP2 aims at simulating ENPs releases in conditions representative of ageing of the paints but also during critical renovation operations, to quantify the flux of ENPs released and to gain insight into the mechanisms of ENPs released. The physical-chemical properties of the ENPs released will be characterised. The so called "aged ENPs" produced will be then be delivered to partners of WP3 and WP4 for environmental fate and toxicological studies.
3	Environmental fate	Nanoparticles released from the outside of buildings directly into the environment are under the influence of physical, chemical and biological processes that determine their fate and behaviour and ultimately their influence on biota and possibly also humans. Aggregation and dissolution reactions may strongly affect the longevity and transport of the ENPs and thus their persistency and distribution to environmental compartments. This WP aims to investigate the reactions of released ENPs in water (WP3-2, in the soil (WP3-3) and in plants (WP3-4) then modelling the fate of the released ENP in the soil-water-plant-human system (WP3-5).
4	Hazard characterization	The mechanisms of action of fine and ultrafine particles on the human health are not yet fully understood. this WP focuses on processes such as translocation, changes in the cell-layer, oxidative stress, pulmonary (and systemic) inflammation. First in ENPs translocation, biokinetics and bioavailability studies in cellular and animal models (WP4-1) then in Specific citotoxicity study of aged ENPs compared to pristine ENPs in order to highlight health effects (allergy and asthma) to be expected of ENP being present in coating (WP4-2). Hazard of the original (pristine) material, but also the product in combination with its solvents (the whole commercial product), and the "aged" product after use and disposure, have to be considered.
5	Safer use and waste management	This workpackage The assessment and properly management of end-of-life of nano-based products are key aspects to be investigated for successfully applying life cycle concepts and for developing eco-friendly products. The main rationale behind this WP is : 1) to estimate the impacts caused by the end-of-life treatment based on a description of state of the art concerning waste management of nano-based applications (WP5-1) and on a determination of geomembrane permeability for ENPs (WP5-3) 2) to provide appropriate solutions for the end-of-life treatment (WP5-2) and propose safety improvement of selected nanoproducts (WP5-4)

Project dissemination, ethics and nanorisks issues (WP6)

The dissemination of the results takes place step by step. Firstly the reports will be the base for dialogues with industries and regulators. Secondly the project relevant information for industrial and private consumers will be disseminated on the NanoHOUSE website. Furthermore, short dissemination reports for the public at large will be broadcasted. An additional part on the risk management will be built up based on the results of the project and made available through the e-learning interactive

software Nanosmile¹ already developed in the frame of NanoSafe2 project and available on the Internet. Finally, the project will give recommendations for the transferability of the methods that have been validated to other application domains where nanomaterials are increasingly used such as cosmetics, automotive, aeronautics and space.



5 NanoHouse Reports and Events

5.1 Progress to date

A report on the potential EHS impacts during the life cycle of façade coatings nanomaterials is achieved and published. This study is disseminated to a large public through the EC DG Environment public web site. New strategies in order to overcome the existing gaps on the life cycle assessment for engineered nanomaterials are being established. A precautionary matrix is established in order to estimate the degree of respect of nanosafety issues. A life cycle model including production, use and end of life is under development. In order to be able to identify emission sources, estimation of ENPs release via the dry and wet route from paint panels provided by the industrial partners in WP2 were performed. Coated panels were exposed to indoor and outdoor aging as UV light, heat and humidity cycles. A Taber standard method for investigating wear resistance to abrasion by "dry route" was chosen. The Taber abrasion device was started and coupled to an aerosol measurement system in order to measure dust released. Estimation of ENPs release induced by abrasion via the dry route before and after UV aging and weathering was achieved for different paints. Experimental and theoretical study of the release of ENPs just deposited from the surfaces and nanoparticles from the product matrix were performed. The particles < 50 nm are trapped on the surfaces by Van der Waals forces. The release of free nanoparticles is seen only when nanoparticles are not perfectly dispersed within the matrix.

A new bench for measuring airborne ENPs released by sanding from paint panels was set-up. The sanding device was coupled to an aerosol measurement system in order to measure dust released. Estimation of ENPs release by sanding is in progress. Collected information and data were used to define a specific protocol for leaching tests for the nano-based products considered in the NanoHouse project. A new bench for measuring ENPs released by leaching was set-up. Leaching tests on painted panels, weathered and unweathered coated panels are in progress.

Radio labelled Ag-nanoparticles and TiO₂ fluorescent nanoparticles have been synthesized. Behaviour of these ENPs in soils was performed. The penetration of TiO₂ and Ag nanoparticles through the sand columns is determined using gamma ray and fluorescent detection. The penetration of TiO₂ and Ag nanoparticles inside the plants and the transfert of NPs in plants was estimated by different techniques.

In vitro cytotoxicity using Ag, TiO₂ and SiO₂ nanoparticles was determined. The effect of these Nanoparticles on human bronchial epithelial cells was estimated as well.

"Aged" ENP's (TiO₂, SiO₂) in high quantities (few grams) for environmental fate experiments (WP3) and toxicological tests (both *in vivo* and *in vitro*) (WP4) were supplied. The size and

distribution of the particles/agglomerates obtained were evaluated through SEM, DLS and Laser Granulometer. The aged ENP behaviour will be compared with the pristine ENP in the environment and toxicology.

A detailed literature research concerning release of nanoparticles from the end of life of nano-product was performed. End of life treatment solutions for waste paints containing ENP are currently under development. A protocol for leaching through waste containing nanoparticles was established. Leaching tests are in progress from paint waste containing nanoparticles.

Finally, two websites (internal and external) have been designed (www.nanosmile.org, <http://www.nanohouse.cea.fr/scripts/home/publigen/content/templates/show.asp?P=55&L=EN&ITEMID=2>)

5.2 Events

5.2.1 Month-18, 24 meeting

The Month-18 meeting was held in Paris France the 27th-28th June 2011. The Month-24 meeting was held in Leuven Belgium the 24th-25th November 2011.

5.2.2 Other workshops

The NanoHOUSE project has been represented in the "Nanosafety Cluster" in Lausanne 03/2011 (Peter Wick), Barcelona 05/2011 (Roland Hischer), Budapest 05/2011 (Peter Hoet), Rome 11/2011 (L. Golanski, P. Hoet). NanoHouse has been represented at the 21st SETAC EUROPE ANNUAL MEETING, in Milan (Italy) May 2011, at the fifth International Symposium on Nanotechnology-Occupational and Environmental Health, August 9-12, 2011, Boston, USA. The midterm dissemination workshop will be organized at Dublin within the QNano conference end of February, 2012.

5.3 Collaboration

NanoHOUSE and NanoSustain projects have established a collaboration which aims to promote a responsible and sustainable development of nanomaterials in the building industry through a Life Cycle Thinking approach. The two projects have agreed to collaborate on weathering and abrasion tests. Anne Thoustrup Saber (DK) from the NanoSustain project sent samples and Dario Cervellati (GFC) carried out weathering tests. This samples will be abraded at CEA by Arnaud Guiot, L. Golanski and release will be estimated before and after weathering.



6 Directory

Table 2 Directory of people involved in this project.

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NanoImpactNet

The European Network on the Health and Environmental Impact of Nanomaterials



Contract Agreement: NMP4-CA-2008-218539 Website: <http://www.nanoimpactnet.eu>
Coordinator: Michael Riediker, Institut universitaire romand de Santé au Travail, Lausanne, Switzerland

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(2)	(Edinburgh Napier University) ²	(NU)	(United Kingdom)
3	Norsk institutt for luftforskning[Norwegian Institute for Air Research]	NILU	Norway
4	National University of Ireland, Dublin / University College Dublin	UCD	Ireland
5	Institute of Energy and Environmental Technology - IUTA e.V.	IUTA	Germany
6	Hospices Cantonaux Vaudois - Centre Hospitalier Universitaire Vaudois	CHUV	Switzerland
7	Deutsche Gesetzliche Unfallversicherung - Institut für Arbeitsschutz	DGUV-BGIA	Germany
8	Technical University of Denmark	DTU	Denmark
9	JRC – Joint Research Centre – European Commission	JRC	Transnational (EU)
10	Institute of Occupational Medicine	IOM	United Kingdom
(11)	(University of Surrey) ¹	(UNIS)	(United Kingdom)
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18	Dublin Institute of Technology	DIT	Ireland
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20	Slovak Medical University	SMU	Slovakia
21	Finnish Institute of Occupational Health	FIOH	Finland
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23	Stichting Dienst Landbouwkundig Onderzoek	Alterra	The Netherlands
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26	Heriot-Watt University ²	HWU	United Kingdom
27	University of Fribourg ³	UNIFRIBOURG	Switzerland

¹Professor changed from partner 11-UNIS to 25-SMUC (11 discontinued).

²Professor changed from partner 2-NU to 26- HW (2 discontinued).

³Professor changed from partner 16-UBERN to 27 UNIFR (16 discontinued).



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1 Summary

Recent technological advances allow the targeted production of objects and materials at the nanoscale (smaller than 100 nm). Nanomaterials have chemical, physical and bioactive characteristics different from those of larger entities of the same materials and from molecular forms of the materials (where these exist). Nanoparticles (NPs) can pass through body barriers, although the detailed mechanisms are not yet understood. This is interesting for medical applications, but raises concerns about their health and environmental impact. NanoImpactNet's objective is to create a scientific basis for ensuring the safe and responsible development of manufactured NPs and nanotechnology-based materials and products, and to support the definition of regulatory measures and implementation of appropriate legislation in Europe. It includes strong two-way communication to ensure efficient dissemination of information to stakeholders and the European Commission, while at the same time obtaining input from these stakeholders about their needs and concerns.

The work focuses on the following areas: Human hazards and exposures, Hazards and fate of nanomaterials in the environment, Impact assessment, Communication, Integration and nomenclature, and Coordination and management. The project lasts four years. Discussions about strategies and methodologies are usually initiated through well-prepared workshops covering the various topics. All researchers and stakeholders dealing with the issues are invited to participate. After these workshops, the researchers collaborate to produce thorough reports and sets of guidelines reflecting the consensus reached. Most of the leading European research groups with activities in nanosafety, nanorisk assessment, and nanotoxicology are represented in NanoImpactNet and they address all relevant exposure routes, major disease classes and impact assessment approaches.

NanoImpactNet coordinates activities within Europe but it is open for worldwide participation and welcomes members from other

continents. NanoImpactNet helps implement the EU Action plan for Nanotechnology and supports the drive to ensure responsible and safe implementation of nanotechnologies in Europe.

2 Background

'Nanomaterials' under its most commonly used definition refers to materials that have at least one structural dimension at the nanoscale, i.e. 1-100 nanometers. Nanomaterials often have chemical, physical and bioactive characteristics different from those of larger entities of material with the same chemical composition, and from molecular forms where these exist. Nano-industries are expanding rapidly and nanotechnology is considered to be a key enabling technology for the 21st century. A wide range of applications are emerging. These new technologies are expected to revolutionize medicine because nanoparticles are small enough to enter individual cells and pass biological barriers inaccessible to molecules or larger materials. The information technology and computer industries are also heavily dependent on nanotechnology for many of their processes and products. Over 800 food and consumer products are already listed in a Woodrow Wilson Institute database, which is currently the largest inventory of consumer products with a declared link to nanotechnology.

Although novel characteristics specific to nanomaterials have led to many exciting new applications, they also raise concerns about their potential health and environmental impacts. Despite recent advances in medical and toxicological research, it is still unclear exactly how nanomaterials interact with biological entities and which characteristics of the nanomaterials drive these responses. Solid NPs and nano-rods (confined in two dimensions) in particular raise potential safety, health and environmental concerns. There is



evidence that some of these materials pass through tissue barriers, including the blood-brain barrier, and cell membranes. There have also been reports of lipid oxidation, granulomatous tissue formation and other adverse responses to interaction with NPs and nanorods.

Little is currently known about the exposure of workers and consumers to nanomaterials, and the effectiveness of existing occupational health and safety measures for industrial processes and consumer products is disputed. This is a challenge for risk and impact assessment studies, and for risk management in laboratories and industry. Even less is known about the environmental fate and impact of nanomaterials. Thus, there are clear knowledge-gaps that need to be addressed as a European priority. Importantly, current environmental and health regulations may not be adequate to ensure the safe environmental dispersion of nanomaterials or to protect human health.

Several national and European projects investigating such risks are already running, about to start or in preparation. However, until recently there was insufficient cross-talk between these initiatives, which posed difficulties both for European researchers and stakeholders. NanoImpactNet was initiated by a group of scientists that wanted to tackle this challenge, and that continues to adapt to the challenge and to develop initiatives to promote cross-talk between projects.

3 What is NanoImpactNet

NanoImpactNet is first and foremost a network and a platform for the exchange of information and ideas. Its unique position has already generated a lot of interest, and the initial 24 partner institutes have been joined by several hundred members, mostly from Europe, but also from the Americas, Asia and Africa. By coordinating research between these scientists from over 30 countries, NanoImpactNet will help to harmonise methodologies and communicate results, initially across Europe, but later worldwide, boosting international cooperation.

The network is composed of researchers from fields spanning the health and environmental impacts of nanomaterials. It receives contributions from researchers and their institutes, representatives of major research projects (European, but also national and non-European) and experts from stakeholders such as industry, civil society and government. Members of NanoImpactNet are leading experts in a wide variety of fields including detection and quantification of nanomaterials, environmental effects, occupational, environmental and consumer health, impact assessment methodologies, materials science, pharmaceutical and medical sciences, and ethics and public engagement. Furthermore, every leading European research group with activities in nanosafety, nanorisk assessment, and nanotoxicology is represented within NanoImpactNet. In synergy, this means that all exposure routes, all major disease classes and all impact assessment approaches are represented within the network.

A better knowledge of the risks that nanomaterials might pose for health and the environment will form a solid foundation upon which to maximise the benefits from nanotechnology, whilst avoiding unnecessary human and environmental harms, and circumventing the possible loss of investments, thereby allowing

for the sustainable development of nanotechnology industries and markets.

Research institutions from countries outside the EC may also participate in NanoImpactNet. We encourage this, in particular from International Cooperation Partner Countries (ICPC) and countries with which the EU has a Scientific and Technological cooperation agreement.

NanoImpactNet workshops provide the opportunity to share and discuss existing knowledge in order to identify knowledge gaps; define strategies to address these gaps; and train staff and students. The workshops provide the opportunity to initiate discussions about strategies and methodologies and external researchers and stakeholders are invited to participate. Following the workshops, the researchers involved collaborate to produce thorough reports and/or guidelines reflecting the consensus reached.

3.1 Summary of NanoImpactNet's key strengths

- NanoImpactNet is committed to open communication, and reports are accessible to all.
- NanoImpactNet communicates stakeholders' needs directly to researchers.
- NanoImpactNet ensures stakeholders receive the information they need and in a format that is of direct use to them.
- NanoImpactNet has become the focal point for exchange of information between the scientific community and stakeholders in the EU and beyond.

4 Organisation of NanoImpactNet

NanoImpactNet's overall goal is efficient exchange of rapidly evolving knowledge, as well as identification of gaps in knowledge regarding the health and environmental implications of NPs. NanoImpactNet develops tools, and training and communication materials to disseminate the current scientific knowledge to researchers, stakeholders and the general public.

NanoImpactNet has a strong commitment to openness and explicitly invites all researchers and stakeholders to participate in the planned activities within the NanoImpactNet co-ordination action. For this purpose, an adaptable two-layer structure was required to manage the complexity and scale of the project: a small coordination group (= NanoImpactNet-consortium) organizes workshops, reports, training material and training schools, while a much larger member-layer (currently consisting of over 1000 people from about 30 countries) comprises experts from science, industry, governments and interest groups who declared their interest to collaborate with the NanoImpactNet consortium (=NanoImpactNet members).

The NanoImpactNet work plan is broken down into six inter-related work packages (WPs, see table below) and obtained funding from 2008 to 2012. Interaction and communication between the WPs is a primary goal of the program to ensure that the consensus of one WP reflects the views, findings and best-practices of the other WPs. Where possible, workshops are held



jointly between WPs to ensure this cross-talk, and the annual Integrating Conferences also serve to promote this agenda. All WPs take existing data into consideration and every attempt is

made to include results and contributions from other ongoing projects.

Table 1 Workpackages (WP) of NanoImpactNet

WP	Title	Topic
1	Human hazards and exposures	This WP is divided into two distinct but related areas: human hazards and human exposure. It helps coordinate efforts to evaluate the safety of nanomaterials towards human health, with particular emphasis on a toolbox of tests to explore key issues, such as the most relevant metric for nanomaterial characterization, methods for dispersion of different nanomaterials, and for assessing persistence, toxicity, and human variability in response to nanomaterials. A second focus is on the determination of any possible exposure of humans throughout a material's (product) lifetime.
2	Hazards and fate of nanomaterials in the environment	This WP helps coordinate activities on environmental fate and effects of nanomaterials released into the environment, the diversity of environments into which they can be released, their behaviour in them and their impact upon organisms within these environments. Issues include compartments most likely to be exposed to or to accumulate nanomaterials of different types, routes of release and potential target species. Computer simulations relating the physicochemical characteristics of nanomaterials to their fate, behaviour and hazard in the in the environment are also addressed.
3	Impact assessment	This WP addresses life cycle aspects of nanomaterials, from production through to the final disposal of products, as well as environmental impact factors for particles and methodological approaches for a comprehensive multidimensional classification of NPs and larger particles that takes into account the many different characteristics of particles. Experts from WP3 will work closely with those from WP1 and WP2 in order to integrate their knowledge, methodologies and achievements into an assessment of the overall impact of nanomaterials.
4	Communication	This WP supports the communication within the NanoImpactNet research community and stakeholders with demonstrated involvement or research knowledge in the field. NanoImpactNet is in contact with over 1000 stakeholders from industries, specialists in technological development, governmental agencies, and the civil society. The network of stakeholders is very interdisciplinary, enhancing the flow of information. This WP seeks input from stakeholders about their information needs and ways to use existing data that is not readily available. Several tools are used, mostly electronic media but also a special session for stakeholders at the NanoImpactNet annual integrating conference.
5	Integration and nomenclature	This WP initiates, stimulates and facilitates cross-talk and collaboration between NanoImpactNet's other WPs. It works towards the creation of joint deliverables and includes the partners in this process from an early stage. This WP organizes an annual integrating conference with the goal to further the exchange of ideas, to share newly gained insights and to discuss strategies to address gaps in knowledge or coordination. It also maintains a research protocols database and a nomenclature database to ensure that a uniform terminology is used within NanoImpactNet reports and documentation.
6	Coordination and management	This WP deals with administrative issues and the mandatory management related to the consortium contract and the contract with the European Commission.

5 NanoImpactNet Events and Reports

NanoImpactNet is approaching the end of its four-year project. Throughout its duration it has organized a series of events, workshops and training schools, and produced several associated reports described below. These illustrate the wide range of questions addressed within NanoImpactNet.

5.1 Events and associated reports

5.1.1 The Dublin Workshops, Ireland

NanoImpactNet held its first two workshops on 19-20 June 2008:

1. Standardization of materials and protocols
2. Most relevant material metrics for different needs (for both hazard and exposure assessment).



Despite being only 2 months after the project's kick-off meeting, over 50 participants - researchers from all over Europe and from the USA - attended each of these workshops. Day one's discussions focused on priority particles for further characterisation, knowledge on dose response relations, the most relevant measurements for characterisation of nanomaterials, and how to determine these in order to conduct a hazard assessment. On day two the need for standard and reference materials and protocols was emphasized. It became clear that robust and established protocols are needed for a new, rapidly developing field of science that is under heavy societal scrutiny. Both workshops struck a good balance between excellent introductions from two respected speakers and ensuing lively discussions and debates.

Approaches to standardization of materials and protocols

All new technologies have an inherent risk, which is typically assessed alongside the development of that technology's applications. This is the case for nanotechnology: a significant portion of the investment in nanotechnology is focussed on its safe and responsible development, and assessment of whether the current regulatory framework is sufficient to handle any additional biological impacts associated with or emerging from the use of engineered nanomaterials (ENMs). Additionally, it is emerging that nanomaterials (NMs) may interfere with the processes behind many existing toxicity tests, and as such, a significant validation procedure is required to ensure that standard chemical toxicity tests are suitable for application to nanosafety assessment. Thus, a widely supported scientific basis and sufficient high quality data upon which to base regulatory decisions are urgently required.

A report entitled 'First approaches to standard protocols and reference materials for the assessment of potential hazards associated with nanomaterials' presents the outcome of the discussions of over 50 experts in the field of safety assessment of manufactured NMs from academia, industry, government and non-profit organizations. It covers some of the critical issues pertaining to the development of standard protocols and reference materials for assessment of the potential hazards associated with NMs.

The major conclusions drawn from the workshop include:

1. Urgent need for nanoparticle (NP) reference (test) materials. However, the validity of positive and negative control reference NMs was questioned, given the very significant variability for NPs.
2. Urgent need to share protocols and best practice. NanoImpactNet offers an excellent platform to achieve this, and the internet based forum should develop a structured approach to method development.
3. Recommendation: OECD could/ should provide templates for the type of data and supporting documentation that it requires for the validation of protocols, and could also provide some training workshops. NanoImpactNet could facilitate this, via one of its training schools.
4. Need for another workshop on this topic closer to the end of the NanoImpactNet project, as clearer consensus of the best practice and recommendations should be significantly advanced by then. Additionally, a second workshop would give the consortium a chance to reflect on the role of NanoImpactNet in facilitating the onward

development and framing of the field in relation to human health impacts assessment

5.1.2 The Zurich Workshops, Switzerland

Over 40 delegates attended a first workshop entitled, 'Strategies to standardize nanomaterials for environmental and ecotoxicological research', on 3-4 September 2008 at ETH Zurich, Switzerland. Workshop delegates were provided with key publications on which to base the discussion in order to allow progression of existing knowledge rather than a reworking of previously published ideas. They focused on three key questions specifically and solely focused on environmental studies:

1. What properties should be characterised for nanomaterials investigated in environmental and ecotoxicology studies?
2. What reference materials should be developed for use in the area of environmental and ecotoxicology studies?
3. Is it possible to group different nanomaterials into categories for consideration in environmental studies?

The workshop participants, through a series of discussion and reflection sessions, generated the following conclusions:

1. The physicochemical characterisation information identified as important for environmental studies included indicators of aggregation/agglomeration/dispersibility, size, dissolution (solubility), surface area, surface charge and surface chemistry/composition.
2. The reference materials identified as being useful for ecotoxicology / environmental studies included TiO₂, polystyrene beads labelled with fluorescent dyes, and silver.
3. No clear consensus was reached regarding division of NMs into categories to aid environmental studies. It was suggested that additional work may be required to derive criteria that can be used to generate such categories.

The workshop therefore allowed identification of priorities for physicochemical characterisation and for the use/development of reference materials.

NanoLifeCycle - Development of approaches and methodologies for assessing the whole life cycle of nanomaterials and nanoproducts

The second workshop's topic was the development of approaches and methodologies for assessing the whole life cycle of NMs and nanoproducts. In particular the workshop considered:

- Current knowledge on release of NPs during production, use and end-of-life: modelling and theoretical approaches
- Current knowledge on release of NPs during production, use and end-of-life: experimental approaches

The workshop's goals were to:

- Support the development of approaches and methodologies for assessing the impacts of NMs during their life cycle in the environment, by assessing the fate and behaviour of NMs in the environment.



- Elaborate upon current data regarding health and environmental exposure to NMs throughout the life cycle of nanoproducts
- Provide advice regarding the identity, quantity and properties of relevant NPs released into different compartments

Group discussions were an important aspect of the workshop. The participants were split into three groups and each discussed several pre-defined questions including: the different meanings of the term 'life cycle', what methods exist to determine life cycle impacts of nanomaterials, and what relevant knowledge is currently available for the overall assessment of the impact(s) of nanoproducts. Another question was how risk assessment methods, and methods based on a life cycle perspective, can complement each other. One group also focused on life cycle assessment methods and on the main elements of life cycle assessment missing in relation to nanotechnology products.

Nanomaterial Environment, Health and Safety Research in the EU: Building a sustainable multi-stakeholder dialogue

The third workshop was a multi-stakeholder dialogue. It built upon targeted phone calls prior to the workshop, during which knowledge gaps and the necessity for further data had been mentioned. Specific discussion items included:

- the potential toxic and safety hazards of NMs throughout their lifecycles;
- the fate and persistence of NPs in humans, animals and the environment;
- the associated risks of NP exposure;
- greater participation of the wider stakeholder group in the preparation of nomenclature, standards, methodologies, protocols and benchmarks;
- the need for development of best practice guidelines for all aspects of nanosafety assessment;
- the need for voluntary schemes on responsibility;
- the need for databases of materials, research topics and themes, but also of expertise.

The first part of this workshop provided an overview of the main stakeholder perspectives, including presentations from representatives of industry, regulatory authorities, NGOs, insurers and the European Commission. During the break-out groups which followed, stakeholders contributed actively to discussions about information needs, communication, safe use of NMs, whether more or other regulation was needed, and whether enough information was available to make informed decisions regarding the safety of NMs and products containing them.

The discussions first confirmed the needs identified in the targeted phone calls. They suggested that reporting should be enhanced, although commercial confidentiality and economic competition were identified as major obstacles. Expertise is needed in the areas of commercial law and economics for a well informed treatment of this communication issue. Further discussion was focussed on the issues of safety and regulation, as follows:

Can engineered nanomaterials be used safely? The idea that NMs are probably safe because some of them have been produced 'for a

long time' was questioned. New legislation like REACH could help address this issue. It was also noted that there is no such thing as a perfectly safe material, but only boundaries, and at this moment we do not know where these boundaries lie. The matter of labelling of products containing NMs was raised, as in the public mind safety and labelling are connected. This may need to be addressed soon as the issue of NMs in food, drink and food packaging may be the first safety issue to attract public and media attention.

Do we need more or other regulation? Any decision making process should accommodate the changing level of uncertainty. To address uncertainties, adaptations of frameworks such as REACH may be necessary for NMs. Even if voluntary measures are welcome, regulation is often needed in order to mitigate the effects of competition between industries.

NanoImpactNet continues an active stakeholder dialogue to further promote interdisciplinary relationships, and to build towards a healthy future with nanotechnology.

5.1.3 The NanoImpactNet Integrating Conference with Training School and Workshops in Lausanne, Switzerland

In March 2009, scientists, policy makers and representatives of civil society and industry from around the world converged at the University Hospitals of Lausanne, Switzerland, to discuss the challenges and limitations of exploring and characterizing NMs. The conference had 5 plenary sessions (1. Human health and exposure; 2. Environmental fate and effects; 3. Life cycle and risk assessment; 4. From research to policies; 5. Connecting the dots) and featured over 30 presentations from leading experts providing insight into the latest nanotechnology research. Back-to-back with the 2-day conference, a training school for young scientists and 2 workshops were organised.

Training School – Handling protocols and toxicological testing strategies

This training school was aimed at PhD students and postdoctoral fellows working on any of the topics related to the assessment of the health and environmental impacts of NMs. The focus was on protocols for handling NMs and protocols for toxicology testing. Issues tackled included controlled dose (understanding of aggregation of NPs in the presence of biological fluids), controlled presentation of NPs to a test system, and development of appropriate testing strategies taking into account the novel aspects of NMs which can influence that testing. The training was thus divided into three sub-sections (1. Nano-object dispersion in media; 2. Introduction of nano-objects into cells, tissues, animals; 3. Toxicological testing strategies), with a plenary opening lecture by Prof. Kenneth Dawson (UCD) on 'Controlling nanoparticle dispersion and presentation is key to rational nanosafety assessment'. The participants were then divided into three in order to ensure that the group size was optimal for encouraging discussion and engagement of the students. Each group attended each of the three training sessions.

Workshop - Protocols for assessment of biological hazards and biological responses

Large numbers of publications are emerging in the literature assessing the hazards of NMs in cells and animals. However, it is becoming increasingly apparent that NMs can interfere with the



read-outs from some test methods, leading to false positives or negatives, as well as inconclusive results. Approaches that are adapted to NMs need to be established and validated. The discussions focussed on three different domains, *in vitro*, *in vivo* and *ex vivo* testing strategies.

A consensus report detailing the proceedings and recommendations of this workshop, entitled "Protocols for assessment of biological hazards of engineered nanomaterials" is available on the NanoImpactNet web site.

Workshop - Development of strategies to assess occupational health effects

One limitation for determining the health and safety impacts of NMs is the lack of methods to determine or quantify levels of occupational exposure over long periods and to investigate the health of potentially affected populations. Currently, there is no Europe-wide system to register occupational health related to NM exposure. Occupational Health reporting strategies were discussed and the ethical, legal and social limitations of such reporting strategies were considered. The workshop began with overviews of the strategies currently used to assess occupational health effects in workers, including health surveillance and occupational health reporting schemes, such as the UK's health and occupational reporting network (THOR). Participants then divided into break-out groups to consider how to develop and apply different approaches within the nanotechnology field. These groups came back together at the end of the day to discuss the best ways forward for occupational health assessment in this arena.

The result of this meeting was a consensus report by Gibson et al. entitled "Strategies for assessing occupational health effects of engineered nanomaterials". This is available on the NanoImpactNet web site.

Stakeholder Workshop - How to make industrial data available

Industry data is clearly proprietary information and can be very sensitive because if it were to 'fall into the wrong hands' valuable investments could be damaged. Firms legitimately put great thought into which partners they might be willing to share their data with. Researchers have to maintain a dialogue with industry to create good faith and trust. From the academic's point of view, it would constitute a great leap forward if industrial scientists could be convinced to share more of their knowledge in public communications or peer-reviewed journals, so as to enable comparative assessments.

Academics are interested in core industry data on exposure, dose response, etc. By bringing industrial and non-industrial researchers and other stakeholders around the same table, this workshop aimed to assess how much information industry is willing to share and what company policies are. The idea was that industry speakers would bring ideas for a common strategy for making industrial data available and what conditions would be necessary for this to happen: case by case, voluntary code, industry rules, existing regulations and/or new nano-specific laws. Additionally, an assessment of the minimum amount of data that would be required for this exercise to be useful was considered necessary, while balancing the needs of industry to protect formulation and other key product-specific information. After a brief introduction and presentations from industry and a regulatory expert, other stakeholders stated their prime, concise question regarding access to industry data to the nano industry participants.

A short stakeholder report entitled, "How stakeholders can be involved in NanoImpactNet and how companies can make data accessible" is available on NanoImpactNet's web site.

5.1.4 The Bilthoven Workshops, The Netherlands

Three interlinked workshops took place in Bilthoven 5-7 October 2009. They focused on the following questions:

Nanoparticle metrics in the air, exposure scenarios and exposure routes

Particle number and particle size distribution are *de facto* standards to describe NP exposure, but other metrics might also be relevant. Furthermore, exposure measurements are more useful for risk assessment if they are linked to exposure scenarios and routes. This workshop focused on measurement metrics relevant for various environments, scenarios and routes, and what needs to be done for a qualitative and quantitative exposure assessment.

Development of standardised protocols to determine fate and behaviour of NPs in the environment

Besides evaluating the state of knowledge regarding the environmental fate and behaviour of NMs, this workshop addressed the problems identified in terms of applying the current chemical exposure assessment framework (i.e. as outlined in the Technical Guidance Document for Risk Assessment of Chemicals in the EU) to NMs. The focus was on carving out solutions through interdisciplinary discussions.

Risk assessment of nanomaterials

This workshop addressed the latest scientific and technical progress across relevant disciplines, with the aim of identifying the issues that are essential for the risk assessment of NMs. It brought support to the integration of existing knowledge and newly gained insights to aid in the development of risk assessment methodologies adequate for NMs.

Two of three reports on these workshops are available on the NIN web site, and one is being merged with a later report.

5.1.5 The Bratislava Training School, Slovakia

This training school for young researchers on "Life cycle-based methods for assessing nanomaterials" took place 9-11 November 2009.

Increasing production and use of engineered NPs raises concerns over their safety to human and environmental health. The training school focused on life cycle-based methods addressing the importance of the whole life cycle concept of nanoproducts, primarily in assessing the hazard and risk of NPs. A basis for the development of adequate methodologies, tools and indicators for assessing the life cycle of NMs was presented. Students got an overview of life cycle methods together with practical training in using life cycle-based tools. The target audience was young researchers, PhD students and junior scientists from different fields with an interest in the fate and life cycle of NMs.



5.1.6 2nd NanoImpactNet Integrating Conference and Training School in Lausanne, Switzerland

From 10-12 March 2010, Lausanne's University hospitals, once again welcomed representatives of academia, regulatory authorities, government departments, civil society and industry to discuss the challenges and limitations of NM safety. The conference had 6 plenary sessions (1a. Interaction between nanomaterials and biological barriers – barriers in the human body; 1b. Barriers in the environment and different species; 2. Nanomaterial behaviour with regard to environmental and physical barriers; 3. Quality control in nanomaterial research; 4. Nanotechnological tools for impact assessment; 5. From research to policy) and featured over 40 presentations from leading experts providing insight into the latest nanosafety research. Over 300 delegates attended this conference, representing over 100 universities and research institutes. A one-day training school took place before the conference on Tuesday 9 March.

Training School – Handling protocols and standardization of nanomaterials in toxicological research

The school addressed higher level issues of 'best practice for safe handling of NMs' to ensure that NanoImpactNet PhD-students and postdoctoral researchers working with them are up-to-date with international best practices. It also aimed to identify the needs of regulatory agencies and develop appropriate strategies. This ensured that NanoImpactNet outputs help these agencies to design and implement nanotechnology regulations. This is key to ensuring that NanoImpactNet plays a leading role in providing the scientific evidence required for potential European nano-regulations.

Prof. Iseult Lynch (UCD) introduced a first session on current international best practice in NP handling, with regulatory (NIOSH), industrial (Intel) and academic (EPFL) experts. Break out groups then used these real-world examples to attempt to determine the minimum safe handling practice that should be observed in their own research laboratories. The second plenary session was on closing the gap between research and regulation. Speakers from the International Organisation for Standardisation, the European Food Safety Authority and the European Medicines Agency, showed how the scientific community's research and reporting of information can contribute to the development of regulations. Guidelines for experimental design and results templates could maximise effectiveness of input to standards & regulation.

Special stakeholder session - "Wrapped up in nano: how to inform the public about nano enhanced food contact materials"

From NGOs to the European Parliament, rising concern about the possible health impacts of NPs in food and NMs in food related products led to the development of this session. Led by Prof. Geoffrey Hunt (SMUC), NanoImpactNet invited stakeholders to contribute to the debate on how this sensitive and controversial issue can be communicated to the public in the near future. Invited speakers from industry (Coke Cola/Confederation of Food and Drink Industries), regulators (European Food Safety Authority) and legislators (EC Directorate General for Health and Consumers), and civil society (Federation of German Consumer Organisations) gave their views. This was followed by a lively debate including questions for conference delegates.

A short report of this stakeholder session is available on the NIN web site and its conclusions may also go towards a peer-review paper.

Conference Sessions

Session 1a focused on Interaction between nanomaterials and biological barriers in the human body. This looked at how NPs interact with barriers in the lungs, gut, brain and other barriers, but also at aspects of NM uptake, intracellular localisation, and cellular fate in the specific cell types found in these organs.

Session 1b looked at the interaction between NMs and the biological barriers in the environment and in different species. It focussed on how NMs in several different media and situations, how uptake differs between species, how NMs enter species (exposure routes) and where they locate within organisms following that exposure and uptake. Large numbers of publications are emerging in the literature assessing the hazards of NMs in cells and animals. However, it is becoming increasingly apparent that NMs can interfere with the read-outs from some test methods, leading to false positives or negatives, as well as inconclusive results. Approaches that are adapted to NMs need to be established and validated. The discussions focussed on three different domains, *in vitro*, *in vivo* and *ex vivo* testing strategies.

Session 2 was on Nanomaterial behaviour with regard to environmental and physical barriers. It focussed on NM exposures in the environment, taking into consideration the ability of NMs to be transported through different environments based on their different surface properties, and also on how NPs are able to cross physical environmental barriers and engineered barriers, such as protective equipment.

Session 3, on Quality control of nanomaterial research looked at NM suspension and exposure, agglomeration and de-agglomeration of NMs in both air and liquids, issues surrounding protein absorption to the surface of NMs, and quality control of NM suspension and exposure systems. This included the physico-chemistry of interactions between NPs and biomolecules.

Session 4 looked at Nanotechnological tools for hazard identification and/or risk assessment, thus addressing how research in the area of nanotechnology safety is facilitating the implementation and use of these products by society. This involved topics such as measurement, imaging and delivery of NPs.

As part of NanoImpactNet's efforts to be a platform for communication and networking, it offered delegates the chance to run their own sessions which took place in parallel with the Stakeholder session. These looked at: Promotion of good practices in research laboratories (lead by ICON, IST and NIOSH); The Precautionary Matrix for Synthetic Nanomaterials (Swiss Federal Office of Public Health); the Fate and toxicity of engineered NPs in the aquatic environment (Italian National Research Council and a number of Italian universities); and NPs and the Immune system (Uni. Salzburg).

5.1.7 Bratislava Training Schools, Slovakia

From 19-23 July 2010, the Slovak Medical University hosted two NanoImpactNet training schools for young researchers.



The first, 19-21 July, was, “*Environmental fate and behaviour of engineered nanoparticles – what’s known and what it would be nice to know*”, prepared and organised by Anders Baun (DTU).

Students learnt how much effort has recently been put into (eco)toxicological research of NMs, and how risk assessment of the environmental fate and behaviour of engineered NMs is also important. This area is less studied and there are important gaps in knowledge in the fields of transport pathways, distribution, degradability, and accumulation of engineered NMs in the environment. This school provided an update on the present state of knowledge in environmental fate and behaviour of engineered NMs and enabled the participants to actively contribute to the ongoing scientific discussions currently taking place in this important area.

The second school, 21-23 July, was entitled, “*Risk assessment for nanomaterials: How can we integrate data for nanomaterials?*”, prepared and organised by Maria Dusinska (NILU).

The school addressed the basic principles of risk assessment and further focused on the most important issues necessary for the development of a framework for the risk assessment of NMs. Participants obtained an overview of the latest insights on the integration of exposure estimation, life cycle analysis, hazard assessment with both human and ecotoxicological data. This is necessary in the development of risk assessment methodologies adequate for NMs. The event provided an update on the present state of knowledge in risk assessment of engineered NMs and also gave the participants the opportunity to actively contribute to the ongoing scientific discussions in this important field. Particular examples were given using presentations of consumer products, food, asbestos, carbon nanotubes, occupational exposures and critical evaluations of *in vitro* and *in vivo* data.

See below for reports, results and conclusions of these training schools.

5.1.8 The Dublin Workshops, Ireland

From 6-9 September 2010, University College Dublin hosted three NanoImpactNet workshops for both young and experienced researchers.

Workshop 1, 6-7 September, was organised by Richard Handy (UoP) and was entitled, “*Hazard assessment of Nanomaterials in Biota – Recent advances in methodology and challenges ahead.*”

This technical workshop brought together experts with “hands on” bench experience of NMs to discuss recent advances in methodology, share current experiences, identify the problems and potential solutions for studying the biological effects of NMs. The purpose was to find agreements on details of ecotoxicity methods for fundamental research, as well as more applied aspects in regulatory testing, using the OECD as an example. The workshop provided key presentations on issues relating to ecotoxicity protocols, but lots of time was devoted to creative scientific discussion. These covered soil and sediment organisms, microbes, terrestrial plants and vertebrate animals, as well as aquatic ecotoxicity on algae, invertebrates and fish in freshwater or marine chemistry.

Results and conclusions of this workshop and its associated training school in Bratislava (see above), entitled “Practical Considerations for Conducting Ecotoxicity Test Methods with Manufactured

Nanomaterials: What Have We Learnt So Far?” has been submitted to the peer review journal *Ecotoxicology*.

Workshop 2, 8 September, was organised by Maria Dusinska (NILU) and was entitled, “*Impact assessment of Nanomaterials – Nanomedicines and nanotechnology: two sides of the same coin.*”

The second workshop addressed the impact of NMs on human and environmental health, discussing all aspects contributing to the health and environmental risks and benefits, using the challenges facing nanomedicine as a frame. This rapidly developing area has created new tools and methods that significantly affect existing conservative practices. Students learnt about how nanomedicine exploits novel physical, chemical and biological properties of nanometer-scale materials: drug delivery, imaging and diagnostics, cancer therapy, surgery, tissue engineering, NM interactions with living tissue, etc. They also learnt about the flip-side: the potential toxicological problems of NMs. Students discussed the gaps in existing knowledge and how to improve communication between pharmacology industry, nanotoxicologists and how to integrate knowledge from all relevant fields. A technical report of the workshop is in production and will go towards further deliverables.

Workshop 3, 9 September, was organised by Markus Berges (DGUV-BGIA) and was entitled, “*Nanoparticle metrics in the air, exposure scenarios and exposure routes.*”

Particle number and number-size distribution are the de facto standard to describe exposure to nano-objects. Students learnt about current measurement devices like big, bulky SMPS or ELPI that can only be operated by trained personnel and about smaller portable monitors close to market. Neither type of instrument gives information on the morphology or the chemical identity of the nano-objects, however. A packed programme explained the use of these NP samplers, sampling and analysis of NPs onto electron microscopy grids, electrostatic sampling of NPs, and the challenges of imaging.

An overview of existing and proposed strategies for measuring exposure was given. Typically grids are sampled for further electron microscopy analysis to resolve this question and to allow for background distinction. This presents a challenge in itself as no standardized methods for the sampling are available.

Given current measurement devices, students learnt the crucial point of applying a suitable measurement strategy to assess and to control NP exposure via inhalation and attempts to solve the problem of missing background distinctions by the instruments via these strategies. They learnt that there is currently no unified agreed approach and no international harmonized exposure database. The advantages of the NP emission assessment technique were given and the inherent challenges of data interpretation. A representative of BASF explained his company’s global strategy for monitoring nanoscale aerosols on its sites.

The workshop and the planned report aimed to close or at least narrow this gap and work on common documents towards agreed approaches. The report will contribute to the Work Package’s final report.

5.1.9 Prague, Czech Republic

As an extra deliverable and in association with the British Embassy (Science and Innovation Network of the UK Government), IOM and IST led a high-level meeting on nanotechnology safety in Prague.



Top policy makers and scientists from the Czech Republic, Europe and the USA met, 29-30 November 2010, at the Academy of Sciences.

Speakers described their areas of expertise in light of the specific complexity and uncertainty which nanotechnologies bring. Delegates learnt about transatlantic dimensions to nanosafety, including how the US National Nanotechnology Coordination Office is creating strategic focus to minimise uncertainty and complexity in nanotechnology safety research, and details of the US EPA's experience in assessing the risk of MNMs.

High-level presentations were given by the EC, JRC, WHO, UNITAR and esteemed European academic researchers. Representatives of industry explained their take on complexity and nanosafety, and Dr Wolf-Michael Catenhusen presented Germany's national dialogue on nanosafety and the views of the most important stakeholders in safe nanotechnologies – citizen-consumers.

Analysis of the risks posed by MNM must cover their entire life cycle - from production (workers may face significant contact with new nano-enhanced goods), to use (consumers take advantage of nano-benefits), to elimination (somewhere in our environment). Nothing can be taken for granted when it comes to nano-safety. This has been reflected in peer review article by Hunt & Riediker published in 2011 in *Nanotechnology Perceptions*: "Building expert consensus on problems of uncertainty and complexity in nanomaterial safety."

5.1.10 3rd NanoImpactNet Integrating Conference and Training School in Lausanne, Switzerland

From 14-17 February 2011, Lausanne's University hospitals, once again welcomed representatives of academia, regulatory authorities, government departments, civil society and industry to discuss the challenges and limitations of NM safety. The conference had 6 plenary sessions (1. Opening session: Setting the scene – Creating bridges between nanomedicine and NanoImpactNet research; 2. Nano-pharmacological input to research on the human and environmental impact of nanomaterials; 3. Lessons from Nano-Immunology on the impact of nanomaterials; 4. Human impact of engineered nanomaterials and lessons for the nanomedical field; 5. Implications from environmental fate and behaviour research for the field of nanomedicine involving nanomaterials; 6. The future of the NanoImpactNet community) and featured over 29 presentations from leading experts providing insight into the latest nanosafety research. Over 240 delegates attended this conference, representing over dozens of universities and research institutes. A one-day training school took place on the day after the conference on Thursday 17 February. *Training School on Reproducible Uptake and Quantification of Nanoparticles in vitro (and in vivo)*.

The school was split into 2 parts. Session 1 covered stereological counting of NPs in cells. This included the preparation and optimisation of samples for TEM imaging and stereology, and the general principles of this method. Students carried out some practical exercises on the quantification of uptake via stereology of TEM images. Session 2 was on quantitative NP uptake. It covered different approaches of studying and quantifying uptake kinetics of NPs in vitro, using flow cytometry and imaging techniques. Students also learnt about Time-resolved analysis for splitting random trajectories (an approach for understanding NP uptake

and dynamics in cells). They then carried out quantification exercises of uptake and dynamic in cells.

Conference Sessions

The Opening session focused on creating bridges between nanomedicine and NanoImpactNet's research. It featured presentations from the director of Lausanne's university hospital; of the opportunities and risks of nanomedicines by a research clinician; and about EU FP7 policies for nanoSafety and nanomedicine research.

Session 1 looked at Nano-pharmacological input to research on the human and environmental impact of nanomaterials. Presentations covered whether nanotechnology can solve solubility problems in pharmacology; identification of protein-nanoparticle interaction sites; blood clearance and tissue distribution of NPs; and nanostructured drug delivery to the brain via the nose..

Session 2 looked at lessons from the field of nano-immunology on the impact of nanomaterials. Subjects covered included understanding the interactions of engineered NMs with the immune system; the effects of NPs on immune response of lymphocytes, identifying immune related gene-markers following interaction of engineered NPs with human intestinal epithelial cells; an immunosafety of engineered NPs and implementing methods for developing nanomedicines.

Session 3 looked at the human impact of engineered NMs and lessons for the field of nanomedicine, Presentations covered silicon nitride porous membranes for NP translocation in vitro assays; suspension and aerosol exposure scenarios of lung cells in vitro to zinc oxide; how copper oxide NPs at via a Trojan Horse mechanism; and a new screening too for NP toxicity experiments.

Session 4 looked at the Implications from environmental fate & behaviour research for the field of nanomedicine involving nanomaterials. Subjects covered included: how NPs behave in biological fluids; how to make highly fluorescent silica NPs to trace their intracellular fate; assessing exposure risk, persistence and accumulation on NP silver in aquatic and marine environments; and how all these impact nanomedicine.

Session 5 looked to the future of the NanoImpactNet community, the project's final year of operation and how certain nanosafety communication activities might continue in the future.

Special stakeholder session - "Involving stakeholders in setting research priorities"

Invited speakers from industry (Novartis), a consumer group (ANEC) and the Centre for Bioethics and Emerging Technologies reflected on whether nanotechnologies will transform medicine, how it might do so and what it might actually offer in the next 10-15 years. They also looked at whether the benefits will be worthwhile both in terms of development costs and in terms of prevention and therapy for patients, and whether those benefits outweigh potential risks, or poor perceptions. Led by Profs. Geoffrey Hunt (SMUC), and Alain Kaufmann (Uni. Lausanne) and with active participation from delegates, discussions covered potential disease and population groups which might benefit.

The results of this session and two parallel sessions on characterisation and communication were combined to make



NIN's 4th report on stakeholders and their interests, available on the NIN web site.

As part of NanoImpactNet's efforts to be a platform for communication and networking, it offered delegates the chance to run their own sessions which took place in parallel. These looked at: Kicking off an Immunosafety Task Force (Italian National Research Council and Uni. Salzburg); Responsible NP use to ensure marine ecosystem sustainability (Italian National Research Council and Uni. Siena); NPs in paints (Danish National Research Centre for the Working Environment and Uni. Copenhagen); The current status, needs and challenges facing NP characterisation (JRC). –

5.1.11 The Leiden Training School, The Netherlands

This training school for young researchers took place from 28-30 September 2011, and was entitled "Risk Assessment Issues of Nanomaterials in the Aquatic and Terrestrial Environment". It aimed to allow participants to gain experience in risk assessment of manufactured nanomaterials. Its main goal was to acquaint them with nano-specific issues regarding fate and effect assessment of nanomaterials, taking the specific physico-chemical properties of the test media into account. Current testing protocols (like the broadly used OECD protocols for testing "regular" synthetic compounds) were the assessment starting points and emphasis was laid on tailoring the protocols for use in nano-specific assessment. Key issues dealt with included:

1. Characteristic differences between nanomaterials and "regular" chemicals, determining distribution and fate, as well as toxic effects of nanomaterials. The keyword in this respect is speciation of nanomaterials;
2. Physico-chemical soil and water properties affecting nanomaterials speciation;
3. Analytical aspects, with focus on experimental test systems;
4. Performing risk assessment of nanomaterial

Results from this training school will go towards the Work Package's final report and will be made available on NanoImpactNet's web site.

5.1.12 The London Workshop, United Kingdom

King's College London hosted a workshop entitled "Nanoparticle metrics and exposure: From exposure to effects", 17-19 October 2011, and it was attended by over 40 experts in the field.

With more and more information on the release, exposure and possible toxicological effects of nano-objects being produced, the understanding of fundamental and general aspects in these fields is steadily increasing. The workshop brought together over 40 experts in these different research fields to build a bridge from exposure to health effects for manufactured nanomaterials. Discussions began with a presentation of the state of the art and future needs. It moved on to cover all the relevant aspects bridging the fields of release and exposure with toxicological studies.

Sessions looked at: Release to exposure scenarios and dose; Measurement of nanomaterials in gas and liquid phase: difficulties and bridging the definitions; Strategies towards a close(r)

integration of exposure measurements and toxicology; and a general discussion on Integration of Exposure measurements and Toxicology and how this improves future risk assessments

On the third day organisers and rapporteurs put together the first draft of a report which will serve as the Work Package's final report and will be submitted as an article to a peer review journal.

The workshop report will contribute to the Work Package's final report and will be made available on the NanoImpactNet web site..

5.1.13 The Bratislava Training School, Slovakia

This training school for young researchers took place 12-14 October 2011, at the Slovak Medical University, Bratislava. It was entitled "Impact Assessment of Nanomaterials" ..

The school aimed to provide participants with the necessary knowledge and skills to assess the impact of manufactured nanomaterials on human health and the environment.

As an example of the rapid developments in the application of nanomaterials, the focus was on the health and environmental risks and benefits of nanomaterials in medicine. Following on from the results and discussions of the workshop on the impact assessment of nanomedicines, held in 2010 in Dublin, the school provided an overview of the broad range of possibilities for application. But a cause for concern is that many of the properties advantageous for medicine can potentially cause problems for human health. Finding the right balance between the opportunities that new technologies can bring and level of acceptance of the remaining risks is crucial. The school helped participants to develop methodologies to assess the costs and benefits of nanomaterials in medicine.

A half day was dedicated to new in vivo data obtained from the NanoTEST project (Development of testing strategies for testing nanomaterials used in nanomedicine) on rats exposed to TiO₂ and Fe₃O₄ NPs. In vivo data was discussed in light of in vitro findings for the same NPs, and using the same biomarkers of oxidative stress, inflammation, immunotoxicity and genotoxicity.

Reports with results and conclusions of these training schools are currently in preparation and will go towards the Work Package's final report and will be made available on the NanoImpactNet web site..

5.2 Further Reports

In addition to reports directly associated with specific events, workshops and training schools, NanoImpactNet has produced a number of reports that are available on its web site.

These include four "Major Information Packages" (another will be ready for the end of the project) which contain press releases for specialised and general media and syntheses of the events organised and reports drafted by the Work Packages on Human hazards and exposures, Hazards and fate of nanomaterials in the environment, and Impact assessment, adapted to be less technically detailed for various stakeholders in nanomaterials and health, safety and the environment.



Reports on the communication and dissemination aspects of the NanoImpactNet project include: a 2008 report on "Stakeholders and their Interests"; a short 2009 stakeholder report on "How stakeholders can be involved in NanoImpactNet and how companies can make data accessible";

NanoImpactNet has disseminated regular informative newsletters by email to keep its many stakeholders in touch with the projects

results. These newsletters also include the individual successes of its members in the field of nanosafety and relevant news and related events .

Other reports and publications include a supplementary final year report and a NanoImpactNet nomenclature document.

6 Directory

Table 2 Directory of people involved in this project.

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NanoLyse

Nanoparticles in food: Analytical methods for detection and characterisation



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5	The Secretary of State for Environment, Food and Rural Affairs	FERA	United Kingdom
6	University of Alberta	UAlberta	Canada
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1 Summary

The NanoLyse project focusses on the development of validated methods and reference materials for the analysis of engineered nano-particles (ENP) in food and beverages. The developed methods aim to cover all relevant classes of ENP with reported or expected food and food contact material applications, i.e. metal, metal oxide/silicate, carbon and organic (encapsulate, carrier) ENP. Priority ENPs have been selected out of each class as model

particles to demonstrate the applicability of the developed approaches, e.g. nano-silver, nano-silica, fullerenes and organic nano-carriers systems. Priority is given to methods which can be implemented in existing food analysis laboratories. A dual approach is followed. Rapid imaging and screening methods will allow the distinction between samples which contain ENP and those that do not. These methods are characterised by minimal sample preparation, cost-efficiency, high throughput and will be



achieved by the application of automated smart electron microscopy imaging and screening techniques in sensor and immunochemical formats. More sophisticated, hyphenated methods will allow the unambiguous characterisation and quantification of ENP. These will include elaborate sample preparation techniques, separation by flow field fractionation and chromatographic techniques as well as mass spectrometric and electron microscopic characterisation techniques. The developed methods will be validated using the well characterised food matrix reference materials that are produced within the project. Small-scale interlaboratory method performance studies and the analysis of a few commercially available products claiming or suspect to contain ENP will demonstrate the applicability and soundness of the developed methods.

The project has a duration of 45 months (2010 – 2013).

2 Objectives

“The Scientific Committee makes a series of recommendations; in particular, actions should be taken to develop methods to detect and measure ENMs [engineered nanomaterials] in food/feed and biological tissues, to survey the use of ENMs in the food/feed area, to assess the exposure in consumers and livestock, and to generate information on the toxicity of different ENMs.”. (EFSA, Scientific Opinion of the Scientific Committee on the Potential Risks Arising from Nanoscience and Nanotechnologies on Food and Feed Safety, The EFSA Journal (2009) 958, 1-39)

In addition to research institutes busy with development and toxicological evaluation of nanomaterials, there is an urgent need both for official food control entities and industry for analytical methods that allow the routine detection of engineered nanoparticles in food, as well as for reference materials for the validation of analytical methods and for proficiency testing of laboratories. The NanoLyse project addresses these needs by the following objectives.

- Development of reference materials for the analysis of nanoparticles in food and beverages matrices
- Development of sample preparation methods for the detection of nanoparticles in food
- Development of rapid imaging and screening methods for nanoparticles in food
- Development of analytical methods for the identification, characterisation and quantification of inorganic and organic nanoparticles from the food matrix
- Dissemination and training of the new methods to relevant stakeholders

PRODUCTS

According to the objectives of the project NanoLyse will deliver a number of different products to the general public, the scientific community and in particular to specific stakeholders involved in the risk assessment and legislation for the use of nanoparticles in food as well as in the analysis of engineered nanoparticles in these matrices for exposure assessment, monitoring and quality control purposes. The deliverables include:

- Protocols for the analysis of different engineered nanoparticles in food and beverages (WP2, WP3, WP4), including sample preparation methods
- Reference materials for nanoparticles in food matrix (for use within the project, available to external laboratories which participate in the interlaboratory method performance studies) (WP1)
- Protocols for the reproducible production of such reference materials (WP1)
- Method validation concept for engineered nanoparticles in food (WP1)
- Publications in international journals on the scientific results of the project (WP1, WP2, WP3, WP4)
- Open days for stakeholders and the interested public (WP5)
- Training workshops for the transfer of the developed methods to stakeholder laboratories (WP5)

3 Concept and structure

CONCEPT

Basically the concept of NanoLyse is to merge the technologies which are available for engineered nanoparticles (ENP) analysis in other disciplines, e.g. materials and environmental sciences, into the analytical strategies and procedures characteristic for the food safety area, taking into account the very specific physico-chemical properties of nanoparticles as compared to their macro-scale or dissolved analogues.

A current analytical strategy in food safety monitoring is a dual approach comprising screening (fast, qualitative) and confirmatory (high standard, quantitative) methods. Screening methods are designed to sort out negative samples in a fast, cost-efficient, automated high-throughput approach. The samples identified as suspect positives are subjected to a more sophisticated method which allows the unambiguous identification and quantification of the target analytes.

NanoLyse adopts this approach by the development of two levels of methods:

- imaging and screening methods for a rapid decision on the presence of ENP in food samples and
- methods for the full identification, characterisation and quantification of ENP in food.

Rapid analysis will be achieved in WP2 by electron microscopy (EM) imaging as well as by two screening assays: a sensor assay for automated high-throughput analysis and an immunoassay in ELISA format for direct implementation of ENP analysis even in basic food laboratories. For the precise characterisation and quantification the most suitable separation (i.e. flow field fractionation, hydrodynamic and size exclusion chromatography) and detection techniques (e.g. EM and mass spectrometry) will be coupled into hyphenated methods in WP3 (inorganic ENP) and WP4 (organic ENP). The developed methods will be validated using



the well characterised food matrix reference materials that will be produced within the project (WP1).

STRUCTURE

The project is structured into four RTD and two supporting work packages (fig. 1). All WPs are closely linked with each other to ensure maximum synergies. WP1 supplies all method developers with characterised ENP dispersions and test and reference materials for method development and validation. All method development WPs (2-4) collaborate very closely on the sample preparation via the respective inter-WP working group (WG), especially in the first phase of the project. In the same way WP 3 and WP4 collaborate on the analytical separation techniques. All RTD WPs (1-4) contribute to the dissemination and training activities which are organised by WP5. WP6 supplies all information necessary for the successful execution of the project to all WPs and collects all data needed for the regular reporting and the monitoring of the progress of work.

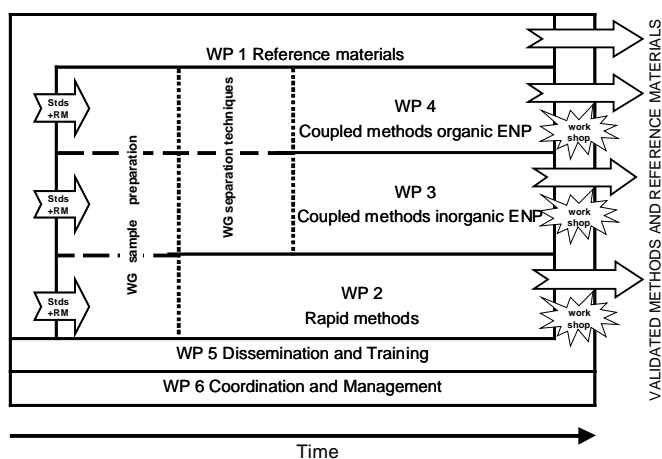


Figure 1: Interrelations between Work packages (WP) of NanoLyse

4 Project tasks and results

4.1 WP1: Reference Materials

OBJECTIVES

Reference materials are essential to calibrate analytical instruments, develop and validate test methods and assess the performance of individual laboratories. Up to date only few reference materials are available (as aqueous suspensions) for nanoparticles (most of them not relevant for food related applications). Therefore, Work package 1 has three main objectives, namely the production of reference materials for method development and method validation, the development of a solid and sound approach for method validation and compilation of the knowledge gained in the project into a document outlining a reproducible preparation of ENP containing food reference materials, including information on processing, homogeneity and stability.

- Supply of well defined and characterised engineered nanoparticles and labelled analogues

- Production and characterisation of engineered nanoparticles reference materials
- Development of a metrologically robust method validation approach for engineered nanoparticles in food

ACTIVITIES

WP1 supports WP2, WP3 and WP4 by preparing and supplying suspensions of labelled and non-labelled engineered nanoparticles (ENP) and food materials spiked with suspensions of ENPs. The suspensions will be characterised for their purity and ENP concentration, and the ENP size distribution.

A number of relevant ENPs has been purchased or produced, and processed into appropriately characterised ENP suspensions. Four different types of model particles have been chosen, namely metal nanoparticles, metal-oxide nanoparticles, fullerenes and organic nanoparticles. These particles have been sourced and, in the first stage of the project, aqueous suspensions of these four types of particles have been prepared. These materials are used by the other project partners for spiking experiments during method development. The aqueous suspensions are tested for homogeneity and stability, thus ensuring that the materials fulfil all requirements for reference materials.

In the second phase of the project, food materials will be spiked with the same materials. Also these spiked food materials will be tested for homogeneity and stability. These food reference materials will finally undergo characterization by intercomparison, thus allowing assessment of trueness of the methods involved.

A metrologically robust method validation approach for the analysis of ENP in food will be developed in WP1 with input from the advisory board on validation and standardisation requirements. The protocols and reports on the production of reference materials will be available for future use in e.g. proficiency testing of laboratories for the detection of ENPs in food.

RESULTS

Silver and silica nanoparticles were obtained from several commercial suppliers and the most suitable ones in terms of size, dispersibility and stability were chosen. The selected materials were silver particles (43 ± 15 nm, spherical, PVP stabilised) and food grade silica (particle size 150 ± 63 nm). Fumed silica is an approved and widely used food additive (E551) and the chosen material is similar to OECD testing material NM203. Dispersions of defined concentration levels were prepared. They were distributed to the project partners and will be used in the development of reference materials.

Tomato soup has been prepared from basic ingredients (fresh onions, tomatoes etc.) to ensure absence of engineered nanoparticles. This soup was spiked with silica nanoparticles to achieve final mass fractions of 1 and 4 %. This soup corresponds to for example soups in powder form containing silica as anti-caking agent, which may also be small enough to fulfil the definition of nanomaterial. Rapeseed oil was spiked with fullerenes at mass fractions of 10 and 100 $\mu\text{g/L}$. These materials have been distributed to the other project partners.



Figure 2: Reference material: Tomato soup spiked with silica

Experiments to stabilise meat, the target matrix for the silver particles, have been executed: freezing is not possible because of potential change and agglomeration of particles. Steam sterilisation was also not successful, but gamma-irradiation was found to prevent degradation. Ag-nanoparticles in aqueous suspension remained unchanged by irradiation, hinting that it should not cause any problems also for the matrix material. Currently, meat spiked with Ag nanoparticles, mimicking e.g. diffusion from Ag-impregnated cutting boards, is being prepared.

Labelled particles have been synthesised and characterised: Silica nanoparticles containing a small amount of GeO₂ and Ag nanoparticles containing a small amount of Au were produced. The materials were characterised with respect to their particle size, dispersion behaviour and Au/Ge mass fraction. The particles show sufficient stability and are intended as internal standards and/or as recovery control for the methods being developed

Finally, a common validation approach for the determination of nanoparticles in food was developed. The proposed approach will be published as a basis for discussion among scientific and regulatory communities and the iterative development and advancement of robust and widely accepted validation guidelines.

4.2 WP2: Rapid imaging and screening methods

OBJECTIVES

Presumably, many foods will not contain any engineered nanoparticles. Applying rapid, cost-efficient and robust methods to distinguish the samples which actually contain engineered nanoparticles from the majority which doesn't, would allow to focus more laborious quantitative methods on those samples. The objective of WP2 is to develop such rapid analytical methods, based on imaging and screening techniques, for providing qualitative and semi-quantitative data on engineered nanoparticles in different food matrices. The developed methods should enable a rapid decision if any target particles are present or absent in a food sample.

- Sample preparation methodology tailored to imaging and screening methods for engineered nanoparticles in foods
- A simplified electron microscopic imaging tool with automated smart image analysis for the rapid detection of engineered nanoparticles presence in different food matrices

- Screening assays for engineered nanoparticles in sensor and ELISA format

ACTIVITIES

In order that the developed techniques can be applied broadly in the future, the work package will explore a range of engineered nanoparticle types that are relevant to food such as: metal-based (Ag), metal oxides (SiO₂), and organic nano-carrier systems.

Electron microscopy: A limited number of imaging methodologies (SEM, TEM, including inherent characterisation of elemental composition by EDX, EELS) will be compared initially for aqueous engineered nanoparticles dispersions. The most suited will be selected for further method development. A major challenge will be the preparation of the food materials for analysis. The work package will therefore explore sample preparation techniques for a range of matrices starting with non-complex systems (i.e. water), and finally moving to more complex matrices. Work will be focused on easy to use and low-cost techniques e.g. resin embedding. For more complex samples a range of more sophisticated techniques will be available, e.g. capsules to enable imaging under fully liquid conditions. Finally the most successful sample preparation and detection methods for different engineered nanoparticle types will be combined into fully validated methods. In order to achieve automation and high throughput automated object-based image analysis will be explored and further developed.

Screening assays: Two approaches will be followed:

- (i) an ELISA approach for engineered nanoparticles for direct implementation in basic food labs,
- (ii) a sensor approach based either on bio- or physico-chemical recognition of functionalised, encapsulate or metal(oxide) engineered nanoparticles, respectively, for automated high throughput analysis. Both methods will be validated according to the standards for screening methods.

The validation will include the analysis of a limited number of real samples from the market, claiming or suspect to contain engineered nanoparticles.

RESULTS

Concerning electron microscopy, a number of sample preparation methods for nanoparticle samples both in liquid dispersions and food matrices have been tested. These include air drying, blotting, freeze drying, ultracentrifugation, chemical drying, resin embedding, and freezing for imaging in liquid conditions. Of the methods tested so far, ultracentrifugation has been identified as the most appropriate method for preparation of samples at low concentrations of ENPs. The method minimises aggregation of ENPs during processing. Blotting is another rapid and cheap method for working with untreated samples as it does not cause any major changes to ENPs in comparison with other drying-on-grid methods.

An initial prototype object-based software system for analysis of nanoparticle EM images in food matrices has been developed for semi-automated analysis of electron microscopy images of nanoparticles in food matrices.

Antibodies have been raised against cross-linked gelatine nanoparticles that are a component of nano-sized carriers for

delivery of nutrients and supplements in food products. A prototype ELISA assay for these organic nanoparticles has shown high specificity and sensitivity.

A screening assay for silver ENPs in food has been developed. The Surface Plasmon Resonance (SPR) sensor based assay uses a metallothionein (MT) protein immobilised on the sensor chip surface. The sensor has shown sensitivity at ppb level for Ag-ENPs in different food matrices.

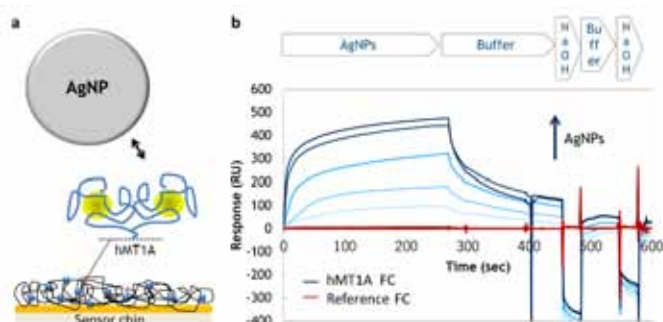


Figure 3: The SPR biosensor for silver nanoparticles; a: principle, b: analysis cycles for different Ag NP concentrations

4.3 WP3: Coupled separation / characterisation methods for inorganic nanoparticles

OBJECTIVES

If engineered inorganic nanoparticles are present in foods their identity and quantity needs to be determined, e.g. for proper exposure assessments or the testing for any (future) legal limits. The goal of WP3 is to develop methods for the unambiguous characterisation and quantification of inorganic nanoparticles in food, including sampling, sample preparation, analytical separation and instrumental detection. Separation and detection will be coupled on-line into reliable quantitative methods.

- Sampling and sample preparation methodologies tailored to the quantitative detection of inorganic engineered nanoparticles in foods
- Validated methods for the determination of inorganic engineered nanoparticles in food extracts, based on size separation (HDC, FFF), size determination (light scattering) and specific detection (ICP-MS)

ACTIVITIES

For quantification purposes most inorganic engineered nanoparticles (ENPs) will require a sample preparation step to isolate them from the matrix. Potential sample preparation techniques include physical separations, wet digestion or thermal treatments. Due to the presence of residual matrix components in sample extracts an additional analytical separation of the engineered nanoparticles will be inevitable and field-flow fractionation (FFF) and hydrodynamic chromatography (HDC) have already been recognized as highly suitable for this. In cases of very small particles which do not aggregate, size exclusion chromatography (SEC) may be a third option. Light scattering techniques and spectrometric techniques (ICP-MS, ICP-OES, UV-

DAD and fluorescence) will be used for particle sizing and detection.

A protocol for representative sampling of engineered nanoparticles containing food will be developed on a statistical basis taking into account the size distribution and number density of ENPs at a range of concentrations above the detection limits. The sample preparation methodologies will follow the track of

- 1) reducing complexity of the sample matrices with minimum alteration of the virgin engineered nanoparticles, including chemical or enzymatic matrix digestion, followed or accompanied by
- 2) a physical separation step as ultracentrifugation, ultrafiltration, density separation, liquid/liquid extraction, preparative SEC and the split-flow thin cell (SPLIT) technique to finally
- 3) transfer the engineered nanoparticles into a state compatible with FFF and HDC.

Analytical fractionation methods for ENPs isolated from the food matrix will be established based on HDC and FFF. Suitable detection methods will be selected (e.g. UV-DAD, static/dynamic light scattering and ICP-MS or -OES) and further optimized for the ENPs received from WP1 in simple matrices. Performance characteristics will be determined. The following criteria will be used for further consideration of a given methodology: recovery of ENPs, fractionation efficiency and detection selectivity, repeatability, sensitivity. The selected hyphenated methods will be fully validated. The validation will include the analysis of a limited number of real samples from the market, claiming or suspect to contain ENP.

RESULTS

Principles for nanoparticle sampling procedures from food matrices based on statistical considerations have been established. An Excel spread sheet was developed which helps estimating the required sample sizes based on statistical methods.

Suitable sample extraction procedures have been identified. These are based on enzymatic and chemical digestion as well as surfactant extractions and density separation.

Separation methods based on asymmetric flow field flow fractionation (AF⁴) were developed for different Ag and SiO₂ nanoparticles.

Various detection devices coupled to Field Flow Fractionation (FFF) have been evaluated. These included UV vis, multi-angle laser light scattering (MALLS), dynamic light scattering (DLS) and inductively coupled plasma mass spectrometry (ICP-MS). Optimizations were carried out towards analysis of silver and silica nanoparticles in food matrices, resulting in recommendations concerning the type of detector and the setting to be used. ICP-MS and light scattering detection present the most promising outcome.

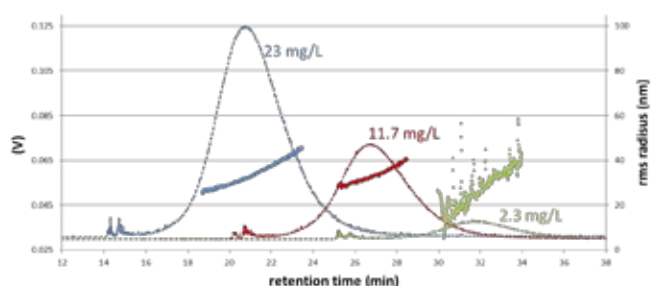


Figure 4: AF4 fractogram of 100 nm SiO₂ with light scattering detection with different injected volumes (blue 100µL, red 50µL, green 10µL), and respective rms radius fit (triangles). The retention time of the 50 and 10 µL were artificially shifted for better readability.

4.4 WP4: Coupled separation / characterisation methods for organic and functionalised ENPs

OBJECTIVES

In the case that engineered organic nanoparticles are present in foods their identity and quantity needs to be determined, e.g. for proper exposure assessments or the testing for any (future) legal limits. The aim of WP4 is to develop respective methods for the detection and characterisation of organic nanoparticles in food, including sampling, sample preparation, analytical separation and instrumental detection.

- Sampling and sample preparation methods for organic and surface functionalised engineered nanoparticles in food matrices
- Validated combined separation and detection methods for organic/functionalised engineered nanoparticles in food matrices based on flow separation techniques and mass spectrometry

ACTIVITIES

The detection and identification of organic, and functionalized, engineered nanoparticles (ENPs) in food items is difficult since the shell of organic engineered nanoparticles is often of a similar nature as that of many food constituents (e.g. proteins, lipids, carbohydrates). A first necessity is therefore the availability of a technique capable of discriminating between organic engineered nanoparticles and residues of matrix constituents. Techniques with that potential will be selected and sampling, sample preparation and separation/fractionation methods for organic engineered nanoparticles from food will be developed from there. Finally, a separation and detection technique will be combined and validated as a complete method for the detection and characterization of organic engineered nanoparticles in food.

RESULTS

A liquid chromatography method with mass spectrometric detection (LC-MS/MS) has been established for fullerenes in food samples.

A differential mobility analyser (DMA) to separate nanoparticles on size is operational. Nanoparticles in suspension are nebulised and ionized with a system that is comparable with electron spray

ionisation in LC/MS analysis. The resolution of the system appears to be good, also for small (>20 nm) particles.

At the same time a nanoparticle database for MALDI-TOF is being prepared and additional particles are tested to add them to the database. Suspensions of these particles, and later on sample extracts, are separated using hydrodynamic chromatography to isolate the fraction containing the particles of interest. These are then off-line analysed with MALDI-TOF.

In addition to that, if the MALDI-TOF is not specific enough, or if compounds are not ionised in MALDI-TOF, mass fingerprinting using TOFMS is being studied. First results with casein as a model particle show that it can be characterised by MALDI-TOF, but much more in detail (type of casein) by fingerprinting/TOFMS.

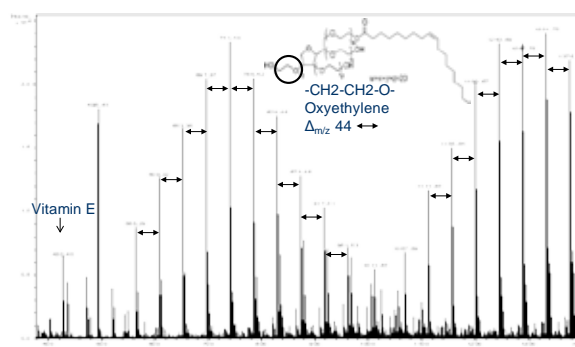


Figure 5: MALDI-ToF spectrum of a polysorbate micelle loaded with vitamin E

4.5 WP5: Dissemination and training

OBJECTIVES

The NanoLyse project intends to exploit the knowledge which is generated within the project in the most beneficial way in various aspects. This includes consumer food safety, competitiveness of the European economy as well as scientific progress. Main goal of WP5 is to ensure that the knowledge and methods which are developed within NanoLyse are distributed to stakeholders and exploited in a proper way.

- Active dissemination of results to stakeholders and scientists via website, newsletter, publications and presentations at stakeholder and scientific events
- Technology transfer and training to consortium members and external end-users (governmental, education, industry)

ACTIVITIES

WP5 addresses the key knowledge transfer activities: transfer to the scientific community, to risk assessors and policy makers (e.g. EFSA, DG Sanco, national Food Safety Authorities) and potential users of the developed analytical tools (statutory laboratories, food analysis contract laboratories, food industry, SMEs), but also within the project consortium.



Core elements of the dissemination strategy are

(i) The public NanoLyse website (www.NanoLyse.eu)

The website is a main portal for the dissemination of the results dedicated to general public, official authorities, food and feed sectors and scientists.

(ii) The e-Newsletter

Bi-annual e-newsletter is distributed actively to stakeholders and other interested parties. The newsletter informs on the scientific progress in the analysis of engineered nanoparticles in food and beverages within NanoLyse, as well as on upcoming events relevant for this field.

(iii) Presentation of results

The scientific outcome of the project will be published in peer reviewed international journals and presented at international scientific conferences, after careful consideration of IPR issues. In addition, two "NanoLyse Open Days" are organised to present the approach and the outcome of the project to a wider public, focusing on potential stakeholders of the developed methods.

(iv) Training workshops

will be organised at the end of the project. Goal of these hands-on workshops will be the technology transfer of the developed tools to laboratories which have or will have the need to analyse food and beverages for presence and levels of engineered nanoparticles. In the first place laboratories involved in the risk assessment and in the (future) monitoring of food for engineered nanoparticles will be addressed, but interested parties from food industry and private contract food laboratories will also be invited.

5 NanoLyse Project outcomes

5.1 Scientific publications

Thomas P.J. Linsinger, Gert Roebben, Conxita Solans, Roland Ramsch (2011), Reference materials for measuring the size of nanoparticles, *Trends in Analytical Chemistry* 30:18-27

Agnieszka Dudkiewicz, Karen Tiede, Alistair B.A. Boxall, Katrin Loeschner, Louise Helene Soegaard Jensen, Eric Jensen, Rafal Wierzbicki, Kristian Molhave (2011), Characterisation of nanomaterials in food by electron microscopy, *Trends in Analytical Chemistry* 30:28-43

Ruud Peters, Guillaume ten Dam, Hans Bouwmeester, Hans Helsper, Günter Allmaier, Frank vd Kammer, Roland Ramsch, Conxita Solans, Monika Tomaniová, Jana Hajslova, Stefan Weigel

(2011), Identification and characterization of organic nanoparticles in food, *Trends in Analytical Chemistry* 30:100-112

Frank Von der Kammer, Samuel Legros (2011), Separation and characterization of nanoparticles in complex samples (food/environment) by FFF, *Trends in Analytical Chemistry* 30:425-436

Günter Allmaier, Anne Maißer, Christian Laschober, Paul Messner, Wladyslaw W. Szymanski (2011), Parallel differential mobility analysis for electrostatic characterization and manipulation of nanoparticles and viruses, *Trends in Analytical Chemistry* 30:123-132

5.2 Open days

The first NanoLyse Open Day had been organised on the 2nd November 2011, during the 5th International Symposium on Recent Advances in Food Analysis (RAFA 2011), held from the 1st to the 4th November 2011 in Prague, Czech Republic.

The Open day was attended by more than 50 participants from various countries and different sectors as well as from national and European authorities. They actively engaged in discussions with the present NanoLyse scientists. The level of interaction was remarkably high and there was an intense exchange not only on the presented goals and results of the project, but also about future needs and possible collaborations.



Figure 6 : Impression of the NanoLyse Open Day

5.3 Training workshops

Training workshops are scheduled for the second half of 2013 for the technology transfer of the developed methods to potential end-users of the new methods for the analysis of engineered nanoparticles in food and other complex matrices.

6 Directory

Table 1 Directory of people involved in this project.

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1 NANOMICEX

Mitigation of risk and control of exposure in nanotechnology based inks and pigments

Contract Agreement: Under Negotiation Website: <http://www.nanomicex-fp7.eu>
Coordinator: Carlos Fito, Packaging, Transport and Logistics Research Center , Valencia, Spain

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3	Institute of Occupational Medicine	IOM	United Kingdom
4	Heriot-Watt University	HWU	United Kingdom
5	Yeditepe University	Y.U.	Turkey
6	University of Aveiro	U.A.	Portugal
7	Nanotechnology Industries Association	NIA	Belgium
8	Tec Star S.r.l.	TECStar	Italy
9	Ardeje	Ardeje	France
10	Pinturas Montó, S.A.U.	MONTÓ	Spain
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Project Duration: 1 March 2012 – 29 February 2014

Project Funding: 3.5 Mio. EUR

1 Summary

Nanotechnology and in particular, the use of nanoparticles in ink and pigment formulations have a great potential for new applications, leading to products with new or enhanced properties, and opening new market opportunities. Consequently, many promising applications emerge nowadays, based on the use of nanoparticles such as Fe₃O₄, TiO₂, ZnO, Quantum dots or Mixed-metal oxides at the nanoscale, which confer a wide range of properties to the final products, covering the most requested properties in pigment/inks applications for the nearest future.

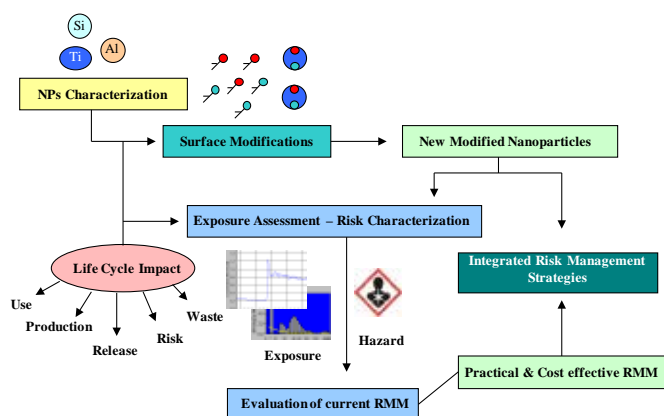
Due to the extraordinary possibilities derived from the application of nanotechnologies the use of engineered nanoparticles is steadily increasing, however their use raise many questions and generate concerns due to the fragmentary scientific knowledge of their health and environmental impacts. The uncertainties are great because the properties exhibited by such particles are often exceedingly different to those demonstrated by bulk forms, affecting their physicochemical and biological behaviour, which results, in more toxic properties. In this sense, It is known that exists a causal association between exposure to NPs with human diseases, as well as environmental pollution, considering that NPs can be released to the ecosystem. Moreover, airborne NPs can be



inhaled, enter the bloodstream and translocate to cells. Despite such hazards, it is not possible to predict their impacts and there are currently no exposure limits specific to NPs nor any national or international consensus standards on hazard assessment and measurement. In addition, there is a major debate on nanotechnology future implications, including concerns about effects on global economics and consumers' acceptance.

In order to address these major concerns and considering the project concept, **the main objective of NANOMICEX project is to reduce the potential risk upon worker's exposure to engineered nanoparticles** through the modification of nanoparticles properties with effective surface modifiers and the characterization of practical and cost effective risk management strategies in the particular operative conditions of the inks and pigments industry.

To achieve such objectives, the engineered NPs under the scope of the project will be studied in detail in order to identify the mean parameters that may influence their chemical and physical properties. The hazard of these materials will be tested using both human and environmental models. Once characterized, nanoparticles will be developed using different biotechnological surface modifiers in order to obtain less hazardous and more stable engineered nanoparticles. The methodologies used in the formation of less risk-posing nanoparticles will be relatively simple allowing them to be easily reproduced in a common laboratory in order to ensure the effectiveness of the methodology in the industry. In a second stage, levels of exposure for workers who are exposed when handling the nanoparticles will be determined in order to develop real exposure scenarios. These scenarios will be studied at all stages of nanotechnology inks and pigments production, use and disposal, considering the engineered nanoparticles as such or as a component of complex ink-pigment formulations. In the third stage of the project, the exposure scenarios will be reproduced in the laboratory clean room, in order to assess the effectiveness of the risk management measures and engineered controls. To this end, the effectiveness of the personnel protective equipment, ventilation, filtration and other controls will be checked in the simulated conditions. As a result, the studies will determinate the most effective techniques to reduce and mitigate the hazard and exposure, and therefore minimize the risk, focussing on safe use. Finally, in the last stage of the Nanomicex project, the modified nanoparticles and cost effective risk management strategies will be tested in case studies with the aim of validating the strategies in the real operative conditions for preparing inks, where the nanoparticles can have a uncertain behaviour.



2 Background

The ink and pigment industry all over the world is being driven by innovation, which allows manufacturers to develop new and innovative products for hundreds of industrial applications and billions of people who use them every day. According to the Ecological and Toxicological Association of Dyes and Organic Pigments Manufacturers (ETAD), pigment applications demand properties such as dispersibility, color strength, light and weather fastness, migration resistance, color shade or hiding power. These properties depend on the chemical composition of inks and pigments and on the size and morphology of their particles. Therefore, nanotechnology and in particular, the use of nanoparticles in ink and pigment formulations have a great potential for new applications, leading to products with new or enhanced properties, and opening new market opportunities. Consequently, many promising applications emerge nowadays, based on the use of nanoparticles such as FexOy, TiO₂, ZnO, Quantum dots or Mixed-metal oxides at the nanoscale, which confer a wide range of properties to the final products, covering the most requested properties in pigment/inks applications for the nearest future.

Along with the benefits there are also concerns that a variety of the characteristics possessed by nanomaterials, such as small size, high aspect ratio, shape, surface reactivity, solubility or dustiness, relate their potential hazard and risk.

However, despite such situation, due to the extraordinary possibilities derived from the application of nanotechnologies in different industrial sectors, the use of engineered nanoparticles is steadily increasing and the number of workers dealing with nanoparticles is also on the rise. For example, the organic pigment industry, in which nano-additives are used, employs more than 100,000 staff and achieve sales of 10 billion Euros. Similarly, the production of nano-structured inks represents both the largest and faster-growing market for advanced ink formulations.

On the other hand, significant regulatory concerns from the European Commission have arisen about unforeseen risks likely to arise from nanoparticles. In this sense, the communication from the commission to European parliament (SEC 2008, 2036) provides a description of elements of selected EU legislation that seems most relevant and likely to apply to nanotechnologies and nanomaterials. At the moment, the most important piece of legislation in the area of health and safety at work is the Framework Directive 89/391/EEC "on the introduction of measures to encourage improvements in the safety and health of workers", which fully applies to risks associated with nanoparticles. This Directive places a number of obligations on employers to take measures necessary for the safety and health protection of workers, considering also the risk mitigation as a recommendation when it is not possible to eliminate the risks. At the same time, the REACH regulation, which is the main legal instrument to ensure the safety use of chemicals in the European market, establishes the need to ensure the safety of substances, such and those included into mixtures (e.g. inks). Even if there is no specific regulation to nanomaterials, REACH regulation applies to all substances and mixtures supplied in the European Union, whatever size, shape or physical state. Nonetheless, in the absence of specific regulations, the precautionary principle should be first applied.



3 Concept and Objectives

3.1 Project Concept

The concept of NANOMICEX stems from the need to ensure the safety of workers dealing with the production or handling of engineered nanoparticles employed in the pigment/ink industry, as well as the need to provide the workers with integrated, cost effective and appropriate strategies to control the exposure to engineered nanoparticles.

On the basis of this concept, the following activities have been planned:

- a- Development of novel methods based on nanoparticle functionalization to reduce hazards caused by potential nanoparticle emissions during ink/pigment-based products life cycle.
- b- Toxicological and ecotoxicological evaluation of nanoparticle impacts, selecting methods that are reproducible, simple, non-expensive and reliable.
- c- Characterization of exposure scenarios in terms of REACH regulation, including exposure assessment
- d- Assessment of the effectiveness of the personnel protective equipment, ventilation, filtration and other control systems in simulated conditions (cleaning room laboratories)
- e- Validation and implementation of simple risk management strategies, involving industrial partners.

3.2 Project Objectives

The main objective of NANOMICEX project is to **reduce the potential risk upon worker's exposure to engineered nanoparticles** through the modification of nanoparticles properties with effective surface modifiers and the characterization of practical and cost effective risk management strategies in the particular operative conditions of the inks and pigments industry. New surface modifiers will be designed to obtain less hazardous and more stable engineered nanoparticles, suitable for their use on inks and pigments compounding. An exhaustive exposure assessment will be carried out in order to control the surface modified nanoparticles in the real operative conditions, including the evaluation of the current risk management strategies. Additionally, a practical and cost effective risk management strategy will be developed, which can be used in combination with the surface modifiers as a consistent and integrated approach for mitigation of workers risks. Additionally, NANOMICEX must cover these actions, including the fulfilment of the current regulation in terms of worker safety and consumer health, avoiding workers exposure to nanoparticles in the current industrial settings.

Related to the nanoparticles considered, the project is focused on those nanoparticles employed in large scale by ink and pigment industries, covering an extensive range of high-tech applications and added value properties (semiconductor, insulator, luminescent, catalytic, refractive and magnetic properties). Such criteria are satisfied by several **metal oxide nanoparticles** (ZnO, TiO₂, Al₂O₃ and Fe₃O₄), **Ag metal nanoparticles**, **CdSe Quantum Dots** and the mixed metal oxide **Cobalt Aluminate spinel**, therefore, these nanometer-sized particles will be studied within NANOMICEX project.

4 Overall view of the Workplan

NANOMICEX consists of 9 complementary Work Packages (WP), summarised in Table 1. For each WP, a complete description is presented below, including the objectives; the WP leader (in bold) and team members; the Hypotheses and Methods; the Deliverables; and linkage to other Work Packages.

Table 1: Work Packages of NANOMICEX

WP n°	WP Title	WP Leader
1	Characterization of engineered nanoparticles	UA
2	Development and selection of functional modified nanoparticles	YU
3	Hazard Assessment	HWU
4	Exposure Assessment	IOM
5	Risk Management and Control Measures	ITENE
6	Nano SLCRA: Adaptive Streamlined Life Cycle / Risk Assessment of nanoparticle-based inks and pigments	LEITAT
7	Industrial Case Studies	ARDEJE
8	Project Coordination and Management	ITENE
9	Project dissemination and training	NIA

This work plan has been split into 4 types of activities and based on the combined experience of the consortium members. The activities are explained below:

1. Scientific and Technological development

These activities cover the scientific tasks to be conducted to achieve the project objectives. A detailed description of these tasks is described on table 2.

2. Validation and Demonstration activities

The main objective of these activities is to prove the viability of the solutions proposed in the industrial settings. It will be conducted under the scope of WP 7, checking the surface modifications in industrial case studies. The exposure scenarios and risk management measures will be implemented and monitored to ensure their correct application.

3. Project Management

This work includes the tasks to be completed by the project Coordinator and contains the tasks required to successfully manage the project. The coordination activities will be undertaken by the ITENE.

4. Dissemination Related Activities

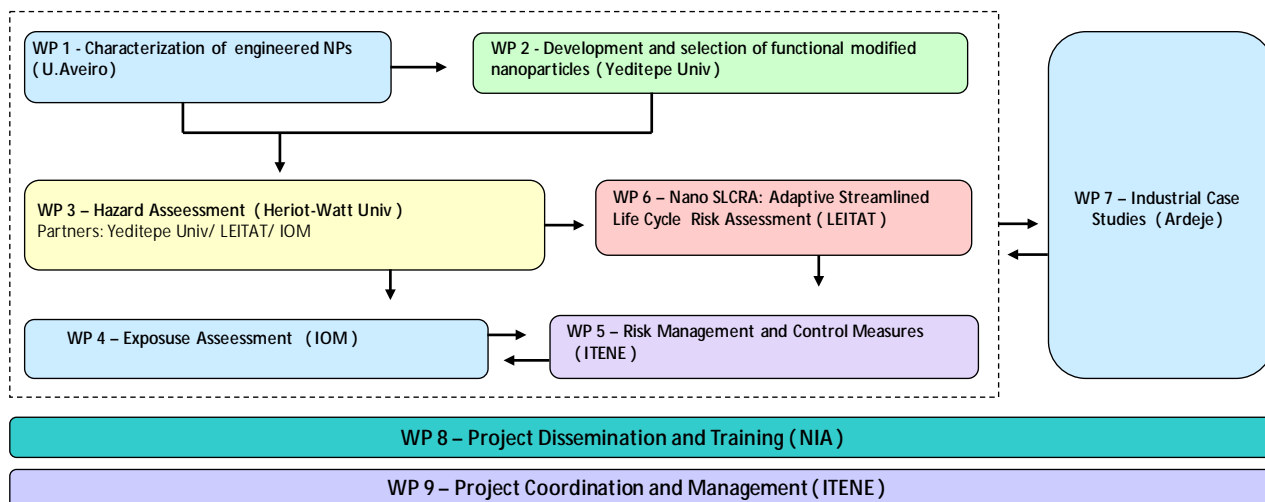
In order to achieve an optimal use of the Project across the EU, dissemination, training and exploitation are essential to the success of the NANOMICEX project. These activities will be conducted within WP 9.



Table 2 Technical & Scientific Workpackages (WP) of NANOMICEX

WP	Title	Description
1	Characterization of engineered nanoparticles	Workpackage 1 (WP1) will focus on the characterization of the nanoparticle panel, identifying the specific types of the metal oxide nanoparticles, AgNPs, CdSe quantum dots and the mixed-metal oxide CoAl ₂ O ₄ employed in the pigment and ink industry. Once identified, a full characterization, in terms of size, shape, mass, surface area, chemical composition, physical and optical properties, will be conducted
2	Development and selection of functional modified nanoparticles	Workpackage 2 (WP2) will select the surface modifiers and derivatize the nanoparticles with the selected modifiers. A systematic study will be carried out in order to design and develop surface modifiers, which will be custom designed from bimolecular structures, hydrophobic organic stabilizer and PEGs. The designed modifiers will be attached through several routes available in the literature or/and newly developed within the project. The new NPs synthesized will be characterized by imaging techniques such as SEM and AFM along with other characterization techniques.
3	Hazard Assessment	Workpackage 3 (WP3) will evaluate the current literature on the environmental fate of nanoparticles used in the ink and pigment industry, and will assess the toxicity and ecotoxicity of the NPs characterized in the WP1. The toxicity profile will be assessed by in vitro assays using a range of cell lines that represent significant exposure and target organs and cell types in the human body. This in vitro approach will be combined with a small number of organism studies to confirm key in vitro observations, as well as to assess ecotoxicity. In addition an analysis of relationships between nanoparticle properties and effects will be undertaken to allow collation of data and reduction in future testing requirements.
4	Exposure Assessment	Workpackage 4 (WP4) will be focused on the exposure assessment. At this stage levels of exposure for workers who are exposed when handling the NPs will be determined in order to develop real exposure scenarios. These scenarios will be studied at all stages of nanoparticles production, use and disposal, considering the nanoparticles as such or as a component of the ink/pigment formulations.
5	Risk Management and Control Measures	Workpackage 5 (WP5) will be focused on the assessment of the effectiveness of the Risk management Measures. The workplace controls as personnel protective equipment, ventilation, filtration and other controls will be checked in controlled conditions in order to determinate the most effective techniques to reduce and mitigate the hazard and exposure, and therefore minimize the risk, focusing on safe use.
6	Nano SLCRA: Adaptive Streamlined Life Cycle / Risk Assessment of nanoparticle-based inks and pigments	Workpackage 6 (WP 6) will assess the potential impact and evaluate the risk posed by NPs on workers. The risk assessment will be done at the different exposure scenarios defined on WP4. Furthermore, a novel methodology based on the combination of the life cycle assessment (LCA) and the risk assessment of nanoparticle-based inks and pigments will be conducted in order to establish their potential health and environmental impacts along their life cycle. This WP will include the development of novel strategies for the management of inks and pigments waste containing nanoadditives.

The workpackages and their interdependence are shown schematically below:



5 Advances over the state of the art

NANOMICEX project will provide an integrated approach to manage the risk posed by nanoparticles, dealing with the current limitations in relation to the worker protection strategies, considering the risk assessment methodologies and risk management measures. The current handbooks, guides and reports of research projects are not focused on specific nanoparticles used in the current industrial setting of the ink and pigment industry, which can differ enormously from another industrial process involving the use of nanoparticles.

In relation to the progress beyond the current state of the art, NANOMICEX will work on the design of functional groups to modify the properties of the engineered nanoparticles employed in the pigment and ink industry in terms of toxicological profile, cell interaction and surface reactivity, but without causing significant changes in the nanoparticles properties, reproducible applications in real conditions and using modification techniques that are easy to implement by non-expert personal. In this sense, the surface modifications of metal oxide nanoparticles, AgNPs, CdSe quantum dots (QDs) and mixed-metal oxides (CoAl₂O₄) NPs are aimed for their inclusion into inks and pigment formulations, reduce their hazardous properties and adverse effects on living systems, without compromising their further application in their current industrial pigment/ink formulations.

Regarding the potential hazards posed by nanoparticles, NANOMICEX will assess the cytotoxicity (in vitro approach), sub-lethal toxicity and dermal effects, considering the relevance of such aspects in relation with the occupational exposure to nanoparticles. This work will allow the determination of the toxic responses in the worker place, and also allow a comparison of the effects of modified and unmodified particles. In addition, the work developed will provide the stakeholders with scientific and consensuated data to conduct regulatory actions (e.g. Occupational Exposure Levels- OELs based on LC50/EC5 data). Similarly, regarding the environmental impacts of the

nanoparticles, NANOMICEX will provide new knowledge in relation to the environmental fate and behaviour of nanoparticles relevant to the pigment and ink industry. A comprehensive ecotoxicological study based on selected OECD test models will be conducted, including the assessment of acute toxicity, sub-lethal ecotoxicity and bioaccumulation. In addition, NANOMICEX project will work in the computational analysis of data obtained from hazard studies, with the aim of determining structure activity relationships in order to provide valuable data to improve the current QSAR tools or create new ones.

In terms of exposure assessment, the research activities within the NANOMICEX project will further develop real exposure scenarios in order to assess the exposure in the real operative conditions of workers dealing with engineered nanoparticles. Once developed, such scenarios will be modelled and reproduced in controlled conditions, improving the knowledge about the background effects and interactions between the engineered nanoparticles and their environment, and how such interactions modify the exposure patterns of the engineered nanoparticles in real conditions. Furthermore, the state of the art real time measurement devices will be tested in controlled conditions, improving the knowledge to interpret the data obtained.

In relation to the protection strategies, during the NANOMICEX project several protective measures will be assessed, evaluating the effectiveness of the existing technical and management exposure control strategies, providing the ink and pigment industry with the most appropriate measures to control the exposure to engineered nanoparticles and therefore to minimize the risk.

Finally, the NANOMICEX project will conduct a life cycle assessment combined with risk assessment, studying the health and the environmental impacts of NP-based inks and pigments at all the stages of their life cycle. The availability of data on the nanoparticles release to the environment, and consequently to



humans at all the stages of their life cycle, which is one of the current most critical limitations to perform LCA of NPs, will allow the improvement of the accuracy of the LCA analysis in comparison with existing attempts. In addition, concerning the disposal of nanomaterial-based products, NANOMICEX will propose novel strategies for the management of the waste produced along the life cycle of inks and pigments containing nanoadditives.

6 Impact

At the moment, much of the research and development work is concentrated on the development of innovative products, considering new properties that improve the final application, while at the same time ensuring the safety at all stages of nano-products manufacturing, use and disposal. Overall market value will benefit from important consumer preferences toward safe and environmentally friendly products, which will support consumption of high-performance inks and pigments. **Major improvements in occupational, consumer and environmental risk assessment are expected** in NANOMICEX through the design of less hazardous engineered nanoparticles and the implementation of tested integrated strategies to mitigate the exposure to nanoparticles in an industrial setting.

By the development of this project, **novel integrated strategies for mitigation of the risk of workers dealing with nanoparticles and nanostructured products will be developed** with the main goal of guarantee a safe worker environment to the ink/pigment industry in particular, and to the European industry in general.

7 Directory

Table 3 Directory of people involved in this project.

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**NANOMMUNE****Comprehensive Assessment of Hazardous Effects of Engineered Nanomaterials on the Immune System**

Contract Agreement: NMP4-SL-2008-214281 Website: <http://www.nanommune.eu>
 Coordinator: Prof. Bengt Fadeel, Karolinska Institutet, Stockholm, Sweden

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2	Royal Institute of Technology	KTH	Sweden
3	Uppsala University	UU	Sweden
4	University of Cologne	UC	Germany
5	University of Turku	UT	Finland
6	Swiss Federal Laboratories for Materials Testing and Research	EMPA	Switzerland
7	Institute of Occupational Medicine	IOM	United Kingdom
8	University of Pittsburgh	UP	United States
9	National Institute for Occupational Safety and Health	NIOSH	United States
10	North Carolina State University	NCS	United States

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1 Summary

Engineered nanomaterials (ENs) present tremendous opportunities for industrial growth and development, and hold great promise for the enrichment of the lives of citizens, in medicine, electronics, and numerous other areas. However, there are considerable gaps in our knowledge concerning the potential hazardous effects of ENs on human health and the environment. The NANOMMUNE consortium is committed to filling these knowledge gaps through a comprehensive assessment of ENs, with particular focus on effects on the immune system. The immune system is designed to respond to pathogens and foreign particles, and a core concept underpinning the current project is that the recognition versus non-recognition of ENs by immune-competent cells will determine the distribution as well as the toxicological potential of these materials. Our international, multidisciplinary consortium will focus on the procurement, synthesis and detailed physico-chemical characterization of representative categories of ENs, and the monitoring of potential hazardous effects using an array of *in vitro* and *in vivo* systems, as well as transcriptomic and oxidative lipidomic profiling strategies to determine specific nanotoxic profiles (signatures) of these materials. The final and integrative component of our research project is modeling and risk assessment of potential adverse effects of ENs on human health, and the dissemination of our findings. Through our comprehensive approach, which combines analytical procedures from many different disciplines and leading experts from several national institutes devoted to occupational and environmental safety, we aim to establish a panel of read-out systems for the prediction of the toxic potential of existing and emerging ENs, thus enabling a continuous and sustainable growth of the nanotechnologies. Overall, the results generated through this international program will contribute to the understanding and mitigation of possible adverse effects of nanomaterials.

2 Overview of project

2.1 Introduction, scientific/industry needs, problem addressed

Nanotechnologies are viewed as being the driving force behind a new industrial revolution which is expected to have profound socio-economic effects (Royal Society and Royal Academy of Engineering, 2004; United States Congress Joint Economic Committee, 2007). Nanotechnologies comprise a disparate array of technologies that cut across many traditional scientific disciplines, including chemistry, material science, engineering, physics, biosciences, medicine, and environmental sciences. The only unifying feature is the nanoscale dimensions at which the material concerned is being manipulated. Nanoparticles have all three dimensions in the nanoscale, whereas nanotubes have two dimensions in this regime, and nanosurfaces have one dimension in this regime. It is important to note that nanomaterials can be on

the same scale as elements of living cells, including proteins, lipids, nucleic acids, and organelles (Shvedova et al., *Annu. Rev. Pharmacol. Toxicol.* 2010). Therefore, one must focus particular attention on how ENs can interact with or influence biological systems, which may be desirable for certain medical applications, but may cause unanticipated hazardous effects upon occupational or environmental exposure to nanomaterials.

The properties of materials are different on a nanoscale for two reasons. First, ENs have, relatively, a larger surface area than the same mass of material produced in a larger form. This can make materials more chemically reactive, and affect their functional properties such as mechanical strength or electrical properties. Second, below 50 nm, the laws of classical physics give way to quantum effects, provoking optical, electrical, and magnetic behaviors different from those of the same material at a larger scale. However, the very same properties that make ENs so uniquely useful, such as a high degree of chemical reactivity and the ability to cross biological barriers may also be associated with unforeseen adverse effects on health and the environment. Moreover, small size *per se* may contribute to the failure of immune recognition and hence to adverse or unexpected effects of nanoparticles. Indeed, numerous physico-chemical attributes, including size, shape, surface area, surface chemistry, solubility, charge, porosity, etc have been suggested to be associated with the potential adverse effects of ENs. However, much more research is required to ascertain the relevance of a given physico-chemical parameter for EN-associated toxicity following human exposure.

One of the members of our consortium co-authored the original proposal for a new subcategory of toxicology, namely *nanotoxicology*, to be defined to address gaps in our knowledge and to focus on the specific problems that are related to ENs (Donaldson et al., *Occup. Environ. Med.*, 2004). Maynard, Tran and other leading scientists have also proposed that the pursuit of responsible and sustainable nanotechnologies can be tackled through a series of grand challenges to stimulate the global research community, including the development and validation of methods to evaluate the toxicity of ENs, and the development of risk assessment models for predicting the potential impact of ENs on human health and the environment (Maynard et al., *Nature*, 2006). Indeed, despite the tremendous growth potential of the nanotechnologies, there is still a considerable lack of information on bioaccumulation, biotoxicity, and biodegradation of ENs in humans as well as in other species. However, previous epidemiological studies have documented a strong association between so-called ultrafine air pollution particles and respiratory and cardiovascular morbidity and mortality in humans. Some, but not all of these effects, may be related to indirect actions of particles on components of the immune system, for instance through modulation of inflammatory cytokine secretion. Indeed, the comprehensive review of nano-immunotoxicological research, published in *Nature Nanotechnology* (Dobrovolskaia and McNeil, 2007) underscores that ENs can either stimulate or suppress immune responses; moreover, these authors suggest that one of the fundamental questions in the field concerns the mechanisms



through which nanoparticles are recognized by the immune system.

2.2 Scope, objectives

Engineered nanomaterials present tremendous opportunities for industrial growth and development, and hold great promise for the enrichment of the lives of citizens, in medicine, electronics, and numerous other areas. However, there are considerable gaps in our knowledge concerning the potential hazardous effects of ENs on human health and the environment, as pointed out in a recent report from the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) of the European Commission (SCENIHR, 2007). Our research consortium, which we have designated NANOMMUNE, is committed to filling these knowledge gaps through a comprehensive assessment of ENs, with particular focus on effects on the immune system, our primary defense system against foreign invasion.

One challenge in evaluating risk associated with the production and application of nanomaterials is the diversity and complexity of the types of materials available, and the many different routes of entry and possible sites of interaction with biological systems. Our interdisciplinary project will focus on the manufacturing and detailed physico-chemical characterization of several representative classes of nanomaterials, and the monitoring of deleterious effects of these nanomaterials on the immune system, using an array of *in vitro* and *in vivo* methodologies, as well as state-of-the-art *in silico* approaches for the assessment of genomic and oxidative lipidomic "nanotoxicity-signatures". Our studies will also include several examples of commercial ENs that are currently on the market. Moreover, we also aim to modify specific features

of various classes of ENs, in order to mitigate toxic responses to these materials.

The immune system, present throughout the body, and on constant surveillance, has the capacity to respond to invasion by pathogens and foreign particles. The core concept underpinning the current project is that the recognition versus non-recognition of ENs by immune-competent cells will determine the distribution as well as the toxic potential of these novel materials. Moreover, we will assess whether ENs interfere with key functions of the immune system *in vitro* and *in vivo*, such as macrophage engulfment of apoptotic debris and antigen-presentation or exosome production by dendritic cells to lymphocytes. Through our comprehensive approach, which combines analytical procedures from many different disciplines, we aim to establish an array of read-out systems for the determination of toxicity not only of currently existing ENs, but also for the prediction of hazardous effects of new ENs that are being developed, thus enabling a sustainable growth of the nanotechnology-based industries.

Moreover, because the assessment of hazardous properties of ENs is a global concern, our NANOMMUNE consortium will strive to harmonize toxicological testing and risk assessment efforts between Europe and the United States, through a balanced participation of investigators from EU member states (Sweden, Finland, Germany, United Kingdom), associated countries (Switzerland), and the United States. Reinforced international cooperation and sharing of data is of critical importance because a reliable basis for the assessment of safety of nanomaterial-based products and technologies requires the production and implementation of standardized test materials, toxicity assays, and risk assessment strategies.

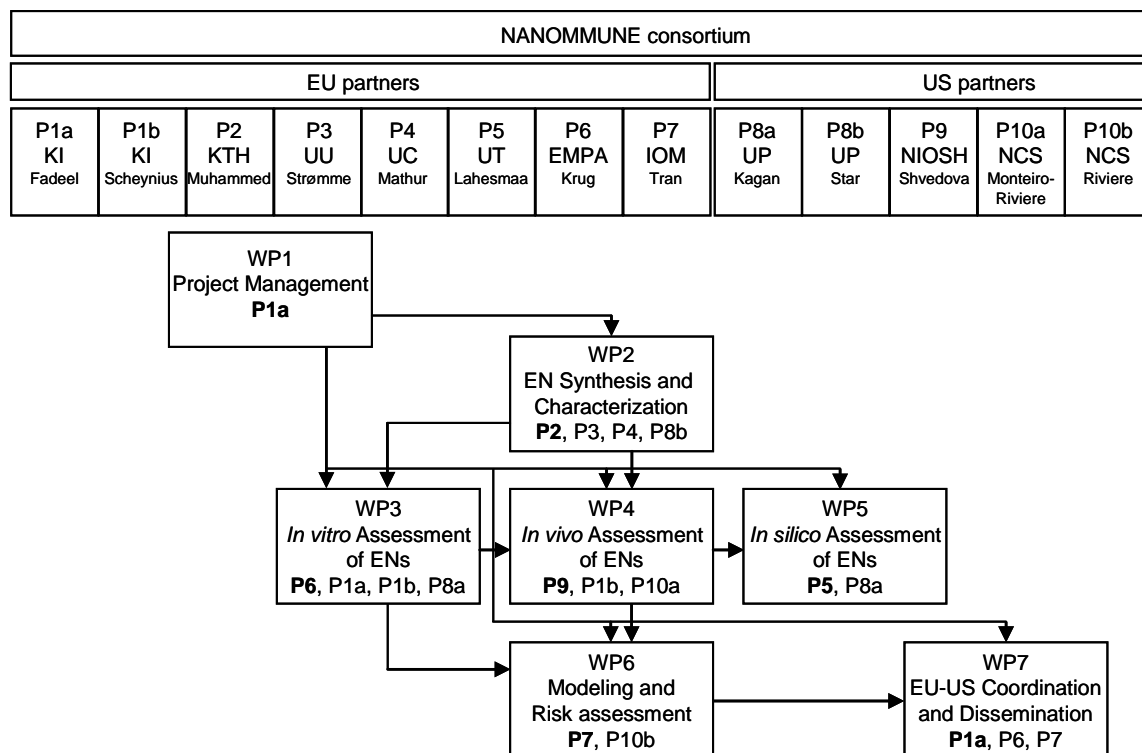


Figure 1. Schematic representation of the consortium and work packages.



2.3 Technical approach, work description

2.3.1 Synthesis and characterization of nanomaterials

Our studies are focused on three categories of nanomaterials of technological and/or biomedical importance: a) metallic nanoparticles, eg. gold; b) oxide nanoparticles; iron, silica, titania, zinc; and c) carbon nanotubes; single- and multi-walled (SWCNT, MWCNT).

Gold nanoparticles have been selected because they are considered for use in a range of biomedical applications; moreover, gold surfaces readily bind proteins and DNA, and gold nanoparticles have photothermal properties that may be of use for localized drug release increasing their potential for therapeutic applications. However, the issue of *in vitro* and *in vivo* toxicology of gold nanomaterials, and in particular the putative effects on the immune system has not been fully addressed. TiO₂ is common in numerous consumer-based and other products, and FeO-ENs have been applied for various biomedical applications, including their use as magnetic resonance imaging (MRI) contrast agents for almost 20 years. Mesoporous silica nanoparticles are of fundamental and applied interest for their potential in diverse applications in areas ranging from catalysis to photonic crystals, biomimetic engineering to sensor technology and drug delivery. They offer several attractive features for hosting molecules of various shapes, sizes and functionalities. However, it remains to be determined whether these materials also may exert adverse effects on immune-competent cells.

Carbon nanotubes, particularly SWCNT, have found numerous applications in different fields of industry due to their excellent strength and high electrical conductivity; moreover, functionalized (surface-modified) CNTs are emerging as novel components in nanoformulations for delivery of therapeutic molecules. The spread and distribution of CNTs in the body is dependent, to a large extent, on their specific interactions with cells of immune system. Indeed, we hypothesize that the recognition or non-recognition of ENs by the immune system will determine the toxicological potential of these nanomaterials, as well as their distribution in various tissues and organs. Systematic study of the therapeutic efficacy of CNTs is anticipated in the near future; however, detailed investigations of potentially hazardous effects on cells of the immune system remain to be performed, and are of paramount importance for the successful application of CNTs in nanomedicine. Moreover, there is a pressing need for careful consideration of the hazardous effects of CNTs for industrial workers.

Our consortium will standardize the synthetic methods as well as the characterization techniques of various classes of nanomaterials. Commercially available nanomaterials of various sources will also be procured and characterized, for benchmarking purposes. Of note, members of our consortium have highlighted the need to avoid contamination with lipopolysaccharides (LPS) in the production of ENs, since LPS can interfere with the *in vitro* assessment of biological responses of immune-competent cells (Vallhov et al., Nano Lett., 2006). The data obtained by the different partners within WP2 and the *in vitro* (WP3) and *in vivo* (WP4) work packages (see Figure 1 for a schematic representation of the different work packages) will be summarized as standard

operation procedures (SOPs), and compiled into the NANOMMUNE Quality Handbook (QHB), which will be made available to academic researchers and industries. The consortium will also develop methods for tuning (controlled modification) of the various physico-chemical properties (crystallinity, size, morphology, surface area, charge, hydrophilicity/hydrophobicity, coating molecules, etc) of custom-designed and commercial nanomaterials, to determine which properties are driving the immunotoxic responses. Finally, we aim to generate nanomaterials that are modified in terms of texture properties, composition and structure, in order to improve their biocompatibility. For instance, the presence of nitrogen in carbon nanotubes can promote the biodegradation of such materials, and our studies may thus aid in the safe development of ENs for medical and other purposes.

2.3.2 In vitro effects of nanomaterials: focus on immune-competent cells

There are several routes for nanomaterials to come into contact with living organisms. Inhalation, ingestion, and dermal routes are the most relevant for most occupational exposure scenarios. In addition, nanomaterials are produced for medical applications or may be released from medical implants upon mechanical stress. Such internal exposure (intentional or non-intentional) will lead to an increased number of nanoparticles within the bloodstream.

The immune system protects us from foreign materials that enter our body and we will therefore focus on possible adverse effects of nanoparticles on immune-competent cells. This is of particular importance, since immunotoxic effects of ENs have not been addressed in much detail to date. An excellent, recent review on this topic summarizes aspects of immunotoxicity of nanomaterials, but deals mostly with those materials that are produced for medical use, such as dendrimers or polymeric nanoparticles (Dobrovol'skaia and McNeil, 2007). Indeed, other more technologically relevant nanomaterials, including metal oxides or carbon modifications are not well investigated to date with respect to immune effects. Nevertheless, two very important conclusions are drawn in this review: first, there is no universal guide for immunotoxicity of ENs and there are currently no agreed-upon guidelines for assessing their immunotoxicity, and second, more mechanistic studies are required to understand how the immune system handles non-biodegradable ENs. This includes the important question of how nanomaterials are recognized and internalized by cells of the immune system.

Thus, the first issue that one needs to consider is the contact of ENs with immune cells and the uptake or internalization of these materials. Specific activation of membrane receptors in various cell types has been suggested to be important for the uptake and the subsequent inflammatory signalling provoked by ENs. On the other hand, no consensus exists with respect to the mechanism(s) of immune recognition of nanomaterials. To further complicate matters, ENs may be opsonised by proteins, and this so-called corona of proteins and/or lipids or sugars could play an important role in particle recognition and subsequent biological responses of phagocytic cells (Nel et al., Nat. Mat., 2009). Moreover, some nanomaterials may escape immune recognition, which could lead to the exacerbation of toxic effects of these materials.

The second major issue is whether important functions of immune cells are perturbed by nanomaterials. Programmed cell death (apoptosis) of immune cells could be affected by ENs, leading to



immunosuppression. Moreover, the clearance of particles or apoptotic cell bodies is a crucial reaction performed by macrophages, and we will investigate whether ENs interfere with this homeostatic process. In addition, antigen-presentation, performed by dendritic cells of the immune system, may also be affected by nanoparticles, which may be beneficial, if controlled, or dangerous, if non-intentional. However, there is no systematic investigation to date of these effects of ENs on immune-competent cells and we have very little information regarding which of the physico-chemical properties of nanomaterials that are important for such toxic responses. Our studies will address the *in vitro* effects of various categories of ENs on immune cells, including murine and human cell lines, primary immune cells (macrophages, dendritic cells, T cells, B cells, and others) and more complex co-cultures of cell lines and/or primary cells. Exosomes are recently discovered endogenous nano-sized vesicles produced by most immune-competent cells (Admyre et al., Allergy, 2008). These nanostructures have been shown to act as immune-regulatory agents depending on the state of the originating cell. Our studies will aim to determine whether ENs interfere with exosome-driven inter-cellular communication. These studies will not only inform us on the toxic potential of ENs, but may also provide novel insight into this mode of communication in the immune system. Taken together, these *in vitro* studies will form an important basis for *in vivo* studies and subsequent risk assessment, and may also generate fundamental insights into the handling of foreign materials/particles by the immune system.

2.3.3 In vivo effects of nanomaterials: focus on immune responses

Inhalation and deposition on the skin are the most likely routes of exposure to ENs in the environment. Indeed, one of the most important target organs for airborne particles, for obvious reasons, is the respiratory system. Members of our consortium have reported a remarkable degree of pulmonary granuloma formation and fibrosis in mice upon pharyngeal aspiration of engineered carbon nanotubes (CNTs) (Shvedova et al., Am. J. Physiol. Lung Cell Mol. Physiol., 2005). These studies suggest that if airways of workers are exposed to CNTs at the current permissible level (for graphite particles), they may be at risk of developing some lung lesions. Our project aims to further understand the potential for hazardous effects of ENs on the lung, focusing on specific interactions of ENs with cells of the immune system, and to use these data as the basis for risk assessment of hazardous effects of ENs for humans.

The *in vivo* work package will increase our understanding of the ability of selected and well-characterized nanomaterials to induce or affect immune responses following inhalation/aspiration exposure. The project will also study the effects of ENs on host immune responses towards infectious agents, using real-time *in vivo* monitoring.

2.3.4 Novel biomarkers of nanomaterial exposure: genomics and lipidomics

Microarray technologies for gene expression profiling (transcriptomics) can be used for identification and characterization of toxic responses, and could provide for more sensitive and earlier detection of adverse effects in, for instance, animal toxicity studies. Moreover, such studies could yield novel

hypotheses concerning the mechanisms underlying nanotoxic responses. Recent developments in genome-wide technology platforms have been extremely rapid. Hence, it is now possible to carry out genome-wide gene expression profiling on a very small sample size starting only with 100 ng of sample material. Moreover, the data analysis and mining tools have made it possible not only to discover individual genes or gene lists that are influenced by a particular treatment but to obtain insight regarding the molecular pathways that are activated or shut down. Members of our consortium have exploited a number of techniques and functional genomics tools that enable holistic approaches to identify known and novel genes involved in the regulation of T cell, DC and macrophage responses. Some studies are available on differential gene expression in EN-exposed cells; however, the full potential of these powerful technologies has yet to be exploited in the nanotoxicological setting, and we expect that genome-wide transcriptomics will provide a useful and unbiased approach to detect EN-induced immune responses *in vitro* and *in vivo* (in fact, we propose the term “nanotoxicogenomics” to describe this emerging area of research).

Oxidative stress has been suggested as a common paradigm of EN-induced toxicity at the cellular level (Nel et al., Science, 2006). Indeed, although not all nanomaterials have electronic configurations or surface properties to allow spontaneous ROS generation, particle interactions with cellular components may still be capable of generating oxidative stress. Furthermore, global assessment of cellular lipid profiles (lipidomics) may provide important information. However, this area of research has lagged behind in comparison to genomics, which is due in part to technical shortcomings in terms of quantification of lipids, but also because lipids can undergo oxidation, thereby giving rise to a tremendous number of oxidation products, which may have distinct signalling properties. The global assessment of lipids and oxidized lipid species, termed oxidative lipidomics is a novel approach that was pioneered in the past few years by one of our consortium participants. Using this approach, a “snapshot” of the cellular lipidome and changes in response to a given treatment or process is produced. The mass spectrometry-based protocols enable the simultaneous identification and analysis of the full range of oxidized and non-oxidized phospholipids from a complex mixture (Kagan et al., Nat. Chem. Biol., 2005). Developments in the field of gene expression profiling and oxidative lipidomics have thus provided increasingly valuable and feasible approaches to search for potential mechanisms underlying physiological or cellular processes, to generate new hypotheses concerning the mechanisms involved, as well as to identify novel biomarkers characteristic for toxic responses. We will apply these protocols to the assessment of cells (WP3) and tissues (WP4) exposed to various classes of nanomaterials.

2.3.5 Risk assessment of nanomaterials: steps towards safe management

Epidemiological studies on ambient particles incidentally produced in industrial processes and from traffic have demonstrated a correlation between ambient air concentration of particles and respiratory and cardiovascular morbidity and mortality rates. These adverse health effects of particles highlight the urgent need for research also on nanoparticles that are intentionally produced. This is also the final, integrative component of our current NANOMMUNE project i.e. guidelines for safe handling of



nanomaterials. Risk assessment will be performed in close collaboration between all consortium members. Members of our consortium possess considerable expertise in Physiologically-Based-Pharmacokinetics (PBPK) modelling. These models can be extended to incorporate the variability seen in animal data and the uncertainty due to lack of knowledge, an important feature of risk assessment. PBPK models have been used in describing the distribution of the internal dose across different target organs. The target organ dose is better correlated with the biological responses than the external exposure. As acknowledged by SCENIHR (2007), there is currently no established PBPK model for the distribution of nanoparticles in the body. In this project, we plan to extend this model, based on the inhalation mode of exposure, to other exposure routes such as intravenous injection and dermal exposure, thus taking it beyond the current state-of-the-art. (Q)SAR [(Quantitative) Structure-Activity Relationship] is the quantitative correlation of the biological (ecological, toxicological or pharmacological) activity to the structure of chemical compounds, which allows the prediction of the so-called "drug efficacy" of a structurally related compound. (Q)SAR is highly desirable as an approach which could replace extensive animal testing. To date, few attempts at (Q)SAR modeling were made for ENs. However, a (Q)SAR-like model, linking the particle physico-chemical characteristics with the immune response to nanoparticles is highly desirable because it helps to better understand the dose-response relationship, and to mitigate hazard with better designs for manufactured nanoparticles, by supplying important information on particle characteristics. Our approach will thus combine the immune hazard data (*in vitro* and *in vivo*) and modelling generated in this project with further information on exposure obtained in the public domain and other ongoing research projects, to develop a strategy for risk assessment of nanomaterials.

In synopsis, our multidisciplinary approach will contribute to the elucidation of the hazardous effects of ENs on the immune system, and will allow us to perform reliable and sound assessment of the risks to human health posed by these materials. Our studies will benefit a) *citizens*, because we address issues related to human health; b) *researchers*, because we will generate new knowledge in material production, and on mechanisms of interaction of nanomaterials with biological systems; and c) *industry* (including SMEs), because we plan to incorporate our characterization protocols and risk assessment guidelines into a Quality Handbook (QHB), which can provide support to interested parties. Moreover, our consortium provides a template for collaborations between European and US institutes as demonstrated by several joint publications.

2.4 Important achievements

In the following sections, some important research results are highlighted (and see list below of selected publications from our consortium).

2.4.1 Interaction of carbon nanotubes with immune-competent cells

Biopersistence, tissue distribution, immune and inflammatory responses to SWCNT are largely dependent on their recognition and uptake by phagocytosing cells. Previous studies on

macrophage recognition of apoptotic cells have revealed that the exposition of the phospholipid, phosphatidylserine (PS) on the surface of apoptotic cells serves as an important recognition signal for phagocytic cells (Fadeel and Xue, Crit. Rev. Biochem. Mol. Biol., 2009). Several partners of the NANOMMUNE consortium have now shown that SWCNT coating with the "eat-me" signal, PS makes nanotubes recognizable by macrophages, including primary human monocyte-derived macrophages and dendritic cells (Konduru et al., PLoS-ONE, 2009). Aspiration of PS-coated SWCNT in mice stimulated their uptake by alveolar macrophages *in vivo*. These studies also demonstrated that PS-coated SWCNT triggered less pro-inflammatory cytokine secretion than non-coated nanotubes.

Enzymatic biodegradation of SWCNT by the plant enzyme, horseradish peroxidase has been reported by US partners belonging to the NANOMMUNE consortium (Allen et al., J. Am. Chem. Soc., 2009). In addition, several members of our consortium have recently demonstrated a novel route of biodegradation of SWCNT through enzymatic catalysis by human neutrophil-derived myeloperoxidase (hMPO) (Kagan et al., Nat. Nanotech., 2010). Biodegradation occurred in primary human neutrophils and to a lesser extent in macrophages. Biodegradation of SWCNT was enhanced when nanotubes were pre-coated with immunoglobulin (IgG) to promote neutrophil internalization of SWCNT through Fc receptors. Furthermore, using an established mouse model of pharyngeal aspiration of SWCNT, it was shown that biodegradation attenuated the characteristic inflammatory responses to carbon nanotubes. These findings strongly indicate that novel biomedical applications of carbon nanotubes may be achievable under conditions of carefully controlled biodegradation. More recently, our consortium has reported enhanced pulmonary inflammatory and fibrotic responses to SWCNT in myeloperoxidase-deficient mice (*in press*). These studies were co-funded by US funding agencies.

2.4.2 Gene expression profiling of nanoparticle-exposed cells and tissues

Monitoring nanomaterial-induced immune responses by changes at the transcription level may be a valuable approach in terms of providing information on signalling pathways involved. One may assume that alterations in nanomaterial-induced gene expression can be rather subtle and, hence, focusing on discovery of biological pathways rather than individual genes is likely to provide a more informative view of the underlying processes. In the NANOMMUNE consortium, both *in vitro* (WP3) and *in vivo* (WP4) models are included for the characterization of immune responses using transcriptomics. The results will be used to generate novel hypotheses for further experimental testing and to describe nanomaterial and cell-specific changes in gene expression, i.e. to define novel "nanotoxicogenomic signatures". In the first study (unpublished), cytotoxicity testing of two commonly used nanoparticles, ZnO and TiO₂ was performed using primary human macrophages and dendritic cells as well as the leukemic Jurkat cell line. Exposure to ZnO nanoparticles triggered a dose-dependent loss of cell viability. Transcriptomics analysis using Illumina Sentrix® HumanHT-12 Expression BeadChips disclosed that the expression of different metallothionein genes was significantly upregulated in all three cell types after exposure to ZnO. These findings are in accordance with the observation that ZnO nanoparticles undergo rapid dissolution in cell culture. Further



assessment of differentially expressed genes is ongoing. In a subsequent study, comprehensive transcriptomics analysis of murine tissues (lung, spleen, and blood) harvested at various time-points post exposure to carbon-based nanomaterials including SWCNTs and fullerenes versus asbestos fibres are performed. These studies, conducted in collaboration between US and European partners, may reveal novel patterns of global gene expression – nanotoxicogenomic signatures – following *in vivo* exposure.

2.4.3 Mapping the surface adsorption forces of nanomaterials

Recently, US participants in NANOMMUNE developed a biological surface adsorption index (BSAI) to predict the molecular interactions of nanoparticles with proteins (Xia et al. Nat Nanotech. 2010). A set of small molecule probes that mimic amino acid residues were allowed to competitively adsorb onto a set of nanoparticles and the adsorption coefficients for the probes were measured. By assuming the adsorption was governed by five basic molecular forces, the measured adsorption coefficients were used to develop descriptors that represent the relative contributions of each of the forces, which, in turn, could be used in an *in silico* model to predict the adsorption of small molecules to other nanomaterials. Moreover, we have reported on the successful measurement of the BSAI nano-descriptors for 16 different types of nanomaterials in a recent study carried out jointly with European and US partners (Xia et al. ACS-NANO, 2011). The 5-dimensional nano-descriptor index describing the five types of molecular interactive forces was reduced to 2-dimensional via principal component analysis, which allows different nanomaterials to be clustered by their surface adsorption properties.

2.5 Conclusions

The NANOMMUNE project spans from synthesis, procurement, and physico-chemical characterization of nanomaterials, to detailed *in vitro* and *in vivo* investigations, using relevant murine and human model systems to assess adverse effects on the immune system, to mathematical modelling and risk assessment. Our project is further augmented by state-of-the-art, high-throughput global transcriptomics and oxidative lipidomics approaches, to obtain “nanotoxicogenomic” signatures, and to define novel biomarkers of immunotoxic responses. Overall, the NANOMMUNE consortium has performed a comprehensive assessment of adverse immune effects of ENs in order to understand how the benefits of the emerging nanotechnologies can be realized while minimizing potential risks to human health. The consortium has collated Standard Operating Procedures (SOPs) in a NANOMMUNE Quality Handbook which we plan to make freely available to all interested parties via the Nanosafety Cluster.

2.6 Dissemination of results

Results of the NANOMMUNE consortium were disseminated as follows:

- A public-access website has been developed as a principal portal of dissemination of our results (at the time of writing, 3930 unique visitors have visited the website).
- Members of the consortium have presented scientific findings at numerous scientific conferences, and scientific findings and reviews are published in international peer-reviewed journals (currently, more than 50 peer-reviewed papers).
- Members of our consortium are actively involved in the organization of international conferences on nanotoxicology, including the 3rd International Meeting on Nanotoxicology, Edinburgh, United Kingdom, 2010 (Dr. Lang Tran); 1st Italian-Swedish Workshop on Health Impacts of Engineered Nanomaterials, Rome, Italy, 2010 (Dr. Bengt Fadeel); 2nd Nobel Forum Mini-Symposium on Nanotoxicology, Stockholm, Sweden, 2010 (Dr. Bengt Fadeel), and the FP7-NANOMMUNE Closing Workshop in June 2011 in Stockholm to highlight key results of the consortium (Dr. Bengt Fadeel, and Project Manager, Dr. Erika Witasp).
- Members of the consortium are also involved in the organization of the Nanosafety Autumn School on San Servolo Island, Venice, Italy (Dr. Lang Tran, Dr. Bengt Fadeel); the first in November 2009, and the second edition in October 2010. The course is open to students, post docs, and participants from government agencies, industry. The third edition was organized in 2011 in the frame of FP7-MARINA.
- Dr. Bengt Fadeel was the main organizer of the 6th Key Symposium on Nanomedicine, Stockholm, Sweden, 2009 (co-organized with the Royal Swedish Academy of Sciences; several NANOMMUNE members contributed as invited speakers).
- In addition, other forms of dissemination were also considered. Hence, the project coordinator, Dr. Bengt Fadeel presented the NANOMMUNE project to a broad assembly of international journalists at press briefings hosted by the European Commission at the EURONANOFORUM in Prague, Czech Republic (2009); and the 6th World Conference of Science Journalists, London, UK (2009).

2.7 Nanosafety Cluster activities

The NANOMMUNE project has participated to the European Nanosafety Cluster, eg. Prof. Bengt Fadeel was involved in the initiation of the *Immunosafety Task Force* with Dr. Diana Boraschi, Italy, and Dr. Alfred Duschl, Austria. The task force is integrated into the WG on Nanomaterial Hazard in the European Nanosafety Cluster.

2.8 Selected publications

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NanoPolyTox

Toxicological impact of nanomaterials derived from processing, weathering and recycling of polymer nanocomposites used in various industrial applications



Contract Agreement: NMP-ENV-2009-247899 Website: <http://www.nanopolytox.eu>
Coordinator: Socorro Vázquez-Campos, LEITAT Technological Centre, Barcelona, Spain

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3	Institut Català de Nanotecnologia	ICN	Spain
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5	L'Urederra Technological Centre	L'UREDERRA	Spain
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Summary

NanoPolyTox main objective consists of monitoring the evolution (nanomaterials properties and toxicity) of three families of nanomaterials (nanotubes, nanoclays, metal oxide nanoparticles) during their life cycle as nanofillers in polymeric hosts. This project will include monitoring of the chemical and physical properties of the nanomaterials and their toxicity from the synthesis, during

processing, aging (use) and recycling to their end of life (disposal) quantifying their migration and/or release to the environment during their aging (use). The biological and environmental fate of these nanomaterials will be studied monitoring their physical-chemical and toxicological properties. The theoretical analysis of the data obtained during the project will lead to the development



of predictive models for the impact of nanomaterials on human health and environment. These studies will include the LCA analysis of nanomaterials included in polymeric host to determine their global environmental impact. Additionally, three recycling strategies will be considered in order to give solutions for the recovery of innocuous nanomaterials toxic. For this purpose, exhaustive evaluations for the selection of adequate dissolving and extraction methods to separate the nanomaterials from the polymeric matrix will be carried out. The strategies proposed for the recycling process will be the following: The direct mechanical recycling of nanocomposites, the recycling of nanomaterials and polymers obtained by novel chemical separation techniques based on nanofiltration using tailored nanofiber-based filters, and the recycling of polymers and immobilization of toxic nanomaterials in inert matrices.

1 Concept and Objectives

NANOPOLYTOX is a small-medium size collaborative project from the FP7 within the topic NMP-2009-1.3-1: Activities towards the development of appropriate solutions for the use, recycling and/or final treatment of nanotechnology-based products (Joint call with Theme 6: Environment including Climate Change).

1.1 Background

The global industry is moving forward taking advantages of the new opportunities and prospects offered by nanotechnology; therefore it is necessary that these developments take place in a safe and sustainable manner. The increasing use of nanomaterials in consumer products has raised concerns over their safety to human health and the environment. Currently, there are major gaps regarding to the health and environment risks presented by the nanomaterials. During the cycle life of a nanomaterial, workers and consumers are exposed to these materials. While workers are exposed during the process of production and the process of recycling or disposal of the industrial nanoproducs, consumers are exposed during the use of the products. Moreover, sooner or later the nanomaterials are free to enter the environment. Therefore, an exhaustive characterization and toxicity evaluation at different stages of the life cycle of nanomaterials used in industrial production is required. NanoPolyTox project proposes to study the evolution of nanomaterials physico-chemical and toxicological properties during their life cycle to evaluate their global environmental impact. Furthermore, NanoPolyTox studies will include the development of innovative strategies for the recycling and disposal of nanomaterials that are included in polymeric matrices.

1.2 Objectives

The main goal of NanoPolyTox is to improve the understanding of the potential environmental/health impact of nanotechnology-based products over their life cycle. Gathering and generating data on the possible impact on human health and/or the environmental impact derived from the use, re-use, recycling and/or final treatment and disposal of nanotechnology-based products containing engineered nanoparticles. The project is focused on

after-production stages and will address the following issues for the products considered: Physical and chemical characterization, hazard characterization (human toxicity and ecotoxicity), environmental and biological fate, transformation, and destiny of nanoparticles. Additionally, this project will provide, at laboratory scale, technological solutions for recycling and final treatment of nanotechnology-based products.

1.2.1 Specific Objectives

- The preparation of nanomaterials from three different families (carbon nanotubes, nanoclays and metal oxide nanoparticles) including adequate tailoring functionalities for their inclusion in three selected polymeric hosts widely used in several industrial sectors
- Generation of nanocomposite samples by processing in double screw extruders and further injection in test specimens
- Weathering of the raw nanomaterials and the nanocomposite test specimens in climatic chambers
- Fully characterization (physical and chemical properties) of all the samples (raw nanomaterials and nanocomposites) during their life cycle
- Collection of toxicological data (*in vitro* and *in vivo* human toxicity and ecotoxicity) for selected samples to evaluate the risks associated with their manufacturing, use, recycling and disposal
- Development of predictive models based on the data obtained for the evolution of the physico-chemical and toxicological properties of the nanomaterials along their life cycle
- Detection and quantification of possible migrations and/or releases of the nanofillers from the polymeric matrices, establishing a relationship between weathering cycles and migration/release of nanomaterials
- Mechanical and chemical recycling for innocuous and toxic nanomaterials including the development of a new, efficient and cost effective chemical recycling technology based on specific nanofiber filters
- Development of new solutions for the disposal of toxic nanomaterials based on the inclusion of specific nanofibers filters containing the toxic nanomaterials, into xerogel matrices by sol-gel processes and sintering
- Evaluation of the global environmental impact of nanomaterials that are highly used in many industrial sectors during their life cycle by LCA analysis specifically complemented with the data obtained during this project and other European projects related to nanosafety

NanoPolyTox will provide important information on a general concern regarding the degradability of polymer nanocomposites and their direct impact on human health and environment. It is expected that these results can prevent or minimize the exposure of workers and consumers, and releases to environment of hazardous nanomaterials.



2 Methodology and Associated Work Plan

Nanopolytox work plan is divided in seven technical work packages (WP), which are described below.

2.1 Synthesis and characterization of raw NM (WP1)

The nanomaterials selected for Nanopolytox studies are the following: Carbon nanotubes (MWCNT), metal oxide nanoparticles (TiO₂, ZnO and SiO₂) and nanoclays (small and large). The selection was based on the analysis of the list of nanofillers used in polymer nanocomposite industry. The nanomaterials selected are the most use in the plastic industry for a variety of applications.

In this WP1, the selected nanomaterials will be prepared using different methods of synthesis (depending on the type of nanomaterial) and tailored with different functional groups to match their surface properties (polarities, chemical functionalities) with three different types of polymeric hosts (selected and described in WP2).

The studies carried out in this work package will include the full characterization of the nanomaterials synthesized. Physical and chemical data of the different nanomaterials will be described in different technical cards. The technical cards will consist of ID cards of the nanomaterial during the project containing all the relevant data about their properties.

2.2 Development of polymer nanocomposites (WP2)

The objective of WP2 is to generate polymeric nanocomposites including the nanomaterials obtained in WP1 using the typical industrial processes such as extrusion and injection techniques. The selection of the polymeric matrices will be based mainly on the polymer industrial uses and in their chemical nature, covering several fields of applications and presenting different chemical properties.

The polymeric matrices will be loaded with a 3% of the nanomaterial, in each case. The nanocomposite pellets obtained from the extrusion processes will be injected to obtain the polymeric demonstrators that will be further studied in the project. The distribution of the nanomaterials in the polymeric matrix and the physical properties of the nanocomposites will be determined by different analytical techniques.

2.3 Weathering of polymer nanocomposites (WP3)

In WP3 the main objective is to simulate the outdoor use of polymeric nanocomposites, evaluating the degradability of the polymers under external conditions (sun-light and climate simulations). Nanomaterials, unmodified polymers and polymer nanocomposites will be aged under the selected conditions. All these materials will be submitted to weathering cycles (hours of ageing in a climatic chamber under the selected conditions) that could then be extrapolated to real aging time based on the results obtained doing aging studies in real time conditions. The materials will be aged in climatic chambers specifically tailored for the collection of any potential releases of nanomaterials from the polymeric matrices during weathering.

The weathered nanomaterials and nanocomposites will be characterized using the same techniques as in WP1 and WP2 in

order to obtain comparable data. The potential released nanomaterials collected during the aging process will be characterized by ICP-MS in order to quantify the released nanomaterials.

2.4 Development of non-destructive separation techniques: Proof of concept of NM recycling and disposal techniques (WP4)

The conventional recycling processes used in the polymer industry are based on mechanical processes obtaining polymers with different properties and consequently will be used in other applications. In the case of polymer nanocomposites, in which the additives are nanomaterials (expensive additives), the main interest will be to recover these additives and reuse them maintaining their properties.

Therefore, in this project the recovery of nanomaterials included in polymeric matrices will be carried out in two steps: First, polymeric nanocomposites will be dissolved by non-destructive techniques and then, the colloidal solution will be filtered to recover the nanomaterials. The chemical nature of the polymer will determine the dissolving method and in the case of high resistant polymers, those will be recycled using mechanical methods.

In a first approach, the nanomaterials will be separated from the polymeric matrices by optimized conventional methods: Centrifugation and membrane nanofiltration. In a second approach, novel organic/inorganic filters based on nanofibers will be generated using electrospinning technology. The filtration capability of these novel filters will be compared with the conventional filters used for nanofiltration. The two techniques will be evaluated in terms of costs and industrial viability.

The properties of the nanomaterials collected after filtration will be evaluated by the analytical methods described in WP1. These data will provide information on the evolution of the physico-chemical properties of the nanomaterials along their life cycle.

2.5 Toxicological and ecotoxicological evaluation of NM at different stages of their life cycle (WP5)

The toxicological studies proposed in Nanopolytox will cover the *in vitro* human health acute toxicity and ecotoxicity in aquatic and terrestrial environments. A preliminary *in vitro* screening will be carried out with the following samples: The raw nanomaterials, the nanomaterials separated from the processed polymeric nanocomposites, the nanomaterials separated from the aged polymeric matrices and the nanomaterials separated from mechanical recycled polymeric nanocomposites. The more toxic nanomaterials obtained from the *in vitro* screening (number determined during the course of the project) will be evaluated by *in vivo* assays to determine their biological and environmental fate.

These studies require the dispersion of nanomaterials in an aqueous medium which it is not toxic itself for the biological systems studied. The dispersant cannot be unified due to the high diverse nature of the nanomaterials studied. Therefore, protocols of dispersion will be developed for all the nanomaterials studied within the project.



The rationale to select the set of cell lines, for *in vitro* human health studies, is based on the way nanomaterials enter inside the body and to represent the main target organs (liver, kidney, skin, gastrointestinal tract, lung and lymphocytes) that could be affected by nanomaterials. All these cell lines will be tested on the following assays: Viability assay, proliferation assay and apoptosis assay. Additional *in vitro* test will be carried out with specific cell lines: The absorption assessment with Caco-2 cells and the evaluation of biodistribution mediated toxicity with a hepatic cell line. In the *In vivo* assays, the nanomaterials will be administered intravenously setting up at least three treatment groups and a control group.

The environmental fate and ecotoxicity of nanomaterials will be investigated using six different assays (*in vitro* and *in vivo*). The investigations will be performed with aquatic and terrestrial organisms. The following studies are anticipated: Fish embryo toxicity (FET) test to evaluate the early life stages of Zebra fish larvae, a fish dietary bioaccumulation study, studying the effect of nanomaterials on soil-dwelling organisms (Collembolan) *in vivo*, distribution of the nanomaterials in soil compartments (adsorption-desorption in soil), aerobic transformation of nanomaterials in water/sediment compartment and aerobic and anaerobic transformation of nanomaterials in soil.

The outputs from these studies will be the inputs in the LCIA analysis of nanomaterials.

2.6 Theoretical studies and LCA analysis (WP6)

In this work package the main objective is to analyze all the data obtained on the physical, chemical and toxicological properties of the nanomaterials over their life cycle and use it for the development of theoretical models to predict the human health and environmental impact of nanomaterials. This theoretical analysis of the data will allow developing predictive models to evaluate the impact of nanomaterials along their life cycle. All the studies will be combined to determine the critical factors influencing: the structural changes, migration and toxicity of the nanomaterials.

Additionally, LCA will be performed in accordance with the ISO standards, which establishes four interrelated basic stages for LCA: the goal and scope, the inventory analysis, the impact assessment, and the interpretation. A comprehensive framework describing the impact caused by engineered nanomaterials included in polymeric matrices (nanocomposites) over their entire life cycle will be obtained. The inputs and outputs collected over the life cycle will be then converted into the corresponding potential environmental impacts. The sum of such environmental impacts will represent the overall environmental effect of the nanomaterial along their life cycle. This will enable a quantitative and qualitative assessment of the overall impacts and trade-offs for nanomaterials. New algorithms will be postulated to obtain the impact indicators for the new characterization factors that have been included in the inventory specific to nanomaterials. These algorithms will be proposed taking into account all the theoretical studies done in this work package.

2.7 Technological solutions for the recycling and disposal of NM included in polymeric matrices (WP7)

Nanopolytox project proposes two strategies for the recycling and one strategy for the disposal of nanomaterials included in polymeric nanocomposites:

- Direct mechanical recycling of the nanocomposites for new applications. The samples obtained after recycling will be fully analyzed.
- Filtration and separation of the innocuous nanomaterials from the polymeric host using nanofiber-based filters specially designed for nanomaterials filtration in WP4.

Both the extracted/separated polymers and nanomaterials will be reprocessed leading to new composites with potential applications in various sectors.

- Filtration, separation and inertization of the toxic nanomaterials in glass matrices: Filtration of the nanomaterials with metal oxide nanofiber-based filters able to react/interact strongly with the toxic nanomaterials, then introduction of the charged metal oxide nanofiber filters in a xerogel by sol-gel processes and final sintering.

2.8 Dissemination and exploitation activities (WP8)

The dissemination plan will be the divulgation of the main innovative aspects evolving during the development of the project, in accordance with IPR restrictions. All partners will be involved in the definition of the dissemination strategy, which will be included in the detailed dissemination plan.

On a scientific level, the dissemination activities will be carried out through publications in specialized journals in the areas of nanotechnology, toxicology, polymers and material science. Wider dissemination will be achieved via a more general strategy for attaining a broad coverage of the project to a wide range of public.

The results of the project will be presented at different events (workshops, technical conferences, fairs and exhibitions) organized by the members of the consortium and in other potentially interesting events that could be planned by other organizations.

Additionally, and to promote the dissemination and collaboration of NanoPolyTox with the four projects financed in 2009 in the area of nanosafety, the active participation on the Nanosafety Cluster by the coordinator and by the members of the consortium is expected. It will allow efforts to be joined on direction of establishing guidelines and providing data about the safety of nanoparticles and nanomaterials within the EU territory.

The partners will analyze and validate the primary and secondary market potential for the developments of the project activities, and structure a market penetration & development plan accordingly.

2.9 Project Management (WP9)

This work package covers the management and coordination of the project. All planned activities will be closely monitored and if necessary, corrections will be performed. The coordinator will be responsible of coordinating the overall running of the whole project and, with the help of the Project Management Team, will ensure that all planned activities are pursued.

3 Current status of the project

Nanopolytox is a 3-year project which started in May 2010; the advances on the project for the last 18 months will be described below.

3.1 Synthesis and characterization of NM

The main goal of WP1 was to synthesize and characterize the nanomaterials to be used for the development of the whole project.

The syntheses of the selected nanomaterials (MWCNT, nanoclays and metal oxide nanoparticles) have been carried out following different methods. MWCNT were synthesized by catalytic chemical vapor deposition (CCVD) processes at high temperatures obtaining CNT with high purities. The synthesis of MWCNT with different surface properties (hydrophobic, amphiphatic and hydrophilic) was performed using wet chemistry procedures. The synthesis of nanoclays (two types of nanoclays with different particle size) was carried out by a two step wet chemistry procedure: Purification of the natural occurring clays and subsequent ion exchange reaction to modify the nanoclays with three different content or structure of quaternary ammonium salts. Furthermore, metal oxide nanoparticles (SiO_2 , TiO_2 and ZnO NP) were synthesized by the flame spray pyrolysis process which relies on the direct introduction of liquid raw materials into a flame. Metal oxide NP have been functionalized by wet chemistry leading to NP with different surface properties (hydrophobic, amphiphatic and hydrophilic). The physico-chemical characterization of all the nanomaterials synthesized was carried out using the following analytical techniques: Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) analyses and X-Ray Scattering techniques (XRS and XRD) and to determine the size crystallography and geometry of the nanomaterials, Dynamic Light Scattering (DLS) measurements to determine the hydrodynamic radius in solution, and BET analyses for the porosity and surface area determination. The chemical characterization included determination of the chemical composition of nanomaterials, their surface functionalities and their stability using Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Fourier Transform Infrared Spectroscopy (FTIR), Ultraviolet-visible Spectroscopy (UV-vis) and ζ -potential, respectively. The data collected was included in the technical card which will be the ID of the nanomaterials through all their life cycle.

3.2 Development of polymer nanocomposites

Polymeric matrices have been selected for the studies of this project; the matrices selected were Polypropylene (PP), Ethyl Vinyl

Acetate (EVA) and Polyamide 6 (PA6). Three polymeric matrices with different polarities were chosen to amplify the range of application of the polymeric nanocomposites. The nanomaterials obtained from WP1 were then incorporated into the polymeric matrices by extrusion processes and subsequently injected to obtain the polymeric nanocomposite demonstrators. The compatibilization and dispersibility of the nanomaterials in the polymeric matrices were evaluated by microscopy analysis (SEM). ICP-MS and TGA analysis were used to determine the concentration of nanomaterial in the polymeric matrices after each step of processing (extrusion and injection). Furthermore, physical characterization of the polymer nanocomposite demonstrators was focused on mechanical properties and thermal resistance determination (DMA, Dynamometric tests, HDT and VICAT, DSC). The results obtained showed that some polymers nanocomposites presented improved properties. However, further characterization analyses are in progress in order to establish relationships between dispersibility of the nanomaterials in the polymeric matrices and the physical properties obtained.



3.3 Weathering of polymer nanocomposites

The climatic chambers were tailored for the weathering tests to be done within the Nanopolytox project. The demonstrators developed in WP2 and the raw nanomaterials, including unmodified polymers were (and still are being) submitted to the selected aging conditions and the potential releases of nanomaterials from the polymeric matrices during ageing processes are collected and quantified.

Therefore, raw nanomaterials and nanocomposites were (and are being) exposed to an accelerated aging in weathering chambers for 1000h and combining climate and sunlight radiation, under the modified normative ISO 4892-2. The nanomaterials released from the polymeric matrices during ageing are extracted from the collected water by freeze-drying (lyophilisation) and then quantified by ICPMS and further analyzed by other analytical techniques (BET, FTIR, TGA, ICP-MS, DSC) to determine their physico-chemical properties.

Aged nanomaterials in the powder form after being submitted to the whole process of ageing were dried and analyzed. After aging, all the nanomaterials except for nanoclays showed alterations compare to the corresponding non-aged nanomaterial. The main difference is the hydration of all the nanomaterials. Furthermore, in some cases, FT-IR indicates the modification of nanomaterial surface chemistry. FT-IR results were supported by the results obtained by BET analyses showing, for those nanomaterials with modified surface chemistries, a significant change in the surface area, pore volume and/or pore diameter of the material. For functionalized nanomaterials, a gain in surface area values indicates a loss of functional groups on the surface of the nanomaterial or a decrease in particle size. However, further

characterization is being carried out, such as ICP-MS and TGA for a more complete analysis.

To evaluate the migration of nanomaterial from the polymeric matrices, nanocomposites will be analyzed by TEM and SEM and compare with those before ageing. These results will be complemented with the data obtained for the nanomaterial recovered from the simulated rain during aging. Furthermore, analysis by ICP-MS is in progress to allow nanomaterial quantification in the nanocomposite after ageing. Degradation of the polymer is being determined by analytical characterization with TGA and DSC.

For comparison, it was designed an additional experiment outdoors with the nanocomposites samples. In this case, 5 specimens of each nanocomposite are being exposed to external conditions for a total exposure time of 1 year. Data obtained will be used to determine the equivalence of the accelerated aging in the weathering chamber with a real ageing process in a Mediterranean climate.

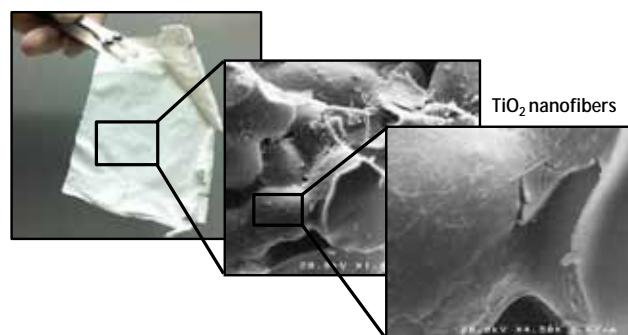
3.4 Development of non-destructive separation techniques: Proof of concept of NM recycling and disposal techniques

The main objective of this WP is the development of techniques for the separation of nanofillers from polymeric matrix without inducing degradation of the nanomaterials under the form encountered in the composites. Therefore firstly, research was conducted in the methodologies to dissolve the polymers without affecting the physico-chemical properties of the nanomaterials. From the different methodologies found, were selected those ones with the mildest conditions and then tested with raw nanomaterials. The results indicated that the most affected nanomaterials were the metal oxide nanoparticles changing their surface chemistry or their degree of functionalization. Even though, these results cannot be totally extrapolated to the effects on the nanofillers of the nanocomposites, because the polymeric matrix can protect the nanomaterial.

These methodologies are applied to the polymeric nanocomposites and the colloidal solution obtained will be then filtered to separate the nanomaterials from the polymeric matrices. Centrifugation and membrane nanofiltration are being optimized for the filtration of those colloidal solutions that are dissolved at room temperature. On the other hand, ceramic nanofiber filters have been developed by means of electrospinning technique and characterized for their morphology and separation efficiency. Optimization of these ceramic nanofiber filters is in progress for the filtration of the colloidal solutions at high temperature.

The nanomaterials collected after all the different filtration processes will be fully characterized and their toxicological profile will be determined.

Free standing TiO₂ nanofiber mat

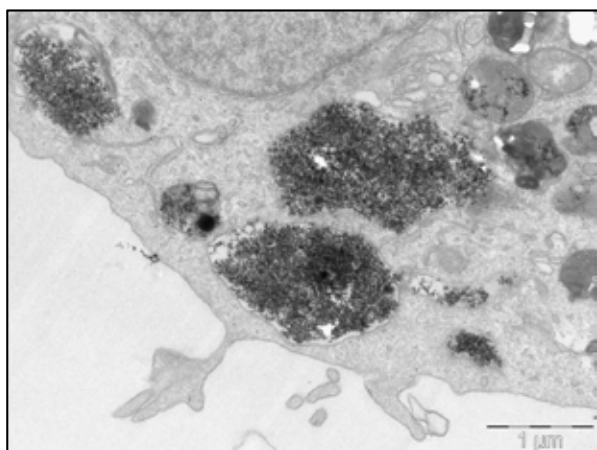
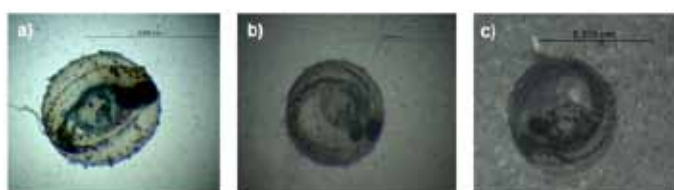


3.5 Toxicological and ecotoxicological evaluation of NM at different stages of their life cycle

The starting point of the toxicological studies was the dispersion of nanomaterials in an aqueous solution. The raw nanomaterials studied in Nanopolytox project have diverse chemical compositions and surface properties making more difficult the selection of the adequate dispersant. The dispersion studies have been carried out case by case and the dispersion protocols have been described for each nanomaterial. The dispersants selected for the studies were Bovine Serum Albumin (BSA), Fetal Bovine Serum (FBS), Tween 20, Sodium Citrate and MilliQ H₂O. The control over the stability of nanomaterials in the dispersion medium was studied with different analytical techniques: UV-vis spectroscopy, ζ-potential analysis and DLS. The data collected was analyzed and the best dispersion medium was selected for each nanomaterial.

The toxicity of raw nanomaterials has been evaluated in a battery of human cell lines and in a fish embryo test and the toxicity evaluation of the aged nanomaterials is now ongoing. A series of mechanistic assays, such as apoptosis induction, cell proliferation and cell internalization, have also been performed. The results showed considerable differences in toxic potential and mechanisms of toxicity among the nanomaterials of the project, but relatively good correspondence between the toxic potential in fish embryos and in human cell lines. In both test systems, nanoclays were the most toxic nanomaterials, and their toxicity seemed to be associated to the organic modifiers used in their functionalization. The toxic potential of zinc oxide nanoparticles was higher in human cell lines than in the standard parameters of the fish embryo test, although they did affect embryo hatching. This suggests that zinc nanoparticles may not be able to cross the yolk sac.

Several ongoing studies are further investigating the toxicity and the environmental fate of the nanomaterials. These studies include *in vitro* and *in vivo* ADME studies, terrestrial toxicity, bioaccumulation in fish, and adsorption-desorption studies.


 TEM picture of cellular internalization of TiO₂ NPs

 a) SiO₂ (1000 mg/L) b) SiO₂-OH (1000 mg/L) c) ZnO-Octyl (1000 mg/L)

3.6 Theoretical studies and LCA analysis

This point has two main sections, the first section involves the establishment of properties-activity relationships, and the second section involves an LCA analysis. Although a considerable amount of data has already been generated in the project, the information is still partial, and has not allowed much progress on the first section. Regarding the second section, the progress has consisted on the definition of the goal and scope, functional unit, and system boundaries, and on the data collection for the synthesis, functionalization, and nanocomposite manufacturing. In addition, we have established the methodology to generate the characterization factors for the relevant impacts associated to the nanoparticles released into the environment, i.e., human toxicity and ecotoxicity. Therefore, the LCA study will include all life stages of each nanomaterial, and the data will be presented as a global aggregate and by life stages, to facilitate transparency and future use of the data.

4 Directory

Table 1 Directory of people involved in this project.

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NanoReTox

The Reactivity and Toxicity of
Engineered Nanoparticles: Risks to
the Environment and Human Health



NanoReTox

Contract Agreement: NMP4-SL-2008-214478

Website: <http://www.nanorettox.eu>

Coordinator: Dr Eugenia Valsami-Jones, Natural History Museum, London, UK*

No.	Beneficiary name	Short name	Country
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2	Imperial College of Science, Technology and Medicine	IMPERIAL	United Kingdom
3	Roskilde Universitetscenter	RU	Denmark
4	Université de Nice Sophia Antipolis	UNS	France
5	Université Catholique de l'Ouest	UCO	France
6	Universidad del Pais Vasco/Euskal Herriko Unibertsitatea	UPV-EHU	Spain
7	Commission of the European Communities – Directorate General Joint Research Centre	JRC	Belgium
8	Universita di Pisa	UNIPI	Italy
9	Ahava – Dead Sea Laboratories Ltd	DSL	Israel
10	King's College London	KCL	United Kingdom
11	United States Geological Survey	USGS	USA
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Project Duration: 1 December 2008 – 30 November 2012

Project Funding: 3.2 Mio. EUR

1 Summary

NanoReTox aims to investigate the potential risks of engineered nanomaterials to the environment and human health by comprehensively addressing five key questions:

[1] How does the environment affect the physicochemical properties and the bioreactivity of nanoparticles?

[2] How does this impact on their ability to interact with and/or penetrate organisms and cells and will bioavailability result in toxicity?

[3] Is there a pattern of cellular reactivity and/or toxicity related to physicochemical properties?

[4] What combination of conditions are most likely to pose a risk to human health and the environment?

[5] How can this information be incorporated in a risk assessment model?

A team of experts from across the EU and the US are working together to address these questions in depth. A description of each group involved can be found at www.nanorettox.eu.



The specific scientific and technical objectives are:

[1] To synthesise and fully characterise a set of engineered nanoparticles with a range of physicochemical properties using industrial and laboratory methods.

[2] To study the abiotic reactivity (transformations) of the synthesised nanoparticles in simulated environmental and biological media.

[3] To investigate *in vivo* uptake of nanoparticles by aquatic species and study mechanisms and paths of internalisation.

[4] To investigate *in vitro* uptake and reactivity of nanoparticles and to discover putative mechanisms of toxicity.

[5] To consider the genotoxicity and carcinogenicity of metal nanoparticles.

[6] To determine whether cellular responses between human cells, mammalian cells, cell lines and invertebrate cells or whole organisms are comparable or different with relevance to screening models.

[7] To establish universal approaches to risk assessment model and risk communication.

2 Background

The physicochemical properties of nano-sized particles are distinct from the properties of equivalent bulk substances. As the use of nanomaterials increases, the research into any potential risks of adverse effects on the environment and/or health must be intensified. Concerns on free engineered nanoparticles have been highlighted in several reports, including those by the Royal Society and the Royal Academy of Engineering in the UK (2004), the EPA's Nanotechnology White Paper (2005), the European Commission's Action Plan for Nanotechnology (2006), and the Royal Commission on Environmental Pollution (2008). All expressed unease over the apparent lack of urgency (at the time of the reports) in identifying the extent of the potential risks.

Indications of the potential toxicity of engineered nanoparticles can be drawn from epidemiological studies of inhaled environmental particulate matter in humans. These show that one of the primary target organs, in this case the lung, cannot necessarily defend other body systems from the effects of inhaling very small, ultrafine, nanosized material. Consequently, the cardiovascular system is also affected. Indications from these human studies are that particles may enter the circulation and translocate to other organs and/or that there are "knock-on" systemic effects due to locally produced pro-inflammatory and pro-thrombotic mediators. Clearly, other mechanisms may exist. Studies in experimental animals suggest that inhaled nano-sized particles relocate to the brain, vasculature, liver, kidney and spleen. Similarly, intravenous nanoparticles can access multiple organs including the fetus¹. Other important portals of entry of exogenous nano-substances are the skin and gastrointestinal tract. Furthermore, there is *in vivo* evidence that activation of specific body compartments by some nanoparticles can initiate both local and systemic reactivity. All these findings may have serious implications for human health. What is not known is which engineered particle(s) induce cellular reactivity, how and where this might occur.

The rapid expansion of nanotechnology means there is a vast array of nanomaterials, many of which are already in industrial production. Because of the wide variety in physicochemical properties amongst different nanomaterials it is not possible at present to predict which elicit human and/or environmental harm. However, until the mechanistic associations between nanomaterial characteristics and putative toxicity are understood, determination of nanorisks will not move forward. Many recent toxicological studies have fallen short of this; furthermore many studies have led to contrasting results and interpretations about risks, possibly reflecting the diverse sources and nature of the test materials. This illustrates the importance of studying commercial and designer particles that have been fully characterised before, during and after toxicity studies. Of the many different types of engineered nanoparticles currently produced, industrially or in the laboratory, environmental risks from non-carbon-based nanoparticles are the least studied. This is despite the rapidly growing use of particles such as TiO₂, ZnO, SiO₂, Ag, CuO, and CdS. The chemical composition of metal nanoparticles may contribute to their having significant additional toxicity, but few studies address this.

Ecotoxicological studies with engineered nanomaterials are currently limited. Most of the studies so far undertaken are simple "proof of principle" tests evaluating the possibility of either toxicity, under high concentration exposures, and/or the visual penetration of cells. There is a clear need for a more systematic approach to evaluating the processes that determine hazard, exposure and risk and for validated models predicting the release, transport, transformation, accumulation and uptake of nanoparticles in the environment and the human body.

3 What is NanoReTox

The overriding objective of NanoReTox is to contribute new knowledge to what will be a global endeavour in addressing the scientific uncertainties related to the health and environmental effects of engineered nanoparticles and to provide a body of new information and a new tool that industry and governments can use to begin to assess the risks of these nanomaterials. Thus, NanoReTox aims to identify the potential risks of free engineered metal nanoparticles to the environment and human health, and address a great deal of the issues described earlier. More specifically:

NanoReTox intends to examine the molecular and cellular reactivity of well characterised nanoparticles on a panel of primary human/mammalian cells and human cell lines originating from different target organs and exposure routes as an indicator of *in vivo* toxicity. The aim is to discover which features of nanoparticles confer reactivity with which cell types/target organs.

NanoReTox will comprehensively address all physicochemical properties of industrially important metal based nanoparticles with a potential to induce toxicity (particle size, size distribution, shape, agglomeration state, crystal phase, chemical composition, surface area, surface chemistry and surface charge). By using labeled particles (with either stable isotopes or fluorescent dyes), NanoReTox will quantitatively determine the accumulated NPs in *in vitro* and *in vivo* systems. These techniques literally resolve the problem of determining nanoparticle bioavailability.



NanoReTox will compare animal models that are likely to be sensitive to nano-materials: animals that pump vast quantities of water across their gills (bivalves) or ingest plants or sediments where nanomaterials are likely to accumulate. NanoReTox will use determinations of biodynamic characteristics, including rate of uptake from water, assimilation efficiency and rate of uptake from food, as well as retention (rate constant of loss), across several species and a range of particles. This screening approach (equivalent to a biological bioavailability probe) will increase the number of particle formulations and characteristics that can be compared. The biodynamic studies will be complemented by longer term experiments with fewer particle types to verify probe results.

By quantifying nanoparticle uptake using labelling, verifying the presence of particles in cells with visual or light scattering approaches, and observing responses of the organism at the cellular and whole organism level, we will assemble the lines of evidence that are necessary to determine if bioavailability and toxicity are feasible expectations for the metal nanoparticles. By systematically making such determinations using a number of different types of particles of well-defined character we will tie together nanoparticle properties with their bioavailability and toxicity in unique ways.

NanoReTox will also provide comparative data between mammalian systems and non-mammalian systems by using isotopic methods in the mammalian systems. This will provide unique data regarding the relationship between particle physicochemistry, bioavailability, cellular uptake and reactivity across a range of relevant target cell systems.

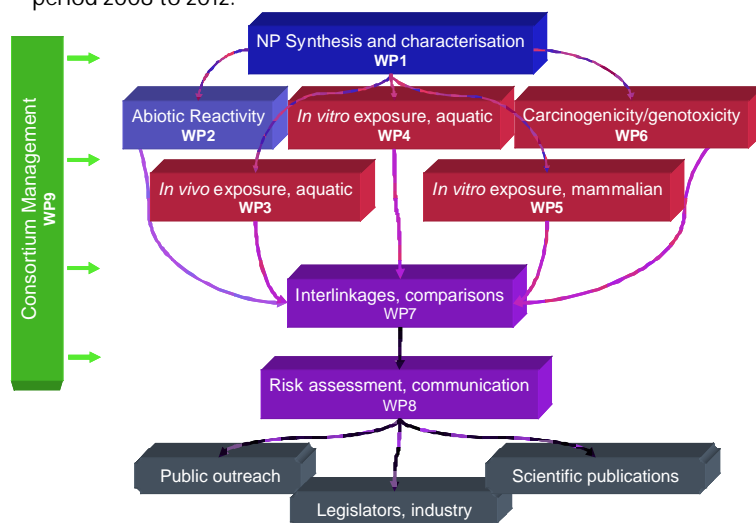
Finally, NanoReTox has been employing the extensive experience within the partnership, and a specific work package devoted to risk assessment and risk communication to develop appropriate criteria, considering factors that have, historically, generated surprising risks in the past.

3.1 The three principles of NanoReTox

- Focus on engineered metal nanoparticles, synthesised and fully characterised by project partners (both research & industry), to provide a coherent well defined study material with controllable properties. The project will not consider natural or other non-engineered (e.g. combustion derived) nanoparticles.
- Use of organisms that are not currently included in standard toxicity tests, but which have (a) greater potential to be affected by nanoparticle toxicity due to the environment where they live or their biology and (b) are potentially valuable indicators.
- Minimal use of animal models in keeping with the 7th Amendment to the EU Cosmetics Directive (European Commission, 2003; Council Directive 76/768/EEC) which aims to reduce the use of experimental animal research.

4 Organisation of NanoReTox

NanoReTox is divided into six inter-related and interconnected work packages (WPs, see graph below) and is funded for the period 2008 to 2012.



4.1 WP 1: Synthesis and characterisation

This workpackage is generating sets of well-characterized nanoparticles using different methods of synthesis. The nanoparticle sets are tailored to display a range of physicochemical properties of interest. In order to establish that the nanoparticles tested by NanoReTox are representative of what is currently and in the future released in the environment, both top down (i.e. nanoparticles produced from bulk materials by milling) as well as bottom up (wet chemical, plasma and microfluidic synthesis), so that many important routes of synthesis are represented. This "in-house" "tailored" synthesis is essential for materials of this nature, because unlike conventional chemical toxins (where a solution of a particular substance will have the same properties regardless of the way it was produced or its source) nanoparticle properties can vary substantially depending on the method of synthesis and subsequent functionalisation. This approach is complementary to that of the OECD Working Party on Manufactured Nanomaterials, by placing emphasis on the controlled variation of properties. The particles being tested are: TiO₂, SiO₂, ZnO, CuO, CdS, Ag (Figure 1) and Au.

The nanomaterials studied in NanoReTox are extensively characterised using analytical and biochemical techniques such as Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Dynamic Light Scattering (DLS) and Zeta Potential Analysis (ZPA), Single Particle Tracing (SPT), Gel Filtration (GF), Fast Protein Liquid Chromatography (FPLC), Scanning Electron Microscopy (SEM), Transmitted Electron Microscopy (TEM), Atomic Force Microscopy (AFM) in both wet and dry mode, X-ray Diffraction (XRD) and surface area analysis (BET). Multicollector ICP-MS is available for analyses of labelled nanoparticle isotopic composition. Focused Ion Beam Scanning Electron Microscopy for visualising the nanomaterials produced and their inner structure. X-ray Photoelectron Spectroscopy (XPS) and Time-of-Flight Secondary Ion Mass Spectrometry (ToF SIMS) for nanoparticle surface composition.

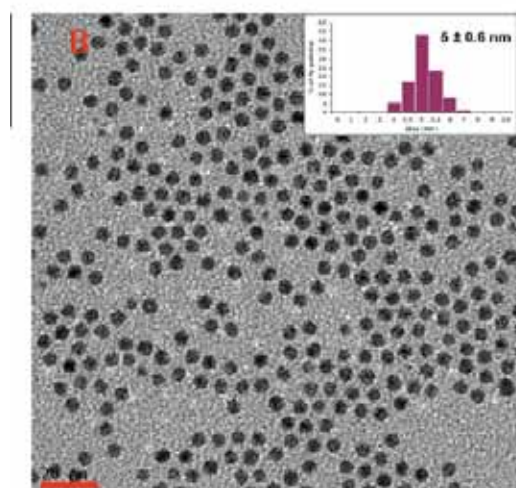


Figure 1. TEM image of in-house synthesised Ag nanoparticles showing good monodispersity.

4.2 WP 2: Abiotic reactivity

Using the nanomaterials synthesised in WP1, this work package has the role of assessing nanoparticle behaviour (i.e. abiotic reactivity and potential transformations) in a variety of media, in order to: (1) select the optimum form and dose for *in vivo* and *in vitro* experiments; (2) prioritise which sets of the synthesised nanoparticles to study; and (3) elucidate nanoparticle behaviour in biological and environmental matrices. Physicochemical properties that will be specifically monitored include: solubility, surface charge, particle size and size distribution, agglomeration/dispersion, surface area and other surface characteristics (roughness, porosity, and appearance), crystallinity and crystal structure.

Behaviour of nanoparticles released in biological or environmental media is currently unknown. It is predicted that nanoparticles in some situations (particularly when present in concentrated suspensions) will tend to aggregate; however there is no evidence to suggest that aggregates, even when formed, behave like larger particles. Another important parameter to investigate will be the stability (in terms of solubility and physical/chemical degradation) of the nanoparticles, to establish how their properties evolve in different media with time. Most physicochemical properties of the nanoparticles, notably size, composition, surface modification and even, in some cases, structure, will change with time. Abiotic reactivity studies of the nanoparticles are carried out in media simulating environmental (hard/soft freshwater, seawater) and biological (simulated body fluid, lung fluid, gastric fluid) matrices. In these series of experiments factors such as pH, eH, temperature, ionic strength and the presence of organic ligands (of biological, e.g. proteins, or chemical, e.g. humic acids relevance) of the model media are investigated.

4.3 WP 3: *In vivo* exposure, aquatic organisms

It is unclear to what extent metals in the size ranges of nanoparticle are accessible for uptake into the tissues and cells of organisms. The goal of this work package is to quantify the bioavailability of different types of nanoparticles and determine if bioavailable nanoparticles exert an adverse response within organisms.

Bioavailability is addressed using particles from WP1, occasionally labelled with artificially enriched stable isotopes and fluorescent labels to quantify biodynamic uptake and loss characteristics. Bioaccumulation will be modelled from biodynamics for a variety of particle formulations, characteristics and compositions. The biodynamic predictions will be verified by longer-term experiments on fewer particle types. The cell and tissue distribution of metal nanoparticles will be investigated in organisms such as mussels and zebra fish by means of autometallography at both light and electron-microscope level, and X-ray microanalysis. The distribution pattern of metal nanoparticles will be compared with that of metals themselves, identifying target cells and tissues for the toxic action of metal nanoparticles.

These experiments will accompany studies of adverse responses at both whole organism and cellular levels. Partners will experiment with different organisms (Figure 2) in order to compare implications of different biological traits. Bivalve molluscs will be compared that filter at different rates and consume different food (*Mytilus galloprovincialis*, *Scrobicularia plana*, *Macoma balthica*). Freshwater and marine snails (*Potamopyrgus antipodarum*, *Lymnea stagnalis*, *Peringia ulvae*) that ingest plant material where nanoparticles might deposit will be compared to animals that ingest sediments (polychaetes *Nereis diversicolor* and *Capitella capitata*). Zebrafish (*Danio rerio*) will be studied as representative model vertebrate aquatic organism. Microscopy techniques and subcellular fractionation of metals within organisms will assess the internal uptake and distribution of nanomaterials. Oxidative stress, genotoxicity, metallothionein induction, DNA damage, lysosomal membrane destabilization histopathology and behaviour (burrowing, feeding rate) are important indicators of stress from metals. Nanomaterials themselves produce similar type responses, *in vitro*. If organisms show such responses to bioavailable nanomaterials, *in vivo*, it is unequivocal evidence that nanomaterial uptake causes the organisms to respond. Visual evidence of nanomaterials present internally, evaluation of internal dissolution and rigorous experimental design is used to determine if responses are due to internal dissolution of the metal oxide particle or due to disruption by the nanoparticle itself.

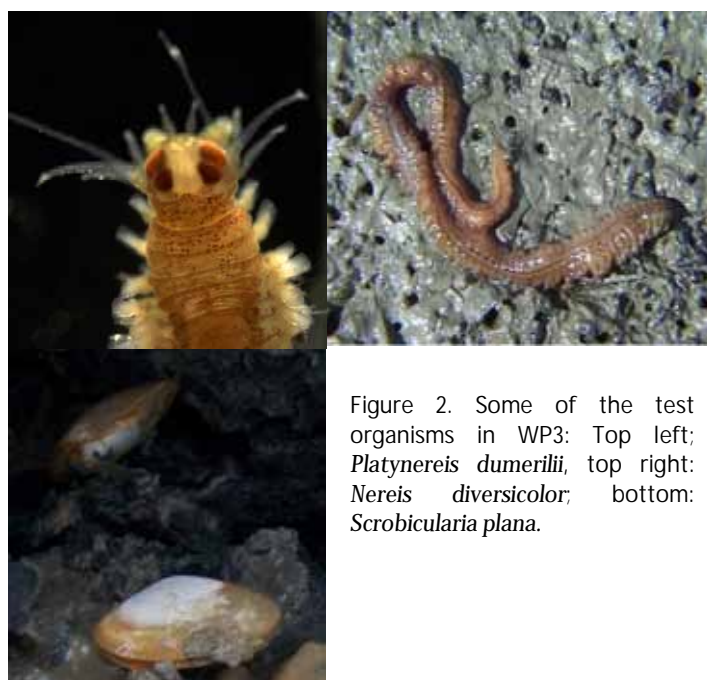


Figure 2. Some of the test organisms in WP3: Top left: *Platynereis dumerilii*, top right: *Nereis diversicolor*; bottom: *Scrobicularia plana*.



4.4 WP 4: *In vitro* exposure, aquatic species primary cells

This workpackage addresses the question whether nanoparticles induce responses that are indicative of a bioactive or potentially toxic material after the particle is taken into the cell of an organism. For example, nanomaterials could possibly be inert within cells, or detoxified by mechanisms in place to fend off foreign particles. In such a case we would expect no response by mechanisms that defend the cell against toxins. However, if we see such responses it is evidence the particle is a potential threat. Furthermore, there are some responses that are well known to be associated with metals or nanoparticles. Although we expect that *in vivo* processes greatly influence nanoparticle bioavailability and toxicity, it is more awkward to study mechanisms of response in whole organisms than in cell cultures. Understanding whether cells recognize and respond to nanoparticles, and how (the exact mechanisms of response) can be efficiently and effectively addressed with *in vitro* cell cultures both in humans and in other animals. Thus *in vivo* and *in vitro* studies are complementary approaches and their combination will help avoid false conclusions about risks from nanoparticles. Most importantly, *in vivo* (WP3) and *in vitro* (WP4) approaches will be co-ordinated using the same aquatic organisms (mussels) and similar endpoints, thus linking interpretation of *in vitro* and *in vivo* responses.

In close connection to WP3, WP4 will determine the *in vitro* effects of nanoparticles in primary cell cultures of mussel haemocytes and gill cells. Haemocytes or immunocytes comprise the main internal defence system in mussels. Effects on this cell type could reflect damage on the immune system, which could have consequences at higher levels of biological organisation, ie, individuals and communities. The *in vitro* experiments with mussel haemocytes and gill cells will be short-term 48 h experiments using the same selected set of particles as in *in vivo* bio-response studies (WP3). In addition to general toxicity tests (cell viability), the emphasis will be to survey a broad range of biological targets that could be damaged by nanoparticle exposure. The goal is to cover as many possible effects as possible in order to identify the most relevant biological targets. These will include oxidative stress (superoxide dismutase SOD, catalase CAT, superoxide anion and hydrogen peroxide), apoptosis (tunel assay) and genotoxicity (Comet assay, micronucleus test, oxidative DNA damage). Further, specific tests for haemocytes (endocytosis, phagocytosis, damage to the actin cytoskeleton) and for gill cells (lysosomal enzyme activity, Na,K-ATPase, multixenobiotic resistance MXR transport activity) will be carried out. These studies, performed in parallel to those in WP3, will allow comparisons between *in vitro* and *in vivo* responses to nanoparticles in mussels. Further, as some of the tests used are identical to those used in human cells (WP5), this will allow some comparison of mechanisms between human cells and mussel cells.

4.5 WP 5: Cellular/molecular mechanisms of action; human

Using *in vivo* models, it is becoming apparent that particles delivered via one system (e.g. lung) can reach, and have detrimental effects on, other body systems/compartments (e.g. vasculature). However, these studies utilise significant numbers of animals, are labour-intensive and are impractical for examining the comparative effects and mechanism of action of a panel of compounds. In NanoReTox we use *in vitro* models to examine cellular responses to nanoparticles; this approach is also in line

with the 7th amendment to the EU Cosmetics Directive (European Commission, 2003; Council Directive 76/768/EEC) to avoid excessive animal testing.

We hypothesise that the cellular reactivity of the particles will critically depend both on the target tissue and the function of the cell type within that tissue. Thus, whilst some nanoparticles may be overtly cytotoxic, even at low levels, others may not, but they may adversely affect cell function, for example stimulating inflammatory mediator release or compromising epithelial barrier integrity. Conversely, the magnitude and profile of the cellular response will depend on the physicochemical properties of each type and format of particle and its exposure dose. We will initially concentrate on Ag, TiO₂, SiO₂, ZnO, CdS (and test all different sets of nanoparticles synthesised), which we expect will have a broad range of activity for comparative purposes. If time permits, or if data from WP2 indicate, we will study other particles. In the following studies we want to know: 1) Which cell types are most vulnerable to nanoparticle exposure? 2) Which cellular functions are affected? 3) Which mechanisms and cellular pathways are involved? 4) What is the cellular fate of nanoparticles? 5) Which physicochemical properties of nanoparticles render them more/less bio-reactive?

This WP aims to investigate the cellular and molecular reactivity of the selected metal nanoparticles in a) primary mammalian and human cells and b) in a panel of established human cell lines. The chosen cells will reflect likely nanoparticle exposure routes. The primary cell work will be performed on lung and skin models. State of the art fixed and live cell microscopy is used to study particle-cell interactions; air-liquid interface models are used for lung epithelial cells. Microscopy techniques include i) high 2D resolution at low light or high speed using a widefield microscope; ii) a widefield with deconvolution high speed microscope; iii) for deep tissue penetration, a multiphoton microscope; iv) confocal for 3D imaging and v) scanning ion conductance microscopy combined with confocal microscopy for topographical, surface imaging.

4.6 WP 6: Cellular/molecular mechanisms of action; human

Occupational and environmental exposures to metals are associated with the development of various pathologies, including cancer; however, the mechanisms of action, especially at the molecular level, are still unclear. Recently, it was shown that exposure to toxic metals may be induced not only by absorption in micro-molecular form but also as nanoparticles. Although metal nanoparticles have been demonstrated to cause pathological responses, the mechanisms of toxicity remain to be explained. Metal-mediated formation of free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) can cause various modifications to DNA bases, enhanced lipid peroxidation, and changes in calcium and sulphhydryl homeostasis and evidence indicates that such ROS and RNS play an important role in the etiology of a number of diseases, in particular neurodegenerative pathologies and cancer. Previous studies on human peripheral lymphocytes, show DNA damage and suggest that some metal nanoparticles might be genotoxic and therefore have carcinogenic potential; one important mechanism involves increased oxidative stress. In this work package, we want to know whether metal nanoparticles possess genotoxic and carcinogenic potential; specifically: 1) Do nanoparticles induce cytogenetic changes and formation of micronuclei? 2) Do nanoparticles cause damage at the



DNA level? 3) Do nanoparticles interfere with cell proliferation? 4) Do nanoparticles induce cell transformation? 5) Do the genotoxic effects of nanoparticles vary between individuals and between species? 6) Which physicochemical properties of nanoparticles render them more/less genotoxic.

The chosen cell models in this WP have been fully characterised and are based on human leukocyte cultures (obtained from healthy volunteers), and on cell lines relevant to occupational and environmental exposure (Figure 3). The A549 (human lung epithelial) cells will model the inhalation processes and the RAW264.7 murine macrophage cell line will model the inflammatory process. *In vivo* studies will concentrate in zebrafish liver as this small tropical fish species is a well-known model for hepatocarcinogenesis. In addition, possible carcinogenic effects are also studied in mussels, where haemic or haemocytic neoplasia and gonadal neoplasia have been reported.

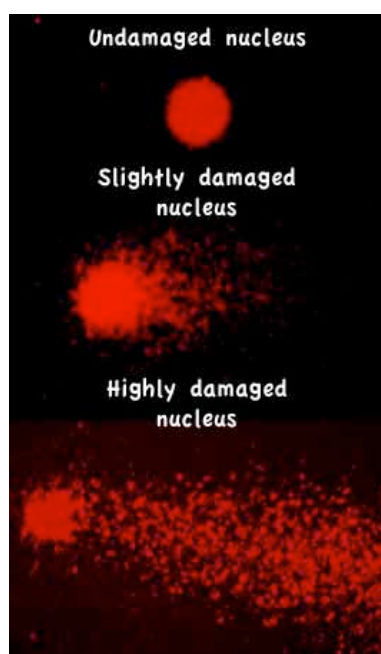
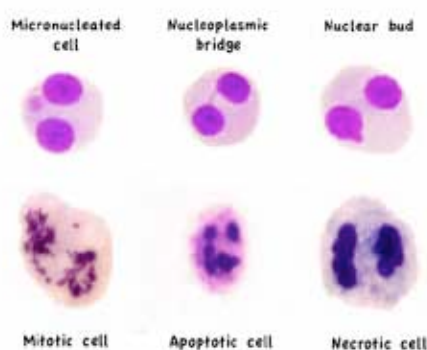


Figure 3. Top: Genotoxicity endpoints evaluated by CBMN Cytome assay in primary human lymphocytes after treatment with nanoparticles. Bottom: damage evaluated by Comet assay in primary human lymphocytes after treatment with nanoparticles.

4.7 WP 7: Interpretative comparison and interlinkage of reactivity, bioavailability and health effects

Using data collected in all previous WPs, this WP compares species, particles of differing nature, as well as human and aquatic organism responses. A variety of datasets will be produced. This work package provides a specific effort dedicated to finding commonalities among the different studies so as to maximize generalizations and applications to risk assessment. For example, many properties of cells are biologically conservative: that is, many similar mechanisms characterize the functioning of cells of all life forms. If there are commonalities in the way humans and other organisms react to nanoparticles then universal methods might be developed to both detect and better understand nanorisks. Specific questions addressed will include: 1) Do organisms differ from humans or among species in their stress responses and/or sensitivity? 2) Can we use abiotic reactivity to predict toxicity? 3) Is in vitro dose response to metal nanoparticles indicative of in vivo responses? 4) Is there a pattern of cellular reactivity and/or toxicity related to physicochemical properties, i.e. a hierarchy of activity?

4.8 WP 8: Risk model and communication

Risk assessment: Ultimately a formal assessment of nanoparticle risks is essential. NanoReTox, in one study, addresses multiple nanoparticle formulations, in multiple media, using multiple species (including humans) and employing in vitro and in vivo approaches. The goal of WP8 is to incorporate this broad set of data from a single study into a risk assessment. Though there is increasing attention toward studying human health risks from nanoparticles, a common framework for conducting risk assessments is lacking. Information on environmental risks associated with nanoparticles, and particularly metallic nanoparticles, is scarce. An important outcome of the project will be development of a conceptual model to guide evaluation of hazards and risks from nanoparticles. Because our studies build a basis for evaluating risks, assessing what our results mean in a systematic way is necessary. The model will be developed to be applicable to the body of evidence that will surely grow quickly as knowledge of nano-materials grows.

Risk communication: The profile of nanotechnology and any associated risk is high in the media; so inadvertent miscommunication is possible. Another goal of NanoReTox is to develop a risk communication strategy that will guide how we release our results, but more important help recipients of our results (government, industry) communicate risks in a balanced, robust manner. It is essential to “get the risks from nanoparticles right” because the technology offers many potential benefits. The costs of over-stating or under-stating risks could be high. Although general risk assessment procedures are well known, there are many unique attributes of nanoparticles that may require new or adjusted methodologies. Communicating new results in an unbiased, balanced and value free way is critical to public credibility. Communicating risks appropriately also requires a holistic view of the issues, as well as a careful, rational and transparent approach.

4.9 WP 9: Project management

All aspects of project management are covered by this WP, including reporting, quality control, progress meetings,



financial/administrative coordination cost control, deadlines, contacts with the EU and dissemination.

5 NanoReTox Results

During its first three years, NanoReTox has produced the following key results:

5.1 Results from WP1 and WP2

WP1 has produced a range of well-characterised sets of ZnO, CuO, TiO₂, SiO₂, Ag, Au and CdS nanoparticles. Some of the particles were produced by the industry partners and some tailored to show properties that vary systematically, so that robust links to toxicity can be made. Reproducible protocols for all the syntheses have been developed and the produced sets of nanoparticles were thoroughly characterised and tested in a variety of (eco)toxicity experiments in subsequent WPs.

WP2 tested the stability of the above particles in a variety of conditions, with the aim to understand their behaviour in biological/environmental media as well as their overall physicochemical response to changes of conditions such as temperature, pH and ionic strength. Major findings include: a) dissolution results, which indicated increased ion release of metal ions from CuO and ZnO nanoparticles compared to their bulk counterparts, while TiO₂ remains relatively insoluble; b) the effect of temperature which appears to be reversible, i.e. an increase in size is observed while the samples are heated, suggesting aggregation, but temperature decrease is sufficient to redisperse the particles; c) the behaviour of nanoparticles in different media shows different patterns depending on the composition of the nanoparticles and the type of stabilisation; however a common feature is aggregation even at modest increases in ionic strength; the presence of organics, humic acid or albumin, induces moderate aggregation.

5.2 Results from WP3 and WP4

The main objective of these two WPs was to test nanoparticle toxicity on a selection of aquatic organisms and conditions *in vivo* and *in vitro*. In WP3 (*in vivo*), a wide range of species belonging to different taxonomic groups and with different biological traits were tested: Bivalve molluscs (*Scrobicularia plana*, *Mytilus galloprovincialis*, *Macoma balthica*), a gastropod (*Peringia ulvae*), two species of polychaetes (*Nereis diversicolor*, *Capitella teleta*) as representatives of the estuarine and marine environment and three freshwater species, two gastropods (*Potamopyrgus antipodarum*, *Lymnaea stagnalis*) and zebrafish (*Danio rerio*, embryos). The experiments included food, water- and sediment-exposure treatments. Toxicity endpoints included both subindividual-level effects (biomarkers) and individual-level effects (mortality/survival, hatching, reproduction, malformations, behaviour, feeding rate). A number of particles from a variety of sources and a wide range of doses have already been tested (CuO, Ag, Au, TiO₂, ZnO) and produced the following findings: (1) a set of preliminary indications of ecotoxicity (oxidative-stress, genotoxicity, cytotoxicity, behaviour impairments, malformations, mortality, reproduction, hatching), as a function of nanoparticle

properties, (2) demonstration that all of the metal forms (ionic, NPs, bulk) were bioavailable and bioaccumulated by some organisms, even of aggregated NPs in seawater; (3) demonstration that metal containing NPs yield bioavailable metals, more so than bulk or micron sized particles; (4) usually in water-exposure treatment, ionic forms were more bioavailable and/or toxic than NPs. Bioavailability of Ag from citrate-capped Ag NPs in *Peringia ulvae* was 2 fold less bioavailable than dissolved Ag. In Zebrafish embryos, ionic silver was the most toxic compound, (5) a detectable release in exposure medium of Ag from lactose-AgNPs has been shown whereas no detectable release of metal was observed with citrate-Ag NPs or CuO NPs (6) a particle size-related differences in bioavailability and toxicity effects has been observed in the case of Ag NPs; the smallest ones were the most toxic in Zebrafish embryos. For mollusks, uptake rates in *P. antipodarum* were faster for the smaller-sized Cu particles. In contrast, the biggest Au NPs induced higher bioaccumulation than smaller ones in *S. plana*. However, AgNPs-size did not affect bioaccumulation in both polychaetes species (*N. diversicolor*, *C. teleta*), (6) differences in bioavailability and toxicity according to the routes of exposure: the response of biomarkers of defence in *S. plana* was more important after dietary than after waterborne exposure to Ag. In the snail (*L. stagnalis*) ⁶⁷Zn was assimilated from ⁶⁷ZnO NPs mixed with diatoms food and the mixture inhibits ingestion rates in animals at higher Zn concentrations, (7) the stable isotope tracing approach is suitable to explore ecotoxicity effects of NPs (⁶⁷ZnO) in environmentally realistic conditions (8) establishing of interspecies differences in bioaccumulation and toxicity effects that may be particle specific, however the species most sensitive to one type are not necessary most sensitive to another type.

5.3 Results from WP5 and WP6

In vitro models were used to examine cellular responses to nanoparticles. The work was based on the hypothesis that the cellular reactivity of the particles will critically depend both on the target tissue and the function of the cell type within that tissue. Cellular and molecular reactivity of selected metal nanoparticles were investigated in a) primary mammalian and human cells and b) in a panel of established human cell lines.

A relatively wide range of tissue sources have been covered, to include the lung, skin, immune blood cells, gut, kidney and liver. A wide range of doses of the nanoparticles were studied, between 0.1 and 100µg/ml for all the studies except those on the human skin organ model, where the doses were increased to 100,000 µg/ml topically, and 800µg/ml systemically, to represent that used in sunscreen products. Key findings include: a) In studies of lung and skin models (Imperial and DSL), which investigated cytotoxicity (MTT assay), oxidative stress (ROS and glutathione levels) and release of pro-inflammatory mediators (IL-6, IL-8, MCP-1 and TNFα), TiO₂ was found to have very little adverse reactivity, regardless of whether it was in the bulk form (BAN, BRU), mixed bulk and nanoparticles (INT), or nanoparticles (TiO₂ 1-4, TiO₂ 10, 50 and 100nm, P25). In studies of lung and skin cells with Au 5, 15 and 40nm, again, the particles had no significant adverse effects with respect to the above indices. b) In a colony forming efficiency assay, CFE, 5 cell lines were studied, A549, Balb/3T3, MDCK, HepG2 and Caco-2 representing lung, kidney, liver and gut. TiO₂ 10, 50 and 100nm, and Au 5, 15 and 40nm, were studied. There was no effect of the TiO₂, regardless of size. Neither was there any effect of Au 15 and 40. Interestingly, Au 5 caused a striking dose-related loss of



CFE by Balb/3T3 cells, where the highest dose resulted in a CFE only 10% that of control. Caco cells and MDCK cells were also affected, but not so severely, and only at the highest doses. A549 and HepG2 cells were not affected. The relative toxicity of Au 5 against Balb/3T3 cells and MDCK cells was determined to be proportional to both increasing Au particle number and nanosize. Also of interest is that cells derived from the key detoxifying organs in the body, lung and liver, are most resistant to Au 5. Importantly, there are clearly significant differences between cells/organs in their response to Au 5 that could be significant *in vivo*, where interstitial fibroblasts, gut and kidney cells might be more susceptible. c) cytotoxicity and genotoxicity studies concentrated on primary PBL cells, RAW264.7 macrophage-like cells and A549 cells. Two assays were used to determine cell viability, the MTT assay and trypan blue dye exclusion assay, where the most significant effects on MTT were observed at 2 hours (acutely) and trypan blue uptake at 24 hours (chronically). Importantly, and surprisingly, PBL cells were

particularly susceptible to TiO₂ exposure, whereas A549 cells were resistant. Au 40 was more toxic, at lower doses, than Au 15. At high doses, Au destroyed almost all the PBL cells. However, the evidence is clear that PBL are particularly susceptible to both nano-TiO₂ and nano-Au. This is important in terms of direct delivery of nanoparticles into the cardiovascular system, or translocation of nanoparticles from the lung, gut or other organs, into the blood, where they may have adverse effects. d) some dispersants studied were found to themselves induce changes in the biomarkers under study.

5.4 WP7 and WP8

Work in these two WPs has only just begun and results are not yet available.

6 Directory

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NanosafePACK

Development of a best practices guide for the safe handling and use of nanoparticles in packaging industries

Contract Agreement: Under Negotiation Website: <http://www.nanosafepack-fp7.eu>
Coordinator: Jose Luis Romero, Tecni-Plasper, S.L., Roca del Vallès (Barcelona), Spain

No.	Beneficiary name	Short name	Country
1	Tecni-Plasper, S.L	Plasper	Spain
2	European Plastics Converters	EuPC	Belgium
3	The Portuguese Association of Plastic Industry	APIP	Portugal
4	Packaging, Transport and logistics research center	ITENE	Spain
5	Institute of Occupational Medicine	IOM	United Kingdom
6	Centro Español de Plásticos	C.E.P	Spain
7	Tec Star, s.r.l.	Tecstar	Italy

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Project Duration: 1 December 2011 – 31 December 2014

Project Funding: 1,6 Mio. EUR

1 Summary

The use of these nanometer sized materials, traditionally known as nanofillers in the polymer industry, improve the volume properties, surface properties, dimensional stability, chemical stability, permeability and other functional properties of the reinforced polymers e.g. photocatalytic, optical, electrical, magnetic and thermal stability. These new properties and the expected development in the near future results in a continuous growth of nanocomposites in the market. However a lack of information exists about the risk posed by the nanoparticles used as nanofillers to human health and the environment. At the same time, while research on developing new nanocomposite materials has prolific for more than a decade, research aiming to improve our understanding of the health and environmental impacts arising from the manufacture and commercialization of nanocomposites is far less advanced, with a particular concern regarding the exposure to NPs at all stages of nanocomposites production, use and disposal. In this sense, since commercial compounding of nanocomposites is typically achieved by feeding the nanoparticles and polymer into a twin screw extruder, the airborne particles associated with nanoparticle reinforcing agents are of particular risk, as they can readily enter the body through inhalation. Moreover, studies of existing nanometer-sized particles of many

materials, including those typically used in the nanocomposite industry, have shown adverse health effects in workers exposed. Similarly, animal studies have shown that ultrafine particles are more inflammogenic and tumorigenic than an equal mass of larger particles of similar composition, . In addition, aerosol control methods have not been well-characterized for nanometer-sized particles, consequently, an evident lack of knowledge exists regarding the potential exposure to workers dealing with engineered NPs d

The aim of the NanoSafePACK consortium is to develop a best practices guide to allow the safe handling and use of nanomaterials in packaging industries, considering integrated strategies to control the exposure to NPs in industrial settings, and provide the SMEs with scientific data to minimize and control the nanoparticles release and migration from the polymer nanocomposites placed on the market.

To achieve this aim, a complete hazard and exposure assessment will be conducted to obtain new scientific data about the safety of polymer composites reinforced using nanometer-sized particles. The proposed work will focus on a selected set of nanometer-sized materials relevant to the packaging sector. Full characterisation



will be carried out, followed by an exposure measurement in order to identify and quantify any potential particle release in the production and processing activities. A comprehensive hazard assessment will allow the evaluation of effects on human and environmental models, including the development of a nanoparticle migration and release index as a hazard indicator. Results from the exposure and hazard assessment studies will be used to compile a risk assessment of the use of nanoparticles in the packaging industry. An evaluation of the effectiveness of risk management measures will be undertaken in order to select and design practical and cost effective strategies, which will be easy to implement in the real operational conditions of industrial settings. In addition, as part of this assessment we will conduct a life cycle assessment of nanocomposites, by evaluating their impacts during the processes of manufacture, use and disposal.

2 Background

Nowadays, the further development of the nanotechnology applied to the packaging industry has enabled the production of functional nanocomposites that are already placed on the market. In this sense, nanoclays have been used as nanoreinforcements in several polymers such as nylons, polyolefins (polypropylene), polystyrene or poly(ethylene terephthalate). Similar aspects can be found in relation to the use of the metal oxide nanoparticles (Ag, TiO₂, MgO, ZnO), used to develop high-conducting and low-leakage porous polymers.

On the other hand, the use of nanoparticles currently raises many questions and generates concerns, due to the fragmentary scientific knowledge of their health and safety risks. The uncertainties are great because such small particles exhibit novel properties that are distinctively different from their conventional forms and can affect their physical, chemical and biological behaviour. In general, the engineered nanoparticles are more toxic than equivalent larger-scale chemical substances. Simultaneously, the use of nanomaterials by workers presents new challenges to understanding, predicting, and managing potential health risks. Primary routes of occupational exposure to nanoparticles include inhalation, trans-dermal absorption and ingestion, however the exposure to nanomaterials is likely to vary throughout the product life cycle (production, use and disposal). Surveys have indicated that nanotechnology – related industry workers have the potential to be exposed to nanoparticles and the degree of skin contamination could not be negligible. In this sense, several studies have determined representative values of airborne nanoparticles in the worker environment due to the ability of nanoparticles to be easily dispersed as a dust (e.g. a powder) or an airborne spray or droplets, resulting in greater worker exposure. In fact, large quantities of nanoparticles are released into the air during the extruder heating phase.

In terms of protection strategies, the current available information is only based on the implementation of engineering techniques, administrative controls and personal protective equipment, without considering specific operative conditions and the unpredictable behavior of the airborne nanoparticles. In this sense, the state of the art shows that the design quality and especially the verification of efficiency are essential factors to ensure adequate protection of workers and nowadays there are no studies in the scientific literature regarding evaluation of the performance of the ventilation equipment used in applications with engineered nanoparticles.

Finally, considering the consumer stage, several studies show a substantial release of nanoparticles from synthetic polymers. Furthermore, nanoparticles may be released when nanocomposites are subjected to wear, such as UV radiation or abrasive uses in the case of packaging nanocomposites. In this sense, there is significant evidence that TiO₂ nanoparticles used in polymeric solutions are detached by natural weathering and the tribological studies on SiO₂/acrylate nanocomposites show that friction leads to the gradual loss of SiO₂ nanoparticles. Amongst these concerns, several strategies have been identified to reduce release and loss of nanoparticles during the service life of nanoproducts, for example the tribomechanical performance of nanoparticle filled polymer composites.

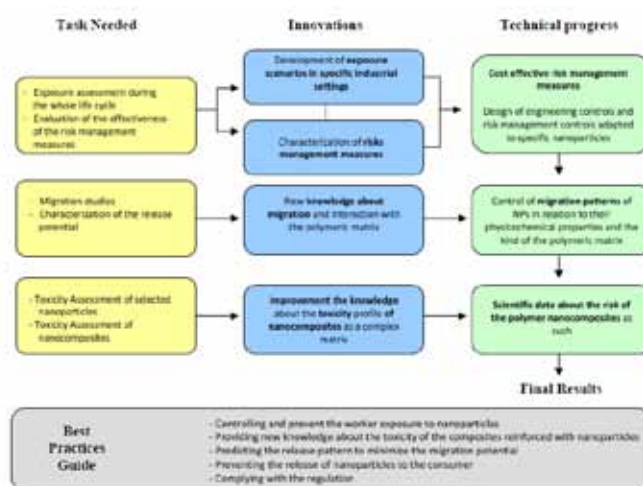
3 Concept and Objectives

3.1 Project Concept

The concept of NanoSafePACK stems from the need to ensure the safety of workers dealing with nanoparticles and to guarantee the safety of the nanocomposites placed on the market, complying with the European regulation and avoiding endangering consumers' health and the environment.

We need to find a cost effective solution to guarantee a safe working environment in the specific nanocomposites production process, as well as to predict and control the release of nanoparticles at all stages of the nanocomposites life cycle, avoiding the exposure of consumers to nanoparticles. We must cover these needs in order to promote the manufacture of innovative products that can compete with the growing composites industry of China and India, improving the competitiveness of our members and the European packaging industry in general. To achieve this, we must ensure the fulfilment of the current regulation in terms of worker safety and consumer health, avoiding workers exposure to nanoparticles and testing the potential release of the nanofillers across of the polymeric matrix in normal conditions of use.

Figure 1. Concept of the NanoSafePACK project



3.2 Project Objectives

The main objective of the NanoSafePACK project is to develop a best practices guide to allow the safe handling and use of



nanomaterials in packaging industries, considering integrated strategies to control the exposure to nanoparticles in industrial settings, and provide the SMEs and industrial users with scientific data to minimize and control the nanoparticles release and migration from the polymer nanocomposites placed on the market.

The detailed objectives of NanoSafePACK are summarized below:

1. To characterize physicochemical properties, toxicological and ecotoxicological profile of the specific nanoclays and metal oxide particles employed in the packaging industry.
2. Hazard characterization of nanoreinforcements including functionalizer agents.
3. To Characterize the particle migration potential in terms of nanoparticle displacement
4. To assess the toxicity of the nanocomposites as such, considering the nanoparticles as a part of the polymeric matrix.
5. To characterize the exposure to nanoparticles through the development of specific exposure scenarios.
6. To characterize the potential release of nanoparticles on consumer products
7. To improve the effectiveness of the risk management measures according to the industrial settings of the packaging industry.
8. To identify suitable methodologies to study the migration of nanoparticles into the consumer products
9. To define appropriate release factors of nanoclays and metal oxide nanoparticles
10. To characterize the most suitable waste management measures
11. To solve problems with waste coming from nanocomposites, including carbon nanotubes (CNT).
12. To develop a cost effective strategy to improve the safety during nanocomposite production use and disposal.
13. To disseminate the research results for a large community of SMEs
14. To study the viability and benefits of the use of nanoreinforcements, taking into account the full product life cycle (production, use and disposal/recycling).
15. To characterize the regulatory aspects concerning the use of each kind of the nanoparticles studied in the project

4 Overall view of the Workplan

NanoSafePACK consists of 8 complementary Work Packages (WP), summarised in Table 1. For each WP, a complete description is

presented below, including the objectives; the WP leader (in bold) and linkage to other Work Packages.

Table 1: Work Packages of NanoSafePACK

WP n°	WP Title	WP Leader
1	Characterisation of Nanofillers	ITENE
2	Hazard Assessment	IOM
3	Development of Exposure Scenarios	IOM
4	Environmental impact of nanocomposites for packaging	ITENE
5	Development of the Best Practices Guide	IOM
6	Field Testing and Validation	ITENE
7	Project coordination and management	Plasper
8	Project dissemination	EuPC

This work plan has been split into 4 types of activities and based on the combined experience of the consortium members. The activities are explained below:

1. Scientific and Technological development

These activities cover the scientific tasks to be conducted to achieve the project objectives. A detailed description of these tasks is described on table 2.

2. Demonstration activities

The main objective of these activities is to prove the viability of the solutions proposed in the industrial settings. It will be conducted under the scope of WP 6, checking the surface modifications in industrial case studies. The exposure scenarios and risk management measures will be implemented and monitored to ensure their correct application.

3. Project Management

This work includes the tasks to be completed by the project Coordinator and contains the tasks required to successfully manage the project. The coordination activities will be undertaken by the Plasper.

4. Dissemination Related Activities

In order to achieve an optimal use of the Project across the EU, dissemination, training and exploitation are essential to the success of the NanoSafePACK project. These activities will be conducted within WP 8.

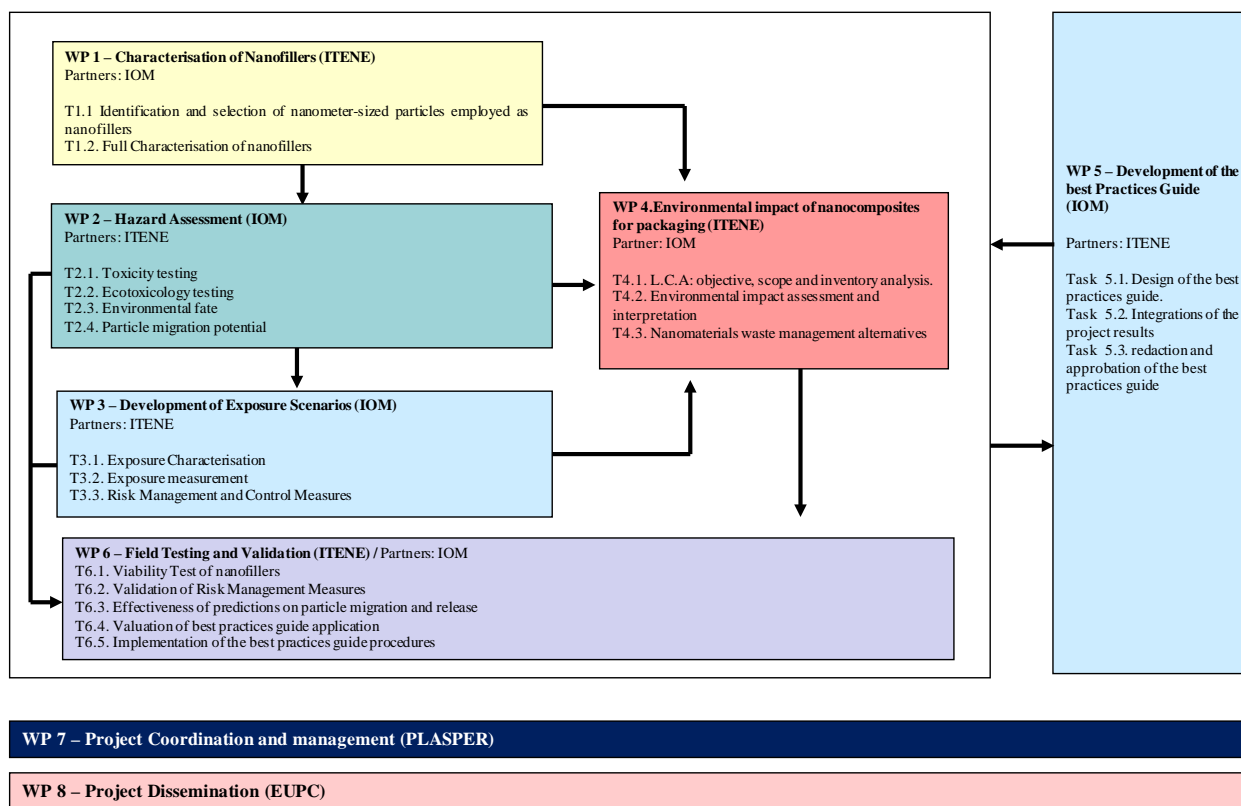


Table 2 Technical & Scientific Workpackages (WP) of NanoSafePACK

WP	Title	Description
1	Characterisation of Nanofillers	The nanoclays and metal oxide nanoparticles employed in the packaging industry will be clearly identified, taking into account the list of endpoints published by the OCDE in relation to the nanomaterial identification and physicochemical properties.
2	Hazard Assessment	To characterize the toxicological and ecotoxicological profile of each nanofiller identified in the previous workpackage. To assess the changes induced by the functionalization of the nanofillers in relation to their toxicological and ecotoxicological profile. To assess the toxicity and ecotoxicity of the nanocomposites as such. To characterize a hazard indicator based on the particle migration potential.
3	Development of Exposure Scenarios	The exposure assessment work package will extend the experience-based understanding of appropriate practices for the safe production, processing and use of nanocomposites based on nanoclays and metal oxide nanoparticles. The objective is to carry out exposure characterisation for the production and processing of nanoclays and metal oxide nanoparticles and their release assessment as a part of the nanocomposites during the consumer stage, this will include: a) A comprehensive set of exposure scenarios in the production, processing and use of nanocomposites. b) Exposure assessments for key scenarios identified, using data obtained from measurements gathered under real operative conditions and information from the literature.
4	Environmental impact of nanocomposites for packaging	Environmental impacts will be analysed applying Life Cycle Assessment (LCA) method for nanoclays and for metal oxide nanoparticles. The LCAs will be developed in accordance with the internationally accepted ISO standards 14040 and 14044. In addition, management alternatives for nanocomposites wastes will be analysed. Environmental information from this WP will be useful for researchers to choose the best options for future development of nanocomposites.
5	Development of the Best Practices Guide	The purpose of this workpackage will be principally the development of the best practices guide thought the integration of the project results. To develop that best practices guide, the following objectives must be covered within this workpackage: Design of the best practice structure and contents, Integration of the project results, Redaction and validation of the guide.
6	Field Testing and Validation	To validate the applicability of the best practices guide in the SMEs (Demonstration). To prove the viability of the solutions proposed in the industrial settings. To evaluate the effectiveness of the risk management measures proposed. To evaluate the improvement of the nanocomposite's safety once applied the best practices referenced on the guide.
7	Project coordination and management	The overall objective is to ensure that the objectives of the different Work Packages are reached and activities complete in accordance with the time schedule and the deliverables and milestones are reported.
8	Project dissemination	The objective of WP8 is to ensure right development and impact of project dissemination and training activities.



The workpackages and their interdependence are shown schematically below:



5 Advances over the state of the art

Due to the variability of the properties of the nanoreinforcements considered and the evident lack of information about the potential effects and impacts of nano-sized materials on human health and environment, we have not been able to identify any manual or guide that can provide valuable advice about the use of layered organoclays and metal oxide particles. This is what the NanoSafePACK project will provide, a specific guide to improve the use of nanoparticles in order to develop functional materials, controlling the exposure to nanoparticles in the worker environment and minimizing the release potential during the service life of the polymer.

Besides the above, the NanosafePACK project will work to progress beyond the current knowledge related to applicability of nanoparticles as nanofillers, as well as the enhancement of knowledge about the health and environmental impacts of the target nanoparticles and nanocomposites. In this sense, the project will take into account the “baseline data” in order to measure our progress, considering also the current research activities related to the field of work of NanosafePACK, avoiding the duplicity of efforts.

The main baseline data to be considered will be:

- Studies and research concerning the applicability of nanoparticles as nanofillers.

- Available data on toxicological and ecotoxicological properties of the target NPs and nanocomposites
- Available data on migration studies
- Available data on exposure assessment methodologies
- Current risk management strategies recommended
- Existing manuals and handbooks

In relation to the existing references, several studies on polymer nanocomposites have been identified in the bibliography, however, none of them represent a direct guide to be applied at all stages of nanocomposites production, use and disposal, and do not take into account the concerns related to workers and consumers safety. In addition, we have not been able to identify any study specifically relating to the potential release of nanoparticles from the polymeric matrix, and even fewer in relation to the methodologies to be employed in migration studies involving nanoparticles.

In relation to the emerging research, we have identified several projects working on aspects related with some of the objectives pursued by our project, e.g. safe handling of nanoparticles, risks associated with the production of polymer nanocomposites and toxicological impact of nanomaterials derived from processing, weathering and recycling of polymer nanocomposites, nevertheless, these projects are not specifically aimed at SME



trade moulders who will not probably be able to readily get hold of this information. Moreover, these projects are very academic orientated thus, they don't fully meet the SMEs needs.

In addition, methodologies to assess the exposure and hazard aren't standardized, so that the results of projects like Nanosafe are difficult to interpret and apply to other conditions. Thus, we aim to develop a best practices guide directly applicable in the nanocomposite industry.

The current handbooks, guides and reports of research projects are not focused on specific nanoparticles used in the current industrial setting of the packaging industry, which can differ enormously from another industrial process involving the use of polymers and nanoparticles. At this stage, our solution is innovative due to the direct application of our members in particular SMEs and industrial settings, as well as providing valuable information to characterize the toxicological profile and potential risk of the new manufactured nanocomposites and also those placed on the market in the recent years.

In relation to nanocomposite safety, the commercial manufacture of nanoparticles is relatively new, so that, there isn't consensus in relation to the potential migration and release of the nanofillers employed on the nanocomposite industry. In this sense, taking into account physicochemical properties of the nanofillers employed, the possibility of migration is likely. Therefore, manufactures of nanocomposites, with special attention to those who manufacture food contact materials, must predict the potential migration in order to protect the consumer's health and comply with the current regulation. At this stage, our proposal will study the aspects concerning the migration and release of nanoparticles in the polymeric matrix, providing the SMEs with scientific and valid data to select the less hazardous nanofillers or predict the potential release to the end user.

After studying the existing solutions and emerging research, the consortium has concluded that the submitted project has a high innovative character and will enhance significantly the state-of-the-art in the nanocomposite area. There is a demand that the current solutions are not able to meet, and the other research approaches are not focused in the specific field of the nanocomposite industry. Therefore we have an opportunity to exploit our innovative approach, including the achievement of the results in a reasonable period of time.

On the other hand, due to the current lack of data in the nanotechnology area, mainly in relation to the adequacy of testing models and exposure measurements, we have conducted a complete contingency plan, including a detailed risk analysis and the contingency actions to guaranty the progress beyond the state of the art, ensuring that the project results will be addressed.

6 Impact

The development of the NanoSafePACK project will have a remarkable impact on the European SME Community and their citizens, clearly identifiable due to the hundreds of applications of the nanocomposite materials every day and the hundreds of people who use them. It's expected a direct impact in the packaging and polymer nanocomposite industry, helping the SME to develop new innovative materials, which provide the end users with new products tailored to their needs. On the other hand, the implementation of the project results will enable the SME Associations, their members and other SMEs across Europe

minimise the health impact and environmental risk from the nanoparticles, providing the information to safely design, manufacture and market nanotechnology enable products.

At the moment, much of the research and development work is concentrated on the development of innovative products, considering new properties that improve the final application, while, at the same time, the safety of the product has been compromised at all stages of nano-products manufacturing, use and disposal. Overall market value will benefit from important consumer preferences toward safe and environmentally friendly products, which will support consumption of high-performance nanocomposites.



7 Directory

Table 3 Directory of people involved in this project.

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8 Copyright

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NanoSustain

Development of sustainable solutions for nanotechnology-based products based on hazard characterization and LCA



Contract Agreement: NMP4-SL-2009-247989

Website: <http://www.nanosustain.eu>

Coordinator: Rudolf Reuther, NordMiljö AB, Sunnemo, Sweden

No.	Beneficiary name	Short name	Country
1	NordMiljö AB [Environmental Assessments]	NOMI	Sweden
2	The Institute of Nanotechnology	ION	United Kingdom
3	National Research Centre for the Working Environment	NRCWE	Denmark
4	Technical Research Centre of Finland	VTT	Finland
5	University of Bremen	UniHB	Germany
6	Veneto Nanotech	VN	Italy
7	European Commission Joint Research Centre, Institute for Environment and Sustainability	EC-JRC	Belgium
8	Kaunas University of Technology	KTU	Lithuania
9	National Institute for R&D in Microtechnologies	IMT	Romania
10	Nanologica AB	NLAB	Sweden
11	Nanogate AG	NGAG	Germany
12	UPM Kymmene	UPM	Finland

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1 Summary

Project Duration: 1 May 2008 – 31 April 2013

Project Funding: 2.5 Mio. EUR

The ultimate goal of the NanoSustain project is to develop new technical solutions that foster the design, manufacture, use, reuse/recycling and final treatment and/or disposal of sustainable engineered nanomaterials (ENM). To reach this goal a comprehensive physicochemical (pc) and hazard characterization (toxicology, dose-response, no-effect levels) campaign has been

started, including exposure (human, environment), risk (RA) and life cycle assessment (LCA) of selected ENM (nanocellulose, nano-TiO₂, CNTs, nano-ZnO) and associated products, also in relation to their fate, transport and transformation.

The following specific objectives build the cornerstones of the project:

1 Hazard identification by finding the most important hazard characteristics, exposures and risks for selected ENM, dose-



response and no-effect levels to humans and the environment for after-production life-cycle phases, such as application or use, recycling, final treatment and disposal, to improve existing risk assessment (RA) methods applicable to selected ENM.

2 Life cycle assessment and preliminary assessment by applying leading edge methodology to identify potential environmental impacts throughout the whole life cycle of a material or product (from production and application/use to final recycling) and by further developing prospective and preliminary LCA into precautionary risk management.

3 Human health and environmental impact assessment by producing and assessing a set of well-defined ENM that represent the main properties and stages during their life cycle, by using critical endpoints, such as the inflammatory reaction as a key event following exposure to ENM in the lung or the comet assay to determine genotoxic effects by Reactive Oxygen Species (ROS) formation, and DNA damage in cells and animals. Also eco-toxicity tests are used and adapted, such as the luminescent bacteria (*Vibrio fischeri*) and white worm (*Enchytraeus sp.*) test, to assess the impact of nanoparticles in aquatic and terrestrial systems and to elucidate toxicity mechanisms that may arise during manufacturing, transport, application, recycling and disposal, or directly from used materials or indirectly via the environment, or by their accidental release.

4 Exploring technical sustainable solutions by testing the behavior of selected ENM during end-of-life phases (recycling by composting, melting), treatment (by incineration) and land-filling, also to redesign materials and products.

2 Scientific / regulatory / industry needs and problems addressed

The behavior and properties of nanomaterials can be quite different to bulk materials, a fact that drives considerable international research and development activities towards exploitation, innovation and commercial application, with a corresponding increase in the number of nanotechnology based products reaching the end of their life-cycle. At the same time, there is increasing concern that the beneficial properties of nanoscale materials and products might also have negative impacts on human and the environmental health. Although much research is now going on, we still do not know how exactly nanoparticles (inter)act in the human body or in the environment, to what extent they are released or leach from products, or how they are transported, transformed, or accumulate in living organisms or environmental systems, like soils or waters, in particular after their consumption, reuse/recycling, final treatment and disposal.

Recent toxicological studies show that nanoparticles have implications on human health inducing, e. g., pulmonary and systemic inflammation, and translocation to different parts within the human body, including the brain, after inhalation. However, reliable data on the (eco-) toxicity of nanomaterials is still scarce, although first studies prove that there are toxic effects on wildlife and potential bioaccumulation in various organisms.

The rapidly increasing amount of nanomaterials produced worldwide raises in particular the question of their final fate when used

in products and released to the environment, and of possible hazards due to accumulation in animals, plants or the human body. Nanoparticles may be extremely resistant to degradation and accumulate in waters or soils, may aggregate or disperse, which will change their properties compared to single nanoparticles to an extent we still do not know. Also for this reason, existing regulation based on mass metrics alone may not be appropriate to quantify the true exposure to nanoparticles, but needs more accurate data on nano-specific parameters, like surface area, degree of dispersion or aggregation, or particle size concentration.

More reliable scientific data is needed on toxicokinetics, exposure and degradability characteristics of engineered nanoparticles to better understand where, in which form, and to what extent these new materials will end up, to develop more accurate impact, exposure and risk assessment models, and to find efficient ways for product design that in turn favor their sustainable use, reuse and recycling and/or safe disposal. Current chemical characterization and biological test methods are often not appropriate to generate the data we need to reliably assess risk and hazard. As a result, there is an urgent need for preliminary assessment at an early stage of product innovation, and to validate and further develop current characterization and testing methods for these new materials in various matrices and compartments, including reproducible test media to which men and ecosystems are exposed, as well as cell lines, body fluids or tissues.

The existing regulatory framework (such as REACH) based on mass (in tons) and concentration metrics may be adequate for areas, where only small amounts of nanomaterials are used (such as research laboratories or small-scale manufacturing shops). However, they may not be applicable for the industrial mass production of nanomaterials, where particle number and/or shape could be more critical for their behavior than for the same bulk chemicals. The applicability of current standardized methods, like those given in the OECD-Guidelines for measuring and testing of hazardous substances, needs to be tested and where necessary adapted or modified, and validated. NanoSustain will in particular evaluate the extent to which existing regulatory and risk management strategies and tools can be applied to after-production stages of nanomaterials, in particular to their recycling, final treatment and disposal.

3 Concept, scope and strategy

NanoSustain is based on the concept of "sustainability" and "scarce resources", which means that the use of new innovative materials, like engineered nanomaterials, must not only consider human needs today but also of future generations, including all possible effects occurring along their life-cycle, and should ensure recyclability and avoidance of dissipative losses of contained nanomaterials. Both concepts are tested and realized by characterizing the properties of representative and relevant nanomaterials and associated products at various stages of their lifecycle in relation to possible impacts on human health and the environment, and by taking their reusability/recyclability and/or ability for safe final treatment and/or disposal, or reintegration into geological cycles into account as requirement for their sustainable development.



The following specific organic and inorganic nanomaterials have been selected and are investigated, including associated LCA relevant test materials (such as prototype products, dusts):

- nanocellulose based materials and products;
- CNT and associated products;
- nano-ZnO based composites, and
- nano-TiO₂ and associated products

NanoSustain considers four main aspects of the life-cycle of selected nanomaterials including, (a) selection and design, (b) manufacture, (c) application, and (d) recycling/disposal. Although most studies still focus on possible toxic effects of nanocomponents after exposure for risk assessment, the potential contribution of these materials to all impacts will be examined, when added to products or processes, to better understand the importance of underlying choices involved in the implementation of this new technology.

The project strategy encompasses the following specific tasks to assess:

- Ø the hazard of selected nanomaterials based on a comprehensive data survey on their properties (physicochemical characteristics, exposure probabilities, etc.) and the adaptation, evaluation, validation and use of existing analytical, testing and LCA methods;
- Ø the impact of selected products by LCA (in relation to material and energy flows);
- Ø the impact of these materials in relation to toxicology, eco-toxicology, exposure, environmental and biological fate, transport, transformation, and destiny;
- Ø the feasibility and sustainability of new technical solutions for end-of-life processes, such as reuse/recycling, final treatment and/or disposal.

4 Technical approach and work description

NanoSustain is structured and organized around 4 technical (vertical) and 2 horizontal Work Packages (WPs), each with distinct tasks, deliverables and milestones:

Work Package 1 (WP1): Project management and scientific-technical coordination

Work Package 2 (WP2): Data gathering, generation, evaluation and validation

Work Package 3 (WP3): Hazard characterization and impact assessment

Work Package 4 (WP4): Life Cycle Assessment (LCA) and preliminary Assessment

Work Package 5 (WP5): Exploring technical solutions for recycling, treatment and disposal

Work Package (WP6): Dissemination and exploitation of results.

So far, the following tasks have been realized during the first 18 months:

WP1: Administrative and operational project management and coordination of the scientific work to ensure regular monitoring, update and control of the quality and progress of work and results achieved, organizing regular consortium and progress meetings, reviewing, editing and archiving of produced deliverables and reports, and establishing and maintaining a smooth communication and information system between partners, with the Commission and main stakeholders (see also WP6).

WP2: (1) A comprehensive data and literature survey on testing and characterization of selected nanomaterials to prepare and organize the (2) design and structure of a materials/product and (3) of a literature database, to systematically collect, store, evaluate and validate already existing and continuously generated new data.

WP3: (1) Production of pure test samples and prototype products from selected nanomaterials, as well as various types of life-cycle dusts from sanding of CNTs containing epoxy-plates and TiO₂ containing painted boards, to simulate and assess human exposure during handling and reworking, and their toxicology. (2) Studies on the effect of weathering and abrasion on emission of selected ENM from glass sheets coated with nano-ZnO and from TiO₂ containing painted boards during sanding, and on testing the suitability of eco-toxicological tests for ENM. (3) Evaluation of newly generated hazard and exposure data to improve existing test strategies and methods for environmental risk assessment.

WP4: (1) Performance of a comprehensive survey of studies and of a questionnaire on manufacture and LCA of selected ENM. (2) Development of two process models for their application/use and end-of-life phase developed.

WP5: (1) Production of nanocellulose-based materials, nano-ZnO coated glass and MWCNT containing epoxy composites and (2) laboratory experiments conducted for their recycling (composting, melting), treatment (incineration) and final disposal (land-filling), in addition to weathering and leaching studies, to explore and develop new technical solutions for the sustainable design, use, reuse / recycling, final treatment and/or disposal of selected ENM.

WP6: (1) A fully functioning and interactive project website and intranet has been established to continuously disseminate the outcome of the project to all stakeholders and to make results available for partners (www.nanosustain.eu). (2) Three dissemination events have been organized to manage and exploit the new knowledge and outcome produced: 2 dissemination workshops (in Glasgow/UK, May 2011, and in Venice/Italy) to present and discuss first results with relevant stakeholders, and 1 LCA training workshop (Bremen/Germany, September 2011) to interact with interested experts.

5 Status of the project

5.1 Work package 1 (WP1)

NanoSustain was launched by a kick-off meeting in Sunne, Sweden, on 25-27 May 2010. All partners agreed to a roadmap and management templates prepared by the coordinator for progress control and a smooth practical implementation of the project, in



particular concerning the timely generation of deliverables and milestones.

Additional regular project meetings have been taken place in Ispra/Italy (16-18 November 2010), in Glasgow/UK (10-11 May 2011) and in Venice-Mestre/Italy (24 November 2011), where partners met to discuss the progress of work and results obtained during the first 18 months, and to prepare the next steps due for the second project period (M19-36) according to the work plan.

The 1st Periodic Report has been prepared and submitted to the EU Commission in December 2011.

5.2 Work package 2 (WP2)

In WP2 a material database has been designed and the main structure, parameters and functions established to collect already existing data on selected ENM provided by manufactures involved in the project, but in particular to store and evaluate the physicochemical and biological data continuously produced (in WP3) and fed into LCA (WP4) and RA (WP3), or used in the exploration of new technical solution (WP5). Before entering the material database, the newly generated data is first collected in Material Data Sheets (MDS) that have been prepared for tested materials and that are online available on the partner intranet, to allow direct data input, use, treatment and validation by project partners, but also to identify significant relationships between material characteristics and biological effects. A comprehensive inter-lab comparison campaign on validation of characterization, measurement and testing, in cooperation with an external reference laboratory, is in preparation and will be implemented in the first half of 2012.

To keep all partners up to date with new research developments and to consider new findings for the ongoing work, but also to integrate the project outcome in a wider context, a scientific database has been established based on a critical literature survey. A regularly up-dated database is available on the project website and equipped with particular search functions (for registered users). Only publications that prove minimum requirements regarding data quality and metrics of tested materials have been considered. Most studies show that characterization of ENM is a key criterion for the success of any subsequent toxicity testing and critical for identifying any significant relationships between metrics and effect endpoints, but also when comparing the toxicity of different studies or when assessing possible risks. The survey also confirmed that false conclusions may be drawn on the toxicity of ENM with similar compositions but different metrics in the absence of a qualified characterization.

Nanosustain is prepared to share data and expertise with other relevant EU FP7 projects, such as NanoFate, NanoHouse, NanoPolyTox and NEPHH, and with the Database Working Group 4 of the NSC, to develop common protocols and harmonize formats to support the build-up of a central EU wide database on nanomaterials risk assessment.

5.3 Work package 3 (WP3)

One main goal of WP3 is to provide not only pure nanoparticles, but also life-cycle relevant test materials for hazard and exposure characterization. For this reason, the following test materials have

been synthesized and treated for characterization and testing: (1) glass sheets coated with and without ZnO nanoparticles, (2) three different types of epoxy plates with and without CNTs, (3) two different paints applied on boards with and without nanoTiO₂, and paper prepared with and without nanocellulose (see Table 1). Five types of life cycle dusts have been also generated by sanding of the produced epoxy-plates (CNTs) and painted boards (TiO₂), and distributed for measurement and testing.

Reference product	Nanoparticle containing product	Nanoparticle
Paint with 36% Nabond TiO ₂	Paint with 12% NanoAmor TiO ₂ and 24% Nabond TiO ₂	NanoAmor TiO ₂ (139.1 m ² /g) Nabond TiO ₂ (28.2 m ² /g)
	Paint with 36% NanoAmor TiO ₂ (excluded due to cracking problems)	NanoAmor TiO ₂ (139.1 m ² /g)
Epoxy	Epoxy with 0.5% CNT	Nanocyl NC7000 CNT (245.6 m ² /g)
	Epoxy (industrial product)	The same
Glass sheets coated without ZnO matrix (100% polysiloxane matrix)	Glass sheets coated with ZnO matrix (30% ZnO, 70% polysiloxane matrix)	ZnO (Zincox™ 10)(53.6 m ² /g)
	pro.Glass Barrier 401 (5% ZnO dispersion used for application on glass sheets)	The same
Paper	Paper nanocellulose with	Nanocellulose suspension (2%)

Table 1: Test materials: Pure nanoparticles and sanding dusts from products (with and without nanoparticles)

A first comprehensive characterization round of the obtained pure nanoparticles has been finalized by using a variety of advanced analytical methods, such as SEM, TEM, XRD, AFM, SNOM, Nano-Raman, Micro-Raman, UV-VIS, SAXS, DLS, zeta-potential, FTIR and BET. To validate the used methods and the quality of the produced data (see WP2), a number of standards and benchmark materials is going to be tested during the first half of 2012.

To assess the emission potential and risk for workers during powder handling, data from simulated work activities (sanding) and dustiness testing (miniaturized EN15051 test) are being generated and evaluated. Characterization of the emission source potential and the dustiness testing of pure nanomaterials is still ongoing and first results are expected during the first half of 2012. Also the feasibility to establish a standard for the miniaturized EN15051 rotating dustiness drum is presently being investigated. Work to evaluate the effect of weathering and abrasion on the emission of selected ENM, and their release from glass sheets coated with and without nano-ZnO and from painted boards with and without nano-TiO₂, is being performed by sanding before and after exposure to weathering and abrasion, in close collaboration with the EU FP7 NanoHouse project and Flügger Denmark. Tests are still ongoing.

The first toxicological testing of pure ENM by exposing mice through single intra-tracheal instillation has been completed and analysis of the occurrence of inflammatory cells in lung and DNA damage in BAL cells is still ongoing. Also RNA purification from lung and liver tissue has been performed and RT-PCR of selected



genes involved in inflammation and DNA repair. Histological investigation of the tissue and of the toxicological testing of life-cycle materials is still ongoing.

Another focus in WP3 is to assess the suitability of existing *in vitro* eco-toxicological test methods for risk assessment of selected ENM. Aquatic toxicity of nanocellulose was evaluated by ISO 213382 using the kinetic luminescent bacteria *Vibrio fischeri* test. Studied samples were not acutely toxic at relatively high concentrations, while a tendency of nanocellulose to form aggregates/agglomerates in the 2% NaCl test medium has been observed. Testing the aquatic toxicity of metal oxide nanoparticles (ZnO, TiO₂) is still ongoing and sample preparation had to be optimized. Also reproduction tests (*Enchytraeus sp.*) are in preparation.

Also in WP3, a critical evaluation of existing strategies and methods on environmental risk assessment has been carried out, to determine where in the product chain nanomaterials are likely to present hazards that are different from bulk chemicals. Basic principles of RA have been summarized and applications in industry given and discussed. Also international guidelines have been reviewed in relation to potential risks with reference to still existing uncertainties. New results from our survey on nanomaterials risk assessment practices in industry are presented.

5.4 Work package 4 (WP4)

NanoSustain is assessing the impact of following organic and inorganic ENMs and products along their whole life cycle: (i) nanocellulose used as paper additive, industrial thickener, or rheology modifier, (ii) nano-TiO₂ used in paint applications, (iii) nano-ZnO used in glass coatings, and (iv) MWCNT used in epoxy plates and solar cells. Based on a comprehensive literature search and a questionnaire sent to all involved manufacturing partners, specific process models for the application / use and end-of-life phases (recycling, treatment and disposal) have been developed, including all relevant material and energy flows and important life cycle steps described and data sources investigated. Modelling, calculation, visualization, and evaluation of material and energy flows have been done by using the Umberto LCA software and results have been reported in 2 separate deliverable reports.

The main bottleneck for LCA still consists in the lack of data for the selected ENM for after-use phases or regarding their possible environmental fate and impact. Individual life cycle steps were defined and described, data sources investigated, specific process models developed and provided in the LCA Umberto software. Also a literature survey on existing emission data has been completed to calculate prospective environmental concentrations needed for developing a specific expo-sure model. Based on the literature survey and established process models, data on all inputs and outputs were collected for the Life Cycle Inventory (LCI), and first life cycle data have been calculated, and methods and results presented at a specific LCA workshop at the University of Bremen (September 2011).

5.5 Work package 5 (WP5)

An internal nanocellulose standard was produced and characterized in WP5 for quality control, validation, and reproducibility check for the planned laboratory studies. Also

nanocellulose based products, such as papers, have been prepared for experiments on dustiness (in WP3), biodegradability and recycling (in WP5).

The biodegradability of nanocellulose was tested and first results show that it forms aggregates in mineral solution, which may decrease the rate of biodegradability. For this reason, the conventional test has been modified and new experiments started (December 2011). Also pilot-scale composting experiments started with paper samples containing nanocellulose, pure nanocellulose-films, and reference paper samples without nanocellulose have been launched at the end of 2011.

Several batches of coating material with and without ZnO-nanoparticles have been synthesized to test the recycling of nano-ZnO-coated glass. The emission potential of nanoparticles was investigated during heating and melting of window glass: coated with nano-ZnO, coated with a sol-gel binder matrix, without any surface treatment, and from heating/melting of the empty furnace crucible without glass. Particle number, mass concentration and number size distribution were measured during heating/melting to mimic the recycling process. Results show that particles are emitted during heating/melting of the glass, but their number, size and mass concentration do not depend on the coating, or on the type of coating. The amount of Zn was almost the same in the controls as in the nano-ZnO-coated glass. Further experiments are planned in 2012.

Multi-walled carbon nanotubes (MWCNT) containing epoxy composites are not suitable for recycling, why incineration at laboratory scale will be tested and the produced particles, bottom ash and gaseous effluents measured and characterized. So far, preparations for the incineration experiments have been made, the aerosol measurement instrumentation for characterization of CNTs in the gas phase qualified, and modifications of the lab-scale incineration facility implemented.

To study the feasibility of land-filling as a final disposal option, the leaching of nanoparticles released from nanomaterials containing waste is studied in WP5. Parameters affecting particles' leaching behaviour have been selected and methods evaluated to detect the expected low concentrations required to draw conclusions regarding release and fate. Experiments and modelling approaches to predict transport in environmental media will start in 2012.

5.6 Work package 6 (WP6)

An interactive project website (www.nanosustain.eu) is providing continuous information and new updates on latest project news, newsletters, and project results. Registered users can access additional information such as the output of dissemination events and other presentations. A partner intranet and database allows all partners to view the technical materials and literature database and all project deliverables (draft and final) in a password protected area.

A first training workshop on Life Cycle Analysis of nanomaterials was held at the University of Bremen and presentations are available on the registered user's area of the project website. Also two dissemination events were held in Glasgow/UK (May 2011) and Venice/Italy (November 2011) to provide a platform for project partners to present the first results of their work, but also to interact with invited stakeholders.



6 Expected Impact

NanoSustain will contribute to improve our current knowledge on hazard, impact and sustainability of nanomaterials and products, in particular in relation to end-of-life stages. The produced new data will help to update and validate already existing databases on materials and methodologies required for reliable and accurate LCA and risk assessment of selected nanomaterials. As almost nothing is known about the release, fate and impact of these nanomaterials during end-of-life processes, new solid scientific data on potential risks and their probability of occurring during reuse/recycling and final treatment or disposal will be produced and made available. For the first time new and innovative technical solutions are explored on a lab-scale for the (1) recycling (by composting and melting) of nanomaterials from waste products, 2) incineration of nanowaste as a safe final treatment option, and 3) land-filling of nanoparticle containing products as final disposal. It is expected that these new technical solutions will help to overcome some still unresolved technical barriers towards the environmentally benign and sustainable design, use and development of nanomaterials based technologies.

8 Directory

Table 1 Directory of people involved in this project.

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7 Cooperation

NanoSustain is represented and actively participating in most Working Groups (WG) of the EU Nanosafety cluster. In addition, the project is closely cooperating with the EU FP7 NanoHouse project and with the Danish Flügger company on the performance of weathering and abrasion tests, with the EU FP7 NEPHH project on LCA-methodology and collection of relevant LCA data, with EMPA/Switzerland on developing an exposure model, with the University of eastern Finland on the incineration of MWCNT containing wastes, and with Health Canada on micro array analyses of tissue from the animal experiments.

NanoSustain will also closely cooperate with other relevant EU FP7 projects, to use and maximize existing synergies, avoid or benefit from duplication of work and increase the overall impact of the project. Additional agreements are in preparation with the EU FP7 Prosuite, NanoFate, NanoPolytox and the ENPRA project.



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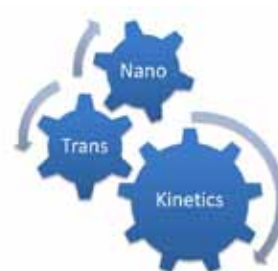
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NanoTransKinetics

Modelling the basis and kinetics of nanoparticle cellular interaction and transport



Contract Agreement: NMP4-2010- 266737

Website: <http://www.nanotranskinetics.eu>

Coordinator: Kenneth Dawson, Centre for BioNano Interactions, University College Dublin, Belfield, Dublin 4, Ireland

No.	Beneficiary name	Short name	Country
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2	Ludwig-Maximilians-Universität München [Ludwig-Maximilians University, Munich]	LMU	Germany
3	Universitat de Barcelona [University of Barcelona]	UB	Spain
4	Rice University*	RU	United States

* EU-US modelling call – project is paired with the project “Nanoparticle Transport: From Cells to Organisms” funded via the the EPA STAR programme (EPA-G2010-STAR-N1 Fate, Transport, and Transformation).

The NanoTransKinetics project is cooperating with ongoing projects in Duke University and University of North Carolina, as well as with the EU modelling cluster (initially ModNanotox coordinated by University of Birmingham).

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1 Summary

NanoTransKinetics is a Small Collaborative Project funded by the European Commission 7th Framework Programme. The project started on November 1st 2011 and will run for 36 months. The project is paired with the US project “Nanoparticle Transport: From Cells to Organisms” funded via the the EPA STAR programme (EPA-G2010-STAR-N1 Fate, Transport, and Transformation). It is also linked with the ModNanoTox FP7 project via a memorandum of understanding, which agrees that the projects will have joint meetings, will share protocols and data, and will support the EU Nanosafety cluster efforts on modeling and databases.

NanoTransKinetics and ModNanoTox held a joint kick-off meeting in London on the 1st and 2nd of December 2011. The meeting was attended by representatives of all partners of both projects, as well as a representative of the US project that is paired with both (coordinated by Prof. Vicki Colvin from Rice University).

2 Introduction

Nanomedicine (and **Nanodiagnostics**) recognize the capacity to treat (and diagnose) most of the remaining intractable disease classes (viral, genetic, cancer). They are based on the central observation that objects of such small size can (uniquely) gain access to, and operate in all parts of the body (including the brain),¹ and within cells. **Nanosafety** acknowledges that (as with blue asbestos - a nano-rod that is non-toxic in bulk form but the agent for the increasingly common cancer, mesothelioma) there exists potential for new, serious and unpredictable diseases originating from the interaction of such small-scale objects with living organisms. There have been, as yet, limited numbers of clearly identified hazards from early phase nanoparticles, but caution is being shown worldwide in developing strategies to address the issues.

It is becoming increasingly clear that Nanomedicine and Nanosafety will rely on the same fundamentally new scientific enterprise, based on understanding (and in the medium term, predicting) the interactions between nanoscale objects and living systems. Indeed, results from experimental projects (such



as those previously lead by Partner 1 - FP6 NanoInteract and FP7 NeuroNano) have produced extensive experimental information that now needs to be integrated in early stage computation models. Early experimental data now begins to clarify the basic scientific issues, and it is clear that we are at the dawn of a new interdisciplinary science (bionanointeractions).

The prediction of all toxicological and biological impacts has, as its basic pre-requisite, the correct prediction of the sites of action and localization of nanoparticles in living organisms - this is the primary purpose of the NanoTransKinetics program. Based on this information, toxicological impacts can potentially be deduced, which is the goal of the project. Thus, we frame models by abstracting the essential, relevant principles of particle-protein (and matrix) interactions, and cellular and barrier transport mechanisms for nanoparticles. Based on preliminary studies, we find a limited number of interactions, particles fluxes (and control parameters) between prescribed sites are sufficient to specify the essential features at each level of description. In all cases, and at each level, these hypotheses are under direct experimental observation.

3 Background

Given that there is currently only limited data regarding the safety of nanomaterials (and validated data takes time to produce) *there is a need to develop approaches based on understanding and mechanism*, rather than overly rely on 'learning sets' which will require much longer to mature. Once this decision is made, it becomes essential to identify the key features and parameters of the arena and experience shows that a *combination of phenomenological and more detailed (semi-microscopic) models*, the latter being directly validated in a detailed manner by experiment is optimal. This approach allows the modeller-theorists to be in direct contact with the experimentalists (and their community) and creates a symbiotic relation that helps shape more usefully the experiments, and their reproducibility. In the lead up to this project, various path-finding efforts were implemented by the partners, and other networks, and the potential for useful interaction was immediately recognized.

Another aspect of higher level objectives and strategy was also considered. Thus, recognizing that a single such program cannot be a total solution, we have considered the issues that are considered most pressing in the field, and organized efforts toward the larger program in a manner *that key larger scale objectives will emerge first*. Understanding the nature of particles that most likely pass the BBB, and bioaccumulate inside non-immune cells are key examples addressed here.

Besides crossing the traditional scientific domains (chemistry, physics, molecular and cell biology, biomedicine, engineering, and toxicology) this field *will above all require a radical shift of scientific paradigm such has rarely been seen in contiguous fields for a generation*. That is, whilst we can (and must) learn from what has been seen in the chemical and (small molecule) drug-organism interactions (for example ADME (Adsorption, Digestion, Metabolism, Excretion) approaches etc.), the underlying scientific processes in the nanoscale are so different as to render these as only of the most general guidance. The

implications of this are deep, and can hardly be overstated, for the development of the program outlined here. Indeed, all the evidence we have suggests that we must return to fundamentals in this arena, and model these new processes at multiple levels of description (nanoparticles surface, cell, biological barriers) in order to develop a model that can usefully integrate emerging biological *in vitro* and *in vivo* data. We conclude that any attempt to press the nano-organism interaction into such a macroscopic ADME framework that is not founded on the appropriate microscopic principles will fail because the conceptual framework is the 'wrong shape'.

From the analysis above, we conclude that it is now urgent to shift the focus of this discussion to a hierarchy of modelling elements that address the real issues of nanoparticle uptake, clearance, and translocation, and some application to examples of toxicology. Our work packages (WP2-4) are thus built around the need for such elements or modules, and involve:

- Modelling of the effect of NP physico-chemical characteristics on interaction with biological fluids – the protein corona (the mediator of biological interactions) as a means to classify nanoparticles;
- Modelling nanoparticle interaction with lipid membranes and extracellular matrix components – effects of NP charge / density / compressibility, lipid structure etc. and cell-cell interactions / degree of confluence etc.
- Modelling kinetics of cellular uptake and inter-compartmental transport and sub-cellular distribution of NPs;
- Modelling nanoparticle passage through biological barriers, including the Blood-brain barrier.

Without doubt, each element of the program is an attempt to reach far beyond the current state of the art. Indeed, we emphasize that the issues highlighted involve such radical paradigm shifts that research in the field is already very ambitious. There is no suggestion that one will be able to immediately produce a model that is predictive *in vivo* - indeed, we consider this an unrealistic short term objective of a single project in the field in its current state. However, we believe that the different elements presented will bring us a very considerable way towards this objective, leaving the way clear for adding in the research of other groups involved in this arena.

Characterization of the 'Biological Identity' of the Nanoparticles

Perhaps one of the most striking (and unforeseen) aspects of the nanoparticles-cell interaction story, that clearly distinguishes nanomaterials from chemicals, is the issue of the 'protein corona'. This arena has been clarified by several authors (including Partner 1 and colleagues from FP6 NanoInteract),^{1,4} and lead to the award of the 2007 Cozzarelli prize of the US National Academy of Sciences to Partner 1 for applications in this arena. In essence, chemicals (again making allowance for great generalizations) interact directly with biological elements, whereas nanoparticles are coated by strongly adhering proteins and lipids whose exchange times are so long that the effective

biological identity of the particles is greatly influenced (in some cases likely completely determined) by the proteins, and not the materials. Figure 1 makes the issue clear by showing the uptake of silica with (and without) serum proteins. The relative amounts are enormous. It is important to note that uptake is dependant even on the type of serum used, and these differences have been studied and linked to different coronas. Clearly, the bare material surface is the wrong parameter. Similar observations are being made for many nanomaterials and situations. It is not possible to explain in great detail, but using new experimental methods it is also now possible to 'read' the corona around particles in organelles inside the cell. Evidently we need to shift considerably towards modelling of the particle and its adhering proteins, and the interaction of this object with biological membranes and barriers in the current program.

Uptake of nanoparticles into cells

Small molecules typically distribute across living organisms such that molecules 'dissolve and distribute' in organs (very crudely speaking) according to near-to-equilibrium physicochemical principles in which *quasi* equilibrium rate constants dominate. Whilst this is a great over simplification, it carries with it the heart of the matter. For example, a small molecule dye will essentially 'dissolve' (diffuse) across a biological membrane. When the source is removed, if there are no highly specific and high affinity interactions in the environment (for example, inside a cell) to retain the molecules, there will be a rapid flow out of the cell (across the cellular membrane again) according to chemical potential considerations. This is all nicely illustrated in a very simple *in vitro* cell model in Figure 2A where uptake and export of a molecular dye are tracked by fluorescence flow cytometry.⁵

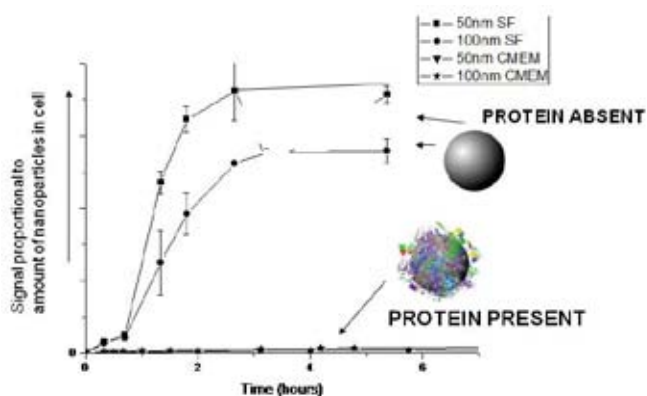


Figure 1. Comparison of endocytosis of 50nm and 100nm SiO₂ nanoparticles at 100ug/ml in the presence (complete MEM) and absence (Serum free Media) of serum proteins. Note the very significant particle uptake in the absence of serum, compared to the much lower uptake in the presence of serum. It has been shown that serum reduces the non-specific interactions between that nanoparticles and the cell surface. Other differences in details of the uptake cannot be discussed here. Data from P1.

On the contrary, nanomaterials are too large to 'dissolve' across membranes in a passive manner, and no such processes have

(so far) been observed in all (our and other) experimental work across many particles types down to sizes of 5nm. On the contrary, nanoparticle uptake across the biological membrane is rapid, and cellular energy dependent (see Figure 1B where we show effects of cell energy depletion on nanoparticles uptake), driven by active biological processes that are currently being uncovered by various EU (including those of the current Partners) and National programs around EU and US. Sufficient preliminary information now exists^{5, 6} for us to identify a broad range of active biological processes (receptor mediated and other) that are responsible for this uptake of nanoparticles. Here it is sufficient to say that particles use a combination of endogenous entry portals (receptors etc) and membrane adhesion (followed by membrane turnover) together producing internalization using the cells own energy.

Trafficking and clearance of nanoparticles at cellular level

Here again, radically new paradigms emerge, for unlike chemicals (which may have wide and distributed access to the intra-cellular space by similar dissolution processes) nanoparticles have limited and managed access using endogenous cellular pathways used to transport proteins and other biomolecules. In some cases these processes lead to nanoparticles being localized at very high concentrations in particular organelles (for example lysosome is typical, as shown in Figure 2C, and later on). Transport occurs only along prescribed pathways, for which appropriate particle surface signals are available - for example, in Figure 2D we show that nanoparticles of a very similar substance to the dye in Figure 2A (but in nanoparticulate form) are not cleared upon removal of the extracellular nanoparticles source, but instead are trapped (as far as we can tell 'permanently') inside lysosomes. This may be visualized in a sequence of confocal fluorescence and EM images from silica nanoparticles (see Figure 3) in which we see events of uptake, and internalization, and final localization into lysosomes. This is a very general paradigm we have seen in many particles, cell types (and higher levels) that must be accommodated in any model.

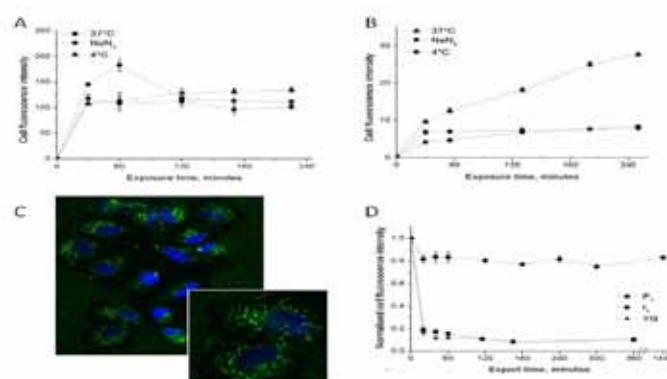


Figure 2. A. Uptake of green fluorescent dye (molecular) by A549 cells – no effect of energy depletion. B. Effect of cellular energy depletion on uptake of 50nm nanoparticles of similar composition to the dye. C. Confocal image showing the localisation of those 50nm nanoparticles in the lysosomes of A549 cells. D. Lack of export of those 50nm nanoparticles from A549 following removal of the particle source (II), compared to rapid release of molecular dye (YG). All data from Partner 1.5



4 Project Description and Organisation

There are several striking features of the program that require particular emphasis and attention in the S/T methodology. These issues, and their impact on methodology are:

- (i) Relative immaturity of the experimental field, lack of clearly validated data, and lack of uniformity on the understanding of the role and methods by which quantitative and reproducible data are acquired.
- (ii) The long time required to generate extensive collections of such data, and the requirement to be more pro-active and constructive in the interim.
- (iii) The need for realistic, achievable outcomes that can be checked at every point.

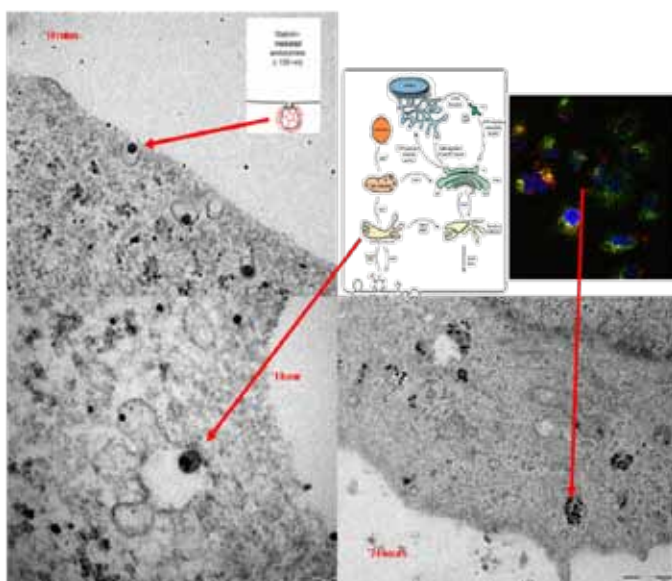


Figure 3. Electron Microscopy images after 10 minutes, 1 hour and 24 hours of exposure to 25 $\mu\text{g}/\text{mL}$ 50nm red-fluorescently labelled SiO_2 nanoparticles. From these images the localisation of the nanoparticles on the surface (some in clathrin and caveolin receptors), early endosomes and finally lysosomes can be observed. Co-localisation of the red-fluorescent SiO_2 nanoparticles with green Lyso-tracker dye is shown in the confocal microscopy image in the top right, confirming the final end point as the lysosomes. Data such as these are now developed to a quantitative level, including quantitative sub cellular localizations. EM and confocal data from Partner 1, sketch of cell re-drawn from Watson et al.⁷

Intimate Collaboration between experiment-modeller-theorist

The current lack of large amounts of data that can be considered reproducible suggests that modellers must rely heavily on the science and understanding, which is now growing rapidly. This understanding of relevant parameters and mechanisms can be gained using a few examples, well studied, and can be widely applied in models. Thus, our approach is based on an implementation of the mechanistic understanding in an interactive manner. Thus, the model when created is

tested using new sets of experimental data, and checkpoints applied to ensure success in a modular approach. Success in this kind of approach (albeit in a limited manner in the early days) is immediate. For example, the simplest uptake model has already been checked experimentally, and the most interesting outcome noted that one had to include the effects of cell division to obtain quantitative agreement. Expansion of this concept allows for a proactive impact on the broader experimental community, from the earliest days, and does not require very large amounts of data before this can occur.

Robust organization to filter data inputs to ensure quality

At all points of the program, the acquisition of high quality data is key, and this is reflected in the accompanying chart, as well as the WP descriptions and the management processes. It is helpful that Partner 1 has an extensive experimental as well as modelling program, as this allows the Unit leaders to be in daily contact, interrogating the experimental information, and models. It ensures such details that the correct parameters and characteristics of the particles are recorded for the models. This allows us to template the process into a more formal management group activity where the young leaders from experiment and theory-modelling are required to evaluate data also emerging from other collaborators outside of the present program. This intimate day-to-day link between modeller and experimentalist we consider as foundation for the success of the project.

Progressive and systematic checks at modular level

Whilst we consider the program highly ambitious, we do feel that it will succeed, and that it is an essential building block on the constellation of such projects. The careful preliminary research and preliminary results in each segment enabled by a series of exchanges and visits in the last year as the program was built certainly gave us much assurance. However, the design and modularity of the project, with the capacity of exposure to the critical evaluation of experimentalists **at each step and at each level** is a key element. Thus, experience of the modellers and theorists in the program suggests that the developments of highly complex models that can only be tested at late stages are risky, and prone to failure. Here, for example, the capacity of a model module to predict the effective interaction between a nanoparticle (complete with corona) and cell membrane can be explicitly tested in a simple experiment. Similarly, the phenomenological model's capacity to model the steady state concentration (for example, in basolateral endosomes in the BBB model) and link that correctly to the macroscopic flux across the BBB barrier can be explicitly checked with live cell imaging, where we have already established the reproducibility of such measurements. Thus, each modular component can be exposed to scientific checks, as well as the usual software validity checks.

The final Integration and Co-ordination tasks (within WP5) ensure that the modules remain in overall conceptual and operational alignment with each other within the program, to allow for a later integration with the future objective of modelling nanoparticle *in vivo* biodistribution. Crucially, this work package also allows for the co-ordination of

communication with a variety of groups in EU and US, and Japan with similar objectives. Key collaborations already exist between the program and US partners. However, key collaborations also exist with other major US and Japanese centres, and these have been aligned to the program (see Section 2.1). Still, we recognise the need to adopt a more flexible approach that takes account of realities on the ground after review of these programs, aligning with those programs that are funded, and newly emerging ones both in modelling, and in collection of experimental data.

We consider that these models, and this methodology, will point the way to the key science, and its relevance for society. A reductionist approach based on interactions and mechanisms, gives the capacity to identify and evolve the key characteristics (size, bare zeta potential, corona composition) of nanoparticles leading to different impacts, and above all, clearly identify the causal link between them. This link is the key to safety by design.

The interaction of the workpackages and the flow of information between them and the external experimental projects is shown in the Pert Chart in Figure 4, below.

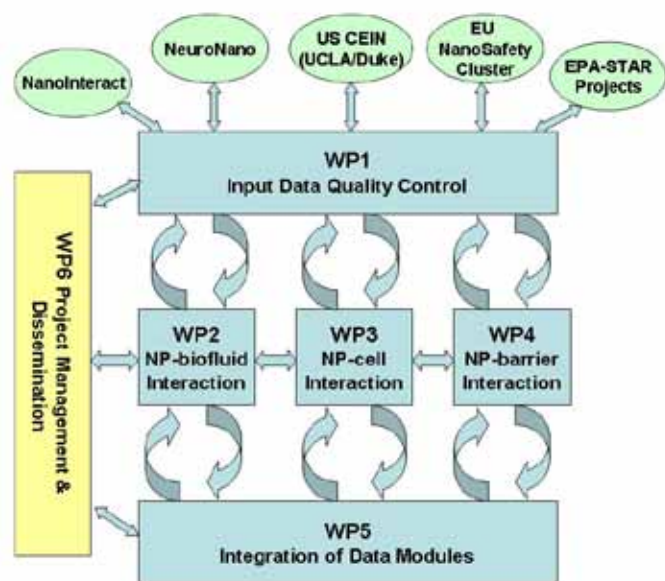


Figure 4. NanoTransKinetics Pert chart.

5 NanoTransKinetics activities

NanoTransKinetics addresses the following objectives:

- To establish techniques for modeling relationships between nanoparticle properties and toxicity (including interactions of nanoparticles with biological systems);

NanoTransKinetics focuses on understanding the mechanisms of nanoparticle uptake into, and sub-cellular transport within cells and through biological barriers with the objective of enabling much more rapid progress towards a screening approach, where predictions of nanoparticle bioaccumulation could be made on the basis of limited in vitro screening data.

NanoTransKinetics is the first integrated effort to develop phenomenological models based on high quality experimental data of nanoparticles interactions with cells and biological barriers. It aims to characterize the hazard posed by nanoparticles in relation to their ability to cross biological barriers, based on nanoparticle concentration fluxes (rather than the traditional ADME approaches which are based on equilibrium properties, which are not applicable to nanoparticles as they interact with cells in a biological manner, and are actively transported within cells. A four tiered approach (interaction with biological fluids, interaction with cellular membranes, interaction with cells in vitro, and interaction with biological barriers, such as the Blood-brain barrier based on in vitro and in vivo data), as shown graphically in the Pert Chart in Figure 4, will ensure sufficient understanding of the role of nanoparticle-protein interactions in mediating nanoparticle-membrane and nanoparticle-protein-cell interactions, whilst allowing sufficient flexibility to be built into the models to allow modeling of data from a wide range of sources, including high throughput data such as High content analysis, thereby also providing a useful route for these data to be integrated into predictive approaches.

- identification of physicochemical properties chosen for establishing groups of structurally similar particles, the characterisation and classification techniques, the test methods, and the relation of structural descriptors to toxicological targets;

One hypothesis of our approach is that nanoparticles in contact with biological systems are immediately coated by a layer of biomolecules which confers to them a "biological identity" which determines how the particles are seen by the cell, and how they interact with the cell. However, a deeper view of this that we have sought to clarify here is that the nanoparticle-environmental interaction cannot be ignored. Partner 2 and Partner 1 were both engaged in a previous EU program (lead by Partner 2) on gene transfer using liposomal and other carriers that though overall successful was striking in illustrating how weak the connection and efficiency between cell level and in vivo predictions was. Considerable investigation revealed that a major element of that was that cell culture takes poor account of the nanoparticle interactions with proteins, extracellular matrix and other biological environmental aspects. Here these elements are built into the program. Learning to predict the biological identities of nanoparticles and to correlate this with uptake, transport and clearance is the only way that we can truly determine a priori, the fate and behaviour of nanoparticles, and their safety implications for human health and the environment. Thus, the key to establishing categories of particles is via their biological identity, or what they actually present to cells. The endpoints that we have chosen to focus on in this programme are thus interactions with biofluids (e.g. plasma / cell culture medium), interactions with biological membranes (involved in uptake processes), interaction with cells (specifically transport kinetics and sub-cellular concentrations) and interaction with biological barriers (to begin the connection to in vivo predictions). Connecting the biological identity of nanoparticles to specific accumulation in certain organelles, and consequently to specific impacts such as apoptosis, enables us to categorize nanoparticles and to begin the process of predicting biological impacts based on biological



identity. The capacity to quantitatively track the particles over long periods of time allows us to determine their biological fate, opening the way for significant advances in our understanding of the transport pathways used by nanoparticles to access the brain, using advanced quantitative dosimetrics, selected and controlled exposure scenarios, long-lifetime radiolabelled nanoparticles, and biophysical approaches such as fluorescently labelling certain proteins involved in the transport pathways and determining co-localization of the proteins and nanoparticles.

- deliver the basis for an inventory of nanoparticles based on potential for exposure, categorising nanoparticles on the basis of physicochemical, structural and toxicological properties:

As above, the capacity to categorise nanoparticles based on their biological identity will offer key advances in our predictive skills, and enable us to connect high throughput screening data to a screening predictive phenomenological models.

- establish relations between experimental (based on available data) and computational properties:

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7 Directory

Table 1 Directory of people involved in the NanoTransKinetics project as beneficiaries*.

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Data from several successful EU FP6 and FP7 projects is being used as the basis for the project (the minimum set of experimental data is already under our control via the NanoInteract, NeuroNano and BioNanoInteract projects which were coordinated by P1, and additional data will be acquired, assessed and implemented via WP5, the NanoSafety cluster and the twinned US projects). As the modelling approaches described are phenomenological initially, they rely directly on the experimental data, in order to reproduce the phenomena, such as protein corona formation, nanoparticles uptake and sub-cellular transport and localisation) and we see a key outcome of the project as being a set of modelling tools that can be linked directly to High Content Analysis assays, such as lysosomal load, in order to correlate impacts with sub-cellular localisation and bioaccumulation potential, for example. We believe that the approach outlined here is the only realistic possibility for understanding and predicting the implications of nanoparticles for living systems, as it is based on understanding the mechanisms of interaction of nanoparticles with cells.

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8 Copyright

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NanoValid

Development of reference methods for hazard identification, risk assessment and LCA of engineered nanomaterials

Project number: 263147 Website: www.nanovalid.eu
Coordinator: Rudolf Reuther, NordMiljö AB, Sunnemo, Sweden

No.	Beneficiary name	Short name	Country
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2	The Institute of Nanotechnology	ION	United Kingdom
3	European Commission Joint Research Centre, Institute for Environment and Sustainability	EC-JRC	Belgium
4	University of Tampere	UTA	Finland
5	University of Salzburg	PLUS	Austria
6	University of Zaragoza	UNIZAR	Spain
7	University of Namur	FUNDP	Belgium
8	University of Ljubljana	UNILJ	Slovenia
9	University of Birmingham	UOB	United Kingdom
10	Fraunhofer Gesellschaft	FHM	Germany
11	Helmholtz Centre for Environmental Research	UFZ	Germany
12	National Institute for R&D in Microtechnologies	IMT	Romania
13	National Research Centre for the Working Environment	NRCWE	Denmark
14	Federal Institute for Occupational Safety and Health	BAUA	Germany
15	Eidgenössische Anstalt für Wasserversorgung, Abwasserreinigung und Gewässerschutz	EAWAG	Switzerland
16	National Institute of Chemical Physics and Biophysics	NICPB	Estonia
17	Federal Institute for Materials Research and Testing	BAM	Germany
18	German Institute for Standardization	DIN	Germany
19	The National Institute of Metrology, Standardization and Industrial Quality	INMETRO	Brazil
20	Federal University of Minas Gerais	UFMG	Brazil
21a	Indian Institute of Toxicology Research (CSIR)	IITR	India
21b	Centre for Chemical and Molecular Biology (CSIR)	CCMB	India
22	McGill University	MCGU	Canada
23	Veneto Nanotech	VN	Italy
24	Nanologica AB	NLAB	Sweden
25	StratiCell	STC	Belgium
26	Grimm Aerosol Technologies	GAT	Germany
27	QUANTIS	QUANTIS	Switzerland
28	Centro Ricerche FIAT	CRF	Italy
29	ARKEMA	ARKEMA	France
	USEPA (no full member of the NanoValid consortium but associated by a LOI to the project)	USEPA	United States



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1 Summary

Project Duration: 1 November 2011 – 31 October 2015

Project Funding: 9.6 Mio. EUR

The growing development, production and use of engineered nanomaterials and associated products will increase exposure of humans and ecosystems to these new materials. However, current knowledge is still incomplete and established test methods inappropriate to reliably assess exposure and risk of materials at the nano-scale. As a result, there is an urgent need to further develop these methods to overcome limitations of current hazard and risk assessment schemes and to generate the data needed for regulative requirements and for safeguarding production, application and disposal of nanomaterials.

NanoValid has mobilized the critical mass of international scientific knowledge and technical expertise required to address these questions. Current analytical and toxicity test methods and models are put to the test and subjected to rigorous intercalibration and validation. Where necessary, methods and materials will be modified, adapted and validated, and new reliable reference methods developed, in cooperation with international standardization bodies and the concerned industries, to support pre and co-normative activities and to make existing risk (RA) and life cycle (LCA) assessment schemes more reliable for ENPs.

The feasibility of validated measurement, characterization and test methods will be assessed by selected case studies to help to improve performance of existing exposure monitoring systems as well as risk management and reduction strategies.

2 Scientific / industry needs and problems addressed

Current knowledge is still incomplete and established test methods inappropriate to reliably assess human and ecosystem exposure to and risk from materials at the nano-scale. There is an urgent need to further develop appropriate methods to overcome limitations of current hazard and risk assessment schemes. NanoValid will address this need through a comprehensive assessment of industrially relevant engineered nanomaterials (ENMs), with particular focus on the development of appropriate reference materials and methods, to manage and reduce associated risks.

The core idea and concept for the project is based on the observation that:

(1) physicochemical properties of nano-sized particles, and hence their biological activity, are often unique and distinct from those of the same bulk materials and often unpredictable,

(2) existing standard methods for measuring and testing that have been developed for macro- and micro-scale material properties may not be applicable to nanoparticles.

These points taken together explain why many current analytical and toxicology protocols developed for bulk materials, may not be suitable for dealing with ENMs, and why a large proportion of the published data may be inaccurate in the context of ENMs and may lead to the drawing of scientifically invalid conclusions.

In particular, new progress will be made in the following fields:

1. Nanomaterials fabrication and characterization

State-of-the-art: At the moment a large proportion of the relevant literature involves poorly characterized, often industrially produced ENPs. The methods for the suspension, preparation and characterization of ENMs prior to biological testing are currently not standardized. Consequently, results from current toxicological tests are not comparable and do not provide the technical framework needed by stakeholders and policy makers as the basis for control and regulatory measures.

It is widely accepted today that the surface chemistry of ENPs is extremely important for possible toxicological effects. Unfortunately, only a few publications are available on the chemical characterization of ENM surfaces, such as nanotubes or core-shell particles. In addition, these studies do not consider standardization or metrology issues that are needed to ensure safe industrial application of ENMs and still no inter-laboratory comparisons or references samples or CRMs exist.

Progress: To produce well defined ENPs for toxicology testing, NanoValid will optimize and extend current synthesis protocols for selected nanomaterials (see lists below) and include the generation of multiple particle sizes, different structural forms, shapes and surface modifications. Synthesis and processing methods will be stringently defined and followed to ensure minimal deviation in the physicochemical properties of the ENMs between batches. Batches of selected ENMs will be also characterized by using a battery of different measurement tools to ensure that any variations are kept within well defined and allowed limits. They will be used in sets of experiments to accurately identify the physicochemical determinants of toxicology and ecotoxicology.

Likewise, the properties of ENP aerosols will be accurately characterized as a function of the ambient conditions to



understand the dynamics of aggregation and propagation that govern their behavior and at the same time limit the effectiveness of control methods, which is decisive in the management of possible risks.

NanoValid will develop and use highly sensitive EN labeling and tracing methods and will design and use a controlled atmosphere dispersion chamber that allows a more precise and reliable monitoring of the behavior of selected nanoparticles.

Initial test materials will include those listed below, although final prioritization will be made in close coordination with relevant standardization bodies and programs and with projects included in the Nanosafety Cluster (in particular with the EU FP7 MARINA, QNANO and NanoDevice project):

Priority 1 test materials: metal oxides (SiO₂, TiO₂, ZnO, CuO), metals (Ag, Au and Pd), CNTs (SWCNTs and MWCNTs) and fullerenes.

Priority 2 test materials: quantum dots (CdSe, CdS, CeO₂), salts (Ca-phosphates, PbS), nanocellulosic materials, polystyrene, dendrimers, ceramics, nanoclays.

2. Human health *in vivo* and *in vitro* models

State-of-the-art: Cellular stress and immune activation have both been reported following exposure to ENMs. However, the lack of validated methods makes it difficult to interpret available experimental and field results. Similar studies sometimes report contradictory effects and often material and methods are insufficiently described to allow a scientifically sound evaluation of data, also regarding exposure to and contamination with bacterial compounds and other stressors.

Progress: NanoValid will establish and implement the following specific tools to address these uncertainties:

- 1) Perform analytical centrifugation and Scanning Electron Microscopy (SEM) on all dispersed ENPs, regardless of the dispersion protocol used and before any *in vitro* and *in vivo* testing.
- 2) Create standardized protocols to follow *in vivo* uptake, interaction, traffic, storage and elimination of ENPs by cells; to study *in vivo* uptake in the lung and elimination through the kidney and the reticuloendothelial system; to define novel endpoints, and to compare *in vivo* effects following uptake by oral, dermal and pulmonary routes.
- 3) Further develop and adapt current cytotoxicity tests to tridimensional tissues, such as reconstructed epidermis, reconstructed lung epithelium or intestine epithelium, to study the possibility of ENP-induced impacts. Progress will be controlled by monitoring correct positive and negative tests and by adapting and modifying further tests. Also existing nanotoxicology tests from classical hepatotoxicology on monolayers of HepG2 cells will be adapted to test and verify effects of ENPs. Other cytotoxicity assays will be optimized to exclude false positives and false negatives more efficiently as compared with current test systems, and novel methods designed to quantify uptake of ENP into cells, together with new protocols to test and assess potential sources of errors.

Although recent publications have shown that fullerenes, ZnO and TiO₂ nanoparticles possess a significant genotoxic potential to human cells, existing data on cellular and molecular interactions of

ENPs with mammalian and bacterial systems are still scarce and inadequate. NanoValid will help to elucidate the exact mechanism of toxicity of ENPs to understand their *in vivo* response in various model systems. In this context, NanoValid will use the most efficient cell-based assays as a basis for the first biological test to reliably monitor work place safety in the industry, which has been exclusively based so far on physical measurements of particle size distributions and concentrations.

A panel of human reporter cell lines will be developed to specifically test *in vitro* ENP effects on cellular stress and inflammation and use in novel *in vitro* reconstructed tissue-based and single-animal models.

A collection device for on-site measurements will be developed which will include a novel Biomodule to allow biological tests for nanotoxicity on-site and which can be used by already available personnel without requiring biological experts.

3. Eco-toxicity

State-of-the-art: Although ENPs are used in many consumer products and industrial applications, their real environmental fate and effect potential throughout their entire life cycle is largely unexplored and reliable quantitative data on toxicological effects of ENPs still scarce even at the single organism level. Eco-toxicological studies on ENPs that have been conducted so far include *in vitro* exposure assessment of vertebrate cells, as well as vertebrates (fish), invertebrates, algae, plants and bacteria. Recent laboratory studies show that aggregated ENPs can be toxic due to solubilization and other specific properties and mechanisms. But there is still no reliable and validated scheme available for eco-toxicological risk assessment of ENPs. One of the key operational bottlenecks is the lack of reliable methods for the characterization of ENPs in exposure test media to account for bioavailability, bio-persistence and bioaccumulation. Another deficiency is the lack of a mechanistic understanding of how physicochemical differences are manifested, which requires well defined cellular systems.

Progress: NanoValid will close these gaps by generating a comprehensive knowledge database on ENPs regarding their life cycle impact on a large range of organisms, which will allow comparison and identification of common mechanisms of effects that are specific for certain types of ENPs. NanoValid will in particular shed light on the behavior of ENPs in exposure media used in OECD and other well recognized regulatory test schemes. Recent studies show that analytical centrifugation needs to be performed before any *in vitro* and *in vivo* testing. NanoValid will use this method to examine the compatibility of various exposure media with *in vitro* models tested, or to determine if and to what degree ENPs are agglomerated, after different treatments, such as gentle sonication, centrifugation or using biocompatible dispersant agents.

NanoValid will further develop and validate a specific model based on fish cell lines to study bioavailability, persistence and bioaccumulation mechanisms in relation to the toxicity of ENPs in fish. By following up and characterizing uptake mechanisms and developing methods for quantification of particle uptake, existing exposure assessment methods will be improved and refined. In addition, interlinking and comparing *in vivo* with *in vitro* results will allow the validation and further development of powerful *in vitro* and *in silico* methods as alternatives to animal testing.



4. Improvement of analytical detection and labeling systems

State-of-the-art: Existing analytical methods have detection limits that are too high to be able to reliably detect low concentrations of ENPs, despite their large surface area conferring a chemical reactivity equivalent to that of a much greater mass concentration of chemically identical, but larger-sized particles. Due to these limitations and challenges, which we face today when working with different ENP characterization techniques, almost nothing is known on the mobility of ENPs in natural environments.

Progress: NanoValid will develop new approaches to increase precision and reproducibility of current analytical detection systems designed for nanomaterials at low concentration in biological and environmental samples, including methods to determine their chemistry, size and morphology, e.g. by advanced secondary electron and optical imaging and spectroscopic techniques. By using these improvements, NanoValid will also assess the applicability of a new system of respiratory exposure assessment that is based on mathematical turbulence models.

State-of-the-art: Also information on reliability and comparability of current biodistribution and bioaccumulation data of nanoparticles is scarce and severely affected by many factors, such as the status of tested nanomaterials, the labelling methods used and sample preparation from animal organs/tissues, which calls for standardized protocols for ENPs labelling, tracing and quantification.

Progress: NanoValid will develop reliable sample preparation and isotope (radiogenic and stable) labeling protocols for selected ENMs, and related analytical protocols for reliably detecting/tracing various ENPs in different animal organs/tissues.

3 Scope and objectives

The main objective of NanoValid is the development of a set of reliable reference methods and materials, including methods for dispersion control and the labeling of ENMs. Based on a comprehensive and critical literature and data survey, the most suitable test materials and methods are currently selected and tested, and new nanomaterials will be synthesized, characterized and stabilized for final method validation.

Already existing industrial or newly designed nanomaterials (ENMs) will be submitted to a comprehensive inter-laboratory validation campaign that includes the currently most advanced methods and instruments for measuring and characterizing of ENMs, to generate accurate and reproducible material data and standardized method protocols, also for labeling, tracing and quantifying of nanoparticles in relation to their size/size distribution, morphology, material identification and other standard physicochemical (pc) properties. The stability and behavior of selected ENPs will be monitored and tested in a variety of relevant biological and environmental samples and test media under both normal and extreme conditions to derive optimum and reproducible fabrication, measurement and test conditions.

The validated pc methods derived from the extensive intercalibration and inter-comparison of selected methods and materials will be used to design well-defined reference materials, which in turn will be employed to validate, and where necessary adapt, modify and further develop current biological approaches

(*in vitro*, *in vivo* and *in silico*) for assessing the toxicity of ENMs and associated risks to human health and the environment. The effects of chronic exposure and of exposure under real-life conditions, where ENPs are likely to act as components of complex mixtures will be taken into account. Finally, appropriate reference methods will be established based on the validated pc and biological methods and their applicability assessed to a variety of industrially relevant ENMs by means of case studies.

Specific objectives are to:

- (1) Test, compare and validate current methods to measure and characterize physicochemical properties of selected ENMs
- (2) Monitor and control their dispersion and stability in various test media and environmental matrices by novel labeling methods
- (3) Generate panels of well-characterized and reproducibly synthesized ENMs, engineered nanoparticles (ENPs) and associated products, designed for further (eco-) toxicological testing
- (4) Test, compare and validate current *in vitro* and *in vivo* methods (for toxicity and ecotoxicity testing) to early identify potential hazards, assess human health effects, including acute and chronic toxicity (oral, inhalation, dermal), and effects to the environment
- (5) Develop a standard test panel according to the mode of action and interaction of ENMs and ENPs with experimental media as used in OECD and other standardized tests
- (6) Identify responsive biomarkers for potential cytotoxic, genotoxic and immunotoxic effects
- (7) Develop further validated methods and materials to reference methods and materials, including Certified Reference Materials (CRMs), for more reliable risk and life cycle assessment (RA and LCA)
- (8) Demonstrate feasibility of validated and established reference methods by means of case studies to assess and improve the performance of methods and systems both during normal operations and for management of accidental risks, evaluation of risk reduction strategies and field detection systems, and for monitoring hazard and exposure to ENPs
- (9) Establish a database on hazard properties of selected ENPs that could be used to support the REACH hazard assessment system
- (10) Build a comprehensive knowledge hub and database to improve existing models on transport and fate of ENPs in the environment, including bioaccumulation, persistence, bioavailability and life cycle impacts onto all forms of biota
- (11) Initiate and support focused efforts to achieve international standardization in cooperation with national (e.g. DIN) and international (e.g. OECD WGMN) organizations.

4 Technical approach and work description

NanoValid's overall strategy is based on (1) a comprehensive and critical review of the existing scientific literature and of relevant material databases, and on (2) a rigorous intercalibration campaign including outstanding test laboratories in Europe and world-wide that participate in the project, to compare and validate current methods and test schemes that have been developed for hazard



characterization as well as exposure and risk assessment of bulk chemicals. Also new methods and schemes will be developed and validated by using relevant and representative industrial and/or newly synthesized NPs and by testing the impact of relevant test media and environmental conditions.

NanoValid is organized in five technical Work Packages (WPs) and three non-technical (management, coordination and dissemination) WPs, as follows:

WP1	Project management
WP2	Fabrication of test materials and selection of test methods
WP3	Validation of pc methods, <i>in vitro</i> , <i>in vivo</i> and computational methods (<i>in silico</i>)
WP4	Application of validated methods to risk (RA) and life cycle assessment (LCA)
WP5	Development of reference methods and certified reference materials
WP6	Case studies to assess the feasibility of validated methods
WP7	Dissemination, exploitation, training, networking and clustering
WP8	Scientific coordination

Although each individual WP has its own distinct focus, function, objectives, tasks, deliverables and milestones, all WPs will closely interact with, support and complement each other in an overarching holistic approach required to match the complexity and multidisciplinary nature of the proposed project. A bottom up approach will be used to gradually link tasks that start with a lower level of complexity (e.g. primary data generation, method and material survey and selection in WP2) with tasks of increasingly higher levels of mutual interaction (e.g. validation of methods and testing their applicability to RA and LCA (in WP3 and WP4), until the intended objectives (verified by specific deliverables and milestones) are achieved and results generated (e.g., establishing reference methods and materials in WP5 and proving their applicability in WP6).

A global dissemination and exploitation strategy (WP7) including internet-based interfaces for all relevant stakeholders (academia, industry, regulatory authorities policy-makers, the public) and events organized at different levels around the project will foster the take-up and exploitation of project results already during the course of the project.

The following Table 1 gives a short overview and description of the work plan broken down into WPs, tasks and methodology and how it will lead participants to achieve project objectives:

Table 1 Work packages (WP) of NanoValid

WP	Title	Topic
1	Project coordination and management	Work package 1 (WP1) will organize and coordinate the consortium and the planned work to achieve project objectives, implement tasks, mobilize the necessary personnel and resources, process and evaluate collected and generate new data, and ensure reporting and quality assurance, according to main topics of the Call, scope of the work, time schedule, and costs. The project management will build upon direct and free communication among participants, and with the Commission, and on a smooth information flow, including regular project meetings and monitoring, progress control and risk evaluation, and strict compliance with milestones and deadlines for deliverables.
2	Fabrication of test materials and selection of test methods	Work package 2 (WP2) will provide the project with a constant and updated review of the existing knowledge and data about nanomaterials fabrication and characterization methods. This will be realized by preparing critical periodic reviews of the relevant literature and of specific databases, which will be distributed to all the partners. Based on the evaluation of obtained results, relevant and most promising test materials and methods will be selected by partners involved in this WP. A group of high-priority industrial nanomaterials will be prepared and synthesized comprising a range of different sizes, structures, coatings and compositions. These materials will be fully characterized in WP2 before being used for validation (WP3), applicability testing (WP4), reference method and material development (WP5) and assessment of their feasibility (WP6). Finally, this WP will provide and evaluate a suite of screening bio-tests for initial profiling of toxicological properties of the prepared NPs.
3	Validation of pc methods, <i>in vitro</i> , <i>in vivo</i> and computational methods (<i>in silico</i>)	Work package 3 (WP3) will be the main validation work package, acting as a link between WP2, where materials and methods will be selected, and subsequent WPs, where the validated materials and methods will be utilized in applications. In summary, WP3 will instigate inter laboratory cross checking of protocols, methods, materials and results (round-robins) aiming to generate cross-referenced and fully validated materials, media and methods across the full spectrum of nanosafety (synthesis, characterization, stabilization, human toxicology and environmental toxicology testing). The focus will be on potentially innovative methods relevant and dedicated to nanosafety. A particular necessity for this task is the participation of several state-of-the-art laboratories with suitable facilities and experience.



4	Application of validated methods to risk (RA) and life cycle assessment (LCA)	Work package 4 (WP4) will evaluate the potential of the methods selected under WP3 to perform hazard and risk assessment (HA and RA) and life cycle analyses (LCA) for the ENMs selected and characterized under WP2. This includes the refinement of test strategies for HA, RA and LCA with regard to ENP, as the respective procedures are developed for chemicals and may need modification/adaptation to deliver meaningful results for ENPs. From the results of HA, RA and LCA, feedback is given to WP5 regarding potential hazards and risks of ENPs as well as to WP6 regarding applicability of test methods validated and selected under WP3.
5	Development of reference methods and certified reference materials	Work package 5 (WP5) will provide reference methods and materials (Certified Reference Materials, CRM) for toxicological testing, ENP exposure assessment, assessment of the ENP impact on human and environmental health in order to underpin hazard and risk assessment related to new and known ENPs. Methods identified under WP2 and validated under WP3 will be evaluated for their potential to be developed as reference methods for a special task. Reference methods developed under WP5 will be specifically characterized by uncertainty considerations and traceability. Traceability for measurands will be established through certified reference materials (CRM) when possible. For those measurands where no CRM is available and for method-defined measurements (which are often in use in the toxicology community), comparability will be established by method specific convention parameters. Agreed convention parameters will be obtained through inter-laboratory comparisons. The goal is to “deliver” these reference methods, which will provide reproducible and comparable data, for use under WP4 and WP6 activities. Finally WP5 results will directly underpin standardization activities under WP7.
6	Case studies to assess the feasibility of validated methods	Work package 6 (WP6) will perform case studies to assess the feasibility of validated methods by applying a battery of test systems and modelling tools to assess the safety of nanomaterials in real and simulated working environments. Tests will be either on-site or will use collected materials to be tested in participating laboratories. The case studies will also include accident simulations to develop proper risk management systems. They will allow making of specific recommendations for managing risks deriving from engineered nanoparticles. These recommendations will be set down in the form of reports, good practice documents and guidelines.
7	Dissemination, exploitation, training, networking and clustering	Work package 7 (WP7) will provide the project results in a suitable format for the wider community to access and use; through regular news and information streams, reports, new standardized protocols, training events, and networking opportunities. It will also afford the wider community (industry, academia, regulatory agencies, relevant NGOs) the opportunity to interact with the consortium and influence the development of the consortium’s work.
8	Scientific coordination	Work package 8 (WP8) will use measures to ensure a high performance quality of the tasks and methodology, equipment and required expertise, to reach scientific objectives by the methods described and developed in WP2-6 (see below) according to the work programme and within the time schedule. The scientific coordinator will produce a quarterly update report on the scientific-technical work in close cooperation with the responsible Work Package Leaders, review and evaluate achievements, and make them immediately available to all project partners by means of short protocols summarizing main results, progress and problems.

5 Status of the project

NanoValid has started on 1 November 2011 and was launched by a kick-off meeting in Rome, Italy, on 16-17 November 2011. This first meeting was coordinated with the kick-off meeting of the EU FP7 MARINA project. Both NanoValid and MARINA discussed during a joint meeting on 18 November 2012 a common strategy to coordinate and synchronize the planned R&D activities, and agreed upon a common action plan to effectively use existing synergies, share data and test materials, and to organize common dissemination events.

Currently (January 2012), an intensive discussion is going on among NanoValid partners, and with MARINA, on the selection and fabrication of a first group of test materials, in accordance with Annex I of the Grant Agreement (Description of Work).

The next regular project meeting is planned on 8-9 May 2012 at the National Research Centre for the Working Environment (NRCWE) in Copenhagen, Denmark, followed by a joint session with the EU FP7 NanoSustain project (www.nanosustain.eu), as about half of the members of the NanoSustain consortium (including the hosting partner NRCWE) are also involved in NanoValid and as



both projects are managed by the same coordinator (Rudolf Reuther NOMI). A half-day common meeting is planned between these two EU FP7 projects in the afternoon of 9 May 2012, to share experiences, exchange data and expertise, and discuss possible ways to cooperate.

6 Expected impact

The project aims to compare and validate current test methods, and if necessary modify and adapt these methods, and/or develop new methods for reliable measurement and testing to improve exposure and risk assessment as well as life cycle analysis of nanoscale materials, with the ultimate goal of establishing reference methods and materials. To achieve these objectives, the project will implement a comprehensive inter-laboratory, inter-comparison and intercalibration campaign between all participating laboratories and a set of case studies to assess the feasibility of the established methods. It is a first step in the generation of reliable quantitative data on the toxicology and ecotoxicology of nanomaterials and the development of accurate methods required to generate the data. The validated methods and new knowledge developed will trigger a step change in the early scientific assessment of potential health, safety and environmental risks associated with nanotechnology-based materials and associated products. It will help to meet existing regulatory requirements and/or develop new legal requirements for safe, responsible and sustainable development.

As a major outcome, the project will provide a set of reliable (pre-standardization) protocols and reference methods that are applicable to a wide range of NPs for early hazard identification, risk assessment and the design of sustainable solutions for safe production, use and disposal of ENMs. NanoValid will generate a comprehensive knowledge and database on the intrinsic properties of particular ENMs and associated ENPs and will contribute to a better mechanistic understanding of their behaviour in various test media, physiological solutions and environmental matrices. The generation of scientifically sound and well-defined reference tools will support standardization efforts to characterize ENPs and their potential effects and hence improve current test schemes for risk management, reduction and monitoring. Providing reliable methods for RA and LCA of ENPs, including new *in vitro* cell panels and other models to assess their bioaccumulation, persistence and bioavailability will help to identify problematic materials early on, which in turn will stimulate the development of safe production processes, novel properties and innovative sustainable products („green nanotechnology“).

The proposed toxicological work will address critical issues, such as the validation of current standardized *in vitro* tests, the follow up of biodistribution, persistence and bioaccumulation of NPs, tracing of NP excretion or immune effects as well as possible genotoxicity and effects on sensory organs or neural tissue. Results from NP testing will contribute to new conceptual and experimental standards for toxicity testing by *in vitro* test systems, which in turn helps to reduce animal testing.

New knowledge on the toxicity of ENMs to particle-ingesting organisms and to those not internalizing NPs, and on mechanisms that control particle solubility and bioavailability, will help to improve the applicability of current RA and LCA systems to NPs. The detection and evaluation of a wide range of different ENMs

under various laboratory and field conditions by various measurement and testing methods will enhance and extend our current understanding of the true nature of the solution, dispersion and agglomeration/aggregation behaviour, persistence and fate of ENMs. NanoValid will develop standardized procedures for labelling and dispersing nanoparticles prior to toxicological testing, which will allow accurate tracing and the conversion of test materials that have been developed and fabricated into standardized stable nanoparticle suspensions.

The development of reference methods and materials will support pre and co-normative activities, such as those required for the implementation of REACH and other relevant EU legislation. It will assist current policy and future decision making, to meet increasing regulative requirements in nanotechnology and the need of relevant stakeholders, such as public authorities, industry, researchers and citizens. Finally, a more reliable measurement, characterization and toxicological assessment of ENMs will support good governance in research and industry and contribute to the future definition of appropriate measures and guidelines in line with the precautionary principle.

7 Dissemination and exploitation strategy

The NanoValid work programme has the aim of developing a number of new collecting, characterizing, and testing methodologies. A number of these have clear commercialization opportunities. Through the participation of industrial partners and partners that have an established background in the commercialization of new devices, it is expected that project results will lead to:

- ü standardized test materials that can be used by different industries to validate the physicochemical properties of ENMs they manufacture or purchase;
- ü novel ENP samplers, e.g. for hot gases;
- ü novel real-time collection and assessment devices which collect airborne ENPs and assay them on an integrated biomodule, thus addressing issues of bioactivity and transport, and ensuing loss or change of reactivity and physical properties;
- ü a multi-compartment cell barrier model to mimic physiological systems and provide a more realistic evaluation of ENP fate and effect in living organisms;
- ü novel environmental models to determine the fate and effect of ENMs to post consumer, that can be employed by different manufacturers to comply with new regulations.

NanoValid is presently establishing (January 2012) a dedicated project website (www.nanovallid.eu) and database, through which project results will be available, and the wider community can be engaged (through feedback and discussion fora). In addition, it will target stakeholders through newsletters and press-releases, and actively engage with individuals through a series of physical and online events. To ensure all relevant stakeholders are reached, the consortium will leverage its own substantial networks and individual membership of international committees and associations. This takes advantage of the range represented in the consortium: academic organizations, industrial SMEs and large-



scale manufacturers, standards organizations, material testing institutes, networks, associations, and consultancy firms.

In addition, NanoValid will assess the commercialization potential of diagnostic tools developed within the project, and propose new standards to ISO/CEN and OECD committees using the expertise within the consortium.

To achieve this, the consortium will make use of established information channels including (but not limited to): the ION

website (over 65 000 nanotechnologists in its database, receives over 100 000 visits and 1 million hits each month), the EU FP7 *observatoryNANO* website (providing analysis of nanotechnology developments and impacts from a European perspective), NanoForum (gateway to European nanotechnology with over 20 000 users registered), and existing news services such as Cordis and Alpha Galileo.

8 Directory

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**Acronym: NEPHH**

**Full Name: Nanomaterials Related
Environmental Pollution and Health
Hazards Throughout their Life Cycle**



Contract Agreement: CP-FP 228536-2 Website: <http://www.nephh-fp7.eu>

Coordinator: EKOTEK S.L.

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1 Summary

NEPHH - NANOMATERIALS RELATED ENVIRONMENTAL POLLUTION AND HEALTH HAZARDS THROUGHOUT THEIR LIFE CYCLE is a Collaborative Project funded under 7th FWP (Seventh Framework Programme) in the research area of NMP-2008-1.3-2: Impact of engineered nanoparticles on health and the environment.

environment and human health of certain “nanomaterials” and “nanoproducts” is not yet fully well understood. Not only should nanotechnologies be safely applied and produce results in the shape of useful products and services, but there should be also public consensus on their overall impact. In fact, the risk assessment of engineered nanomaterials has become the focus of increasing attention. To date the widely accepted view is that there are many unanswered questions on the potential environmental and health risks associated with the manufacture, use, distribution and disposal of nanomaterials.

2 Introduction to NEPHH

While nanosciences and nanotechnologies (N&N) offer a number of beneficial applications, the potential impact on the

In 2005, the Commission requested the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) for an



opinion on the appropriateness of existing risk assessment methodologies. SCENIHR concluded that nanomaterials may have different (eco-) toxicological properties than the substances in bulk form and therefore their risks need to be assessed on a case by case basis and the risk assessment methods and instruments may require further development. There is now a need to assess the suitability of current risk assessment methods in more detail in order to guide how to deal in practice with nanomaterials in an appropriate manner.

Furthermore, opinions by the SCENIHR conclude that current risk assessment methodologies for micro/macroscale chemicals require modification in order to deal with the risks associated with nanotechnologies and, in particular, that existing toxicological and ecotoxicological methods may not be sufficient to address all of the issues arising from nanoparticles as size confers unique properties to nanomaterials. The opinions also indicate that very little is known about the physiological responses to nanoparticles and that there are major gaps in the knowledge necessary for risk assessment. To date, the widely accepted view is that **there are many unanswered questions on the potential environmental and health risks associated with the manufacture, use, distribution and disposal of nanomaterials.**

There is a need for a rapid improvement of the scientific knowledge basis to support the regulatory work. In particular, research is needed in areas underpinning risk assessments and risk management like:

- § Data on toxic and eco-toxic effects as well as test methods to generate such data.
- § Data on uses and exposures throughout the lifecycle of nanomaterials or products containing nanomaterials, as well as exposure assessment approaches.
- § Characterisation of nanomaterials, development of uniform standards and nomenclature, as well as analytical measurement techniques.
- § For occupational health aspects, the effectiveness of a range of risk management measures including process enclosure, ventilation, personal protective equipment like respiratory protective equipment and gloves.

Although Europe is at the forefront of this promising field of science, many knowledge gaps remain in relation to the impact of these technologies on human health and the environment. Concerns over ethics and the respect of fundamental rights are also linked to N&N.

The key motivations for NEPHH project are:

1. In early studies, engineered nanoparticles have shown toxic potentially properties. They can enter the human body in various ways, reach vital organs via the blood stream, and possibly damage tissue. Due to their small size, the properties of nanoparticles not only differ from bulk material of the same composition but also show different interaction patterns with the human body. The risk assessment for bulk materials is therefore not sufficient to characterise the same materials in nanoparticulate form. Information on the bioaccumulation and potential toxic effects of inhalation and/or ingestion of free engineered nanoparticles and their long-term implications for public health is needed. The environmental consequences

associated with the ultimate disposal of these materials also need to be evaluated carefully. There is a dearth of evidence about effects of pollution nanoparticles on environment. Moreover, in common with other chemicals, nanoparticles may reach humans and other organisms by a wide variety of environmental routes.

2. Prioritizing and obtaining materials to evaluate are major challenges when studying nanomaterials. Specific nanomaterials with the highest exposure potentials are not well known, making it difficult to identify the most important materials to study. Obtaining materials is also an impediment. In many cases, information about the nanoscale material is proprietary. Consequently, the EU may be unable to study those materials that pose the highest potential exposure to humans. In other cases, the material may be available, but not in sufficient quantities to allow an adequate hazard evaluation, particularly regarding long term, repeated exposure studies.
3. Characterization of nanomaterials has proven to be more difficult than anticipated for several reasons. First, a standard nomenclature has not been developed. Second, biologists, physicists, and materials scientists working in this area do not always communicate effectively. In addition, an analytical infrastructure to allow characterization is not consistently available or well-located. The high degree of variability in size and surface chemistry of nanoscale materials and in the coatings, crystal structure, shape, and composition used in preparing these materials increase both their complexity and the multiple permutations that must be considered in their evaluation.
4. Adequate methods to detect nanomaterials in cells and tissues also need further development. Some of these impediments could be addressed by, for example, the development of a repository of well characterized model of nanomaterials for use in both toxicological and biomedical research/ reference standards for nanoscale particles targeted for the biomedical and toxicological research. This development would significantly enhance the quality research investigating the health effects of nanoscale materials.
5. Health, safety and environmental risks that may be associated with products and applications of Nanotechnology and Nanosciences (N&N) need to be addressed upfront and throughout their life cycle. Doing complete life cycle analysis on newly developed products, and considering all the ecological as well as the socio-economic components, will help to ensure growth and employment in the European Economic Area (EEA). Furthermore, material science will play an important role in contributing to the solutions for some emerging societal needs and in increasing the quality of life of European citizens.
6. The implications of the special properties of nanoparticles with respect to health and safety have not yet been taken into account by regulators. Size effects are not addressed in the framework of the new European chemicals policy REACH. Although production volumes for the most commonly used nanomaterials are already approaching the REACH threshold of 1 tonne per year per company. This is why nanoparticles raise a number of safety and regulatory issues that governments are now starting to tackle.



3 NEPHH's Approach and Overall Objectives

The aim of NEPHH is to identify and rate important forms of nanotechnology-related environmental pollution and health hazards that could result from activities involved in nanostructures throughout their life cycle, and to suggest means that might reduce or eliminate these impacts. The NEPHH project considers the safety, environmental and human health implications of nanotechnology-based materials and products.

Nanomaterials selected are Silicon based laboratory materials which will be supplemented with nanomaterials from industry. On the one hand, Silicon based nanoparticles including (nano)silica (SiO₂), layered silicates (MMT), glass (nano)fibres and foam-glass-crystal materials have been selected. On the other hand, a total number of three engineering polymeric matrixes have been selected, including polyamides and polypropylenes as bulk materials and polyurethanes as foamed polymeric materials, which will be used to produce nano-induced polyurethane foams. According to this selection, 12 polymer composites will be produced on the combination of all nanomaterials and polymeric matrixes.

Finally, industrial Silicon based nanomaterials from leading companies (Bayer, Honeywell Polymer, RTP Company, Basell, Blackhawk Automotive Plastics Inc, Gitto Global, Akzo Nobel Polymer Chemicals, Laviosa Chimica, Southern Clay and Sud Chemie) will be acquired.

Developed polymer nanocomposites will be used to fabricate macro-scale structural specimens, to be used to simulate Crash Test Laboratories. Dust particles form macro-scale nanostructures will also be obtained from Industrial Silicon based materials, establishing a collection of different samples: which will correspond to laboratory materials including selected Silicon based nanoparticles, polymeric matrixes and polymer nanocomposites resulting from their combination with selected polymeric matrixes and acquired industrial materials.

Considering that for most applications nanoparticles can be surface modified and generally are embedded in the final product and therefore do not come into direct contact with consumers or the environment, NEPHH will be going beyond the primary nanomaterials and looking into the secondary and tertiary nanoproductions in a wide range of typical applications from automotive to household usage. It will look into end-use products – ranging from nanocomposites to energy absorption foams in automotive and aerospace applications.

An integrated holistic life-cycle approach is considered.



Fig 1: Diagram representing different stages of Nanotechnology based Applications / Products' Life Cycle.

The specific objectives of the project are the following:

1. Development of a systematic, continuous practice for selecting and prioritizing engineered nanomaterials in order to assess their safety, environmental and human health impacts. Since currently, there is a substantial interest to develop and introduce into market dispersions of nano-scale reinforcements in polymers, NEPHH will constantly review novel applications and products along with their potential impacts in human health and environment.
2. Contribution to the standardization and validation of test methods and test schemes for nanomaterials as adaptation of the current physicochemical sampling protocols to present research is envisaged.
3. Collection of nanocomposites samples, including laboratory and industrial Silicon based materials. Targeted materials represent an innovative selection supplementing ongoing investigations and setting a basis for future ones.
4. Achievement of a better understanding of the health impacts of the selected nanomaterials.
5. Assessment of the environmental exposure throughout the life cycle.
6. Assessment of the potential of nanomaterials to damage the environment (or human health through the environment).
7. Selection and dissemination of the best practices (in the fields of manufacture and disposal mainly), and actuation guidelines for exposed workers.
8. Contribution to the 'Code of Conduct for Responsible Nanosciences and Nanotechnologies Research' action to ensure that nanotechnologies are developed in a safe manner.
9. Contribution to the regulatory frameworks which are applicable to nanomaterials (chemicals, worker protection, environmental legislation, product specific legislation, etc).

4 NEPHH Project Execution

In order to ensure that the research and innovation objectives of this project are achieved, a clearly defined work-programme has been set up and divided into a number of Work Packages (WP) and Tasks to allow the team of researchers to focus on the development of the project, currently under execution.

Present section includes some of the main outcomes achieved in the course of the first year.

WP1 – Technological Surveillance System

Although nanotechnologies are quite an incipient activity area, they are increasingly produced for use in a wide range of industrial and consumer products and many authors are working in their possible impact in human health and



environment. The rate of generated information is, therefore, very rapid. NEPHH has developed and implemented a Technological Surveillance System to capture, evaluate and disseminate information released about a number of topics related to nanotechnologies, also aligned with the selection and prioritization of engineered nanomaterials as they penetrate into the market for latter evaluation.

Conversely, as activity shifts from research to the development of applications, there exists an urgent need to understanding and managing the associated risks, particularly to personnel working with these materials and therefore the evaluation of in force health and safety procedures currently set in place for the minimisation or elimination of such potential risks provides a global picture of the awareness of Manufacturing Companies, RTD Laboratories and Centres towards this specific issue. This evaluation has been carried out by the execution of an international survey whose main results can be obtained from NEPHH Project's webpage.

Finally, NEPHH Consortium has defined the envisaged procedure for samples production, collection, storage, labelling and transference amongst partners. Such procedure is a relevant highlight of NEPHH, as it is a first trial for the standardization of the testing approaches inside Project Consortium that could be later replicated at a major scale.

WP2 – Working Nanomaterials Supply and Preparation

The production of macro-scale structural specimens involved a number of steps as hereby listed:

- (1) Selection and characterization of Silicon based nanoparticles – (Nano)Silica (SiO₂), Layered Silicates (MMT), Glass (nano)Fibres (GF) and Foam-Glass-Crystal (FGC) materials to be used as nano-reinforcing agents.
- (2) Selection and characterization of engineering polymeric matrixes – Polyamides (PA) and Polypropylenes (PP) as bulk materials and Polyurethane (PU) as a foamed matrix.
- (3) Polymer nanocomposites preparation by using polymers and nanoparticles described in points 1 and 2.

The nanocomposites of PP and PA6 with four types of fillers: organically modified montmorillonite, nanosilica, foam-glass crystal material and glass fibres have been obtained by direct melt mixing in twin-screw extruders. In order to maintain high dispersibility of nanofillers in both apolar (Polypropylene - PP) and polar (Polyamide – PA6) matrix selected nanofillers were used with proper surface functionalization and macromolecular compatibilizer addition providing good compatibility with both types of polymers - nonpolar polypropylene and polar polyamide-6.

On the basis of literature review and laboratory results eight different compositions (PP/MMT, PP/SiO₂, PP/FGCM, PP/GFs, PA6/MMT, PA6/ SiO₂, PA6/FGCN and PA6/GFs) containing 5wt% of nanofiller were selected as a suitable material for preparation of macrosamples that would be further examined in the scheduled physical tests and leaching experiments. Selected filler concentration provided the highest content of additive in

the form of well distributed and not agglomerated nanoparticles. No other additives, eg thermal stabilizer, processing aids... were used in order to avoid their influence on material's toxicity. Macrosamples for physical processing (crash tests) were prepared by compression moulding technique.

Polyurethane foam (PUR) was synthesized (after a set of optimization experiments) in a three-step process comprising (i) preparation of polyol premix with auxiliary components and nanoadditives, (ii) introduction of blowing agent into polyol premix and (iii) addition of isocyanate component. High speed mechanical mixer was applied in order to enhance dispersion of nanoadditives and ensure proper mixing of reagents in the course of synthesis of PU foams. Four types of nanoinduced polyurethane foams were prepared: polyurethane foam/montmorillonite (PUR/MMT), polyurethane foam/nanosilica (PUR/ SiO₂), polyurethane foam/ foam-glass crystal material (PUR/FGCM) and polyurethane foam/glass fibres (PUR/GF) with 5wt% of nanofiller.

Injection moulding bars of nanocomposites and foams have been examined in terms of structure, morphology and thermal properties by WAXD, SAXD, TG, DSC, SEM, POLM and FTIR methods.

WP3 – Dust particles from macroscale nanostructures

The aim of WP3 is to generate nanoscale dust particles from the macro-scale nano reinforced nanostructures fabricated in WP2, to consider the exposure throughout the whole life cycle of nanomaterials in near 'real life' exposure as possible. Mainly due to the fact that the potential exposure in high performance structures (aerospace, automotive etc) is deemed to increase when material fracture occurs WP3 focuses on potential exposures in the transport vehicles accidents, recycling centres (especially composites ones), milling, sawing, machining, manufacture and testing of nanoreinforced composites.

Ageing protocols are also being performed to evaluate the effects of silicon-based NMs on recycling and reclamation at the end of the final product life-cycle. Furthermore, protocols for the evaluation and characterization of nanoparticles release in **incineration** processes are also being performed.

Nanoparticles have been generated by **impacting** polyurethane nanocomposites with different nanofillers (Montmorillonite – MMT-, Nanosilica - SiO₂-, Glass fibres – GF-, Foam Glass Crystal – FGC-) via low velocity impact testing. Also, nanoparticles from polypropylenes and polyamide nano and fibre reinforced panels have been generated by **mechanical drilling**, as impacting of these materials generated low quantities of dust. Both these processes have been performed in special designed chambers are hereby illustrated.

Released particles have been sampled and extracted by suspending them in solution. The solution has been filtrated in several steps and the physical and chemical properties have been characterized by means of scanning electron microscopy (SEM), transmission electron microscopy (TEM) measurements and Dynamic Light Scattering (DLS) technique and Nanosight (NS).

The results show that by different processing methods nanoparticles can be generated. Moreover the characterisation has revealed that the physicochemical properties of the generated particles significantly vary depending on filler and matrix material.

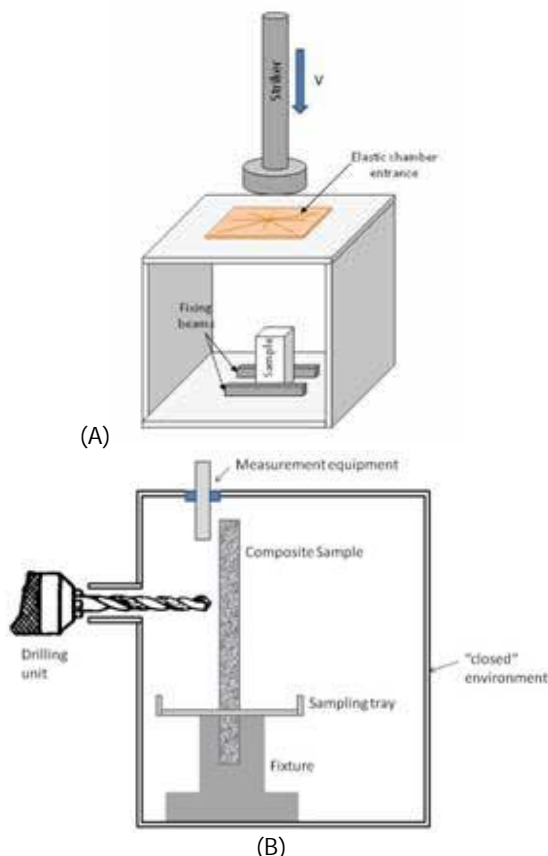


Fig 2: Crash Chamber for nanodust collection during low-velocity impact tests (A) and Drilling process for particle collection (B) – By CRANFIELD UNIVERSITY

NEPHH through WP3 is contributing to critically assess the release of nanoparticles during different stages of the life cycle of polymer nanocomposites by next means:

- Developing methodologies to measure the release of nanoparticles in different stages of the life cycle of nanocomposites as in the cases of physical processing as during product manufacturing (pieces assembly, machining, sawing...) and also by degradation during service and post service (eg landfills, incineration...). These methodologies serve as a basis to assess the **occupational exposure** to nanoparticles associated to different processes (manufacturing, recycling...) of novel Nanotechnology-enabled products entering the market on an increasing rate. Furthermore, developed methodologies are key to evaluate the **exposure rates towards final consumers and the environment**.
- Methodologies developed in the frame of NEPHH for the assessment of NPs release provide a solid basis for **standardization** purposes. In fact, NEPHH is developing appropriate tools and protocols for the

assessment of nanoparticles release from industrial products in premarket stage, therefore promoting the "safe by design" principle for Nanotechnology based products.

- Contributing to filling the actual gap related to the characterization -including chemical composition- of nanoparticles released during the different stages of the Life Cycle of nanocomposites.
- Developed methodologies and assessments performed are the key to define models for the prediction of nanoparticle releases.
- Ulterior WPs (WP4, WP5) determine the potential impacts that released nanoparticles pose towards human health and the environment in comparison with alternative formulations excluding nanomaterials. Such information if correlated with models for NP release can be considered key contributions of NEPHH for Nanomaterials Risk Assessment.
- Results obtained in the frame of NEPHH illustrate new insight into nanoparticle behaviour and advice on a new dimension for nanomaterial risk assessment, since the actual release of embedded NPs has been confirmed but, furthermore, hybrid particles comprising engineering matrixes and selected nanofillers have been detected.

WP4 – Health implications of engineered nanomaterials

The main target of WP4 is to assess the toxicological mechanisms and health impacts of selected nanomaterials and to collaborate in establishing reliable and useful *in vitro* methodologies for the regulatory demands of the safety assessment of nanotechnological products.

For overall product safety assessment, the toxicity data needs to be evaluated in connection with the ability of a given product to release NP throughout the product life cycle. The approach developed in NEPHH based on one scenario can be adopted for assessing the level of NP release from different materials in different scenarios and the related health effect, therefore contributing to LCA for the safety of novel materials.

Test effects of the nanomaterials selected and nanometric residues of nanomaterials physical processing obtained in the execution of WP2 and WP3 are actually being carried out to analyze and model their biohazard potential throughout product's life-cycle.

So far silicon based nanoparticles that have been selected for composites nano-reinforcement have been evaluated for toxicological assessment as those analyses allow establishing and optimizing the experimental conditions for further investigations. A consensus in the strategy for the evaluation of human health impacts in samples originated in WP3 has been reached, with the main target of all project partners evaluating the same (nano)objects.



A number of methods and protocols for assessing NMs toxicity in vitro using different cell lines have been well established, which can be applied to toxicity assessment of NMs from other sources. In detail:

- In vitro protocols for the analysis of lung bronchoalveolar lavage fluid and blood serum lipid and protein oxidation have been developed and adapted to present samples.
- Cell culture conditions including cell seeding density and incubation times for NPs toxicity study have been optimized.
- The methodologies for cell viability study and ROS assay have been adapted to envisaged samples. Ames test methodology for the evaluation of bacterial mutation has been adapted to envisaged samples.
- Characterization method of selected raw nanoparticles in culture medium based on FT-IR has been developed.
- Evaluated cytotoxicity endpoints so far have included cell viability (MTT test), intracellular reactive oxygen species generation (ROS) and cellular membrane damage via LDH Assay.

WP5 – Environmental implications of engineered nanomaterials

The main objective WP5 is the assessment of the environmental life-cycle impacts of selected nanomaterials as alternatives to conventional materials. This analysis also intends to provide a baseline life-cycle assessment (LCA) of the alternative nanomaterials. This evaluation involves studying the persistence, bioaccumulation, toxicity and ecotoxicity of such nanoparticles and, the analysis of their risks and hazards in different abiotic media.

So far, raw nanoparticles that have been selected for composites nano-reinforcement have been evaluated for ecotoxicological assessment as those analyses allow establishing and optimizing the experimental conditions for further investigations. A consensus in the strategy for the evaluation of environmental impacts in samples originated in WP3 has been reached, with the main target of all project partners evaluating the same (nano)objects.

The evaluation of dust suspensions has proven that all dust samples except those incorporating nano-SiO₂ were not toxic to bacteria (E.coli and SODAB mutant) under UV or in the dark after 24H Exposition. However dust from nano-SiO₂ composite (mainly PA and PP) exhibit toxicity. Further assessments are actually on progress.

The evaluation of bioaccumulation and toxicity of FGC as an alternative to conventional insulation building materials has been carried out. FGC is a volumetric material, incorporating nanoscale structural elements, produced by heat treatment (~800-850° C) of amorphous matrix containing crystalline phase in the form of SiO₂, when the crystalline phase in the foaming

process is reduced to nano-sized. This estimate has provided an analysis of environmental risks and hazards of FGC in non-residential buildings. To identify environmental hazards logic and graphical analysis methods such as "tree of failures" and "tree of events" have been used. The studies indicate that FGC products meet European standards for health protection and preservation the environment at all the stages of its production and application which allows using this material in construction, ensuring the absence of toxic substances and danger to human health.

WP6, actually on progress, aims to make available the understanding of the safety, environmental and health implications of nanomaterials in order to define the appropriate measures and minimise the exposure of workers. Guidelines for responsible management of waste nanomaterials are also being developed.

5 NEPHH Project Dissemination

The list of articles released within the second year of NEPHH includes:

- § Kaz'mina O.V. *Effect of the component composition and oxidation - reduction characteristics of mixes on foaming of pyroplastic silicate pastes*. Glass and Ceramics, Vol.67, Nos.3 – 4, 2010, pp. 109-113.
- § Sachse S., Irfan A., Zhu H., Njuguna J. *Morphology studies of nanodust generated from polyurethane/nanoclay nanofoams following mechanical fracture*. Journal of Nanostructured Polymers and Nanocomposites , 2010.
- § Adeel Irfan, Sophia Sachse, James Njuguna, Huijun Zhu*, Ainhoa Egizabal, Krzysztof Pielichowski, Maria Blázquez. *Are engineered nanomaterials safe? Focusing on polymer-nanosilica composites throughout their life cycle*. Accepted, 2010.
- § Kazmina O.V., Semukhin B.C., Mukhortova A.V. *Structural and mechanical characteristics of high performance heat-insulating foamglass material// Construction and Building Materials*, 2011.
- § J. Njuguna, S. Sachse, I. Adeel H. Zhu, S. Michalowski, K. Pielichowski. 'Nanoparticles Released from Structural Nano-enhanced Products Following Mechanical Loading at Low Velocity Impacts: A Case Study on Structural Polyurethane Nanoreinforced Foams' - 'MODERN POLYMERIC MATERIALS FOR ENVIRONMENTAL APPLICATIONS', Vol. 4, Iss. 1, pp. 247-254.

Other Dissemination Materials for NEPHH Include:

- Six Monthly Newsletter.
- Six Monthly Dissemination Bulletin including main outcomes of the Technological Surveillance System.
- Project Brochure and Leaflet.
- NEPHH Project's Webpage.
- Project's Press Releases (both on internet support and printed media).
- NANOLCA 2011 – In a joint effort with NANOPOLYTOX and HINAMOX FP7 founded projects.



6 NEPHH's Expectations

NEPHH will contribute to ensure the generation of quantitative data on engineered nanomaterial toxicology and ecotoxicology and to close the knowledge gap, providing the basis for meeting regulatory requirements for responsible and sustainable

development of Nanotechnology. Validated testing strategies for novel materials are envisaged.

7 Directory

Table 1 Directory of people involved in this project.

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NeuroNano

Do nanoparticles induce neurodegenerative diseases? Understanding the origin of reactive oxidative species and protein aggregation and mis-folding phenomena in the presence of nanoparticles



Contract Agreement: NMP4-SL-2008-214547

Website: <http://www.neuronano.eu>

Coordinator: Kenneth Dawson, Centre for BioNano Interactions, University College Dublin, Belfield, Dublin 4, Ireland

No.	Beneficiary name	Short name	Country
1	National University of Ireland, Dublin / University College Dublin	NUID UCD	Ireland
2	University of Edinburgh	UEdin	United Kingdom
3	University College Cork	UCC	Ireland
4	University of Ulster	UU	United Kingdom
5	Helmholtz Zentrum Munchen	HELMUC	Germany
6	JRC – Joint Research Centre of the European Commission	JRC	Belgium
7	University of Rochester	UR	USA
8	The Regents of University of California / University of California, Los Angeles	UCLA	USA
9	Rice University	RU	USA
10	National Institute of Materials Science	NIMS	Japan
11	Universidade Federal do Ceará	UFC	Brazil

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1 Summary

NeuroNano is a Small Collaborative Project funded by the European Commission 7th Framework Programme. The project started on February 1st 2009 and will end on 31st January 2012 (duration 36 months). NeuroNano drew together a unique team, several of whom have pioneered the preliminary results in this field, and supplements them with the necessary skills and facilities required to address these questions. It is a knowledge-based approach, first probes the questions in the deepest manner, isolating each separate element of the nanoparticle's physico-chemical qualities that control fibrillation and oxidative stress, and access to the brain, determining their consequences separately.

The background that led to the formulation of the NeuroNano project include the following issues:

Nanoparticles may reach the brain –evidence that nanoparticles less than 40 nm particles can potentially pass through the blood-brain barrier.

- Nanoparticles may induce oxidative stress in living systems. Oxidative stress from ambient or combustion particles contribute to cell damage, including DNA damage.
- The large surface area of nanoparticles means that they can modulate the fate of protein fibrillation in solution. Whether this has significance *in vivo* is a key question that will be determined within the NeuroNano project.
- Oxidative stress and protein fibrillation are both associated with neurodegeneration.



Based on these issues, the key questions that were being addressed within the NeuroNano project were directed towards understanding the implications of these initial observations, separately and in combination, on the potential for a role for nanoparticles in neuro-degenerative diseases. Thus, the overall objectives of the NeuroNano project are:

- To determine if engineered nanoparticles present a significant neuro-toxicological risk to humans;
- to assess nanoparticle impacts on oxidative stress and protein fibrillation;
- To correlate nanoparticle access to the brain with induction of oxidative stress and/or protein fibrillation;
- To develop a simple screening and risk assessment matrix for nanoparticles in neurodegenerative diseases.

Significant progress has been made in all areas of the project in terms of clarifying all of the issues, and to date, no clear hazards from the nanoscale have emerged.

2 Background

Neurodegenerative diseases currently affect over 1.6% of the European population, (Alzheimer Europe 2006) with dramatically rising incidence likely (in part) due to the increase of the average age of the population. This is a major concern for all industrialized societies. There is also some epidemiological evidence that Parkinson's disease is connected to environmental pollutants, and it is often noted that historically, reports of Parkinson's symptoms only began to appear after widespread industrialization. There is some general agreement that (for example) pesticides are a significant risk factors (McCormack 2002). There are also persistent claims, based on epidemiology, that pollution may also be a cofactor in Alzheimer's disease, but here the evidence is controversial. The risk that engineered nanoparticles could introduce unforeseen hazards to human health is now also a matter of deep and growing concern in many regulatory bodies, governments and industry. Some comments about the topic have appeared in the more general literature (Ball 2006; Phibbs-Rizzuto 2007).

The NeuroNano project builds on striking published findings, as well as preliminary data from most of the project partners. *Whilst at the present time there is no evidence to suggest an association between neurodegenerative disease and nanoparticles, given this data is prudent to strengthen the confidence that no such link exists.* At present there is at most significant circumstantial evidence that nanoscale particles could impact on such diseases. The program will, mindful of the importance of the issues, exercise extreme caution in interpreting the data, and a process of checking in additional laboratories findings relating to a toxicity due to the nanoscale will be implemented.

There is incontrovertible evidence that some engineered nanoparticles (for example 6nm and 18nm gold nanoparticles), entering intravenously or via the lungs can reach the brains of small animals. (Kreyling 2007) Indeed, they lodge in almost all parts of the brain, and there are no efficient clearance mechanisms to remove them once there. Furthermore there are suggestions that nanoscale particles arising from urban pollution reach the brains of

animals. (Elder 2007) The relevant particle fractions arise from pollution but their structure and size are similar to engineered carbon nanostructures. Secondly, many nanoparticles in contact with tissue induce oxidative stress, (Brown 2001; Xia 2006; Brown 2007; Duffin 2007) as well as various inflammatory mechanisms that could themselves lead to further oxidative stress (Block 2004; Nel 2006). Finally, it has recently been discovered that nanoparticles (many of significant industrial interest) can have highly significant impacts on the rate of fibrillation of key proteins associated with neurodegenerative diseases, (Linse 2007) and we will report here significant new findings for the proteins associated with Alzheimer's (amyloid β) and Parkinson's disease (α -synuclein).

Whilst the precise mechanisms leading to neurodegenerative diseases are not fully clarified, it is broadly agreed that the key effects involve the presence of early pre-fibrillar protein structures, neuroinflammation and Reactive Oxygen Species (ROS)-related processes. Thus, all of the links in the causal chain are now present for a credible expectation that nanoparticles could have impacts on the onset, progression or severity of neurodegenerative diseases. The main ideas and interconnections between them are laid out in Figure 1.

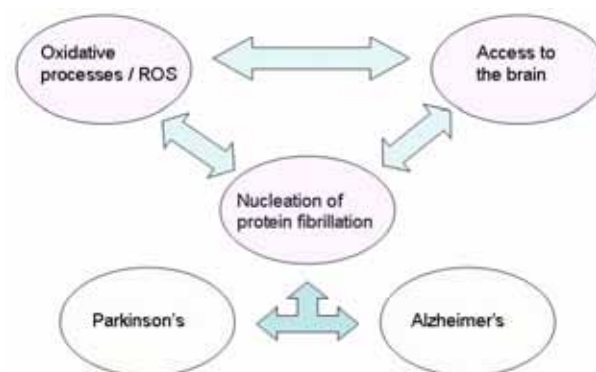


Figure 1. Overview of the interplay between various factors regarding nanoparticle interactions with living systems that could pose a risk for the development of neurodegenerative diseases.

This research program was deeply challenging, and entailed the gathering of entirely new knowledge in a field (neuronanotoxicology) that was itself emerging. It required the marshalling of unique expertise, methodologies, techniques and materials, many themselves completely new, and the whole never before brought together in the required combination.

The overall science and technology objective of this program was to determine if engineered nanoparticles could constitute a significant neuro-toxicological risk to humans for neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases. *The project did not presume neurotoxic hazard, nor indeed hope to discover such hazard.* Thus, a major focus was on the critical evaluation of the entire chain of reasoning leading to the present concerns. This was achieved by the detailed determination of cellular and molecular mechanisms involved a long time whole chain of effects induced by engineered nanoparticle-biological interactions, all in a dose dependent manner. The emphasis on mechanisms is important for it will advance the field of knowledge of neuronanotoxicology, irrespective of whether any clear disease endpoint emerges.

A risk-assessment framework

The generation of large quantities of data is not an end in itself. Instead, the data generated within the program is currently being consolidated into a deeper understanding of the risks posed by nanoparticles in terms of human health, disease and in particular neurodegeneration. A full study, and risk assessment would not be possible within this project, but the information can be prepared, and experiments framed in such a way to usefully inform a risk assessment. Thus, we shall attempt to transfer our scientific finding into a quantitative hazard report. The screening stage is based on the three parameters currently considered most likely to indicate a risk for neurotoxicity: access to the brain (we will determine a critical access threshold, likely $1/1000^{\text{th}}$ of the applied dose); the potential of the nanoparticles to cause oxidative stress (the Free Radical Generation Potential, FRGP), and the potential of nanoparticles to induce amyloid fibril formation (Amyloid Fibril Generation Potential, AFGP), and thereby seek to predict the outcome for several examples. Engineered nanoparticles were planned to be ranked according to these three parameters, and mapped onto the FIBROS map (Figure 2).

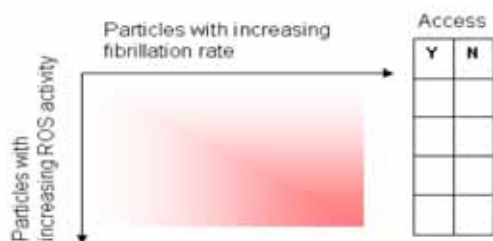


Figure 2. The FIBROS map as a potential screening assessment for nanoparticle risk in terms of neurotoxicity. Engineered nanoparticles will be tested for their oxidative effects, fibrillation effects and access to the brain.

This approach is needed not just for the purposes of informing a risk assessment, but in directing the progress of the Program itself. Thus, nanoparticles that score highly in terms of FRGP, AFGP and access to the brain will automatically proceed to the full-scale animal studies including behavioural and cognitive studies. Selected examples of particles that score on two out of the three parameters (representative examples of each possible combination) will also be tested in the animal studies, in order to validate the FIBROS map as an indicator of neurotoxicity potential. This will enable us to ensure that we do not prematurely rule-out any potential sources of nanoparticle-induced neurotoxicity.

If the assumed correlations are successful, then this type of representation could have immense value. It would indicate those levels of oxidative stress and amyloid load that constitute a serious hazard, and give clear guidance to regulators and industry on the thresholds. In time, if the in-cell determinations of the oxidative stress and amyloid load prove reliable, a single FIBROS map (the original having been validated with animal studies) would be sufficient to characterise the hazard from new engineered nanoparticles. Such a no outcome would represent a durable contribution to science, and a highly significant contribution to society at large.

However, as will be shown in detail in sections 4 and 5, this framework was too simplistic and the hypothesis was incomplete, particularly a round the pre-requisite that nanoparticles need to

access the brain in order to exert an effect, but also in terms of the possibility to rank nanoparticles in terms of the oxidative stress and fibrillation potential. Much more subtle aspects, such as the role of the protein corona, the nanoparticle as a scaffold for proteins and biomolecules, thereby enabling engagement of cell surface receptors and other aspects of the bionano interface also need to be considered.

3 Project Description and Organisation

The NeuroNano project was organised into 5 scientific and 1 management Work Packages, as illustrated graphically in Figure 3. The inter-dependencies of the Work Packages are also illustrated here. The flow of work in the 3 central experimental Work Packages was based on a tiered approach, where experiments are conducted in order of increasing system complexity – experiments in solution, experiments *in vitro*, and finally, experiments *in vivo*. The purpose of this approach was to establish (where possible) *in vitro* methodologies to assess the potential neurotoxicity of engineered nanoparticles, and to reduce the numbers of animals needed in the course of the project, in line with the European Commission policy on alternative methods to animal studies (reduction, replacement and refinement - Directive 86/609/EEC).

The project was a challenging, multi-disciplinary work, which aims to investigate the (potential) role of nanoparticles in neurodegenerative disease. Many of these diseases are themselves quite controversial scientifically, with considerable debate as to the molecular origins of the diseases. We may add the fact that there was essentially no literature on the role of nanoparticles in neurodegenerative disease when the project commenced.

The three main strands of the project were investigation of the potential of nanoparticles to induce reactive oxygen species, to induce protein fibrillation, and to cross the blood-brain barrier. As shown in Figure 3 each of the three phenomena will be investigated at all levels of complexity, from *in vitro* cell line studies to full animal studies (using well designed experiments, where the number of animals used will be minimised, and the maximum information gained from each animal, by utilising the tissue in multiple-level experiments such as the protein corona and “omics” studies, following the translocation and behavioural studies, for example). Thus, for each of the three main strands, the consortium included the relevant and most appropriately skilled partners with expertise at cell-level, animal level, and (where appropriate) at human and disease level.

The tiered approach to the three strands of work relating to the assessment of neurodegenerative disease (in solution studies, in cell studies, and finally in animal studies) represents a scientifically and ethically balanced approach to the work, balancing the necessary level of scientific excellence with the need to reduce the numbers of animal experiments.

The inclusion of novel approaches such as the redox proteomics and transcriptomic and proteomic assessment of cellular and tissue responses to nanoparticles (in terms of oxidative stress, localisation and fibrillation), combined with a wider range of imaging techniques, offers the unique possibility to bridge the cell, tissue and animal studies. Indeed, this combined approach has been one of the key strengths of the project and has been very important in the success of the project overall.



concentrate on radiolabelling of industrially produced ST-01 TiO₂ NPs, which could be irradiated with protons without visible damage to the sample. A specially-designed irradiation capsule

was developed that optimised sample cooling while maximising the specific activity yield.

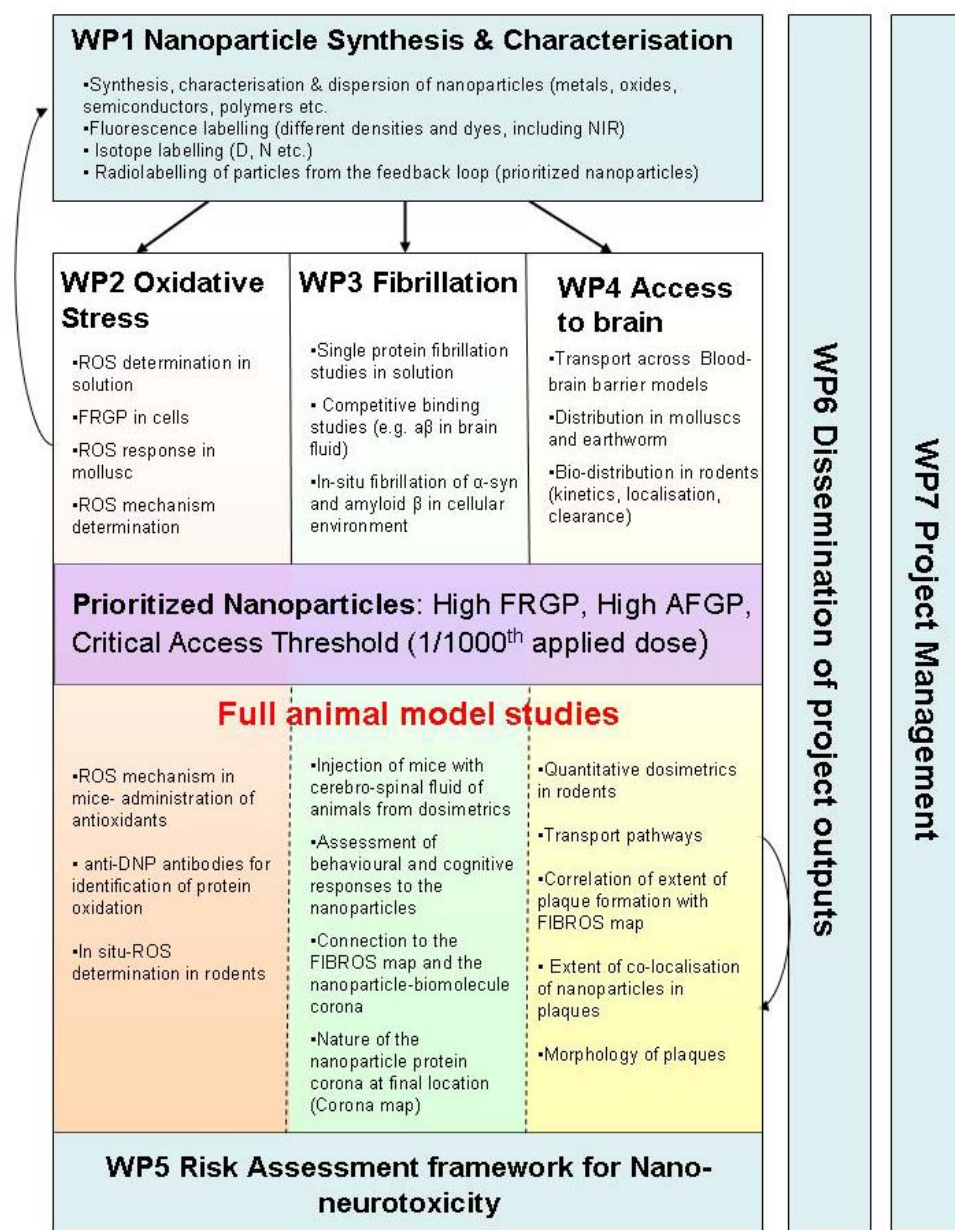


Figure 3. Workflow and ideological layout of the work packages and the information flow within the NeuroNano project.

4 Progress to date

Nanoparticle radiolabelling

Industrial TiO₂ nanoparticles have been successfully radiolabelled in dry form, then dispersed, size-selected, and delivered to HELMUC for *in vivo* studies. The aim was to be able to radiolabel in-house synthesised TiO₂ NPs for which a protocol was available for full dispersion (to primary nanoparticles in suspension). After several attempts it was decided that irradiation of dry TiO₂ NP samples followed by subsequent full dispersion could not easily be achieved. While this goal remains under study, it was decided to

Several test irradiations on different types of TiO₂ were carried out, and XRD structural damage assessment was performed on P25 and ST-01 TiO₂ NPs after proton irradiation. The XRD results indicated that no significant damage was caused, even at activity levels as high as 1 MBq/mg, achieved after 20 hours irradiation. Much time was spent in developing a dispersion and size-selection protocol in order to achieve a “bioavailable” suspension of activated ST-01 TiO₂ NPs. This included several steps of dispersion, leaching, centrifugation, “washing” and filtration. In late 2009 and early 2010, three sets of activated ST-01 TiO₂ NP samples were delivered



to HELMUC for *in vivo* biokinetics studies. The suspension consisted of NPs of approximately 100 nm hydrodynamic size (DLS performed at the HELMUC), that were stably labelled to a radioactivity level of more than 1 MBq/mg. The *in vivo* studies indicated only minimal release, if any, of ^{48}V from the activated nanoparticles.

Gold nanoparticles have been successfully radiolabelled, and the JRC can produce an activated suspension with up to some hundreds of kBq of ^{198}Au per mg of gold nanoparticles, enough for some *in vivo* studies. Higher ^{198}Au activities may be produced by reactor irradiation.

The neutron capture cross sections for stable cerium isotopes are all very low which means that ion-beam activation has to be used for radiolabelling of ceria nanoparticles. For *in vivo* studies the ^{141}Ce radioisotope is probably the most suitable, with a half-life of 32.5 days. It can be produced in significant quantities using the (d,p) reaction on natural CeO_2 . One problem with using deuterons to activate the nanoparticles is that refrigerated helium rather than water cooling has to be used to cool the irradiation capsule, and that the energy deposition of deuterons in the sample is higher than with protons. Thermal and radiation damage are higher than with proton irradiations, but activations are lower beam currents can be used. Deuteron activation tests have been performed on CeO_2 , indicating a yield of 0.05 MBq/mg with an irradiation of 5 hours at 2 μA . At this relatively low activity DLS studies indicated no significant changes to the NP powder size distribution. Leaching studies in water indicated no significant radiotracer release from the activated NPs. Higher level activations have not been tried yet.

Experiments have been carried out on radiolabelling of carbon-based (carbon black, MWCNT, ...) nanoparticles. ^7Be is created via the $^{12}\text{C}(p,3p3n)^7\text{Be}$ reaction in reasonable quantities, with a half-life of 53 days. Calculations and experiments indicated no major recoil problems and the ^7Be remained well attached to activated carbon black NPs and to MWCNTs in water. The activation yield measured on carbon black was 4 kBq/($\mu\text{A}\cdot\text{h}\cdot\text{mg}$), meaning an irradiation of 25 hours at 5 μA would give 0.5 MBq/mg, enough for *in vivo* tracing studies given the relatively long half-life of the ^7Be radiotracer.

An additional development has been the activation of gold electrodes using high energy protons. The (p,3n) reaction creates ^{195}Hg , which decays with a half-life of about 2 days to ^{195}Au . This gold isotope has a half-life of 186 days and is therefore useful for longer term *in vivo* studies. The gold electrodes were provided by HELMUC, activated over several days at the JRC cyclotron, and sent back to HELMUC after an appropriate period of decay (some weeks) to allow the ^{195}Hg to decay to ^{195}Au .

Following on from this unique work, silver electrode activation tests have now also been carried out. Again, high energy protons are required to induce the (p,3n) reaction, producing ^{105}Cd which has a half-life of 56 minutes and decays to ^{105}Ag . This silver isotope has a half-life of 41 days, ideal for *in vivo* radiotracing.

Fe_3O_4 NPs have also been activated directly, using the $^{56}\text{Fe}(p,n)^{56}\text{Co}$ reaction. Subsequent tests showed no thermal or structural damage and *in vitro* uptake tests indicated a similar behaviour to non-activated NPs.

A unique recoil method has been developed to radiolabel NPs that cannot be directly activated with either neutrons or ion-beams – e.g. SiO_2 and Al_2O_3 . Making use of momentum conservation during nuclear reactions, NP samples were mixed with Li_2O and the recoiled ^7Be created from Li via the (p,n) reaction can be used to

radiolabel the target NPs. Initial tests have been very successful and refining of this method is underway

Another radiochemical method was also developed for synthesis of ^{56}Co labelled SiO_2 . An iron foil was irradiated to produce ^{56}Co . The foil was then dissolved and the ^{56}Co radiochemically separated from the iron. The radioisotope was introduced into the precursors for SiO_2 synthesis and the results indicated good integration into the SiO_2 structure and good stability.

Nanoparticle dispersion

Within EuroNano, a novel strategy to disperse titanium nanoparticles in water at physiological pH from dry powders, using molecules of low toxicity, such as gallic acid, citric acid, dopamine and sodium pyrophosphate, which are naturally occurring in the body, and which bind irreversibly to the nanoparticles has been developed. These molecules form complexes with some of the Ti present on the surface of the NP. Since these molecules have a charged end, they increase the Zeta potential of the NP, hence improving their dispersion. The dispersions prepared using these ligands presented good stability over a minimum period of two weeks storing the NP suspension at room temperature. NP suspensions of high concentrations (up to 10 mg/mL) were prepared using this strategy. Although dispersions with the nominal particle size have not yet been achieved, suspensions with a monodistribution of agglomerates of less than 50 nm were successfully achieved, as shown in Figure 4.

The protocol consisted of mixing the titanium with 0.02 M citric/citrate buffer at pH 7, and sonicating it using a probe sonicator for 15 min applying 50 W as 8 s pulses with 2 s breaks between pulses. The large aggregates were successfully eliminated by centrifugation of the samples for 1 min at 4000 rcf. The excess citrate was eliminated successfully by dialysis to levels that were compatible with most *in vitro* studies, and the larger particles were successfully eliminated by a simple centrifugation process.

The EuroNano protocol led to dispersions that were stable for months, rather than minutes like those used in many studies. The limiting factor in the lifetime of these suspensions was the appearance of bacteria rather than agglomeration and sol instability (see Figure 4). These dispersions were obtained from dry powders and provide a technique to study manufactured as well as synthesised samples. This ability to re-disperse powders is important because radiolabelling (for *in vivo* studies), phase/size changes and other modifications can be more readily achieved using nanopowders compared to solution phase materials.

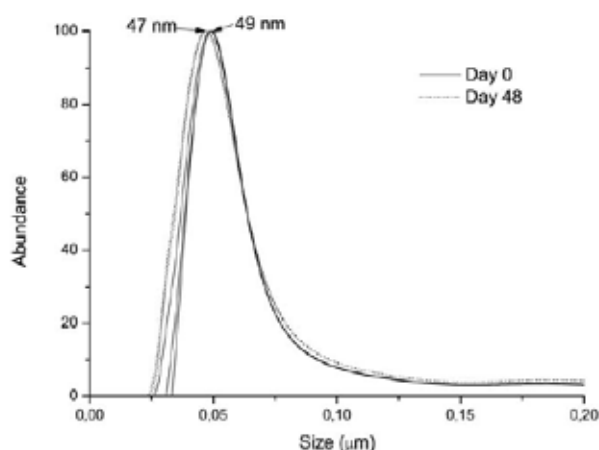


Figure 4. The stability of a 10 mg/mL titania suspension stabilised with citrate (excess citrate was dialysed out). (Ramirez-Garcia 2011)

This is a very simple but effective method that can be easily implemented in a very reproducible manner in any dispersions laboratory. Moreover, the resulting suspensions were stable in cell media with different concentrations of plasma. The protein corona formed on this titania and also onto titania with other crystal structures is currently under investigation in our laboratory.

Alternative routes to oxidative stress?

The classical understanding of oxidative stress is that it represents an imbalance between the production of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of tissues can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Some reactive oxidative species can act as messengers through a phenomenon called redox signaling.

Key observations from NeuroNano regarding the role of nanoparticles in the induction of oxidative stress responses by cells represent significant departures from conventional understanding. The full consequences of these observations are still under consideration, and will be reported in a detailed future course. These observations include:

1. The way nanoparticles are prepared and dispersed, and what they pick up from their surroundings into their biomolecule corona, determines so much of their fate and behaviour. This is reflected in the fact that amine modified polystyrene nanoparticles with different degrees of amination of the surface induced quite different responses - one induces catalases while the other does not.
2. There is emerging evidence that oxidative stress may not be caused only by the formation of Reactive Oxygen Species, and thus, attempts to rank the toxicity of nanoparticles on that basis only will fail. Thus, the FIBROS map requires more detailed mechanisms to identify the oxidative damage, such as the formation of protein lesions such as carbonyls or thiols. The approach of redox proteomics is shedding important light on some very subtle protein lesions, even

using particles that are considered fully benign by all other methods, e.g. COOH-polystyrene nanoparticles. This suggests that nanoparticles that accumulate could induce some effects, even if the particles themselves are entirely passive.

3. Oxidative stress may have signalling origins, and again the biomolecule corona effect on this may greatly affect signalling. For example, a recent paper suggested that binding of fibrinogen to nanoparticles in an orientation that allows it to engage with the MAC-1 cell receptor initiated a signalling cascade resulting in oxidative stress, whereas the same protein bound to large nanoparticles of identical composition did not induce oxidative stress. (Deng 2010)

These findings might also explain some of the differences in data reported in the literature. A key outcome from NeuroNano will be a comprehensive review of the sources and types of nanoparticle-induced oxidative stress.

There is always the concern with *in vitro* studies that the use of immortalised or cancer-derived cells introduces anomalies that are absent from equivalent primary cells or *in vivo*. However, within NeuroNano we have used a high content analysis approach and found no evidence of differential responses to nanoparticles of primary or cultured astrocytes in terms of impacts including oxidative stress.

Nanoparticle access to the brain

In the conception of NeuroNano, the focus was on determining which nanoparticles could potentially reach the brain, and what it was about these nanoparticles, or their protein/biomolecule coronas that enabled them to cross the Blood-Brain barrier or reach the brain via the olfactory nerve. However, it turns out that this too was somewhat simplistic, as it has emerged from work outside NeuroNano, and using the foetal barrier as a model, that nanoparticles have the potential to signal across the biological barriers, without crossing themselves. (Bhabra 2009)

Based on the work within NeuroNano on the *in vitro* blood brain barrier, another more pertinent question may be whether crossing of the barrier is actually not the key issue, but rather whether poisoning of the barrier cells due to endocytosis of nanoparticles and their localisation in the lysosomes of the barrier cells, with consequent signalling impacts, may in fact not be the greater potential harm. The logic for this is that for each nanoparticle that crosses the biological barrier, there are very many more nanoparticles trapped in the lysosomes of the barrier cells, as this has been shown to be the final location for large numbers of nanoparticles in both these and other cell lines. Thus, crossing of the biological barrier will be an extremely rare event, and instead accumulation in the endothelium is potentially the major sink for nanoparticles.

This scenario of a accumulation of these nanoparticles in the lysosomes of the barrier cells is consistent with everything we know at cell level. For that not to happen in barrier would require some very significant biological signalling to redirect the nanoparticles from the endo-lysosomal pathway to the transcytotic pathways. In the same way that we have been unable



to identify exit pathways for nanoparticles from a range of cell types (e.g. lung epithelial cells), we have also

However, discussions at the joint NeuroNano-ESF EpitopeMap workshop on nanoparticle interactions with biological barriers (6th and 7th December 2011) suggested that such large numbers of lysosomes have not been observed *in vivo*. Indeed most *in vivo* dosimetric studies consider the nanoparticle load in the brain endothelium as being in the brain (as distinct from being in the blood). This raises the need for some additional approaches to distinguish nanoparticles contained in the barrier tissue itself from nanoparticles that have actually crossed through the biological barrier into the brain tissue. It also suggests that further identification of the sub-cellular localisation of nanoparticles in tissue *in vivo* might shed some important light on this question.

In vivo data showed that unconjugated Au (citrate stabilised) nanoparticles were rapidly cleared and within 30 minutes were almost all in liver following IV injection. A Apo-E functionalised Au nanoparticles showed a different biodistribution *in vivo*, where the particle load in the liver decreased from 90 to 70% and the particle load in the spleen increased to close to 20%. In contrast, for Albumin-functionalised Au nanoparticles, distribution in both the lungs and spleen was significant, while the particle load in the liver was dramatically low (Kreyling et al, manuscript in preparation). This links well with the findings that the nature of the adsorbed proteins has a dramatic role in determining the uptake and biodistribution of nanoparticles. In terms of access of these nanoparticles to the brain, Apo-E functionalised Au nanoparticles reached the brain by a factor of 10 more than citrate stabilised Au nanoparticles, and the nanoparticle load in the brain was observed to decrease over the subsequent 10 hours. The effect was even more prominent for the Albumin-Au NPs, suggesting that albumin plays a role in determining retention in endothelium or penetration into brain.

Behavioural effects of nanomaterials

A key element of the project was to connect exposure to nanomaterials with any observed behavioural changes. While much of the data is still under evaluation, and as such it is too early to draw significant conclusions, some behavioural effects were observed in two species studied – mice and ragworm.

In the ragworm, exposure to both CuO₂ (1000 ppb) and TiO₂ (2000 ppb) nanoparticles resulted in a distinct decrease in burrowing behaviour, after 10 days of exposure, with some mortality also observed. Histology effects are currently being analysed. The full study is being repeated to determine the dose-response curve and redox proteomics is also underway. It is worth noting that the route of exposure for worms is oral, and that the worm is a model for Parkinson's disease.

Learning, memory, attention, and performance are elements of cognitive function that can be assessed in a variety of tests, such as the water maze or the lever-press response. Behavioural effects following direct injection of relatively high doses of TiO₂ nanoparticles was assessed, and while the data are still being processed, preliminary results suggest that here also effects were persisting over 10 days, which is an unusual response that is never observed with chemicals.

5 Significant outcomes / conclusions

Radiolabelled nanoparticles synthesised by JRC

A key output from NeuroNano is a range of radio-labelled nanoparticles for use in dosimetric / biokinetics or environmental fate and behaviour studies, or other studies where tracking of nanoparticles over specific time periods is required.

Pre-synthesised Au, CeO₂, Fe₃O₄, and TiO₂ nanoparticles can be radiolabelled using ion-beam methods, and Au and Ag electrodes can be activated for subsequent nanoparticle synthesis.

Significant progress towards activation of carbon-based nanoparticles has been made, with direct high-energy proton bombardment and a novel recoil method both achieving good levels of activity in MWNTs. Some further work has to be performed to check for irradiation damage effects. The recoil technique can be used to radiolabel nearly any type of nanoparticle, and successful initial experiments have been performed on SiO₂ and nanodiamonds.

Radiochemical synthesis methods can also be used to synthesise labelled nanoparticles starting from precursors with trace amounts of radioisotopes. In this way, SiO₂ nanoparticles labelled with a Co-56 radiotracer have been successfully created. This route can potentially be used to synthesise many different types of radiolabelled nanoparticle under carefully controlled laboratory conditions.

Access to radiolabelled nanomaterials is possible via the QNano Research Infrastructure (www.qnano-ri.eu), or by contacting the JRC (Neil.Gibson@jrc.ec.europa.eu), assuming that all necessary safety provisions and permissions for handling radioactive materials are in place, that the irradiation/labelling process can be appropriately included in the JRC cyclotron's schedule, and that the requesting institute provides the necessary input (knowledge, assistance, etc.) in the case of new experiments.

Unique protocols developed covering a range of aspects

Protocols covering a variety of aspects, from nanomaterials synthesis and characterisation, surface functionalisation and dispersion, to all aspects of nanoparticle uptake and localisation, apoptosis, assessment of changes in gene expression, oxidative stress and redox proteomics, dosimetrics and biokinetics and assessment of behavioural impacts of nanomaterials, have been developed within NeuroNano. Many have already been published as part of scientific manuscripts, and many others are currently in final stages of preparation for publication.

As part of the wrap-up of the NeuroNano project, these protocols are currently being re-formatted into the template designed by NanoImpactNet and will be made available to the community via the NanoImpactNet protocols database, which will be taken over by QNano and/or the NanoSafety Cluster.

Recommendations for further research

As part of the NeuroNano final meeting (5th December 2011), and in collaboration with the ESF EpitopeMap Research Networking



Programme¹, a joint workshop was held in Dublin on 6th - 7th December 2011 that brought together leading European experts² in Engineered nanoparticle transport across biological barriers to identify the central questions that require scientific and policy attention as a matter of priority. Among the outcomes from the workshop of particular relevance for NeuroNano were:

Accidental 'targeting' of ENP to the brain – need for implementation of "protein corona" screening

Many of the "success" stories to date in terms of using engineered nanoparticles to deliver drugs to the brain have been the result of the ENP surface offering an appropriate scaffold for known blood-brain-barrier receptors, such as the LDL receptor. In fact, one key study shows that having a specific surfactant coating (polysorbate 80 - Tween® 80) that spontaneously binds apolipoprotein E and apolipoprotein A-I from the dispersion medium was as effective at delivering the drug to the brain as the specifically surface-engineered ENP where these apolipoproteins were chemically grafted to the ENP. (Kreuter 2002; Kreuter 2004; Zensi 2010) This raises a very important question as to whether other ENP, which were never intended to reach the brain (or indeed other key organs, or the foetus), could unintentionally bind such targeting protein coatings and then reach delicate organs with very high efficacy.

Regulatory policy recommendation: Interaction of ENP with transporter proteins under competitive conditions could potentially be a n indicator or risk, and could become part of a "safety by design" strategy for design of ENP surfaces.

Need to greatly improve in vitro and ex vivo models to correlate outcomes with in vivo effects in order to reduce reliance on animal studies and address developmental toxicity questions

A surprising outcome of the workshop was how little we know about the extent to which current *in vitro* blood-brain barrier models fully represent the *in vivo* situation, and how much is just taken as common knowledge. Concerns raised include the fact that: barrier models never achieve the same levels of electrical resistance as *in vivo* barriers; imaging of cellular barriers often reveal holes due to incomplete cell coverage, and mono-cell barriers are missing many of the signalling inputs that would be present *in vivo*, such as interactions with co-located pericytes and glial cells (in the case of the blood brain barrier) that also may induce tighter junctions and greater barrier specificity.

This could suggest that *in vitro* barrier models are unlikely to reproduce transcytotic pathways, and may be under-reporting on transport across the barriers, and over-reporting on transport into the barrier and accumulation within lysosomes inside the barrier cells. It was interesting to note that *in vivo* brain endothelial cells appear to have far fewer lysosomes than analogous cells studied *in vitro*. Careful quantification of these differences and an understanding of their significance are needed.

There was uncertainty about whether, how and over what timescale biological barriers are renewed *in vivo*, and the consequences of this for bioaccumulation of ENP, especially in response to chronic low level exposure.

Finally, the question of indirect signalling effects from ENPs localised in or near barriers that do not themselves cross needs to be explored as a matter of urgency, especially with regards to the brain and placental barriers.

Funding recommendation: Research is needed to improve existing and create new barrier models and develop clear *in vitro-in vivo* correlations, including appropriate culture conditions (serum free) to optimise barrier functioning and cell-cell (paracrine) signalling. Research should be directed towards the use of co-culture and 3D cell culture approaches, and towards comparative *in vitro* and *in vivo* studies under conditions that are as similar as possible, and where limitations are documented and understood.

Infrastructure needs for nanosafety and nanomedicine

A key concern relates to large scale facilities and specialised research centres for supporting the safety and efficacy assessment of ENP (for example facilities for radiolabelling ENP and assessment of the biodistribution and biokinetics of radiolabelled-ENP). Funding to support such facilities, and the mobility of EU researchers / post-docs for their wider exploitation, over the coming decade is vital, as many questions regarding safety and efficacy of ENPs can only be answered by the use of not yet available radioactive nanomaterials. In a parallel fashion, support to maintain the intellectual infrastructure (appropriately trained personnel) is required to respond adequately to EU needs in relation to ENP.

¹ EpitopeMap is an ESF Research Networking Programme to understand the role of the protein corona in determining nanoparticle biocompatibility.

² Experts included David Bogley, Jörg Kreuter, Patrick Case, Antonio Pietroiusti, Peter Wick, Wolfgang Kreyling, Christina Schulze, Andreani Odysseos, Marcello Cacace, Margaret Saunders, Luisa Campagnolo, Joseph Brain, Kenneth Dawson, Iseult Lynch, C. Vyvyan Howard and Neil Gibson.



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7 Directory

Table 1 Directory of people involved in the NeuroNano project

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NHECD

Creation of a critical and commented database on the health, safety and environmental impact of nanoparticles



Contract Agreement: NMP4-SA-2008-218639 Website: <http://www.nhecd-fp7>
Coordinator: Professor Oded Maimon, Tel Aviv University, Tel Aviv, Israel

No.	Beneficiary name	Short name	Country
1	Tel Aviv University	TAU	Israel
2	tp21 GmbH	TP21	Germany
3	Joint Research Centre	JRC	Italy
4	IVAM Research and Consultancy on Sustainability	IVAM	The Netherlands

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1 Overview

1.1 Project title

Creation of a critical and commented database on the health, safety and environmental impact of nanoparticles

1.2 Acronym

NHECD

1.3 Start and End date

Start date: December 1st, 2008

End Date: November 30st, 2012

Duration: 48 Months

1.4 Size

Project Funding: 1.4 M€



2 Summary

NHECD is free access, robust and sustainable web based information system including a knowledge repository on the impact of nanoparticles on health, safety and the environment. It includes a robust content management system (CMS) as its backbone, to hold unstructured data (e.g., scientific papers and other relevant publications). It also includes a mechanism for automatically updating its knowledge repository, thus enabling the creation of a large and developing collection of published data on environmental and health effects following exposure to nanoparticles.

NHECD is based on text mining methods and algorithms that make possible the transition from metadata (such as author names, journals, keywords) to more sophisticated metadata (such as whether the paper contains graphs) and to additional information extracted from the scientific papers themselves. These methods and algorithms were implemented to specifically extract pertinent information from large amount of documents. NHECD created a systematic domain model of concepts and terms (i.e., a wide set of domain taxonomies) to support the categorization of published papers and the information extraction process within this project.

The unique features of NHECD allow different user groups - academics, industry, public institutions and the public at large - to easily access, locate and retrieve information relevant to their needs. The creation of the NHECD knowledge repository enriches public understanding of the impact of nanoparticles on health and the environments; it supports a safe and responsible development and use of engineered nanoparticles; and represents a useful instrument for the implementation of relevant regulatory measures and law making.

3 Background

The potential of Nanotechnology to bring scientific advancement and different economic benefits is strictly dependent on the success of the approaches and strategies chosen to guarantee a safe and responsible development, production and use of engineered nanoparticles and nano-technology-based materials and products.

Research on the health, safety and environmental impact of nanoparticles is currently rising due to the enlarged interest of the general public as well policy makers. On one hand, European industry and consumers want to learn more about these issues since a growing number of commercial products containing nanoparticles are currently advertised; on the other hand, environmental groups and ethical committees are asking for the implementation of clear and defined regulatory measures concerning the products development and research experiment involving Nanotechnology and especially nanoparticles.

To meet this growing demand, different institutions such as Universities and National Task groups have started launching electronic information repository, web portals etc. to provide to the public access to all sorts of documents on related topics. However, the majority of the existing databases and content management systems are operated manually, i.e. only a limited

amount of data can be processed, and the taxonomy and ontology guiding the documents' categorization procedure.

Due to the steadily increase of scientific papers and other types of publications within this field, there is an urgent need for a new quality of information management capable of handling and processing large amounts of different types of pertinent documents.

4 NHECD Achievements To-Date

NHECD, currently at the end of month 36, has by now achieved:

- NHECD frontend, the interface of NHECD to the three communities targeted for it, namely nano-tox scientists, regulators and the public. NHECD frontend is a comprehensive solution designed to allow users to search for relevant information using a state-of-the-art graphical user interface (GUI) matching diverse types of users (regular users, sophisticated users and more). The GUI allows for taxonomic search, simple / advanced search, full-text search, intelligent search (a unique method enables researchers to search for the information extracted from the scientific papers) and any combination of the above search methods.

NHECD website is reachable at <http://nhecd.jrc.ec.europa.eu>

- A backend system based on a robust content management system and its accompanying modules such as classification, full text search and more.
- A crawling system intended to navigate selected websites in order to obtain (automatically) all the relevant published material related to NHECD.
- A rich set of computer based taxonomies related to the NHECD target areas.
- A body of classified knowledge consisting of scientific papers related to in-vivo/in-vitro, ecotox and occupational nano-toxicology. The corpus currently contains around 10,000 papers in NHECD subject.
- Online validation tools (IE and crawler) used to train the system to extract information and enhance the quality of the results, as well as to build and maintain the above body of knowledge.
- Information extraction (IE) algorithms and methods especially crafted to create a layer of comments (in the context of NHECD the comments are an information layer on top of the scientific paper itself) to enhance the knowledge found on NHECD's body of knowledge. IE results satisfactory and maturing, yet there are some issues, such as relation extraction, for which the literature is still being developed. NHECD keeps working towards achieving optimal algorithms.
- The infrastructure for the backend, frontend and all related components is established along with administration and maintenance procedures.



- A working relationship with groups of nano-tox related people, including outstanding scientists and regulators.
- The possibility of collaborating with the leading publisher's companies is being considered.

NHECD, soon to be at the end of the development phase, is aiming at the dissemination of the assets obtained (i.e., developed tools), while working on enlarging the repository. All of this is towards achieving a high quality of information and tools.

5 Organization of NHECD

NHECD scientific activities are conducted within five work packages (WPs, see table below). NHECD success is a consequence of the synergetic relationship between the work packages results, themselves a result of the synergy between the partners. Where possible, joint activities that were not initially planned were held to ensure that NHECD achieves its goals in an optimal way.

Table 1. Work packages (WP) of NHECD

WP	Title	Topic
1	Provision of Information Technology	Provide the information technology infrastructure enabling to establish and maintain on an on-going basis an automated retrieval, indexing and extraction of relevant results from scientific publications in common electronic document formats. The data will be categorized as per special domain taxonomy specially designed by domain experts for efficient navigation by novice users. The framework will enable domain experts to comment the extracted results while considering the journal impact factor and the novelty of results.
2	Toxicology	The overall goal of this WP is to provide knowledge in the domain of the impact of nanoparticles on health and environment for setting a data-base. This will be accomplished by providing appropriate taxonomy, prioritizing and ranking of the articles and selection of PDF articles, in addition to the provided meta-data, that will serve for a comprehensive data mining. An additional central goal is to validate, at each stage of development of the tools for data mining, the quality control of the developed process.
3	Tools and environment	To supply and maintain tool and infrastructure including hardware, software and networking services. The primary software includes open source content management, database and website front-end of the project, accessible to all the users per their definitions. On an ongoing basis, to provide system administration and management services including user management (anonymous users, registered users, etc.), security and access management, backup and recovery, and more.
4	Interaction with stakeholders and dissemination	Exchange with relevant stakeholder groups to integrate further information and meet requirements. Provision of purposeful information towards all target groups, and processing of feed-back in order to optimize the system. Comprehensive promotion. Evaluation of options to further operate NHECD after the project end.
0	Project Management	Ensure a smooth flow of the project activities – constant monitoring and steering of the work with the objective to achieve the project goals. Proper and timely execution of all administrative and financial tasks including the contractually required reports. Elaboration and implementation of corrective actions, where necessary. Faithful interaction with the Commission's representatives.



6 Work performed and Results

Following is a detailed description of two important components of NHECD, Information Extraction and NHECD User Interface. These components synthesize the investment of all NHECD work-packages and are reviewed in detail. The detailed description of the other components was given in previous publications.

6.1 Information Extraction

Information Extraction (IE) is a type of information retrieval whose goal is to automatically extract structured information from unstructured and/or semi-structured machine-readable documents. In most cases, this activity concerns processing natural language texts using NLP. Information extraction has numerous potential applications. For example, information available as unstructured text can be transformed into traditional databases that users can probe through standard queries.

6.1.1 IE system flow

The IE component is built in a modular linear flow. Each module can work independently. If needed, any of the modules can be separately changed.

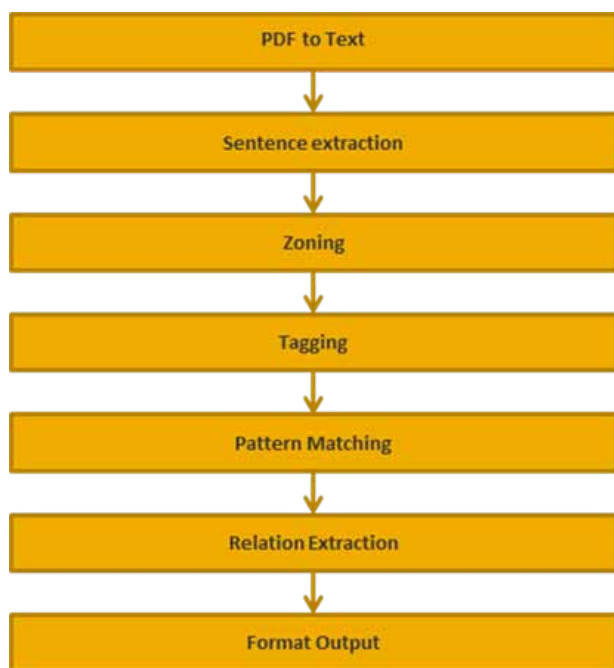


Figure 1 -IE System Flow

6.1.2 The process

The aim of NHECD IE component is to extract, from every scientific paper gathered by the NHECD crawler, a comprehensive, full and precise list of relations. NHECD text mining tasks are in fact information extraction tasks, namely, to extract entities and relations (which are, by nature, structured information) from unstructured Nanoparticle-toxicity related documents. The information extraction system expected results include the following entities or relations: (1) Nano particle, (2) Model – Cell model or animal, (3) Attributes – NP size, Zeta potential, animal

age, etc. and (4) Experiment attributes – mode of exposure, measurement assay etc.

See the following example of the information extraction task.

The examined text:

Under phase-contrast microscope, **HT-1080** cells (control) appeared polyhydric or stellate showing slender lamellar expansions (Fig. 1A) that joined neighboring cells. With increasing concentration of **SNP** (from 6.25 to 50 μ g/mL), cells were seen as less polyhydric; and more fusiform, shrunken and rounded.

The results of **XTT assays** (Fig. 3) showed a **dose-dependent cytotoxicity** for both the cell types with IC50 values of **SNP** working out as **10.6 and 11.6 μ g/mL** for **HT-1080** and **A431** cells, respectively

Cell model
NP name
Concentration
Assay
Result

Figure 2- IE examined text

Should result in the extraction of the following relations:

Chemistry	Cell model	Concentration	Assay	Effect	End Point	Measure Change
SVN	HT-1080	mcg/ml 6.25 to 50				less polyhydric
SVN	HT-1080	mcg/ml 6.25 to 50				more fusiform
SVN	A431			morphological change		
SVN	A431	mcg/ml > 6.25		cell numbers decreased significantly		
SVN	A431	mcg/ml 10.6 and 11.6	XTT Assays	effect on cell-division cycle	EC50	

Figure 3- IE relations result

6.2 The NHECD User Interface

The NHECD user interface offers different possibilities to retrieve efficiently knowledge on the health, safety and environmental impact of nanoparticles.

- A **BASIC SEARCH** interface to perform keyword and/or taxonomy based queries.
- An **ADVANCED SEARCH** interface for experienced users to extend the capabilities of the basic search using advanced search features such as logical operators (AND, OR, NOT), as well as allowing to search through extended metadata.
- The **INTELLIGENT SEARCH** is a unique method especially adapted to the needs of researchers in the nano-science field. This feature includes, among other capabilities, the power to search by model, experiment and nanoparticles attributes. The target is the exact data taken from the results of all experiments described in the corpus (shown in figure 4).



Further functionality allows for:

- Features such as save searches, recall of recent queries, managing a clipboard and organizing the data in collections.
- Tutorials guide the user to efficiently use the interface.

In addition to the search functionalities provided, a variety of further information resources related to the health, safety and environmental impact of nanoparticles is available:

- A White Paper Forum allowing registered
- Users to comment on White Papers entered by a moderator.
- Further features include an introduction to the field, information on corresponding rules and regulations, news and events, and inform the users on relevant developments and activities related to the potential impact of nanoparticles on health, safety and the environment.

The User interface homepage is shown in the next figure:



Figure 4- Frontend website-Home page

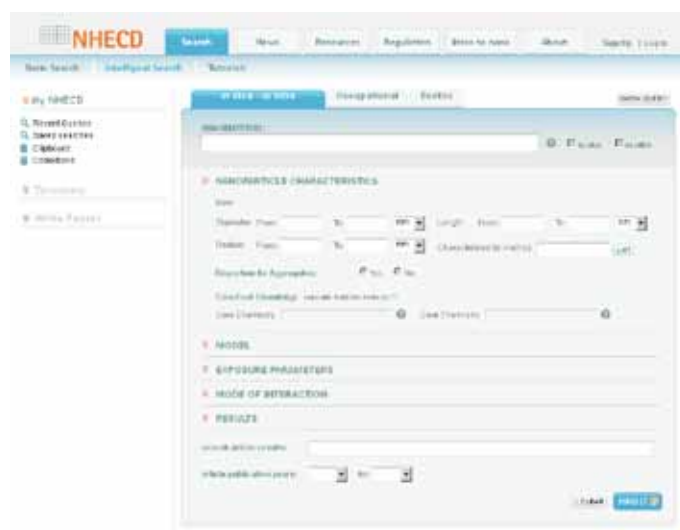


Figure 5 -Frontend website -intelligent search



7 Directory

Table 2. Directory of people involved in this project (partial list)

First Name	Last Name	Affiliation	e-mail
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8 Copyright

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QNano

A pan-European infrastructure for quality in nanomaterials safety testing



Contract Agreement: SP4-Capacities-2010-262163

Website: <http://www.qnano-ri.eu>

Coordinator: Kenneth Dawson, Centre for BioNano Interactions, University College Dublin, Belfield, Dublin 4, Ireland

No.	Beneficiary name	Short name	Country
1	National University of Ireland, Dublin / University College Dublin	NUID UCD	Ireland
2	Natural History Museum	NHM	United Kingdom
3	Institute of Occupational Medicine	IOM	United Kingdom
4	Joint Research Centre of the European Commission	JRC	Italy
5	Federal Institute for Risk Assessment	BfR	Germany
6	Karlsruhe Institute of Technology	KIT	Germany
7	Facultes Universitaires Notre-Dame De La Paix	FUNDP	Belgium
8	Institute for Work and Health	IST	Switzerland
9	University of Leeds	UL	United Kingdom
10	Norwegian Institute for Air Research	NILU	Norway
11	Helmholz Centre Munich	HMGU	Germany
12	Ludwig-Maximilians Universität, München	LMU	Germany
13	Centro de Investigación Cooperativa en Biomateriales	CIC	Spain
14	Uppsala University	UU	Sweden
15	Institut Català de Nanotecnologia - Consejo Superior de Investigaciones Cientificas	ICN	Spain
16	Stichting Dierlandbouwkundig Onderzoek	DLO	Netherlands
17	Wageningen University	WU	Netherlands
18	Deutsche Gesetzliche Unfallversicherung	BGIA	Germany
19	Tel Aviv University	TAU	Israel
20	Slovak Medical University	SMU	Slovenia
21	Vlaamse Instelling voor Technologisch Onderzoek	VITO	Belgium
22	Trinity College Dublin	TCD	Ireland
23	Finnish Institute of Occupational Health	FIOH	Finland
24	University of Exeter	UOE	United Kingdom
25	Edinburgh Napier University	ENU	United Kingdom
26	University Paris Sud	UPS	France
27	L'Institut National de l'environnement industriel et des risques	INERIS	France
28	Heriot Watt University*	HWU	United Kingdom
29	University of Birmingham*	UBrm	United Kingdom
30	Rijksinstituut voor Volksgezondheid en Milieu*	RIVM	Netherlands

* Grant agreement is being amended to add these beneficiaries. Beneficiary 25 withdrew from the project due to movement of key staff involved in QNano to HWU.



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1 Summary

Nanoscale objects interact with living organisms in a fundamentally new manner, ensuring that a fruitful marriage of nanotechnology and biology will long outlast short term imperatives. Therefore, investment in an infrastructure to drive scientific knowledge of the highest quality will have both immediate benefits of supporting the safety assessment of legacy nanomaterials, as well as pointing towards future (safe) applications with the lasting benefits to society. There are immediate priorities, for few doubt that serious damage to confidence in nanotechnology, unless averted, could result in missed opportunities to benefit society for a generation, or more. QNano, as an infrastructure for analysis of nanomaterials for biological safety assessment, will materially affect the

2 Background

Nanoscience constitutes a new scientific frontier in which we can, for the first time, engineer materials on the length scale of some millionths of a millimetre. The potential applications of nanotechnology for the benefit of mankind range from information technology, energy storage and harvesting, to radically new medical technologies. The projected market for nanotechnology incorporated in manufactured goods may be worth US\$ 1.6 Trillion in the forecast period (2009-2013).¹

The scientific issues are fundamental, and durable. Much of the internal processing, passing of signals, and other key functions of living organisms use endogenous processes operating on the nanometer scale. Engineered nanoscale objects (nanomaterials) therefore can interact with organisms in a fundamentally new way (compared to micron scale materials of identical composition), ensuring that the fruitful marriage of nanotechnology and biology will long outlast short term imperatives.² As such, our ability to generate fundamental scientific knowledge of the highest quality to support the safety assessment of nanomaterials for humans and for the environment will be an investment in the infrastructure, and the future, with lasting positive impact. All steps must therefore be taken, as quickly as possible, to ensure that the field is guided towards success, with responsibility. Few doubt that serious damage to confidence in the technology could result in missed opportunities to benefit society for a generation, or more.

outcome, at this pivotal moment of nanotechnology implementation.

The overall vision of the QNano Research Infrastructure for nanosafety assessment is the creation of a 'neutral' scientific & technical space in which all stakeholder groups can engage, develop, and share scientific best practice in the field. Initially it will harness resources from across Europe and develop efficient, transparent and effective processes. Thereby it will enable provision of services to its Users, and the broader community, all in the context of a best-practice ethos. This will encourage evidence-based dialogue to prosper between all stakeholders. However, QNano will also pro-actively seek to drive, develop and promote the highest quality research and practices via its Joint Research Activities (JRA), Networking Activities (NA) and provision of Transnational Access (TA) functions, with a global perspective and mode of implementation.

QNano will also look to the future, beyond the current issues, and promote the growth and development of the science of nanoscale interactions with living organisms. By working with new and emerging scientific research communities from medicine, biology, energy, materials and others, it will seek to forge new directions leading to new (safe, responsible, economically viable) technologies for the benefit of European society.

The QNano project commenced on 1st February 2011 and will run for 48 months.

Despite significant R&D investment over the last 10 years,³ several critical road-blocks to rapid implementation and commercialisation in a safe and responsible manner, acknowledged by all stakeholders, were not fully foreseen. The real (and perceived) unknown hazards and risks of nanomaterials, allied to concerns about the reliability of current testing approaches have been highlighted in all dimensions from science, media, and even to the highest levels of government.⁴ Furthermore, discussions between stakeholders have not always been easy and to some degree the discussion has become polarised, based on opinions, and some erosion of trust has occurred.⁵

Additional complicating issues have arisen because manufacturing standards, and workplace practices, of nanomaterials are not uniform across market sectors, and in different parts of the world. It is clear that, in the absence of an understanding of what constitutes (useful) standards, the reputation of nanotechnology could be affected by the weakest players. Serious issues have already arisen, for example, from issues of impurities, unconventionally sequestered in nanomaterials. The political sensitivity of these issues in a global market, and the need to address them via infrastructural developments such as we propose here, is universally acknowledged.

Compounding this, very significant variability of reported biological and toxicity outcomes on nominally identical materials has caused controversy in science, and the media, and could, if not urgently



reversed, lead to a loss of confidence in science that single force capable of unifying societal views on this topic. Solid, disciplined, evidence based dialogue is urgently required to resolve these issues. The need for scientific opinion, whether academic research, regulatory or industrial, to converge on basic results within a

cohesive framework of structured research (in part based on blind round robin tests) is now critical. Indeed, there are few more urgent or compelling cases to be made than the need for infrastructure now to transform and drive the transition required.

QNano will:

1. Create a neutral ethos of excellence where all nanotechnology stakeholders can focus on concrete science-based outcomes;
2. Establish a core infrastructure to address the critical issues currently hampering the industrial deployment of nanotechnologies across a range of industry sectors;
3. Provide Users with a full range of services from standard nanomaterials, tuition in best practice, laboratory support and training, and a suite of protocols for all aspects of nanomaterials processing and characterisation in a biological context;
4. Push beyond the state of the art in nanomaterials processing, labelling and identification and characterisation *in situ*;
5. Develop novel analytical approaches and tools where most urgently needed to enhance understanding of health and safety issues in nanotechnology;
6. Create a hub to drive the development and implementation of standards across all aspects of nanosafety evaluation and to link with other EU actions (RTD, ERANET, Nanosafety Cluster, OECD, ISO) and international stakeholders;
7. Look to the future - framed with new scientific communities, and new industry sectors, forging new (safe and responsible) applications of nanoscience and implementations of nanotechnology.

QNano will qualitatively change the outcome and potential for successful commercialisation of nano-enabled products at this critical period of nano-implementation.

3 Project Description and Organisation

The vision of QNano is the creation of a 'neutral' scientific and technical space in which scientists from all stakeholder groups can engage, develop, and share the scientific best practices in the field. It is understood that such an organization cannot resolve all of the challenges, nor even address all the important areas of the science, especially at the beginning. In the early days its aspiration must be limited to the creation of an ethos, development of processes, and harnessing of the resources, to allow evidence based dialogue in critical areas to flower. The program will not engage in controversy, nor promote opinions, for in doing so it will lose the trust of one or more stakeholders. Uniquely important in the current situation, the infrastructure will need to patiently display ethical standards in actions and processes if the current uncertain atmosphere is to yield to clarity and unity of purpose. By processes (for example, blind round robins) it will determine (and provide the support to determine) facts, and report them to the scientific community, and stakeholders. Its greatest strength is that these factual results (from well defined studies) even if their implications remain open to interpretation will be trusted by all. The infrastructure will be global in perspective, and implementation. As noted above, some of the challenges do not lie in Europe alone, nor can they be resolved there. Existing warm relations in the United States, Asia and Latin America (and beyond) will be further developed within the framework.

Many issues need to be addressed, short term, and longer term. A scientific culture must be built, and full acceptance of the challenges and difficulties of working in scientific excellence in such a new arena must be argued, and won, step by step. At this period in history, resolving even simple issues, such as the creation and provision of (nanoparticulate) biological end-point positive (and negative) controls, will have profound effects on the way that the User community performs its' work.

QNano is founded on a belief in the potential of nanoscience and nanotechnology. It will therefore look to the future, beyond the legacy of the current debates, and find creative approaches to organising and thinking, implementing new ways to deliver the promise nanotechnology to benefit mankind - safely. The union of nanoscience and living organisms is indissoluble. They have a long way to travel together in the future, as outlined in the vision statement of the European Technology Platform for nanomedicine. Well-conceived infrastructures to support that journey will give lasting value to European society, and far beyond.

Practically speaking, QNano will be **an accessible integrated European resource for research, regulatory, and industry (both small and large) developers in nanoscience and nanotechnology**. It will harness and integrate existing research expertise and facilities from across the EU member states into a cohesive interdisciplinary entity strongly focussed on scientific excellence and quality of execution in all aspects of nanomaterials processing and characterisation for assessment of their biological and environmental impacts. It has consulted with, and will build in alliance with OECD, ISO, the International Alliance for NanoEHS Harmonisation (www.nanoehsalliance.org), the EU FP7 CSA NanoImpactNet (www.nanoimpactnet.eu), and numerous other national platforms.

It will offer a distributed set of transnationally accessible facilities, centrally managed, as with any infrastructure program, *but also offer a range of added-value services to users and stakeholders*. These will include high quality ('approved') Nanomaterials, Training (and certification) in advanced characterisation methodologies and round robin validated protocols for biological assays, as well as Industry-oriented support, using flexibly configured distributed 'hubs' via which different constituencies can interact. Crucially, these hubs will embed existing (and emerging) core constituencies



(via suitable program partners), promoting the concept of infrastructure as a 'learning' organization. Thus, whilst the vision of excellence and quality will be fixed, the means to achieve that end will evolve responsively.

QNano is primarily an analytical infrastructure whose purpose is to drive high quality research and testing practices. Physiochemical and other analytical characterization in the biological and safety contexts is quite different from analysis of nanomaterials for other applications. Some of the important (relevant) physiochemical characteristics are not yet fully understood. Even implementation of known science is not always evident, but in a new industry making its reputation, it is crucial. The fact that engineered structures have access to biological machinery, combined with their unique (for example high-surface area) properties, means that material quality and reproducibility are important, not just for this program, but long term, in industry in general. Details such as the tendency of nanomaterials to secrete difficult-to-remove relatively immobile impurities into an organism, or to sequester contaminants from the environment and transport them into living organisms, for example, can have profound consequences for predicting fate and behaviour in different cell types, tissues, complex matrices, organisms, and all require detailed characterisation to be interpreted correctly. Such aspects are believed to underlie some early negative toxicity reports, leading (in these specific cases) to unwarranted and widely publicised fears. There is a critical need to separate issues of quality from the durable questions of intrinsic nanomaterials safety. The potential for these issues to have negative impact on trust in global trade (where good practices are not universally accepted) are incalculable.

By fostering a new quality-based research and application consensus that values both the durability, and reproducibility of new findings, QNano will qualitatively affect the outcomes in this domain. It cannot address all the challenges, but it will provide the basis for those challenges faced, at what is certainly the most pivotal period in the adoption of nanoscience and nanotechnology in society.

The activities of QNano are summarised as follows:

Networking Activities:

- **NA1** - Management and coordination
- **NA2** - Nanomaterials Hub: an instrument for Quality Assurance testing of nanomaterials via Round-Robin trials, and their provision to the wider User community.
- **NA3** - Training Hub covering all aspects of best practice in nanomaterials for biological testing.
- **NA4** - Working Groups to drive future development and sustainability of the infrastructure.
- **Transnational Access:** Provision of transnational access to the nanomaterials processing, characterisation and exposure assessment facilities of the 15 TA Participants via a single application and evaluation process and 6-monthly calls for applications on the QNano website.

Joint Research Activities:

- **JRA1** - Development of strategies to eliminate and/or reduce variability in nanomaterials batch-to-batch reproducibility and to determine acceptable variability levels for biological applications.

- **JRA2** – Optimisation of traceability of nanoparticles by development of reliable labelling (radioactive, stable isotope and fluorescent).
- **JRA 3** - Development and validation of characterisation tools for nanoparticles *in situ* in biological, environmental or consumer milieu.
- **JRA 4** – Development of optimal modes of presentation of nanoparticles to cells, tissues, organisms and whole animals for quantitative reproducibility.
- **JRA 5** – Towards development of priority alternative *in vitro* tests to replace animal testing.

4 Key Challenges being addressed by QNano

Irreproducibility in nanomaterials leads to irreproducible biological impacts.

There remain genuine scientific challenges in making reproducible nanomaterials using early manufacturing processes. This is not a trivial issue, and it will take some years yet before it is resolved. However, it must be noted in the current context. Thus, because of the enormous surface-to-volume ratio presented by nanomaterials, it is not uncommon for 1 millilitre of dispersed nanomaterials (1wt%, 70nm) to present over 8m² of surface area to the endogenous machinery of biological organisms. The level of care taken by the medical device industry to understand the role, and maintain the quality and reproducibility of medical device implants, with much smaller exposed surface areas, is barely conceivable in nanomaterials preparation. Yet, this is the standard we have to work towards and progress on urgently. Beneath several hundreds of nanometers, the immune clearance system is less effective, and nanomaterial surfaces may be in prolonged contact with biological systems. Thus, irreproducibility in surface quality or properties (more perhaps than variations in absolute surface area) inherent in current, poorly controlled batch nanomaterials synthesis methods can be amplified far beyond that expected based on their usual applications, which is not necessarily all surface-related. Not all variations are expected to be biologically significant. Some known factors include surface charge and crystallinity, but no systematic studies of the biological impacts from batch-to-batch variability have been attempted, in part because of the large variations in the methods themselves.

Paradoxically, even where such variations of surface quality do not present a real hazard, they can lead to a troubling irreproducibility in biological or toxicological assessments that in itself leads to controversy and a general lack of confidence in the capacity to do good science in this field. Attempts to suppress these effects (for example, OECD, IANH, and other large national programs) have been made, choosing one representative batch that is maintained throughout the particular program, with the usual problems of such approaches. With nanomaterials, however, the problems can be more serious. Batch aging, especially in dispersion, is quite serious, and for many materials requires disposal of a given batch after three months, even if the storage conditions are optimal, an organizational issue that is itself challenging, and fraught with unforeseen difficulties. Additionally, chemical purity and surface modifications can introduce further variability in biological responses.

***Unscientific lack of nanomaterial positive and negative controls for biological assays.***

Amongst the most basic requirements of any well defined experiment is the need to have positive and negative controls to demonstrate that the assay is working but is not triggered non-mechanistically, and to present the biological or toxicological outcomes of these in any report. This is part of the basic social contract formed between scientists in all fields, for over a century. Much current nanosafety research is largely carried out in the absence of any such controls, or using non-nanomaterial positive controls (e.g. molecules), simply because there are few (if any) agreed positive control nanomaterials for the various biological end-points (e.g. apoptosis, cell cycle disruption, genotoxicity etc.). In those cases where chemical controls have been used, they generally have a different site of biological action (not using the same endogenous mechanisms) and are therefore of dubious value. This single scientific difficulty has, perhaps, led to the most striking damage to the scientific reputation of the field, in the sight of the broader scientific community, and has deep cultural impacts for the nanosafety and nanobiology communities, limiting aspirations for the level of potential publications, and thereby damaging the careers of young researchers engaged in the field.

Unknown or poorly chosen dispersants for base nanomaterials.

Even the most basic issues, such as how to prepare nanoparticulate dispersion in biological media where they would be studied, are not always well understood. Naturally chemists and physicists have for years studied the dispersibility of nanomaterials for a variety of applications, but most dispersants are at least biologically disruptive, if not downright cytotoxic, at the levels required for good dispersion. In many cases, lack of common training, culture and understanding between nanomaterials scientists, biologists and toxicologists lead to the latter using directly a dispersed material without appreciating that some of the added components could lead to significant biological impacts themselves. Such issues were compounded by the fact that dispersants for specific materials are sometimes commercially valuable information, and in some cases nanomaterials purchased from companies were studied without knowledge of the added components. In such cases there was no opportunity to control the dose of the added dispersant or other additives, and even those that are quite safe under normal application conditions (for example after preparation in paints etc.) could lead to undesirable toxicological outcomes if studied at inappropriate concentrations. Such issues have proliferated to the point where, in the literature, it has become difficult to separate the biological role of nanomaterials themselves, from a multitude of other preparative details, often not clearly known, or reported.

Limited application of characterisation methods (in some cases limited capacity to characterise) to nanomaterials at any stage of their processing and analysis.

The framing of the call text of this particular program (Analytical Facilities) highlights this particular aspect of the challenges facing the nanosafety community, and thereby correctly cuts to the heart of one of the most critical issues of the field. This is an issue at every stage of nanomaterials system preparation, and impinges at every level, from the most fundamental science, to the most practical issues of regulatory outputs.

There are basic challenges that are a legacy from the interdisciplinary origins of the field. For example, many biological and toxicological laboratories are only now acquiring basic fixed light scattering and zeta potential devices and many are not yet fully integrated into the laboratories. Many of advanced characterisation technologies will remain outside of the reach, or indeed reasonable interest, of the User community of biologists and toxicologists, and this must be acknowledged, and addressed.

Nanomaterials tracking, localization and characterisation in living organisms and the environment is relatively unknown as yet, and such limited information as exists has few cross checks and is of unknown reliability.

It is hard to believe that nanotechnology can have arrived at this phase of its development with such a lack of good quality, labelled nanomaterials suitable for biological applications and relevant to the scientific and safety issues at stake. This constitutes a serious bottleneck to progression of the field, and confidence in regulatory decisions.

There is limited access to even existing labelled materials (for example radio-, or isotope labelled materials) which tend to be available only to specific collaborators. Furthermore, the design of labelling strategy is often poorly aligned with the User community needs, which wishes to study nanomaterials of high economic relevance and high usage. Labelling strategies that significantly affect the surface can lead to quite different biological outcomes, and are therefore of more academic interest, and labels of the wrong intensity or misaligned in, for example, wavelength for typical biological instrumentation is also a serious practical problem, in part derived from the fact that labelling is often driven by chemists and physicists only in limited contact with biologists.

Lack of in situ characterisation of the nanomaterial-biomolecule complexes.

There are other serious issues for the field resulting from the lack of *in situ* characterisation of nanomaterials during biological and environmental studies. A key point, often missed in the immediacies of the nanosafety question, is that the future of the field as a true science requires *in situ* characterisation of the nanomaterial-biological complexes. It is now clear that in many biological fluids, nanomaterials (unless specifically designed not to) are coated by a very long lived biomolecular shell ('hard corona') that is sufficiently durable and thick as to determine the early outcomes of translocation, localization in living organisms. Similar issues (although that arena is in an even earlier phase of development) pertain in the environmental context where the nanomaterial surface may be coated by a variety of naturally occurring biomolecules such as polysaccharides from organic matter. Thus, whilst fundamental for the discipline and basics of nanomaterial production and supply, the well known nanomaterial characterisation methods give parameters that may merely be proxies for the 'real' biological identity, that is, what living organisms really 'see'. This is a critical issue for the development of the field. There are practical issues also, for the nature of the plasma or serum used may lead to different outcomes.

Thus, the dispersion of nanomaterials in even the simplest biofluids such as blood plasma or in environmental fluids such as river water requires care, and understanding in practice. Furthermore, there are as yet great unknowns in the structure and evolution of such dispersions, and ongoing nanomaterial-biomolecule aggregation



can affect the bioavailability of the nanomaterials. One cannot in this field expect the scientifically idealized outcome of perfectly stable dispersed materials, but one can at least insist that nominally identical dispersions used by different groups of scientists are indeed identical. Therefore, uncertainties in this arena may impact on the framing of poor, or poorly defined, dispersion protocols in which insufficient parameters are fixed to ensure reproducible dispersion and dispersion kinetics. In all these cases the lack of application of known characterisation methods, and the limited manner in which these have so far been translated for use in this field are currently limiting factors in the onward development and the implementation of regulation. There is also an overarching challenge regarding dissemination of this need for *in situ* characterisation techniques into the User community.

Poorly understood, poorly characterized, without agreed standards or experimental formats for presenting nanomaterials in biological, toxicological, environmental and occupational exposure studies means that dose, and dose rates are poorly understood, rarely uniform, and can lead to widely different 'actual' doses.

The problem of how to present the nanomaterials in a meaningful, reproducible, and bioavailable manner is challenging. Without

specific measures, and when combined with issues of poorly controlled aggregation may lead the intracellular concentration for nominally identical nanomaterial concentrations and biological materials to differ by several orders of magnitude. Though less well understood, similar issues are believed to be relevant *in vivo* and in the environment, where different modes of preparation and delivery combine to lead to different 'presentation' of the nanomaterials. Occupational exposure scenarios are no different in the challenges presented, and (for example) implications for different modes of delivery, and measurement, of carbon nanotubes (CNTs) are poorly understood, and lack any agreed approach.

Poorly structured and poorly supported by infrastructures.

This challenge ranges from the lack of common set of laboratory practices and facilities from which the most expert can support those (often highly expert) biologists and toxicologists that lack expertise in system preparation and characterisation. The challenge is, however, deeper. In the absence of infrastructure, the community is fragmented, and is only slowly forming a vision of what it wishes to be.

Joint Research Activities	Networking Activities	Transnational Access
Category A: Nanoparticle synthesis, cleaning, and pre-dispersion for biological and environmental applications; Consolidation and distribution of existing high quality nanoparticle sources; purification & cleaning processes; research into production of reproducible nanomaterials for positive and negative control in biological experiments. JRA1	NA2	TA Calls A-F
Category B: Nanoparticle labelling, functionalisation and dispersion for biological and environmental applications; Consolidation and distribution of existing fluorescent, radio-, magnetic labelled particles; research into production of reproducible nanomaterials for tracking and biodistribution assessment. JRA2	NA2	TA Calls A-F
Category C: Nanoparticle characterisation <i>in & ex situ</i> in biological /environmental milieu <ul style="list-style-type: none"> • IN PRISTINE REFERENCE SOLVENT; Complete characterisation of nanoparticle size, size distribution, surface, reactivity etc. • in situ IN BIOLOGICAL FLUIDS; Development & complete characterisation of biocompatible nanoparticle/dispersant complexes. • in situ IN THE ENVIRONMENT; Nanoparticle dispersion & complete characterisation in NOM and evaluation via RfL. • in situ IN CONSUMER PRODUCTS; Nanoparticle complete characterisation in formulations & complex matrices; development of <i>in situ</i> detection & quantification methods. JRA4	NA3 / NA4	TA Calls A-F
Category D: Exposure and Dose-response Assessment (Occupational, <i>in vitro</i>, <i>in vivo</i>). BIOLOGICAL STANDARD MATERIALS, Centralisation of standard biological materials (cells, serum, 3D models, test species, etc.); <ul style="list-style-type: none"> • OCCUPATIONAL; Improved measurement strategies for nanoparticles; workplace assessments & instrument calibration services. • IN VITRO ASSESSMENT; High-throughput and alternative approaches. Modes of NP presentation for reproducibility, uptake and trafficking, toxicological & functional impacts. • IN VIVO ASSESSMENT; GLP best practice. Modes of NP presentation for reproducibility, biokinetics, biodistribution, toxicological & functional impacts. • MODELLING; Nanoparticle ADME based on biokinetics /biodistribution: nanoparticle fate & behaviour in air, water, soil, biofluids etc. JRA3 / JRA5	NA3 / NA4 / NA5	TA Calls A-F

5 QNano activities

QNano is founded on three functionally distinct elements to promote high quality and reproducible research on nanomaterials in contact with biological and environmental systems, and build the knowledge on nanosafety. Each of the (three) functional elements is essential, as are the linkages (both in process, and in people) that have been designed into them. The three elements are closely interlinked from an operational and management point

of view, in addition to the close scientific linkages shown in Figure 1. The functional elements are as follows:

Transnational Access (TA): Physical access to 15 of the major nanomaterials processing and characterisation for health, safety and environmental application sites in Europe. Collectively, these sites enable Users to access small to medium scale equipment and facilities (with the appropriate knowledge to apply them in this



context) through to some of the most highly equipped nano-characterization centres in Europe.

Joint Research Activity (JRA): 28 partners (including 14 of the TA partners), have been selected based on their unique contributions in research, where it pertains directly to new or improved methods that contribute to the infrastructure of the field. Several of these are outstanding scientists in particularly relevant research functions.

Networking Activity (NA): To ensure appropriate dissemination of the best practice in nanomaterials synthesis and dispersion in reproducible manners, characterisation of nanomaterials *in situ*, methods of presentation of nanoparticles to living systems, and alternative testing methods (i.e. the JRA topics), QNano has a strong focus on networking activities, such as training of young researchers (through the Knowledge Hub), provision of high quality nanomaterials (through the NanoMaterials Hub), contributing to research road-mapping and priority setting for the field, and supporting the development of internationally agreed archiving and databasing protocols for data generated within EU projects.

Overall Strategy of the QNano work plan

The QNano Research Infrastructure workplan and methodology are linked in terms of their scientific and operational objectives via the three pillars of activity and the four horizontal themes (which map to the four categories of Transnational Access), and each of the JRA and NA work packages (WPs) map onto one or more of the horizontal themes, as shown in Figure 1 below. The information and knowledge flow is also seen in this manner – issues that are at their earliest stage of development are in JRA, and much of that knowledge is expected to flow directly into application via TA (particles, protocols, characterization methods), or NA (characterization and measurement *in situ* in organisms, environment, and workplace, nanomaterials supply hub, training and others).

QNano officially commenced on 1st February 2011, with the kick-off meeting held in Dublin on 15th and 16th April 2011. The project will run for 4 years. During its first year of activity, much of the effort has been quite operational in nature, focussing on establishing the online portal for processing the Transnational Access and the Knowledge Hub, implementing the various management committees, such as the Networking and Research Steering Group, the Transnational Access Steering group and the Nanomaterials Hub Steering Group, and preparing the project support documentation, such as the Transnational Access User guidelines and the project Procedures and Quality Assurance Manual, and preparation for the 1st Annual QNano conference (being held jointly with NanoImpactNet CSA) from 27-29th February 2012.

A summary of some of the main achievements from the first 12 months of QNano activity includes:

Networking Activities:

- **Joint NanoImpactNet-QNano conference** will be held in Dublin from February 27th to 29th 2012 and addresses the topic “From theory to practice - development, training and enabling nanosafety and health research”. This is the four in the series of NanoImpactNet conferences, and will be continued under the banner of QNano for three more years. Topics to be addressed in this years’ conference include:

- Opening session: Setting the scene – From NanoImpactNet to QNano.
- Session 1: Materials for the future
- Session 2: Eco-Hazard Assessment
- Session 3: From Production to Exposure
- Session 4: Characterisation *in situ* following exposure
- Session 5: Beyond non-specific hazards
- Session 6: Stakeholder needs and Risk Assessment

The scientific programme and registration details can be found at <http://www.nanoimpactnet.eu/>

- **QNano Training Schools:** the first two QNano training Schools are being held in conjunction with the QNano for Modelling and Good Laboratory Practice will be held during the joint NanoImpactNet-QNano Conference on March 2nd and 3rd 2012. A full set of learning outcomes and training materials
- A provisional calendar for the upcoming QNano Training activities has been proposed. The provisional schedule is shown in Table 1. Full details of all Training Events will be advertised on the QNano website via the Knowledge Hub, and the information will be disseminated via the NanoSafety Cluster and other appropriate channels. Should demand for the planned courses exceed capacity of the hosts, events will be ran a second time to facilitate the demand. All training materials developed for the QNano Training Schools will be made available via the QNano Knowledge Hub as Open Access documents distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
- **Round Robins:** Round Robins, or interlaboratory comparisons, are an important mechanism to provide quality assurance regarding methods, protocols, data and staff proficiency. Round Robins are performed for various reasons,⁶ e.g.
 - § to validate test procedures,
 - § to certify reference materials,
 - § to assess the competence of laboratories (proficiency testing),
 - § or more generally, to investigate the degree of comparability among laboratories.Within QNano, three types of round robins are being envisaged for all four of these purposes.
- The first Round Robin (RR) for proficiency testing of the Transnational Access Facilities (TAFs) in the performance of simple physico-chemical characterisation using two commercially available well dispersed and relatively monodisperse polystyrene nanomaterials has been completed and the data are being analysed to assess the intra- and inter-measurement variability. Overall, the data from all participants was comparable, which ensures that all researchers who undertake Transnational Access at any of the partner institutions can be assured that simple size and size distribution measurements of their samples on-site at the host



institute can be performed to an acceptable standard, as the baseline measurements prior to undertaking more advance characterisation *in situ* using the state of the art facilities at the host institutions. Partners provided data using Dynamic Light Scattering, Transmission Electron Microscopy, Nanoparticle Tracking Analysis and/or Differential Centrifugal Sedimentation.

- The RR for benchmarking of participating laboratories in their proficiency to maintain *in vitro* cell culture and perform cell viability testing is under way. A second physico-chemical characterisation RR, where protocols are compared to identify the best dispersion protocol for Aerosil 200 silica nanoparticles, is also under way. A RR to validate a potential positive control nanomaterial for apoptosis is planned and will commence shortly.
- **Nanomaterials Hub:** Processes for sourcing commercial and in-house nanoparticles have been developed; 1st Nanomaterials Technical Specifications and Materials Safety Data Sheets are being completed for the QNano selected nanoparticles, including full characterisation data, both pristine and *in situ* in representative biofluids (e.g. cell culture medium containing different amounts of foetal calf serum and in river water containing natural organic matter).
- Terms of Reference and Table of Contents for the first QNano State of Art report have been agreed and responsibilities assigned among the partners for drafting the text. The title of the first report will be *State Of the Art in characterisation of nanomaterials for safety assessment*.

Table 1. Preliminary schedule of QNano training events. Exact dates and titles may change slightly.

	What	When	Where
D3.8	Modelling School #1	Feb 2012	Dublin
D3.9	Training module GLP / best practice #1	Feb 2012	Dublin
D3.2	Hands-on training in nanomaterials characterisation for toxicity testing #2	Apr 2012	Edinburgh
D3.10	Training module for industry / SMEs #1	Apr 2012	Edinburgh
D4.6	Industry needs workshop #1	Apr 2012	Edinburgh
D4.7	Risk Management Hands-on training #1	Apr 2012	Edinburgh
D3.5	Training in best practice for young researchers #1	Jun 2012	TBC
D3.12	Hands-on training in biological assays #1	Jun 2012	TBC
D3.2	Hands-on training in nanomaterials characterisation for toxicity testing (#1 in series)	Mid 2012	TBC
D3.8	Modelling School #2	Jan 2013	Edinburgh
D3.2	Hands-on training in characterisation / tox testings #3	Jan 2013	Edinburgh
D3.11	Training modules for researchers from contiguous areas	Jan 2013	Karlsruhe
D3.5	Training in best practice for young researchers #2	Jan 2013	Karlsruhe
D3.7	Occupational Exposure Management training #1	Feb 2013	Dublin
D4.6	Workshop on assessing industry needs #2	Feb 2013	Dublin
D3.7	Occupational Exposure Management training #2	Jun 2013	Pisa
D3.12	Hands-on training in biological assays #2	June 2013	Pisa
D3.9	Training module GLP / best practice #2	Oct 2013	Edinburgh
D3.2	Hands-on training in nanomaterials characterisation for toxicity testing #4	Oct 2013	Edinburgh
D3.10	Training module for industry / SMEs #2	Oct 2013	Edinburgh



Transnational Access:

- QNano aims to provide approximately 400 Users with access to state of the art characterisation equipment across the 15 *Transnational* Access Facilities shown in Figure 2, and to promote the need for characterisation of nanomaterials in situ in the medium in which they will be exposed to living systems.⁷
- Funding for approved applicants will cover the costs of International travel, accommodation, living costs for the researcher, and the cost of provision of the access for the host Transnational Access Facility. Average visits 5-10 working days.
- Visits are fully supported with the technical expertise in the institute of equipment being accessed, and with protocols and nanomaterials as needed.
- The first Transnational Access call opened November 1st 2011 for 3 months. The second call will open May 1st 2012 and close July 31st 2012. Further calls will be held every 6 months for the duration of the project.



Figure 2: Map showing the locations of the institutes offering QNano-funded Transnational Access to state of the art characterisation facilities for nanomaterials in contact with living systems. Note that a potential User cannot request to visit a facility in the country in which they work.

TAF	Category of Access			
	A	B	C	D
UCD	✓	✓	✓	✓
RHM	✓	✓	✓	✓
JRC	✓	✓	✓	✓
KIT	✓		✓	✓
FUNDP			✓	✓
UNIVLEEDS			✓	
NILU			✓	✓
CIC		✓	✓	✓
UIU	✓		✓	
ICH	✓		✓	
WU	✓	✓	✓	✓
VITO			✓	✓
SNU			✓	
TCD			✓	✓
UoE			✓	✓

A: Nanomaterial synthesis
 B: Nanomaterial labeling & pre-processing
 C: Nanomaterial characterisation *in situ* & *ex situ*
 D: Nanomaterial exposure assessment

Figure 3. Categories of QNano Transnational Access. The categories of access offered by each Transnational Access Facility (TAF) are shown in the graph (left). A legend explaining the categories of access is shown on the right.

- Transnational Access is provided according to four thematic categories: nanomaterial synthesis, labelling and processing, characterisation *in situ*, exposure assessment. As shown in figure 3 the 15 Transnational Access Facilities offer access under one or more of these categories.
- Details of the application process can be found on the QNano website: <http://www.qnano-ri.eu/access.html>.
- Briefly, after contacting the Technology Expert at the host institution of interest the applicant (User) submits a project, which was previously agreed with the Technology Expert, through the online application system. The application undergoes an eligibility check and is afterwards evaluated by an external unbiased panel of experts, the User Selection Panel (USP). The USP provides feedback on the application through the online application system. Successful applicants are notified by the QNano project office, and have 1 year in which to complete their visit. Exact timing of the visit must be agreed between the User and the relevant Technology Expert at the host institution. The process is shown schematically in Figure 3. A User Handbook and Frequently Asked Questions sheet are available on the QNano website, as well as the contact details for each Transnational Access Facility.

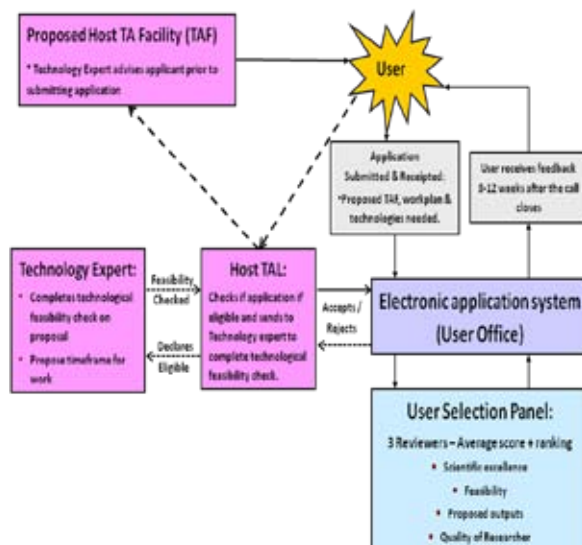


Figure 4. Flow chart of the Application Process. The steps involved in the submission of a proposal and the evaluation process are shown in the graph. Shown are also the expected interactions between the applicant (User) and the Transnational Access Leader (TAL) and Technology Expert.

Joint Research Activities:

- All of the research WPs are underway, with WP meetings scheduled, round robin activities in progress, and first batches of well-characterised nanomaterials available.
- JRA1 - strategies to eliminate and/or reduce nanomaterials batch-to-batch variability: Assessment of sources of variability in widely used synthesis methods is under way and strategies are being developed to eliminate or reduce such sources of variability. Different batches of Silica nanoparticles made via the Stöber synthesis route are being produced to verify the extent of variability among



batches and identify the source of such variability. Strategies to reduce this variability will be developed.

- JRA2 - development of reliable labelling: A reliable strategy has been developed to isotopically label ZnO nanoparticles to allow for detection in *in vivo* experimental models; Fluorescently labelled nanoparticles are currently being synthesised and characterised.
- JRA 3 - Development and validation of characterisation tools for nanoparticles *in situ*: a protocol has been developed and refined for single particle Inductively coupled mass spectrometry (SP-ICP-MS); Training in the application of this protocol will be provided to JRA3 partners (and external researchers), and the protocol will be used to perform a round robin study to quantitatively assess the presence and composition of nanoparticles in complex organic mixtures.
- JRA 4 - Development of optimal modes of presentation of nanoparticles for quantitative reproducibility: A literature survey has been undertaken to identify the most widely used methods to present nanoparticles to *in vitro* cell systems; the most promising are being chosen as candidates for round robin testing in order to determine the method that produces the most reproducible and homogenous dose of nanoparticles across all cells in the culture. Effect of cell division / cell cycle on dose and uptake of nanoparticles is also being assessed.
- JRA 5 - Towards development of alternative *in vitro* tests: This WP is scheduled to commence research activities in February 2012. JRA 5 has secured a session at the ESOF 2012 (European Science Open Forum conference (11-15 July, 2012, Dublin) <http://www.dublinscience2012.ie/>). The session is entitled '*What realistic alternatives are there to animal testing to ensure safe introduction of new technologies?*' and will be a panel discussion targeted at scientists, policy makers and industry. The session is part of ESOF 2012 Theme 5. Science: Reshaping the frontiers of knowledge.
- The vision of QNano is thus a unified and continuous flow of knowledge and information, from discovery to implementation and dissemination, enhancing the overall access, and service available to the research community (the Users), and raising the quality of the research outputs from the whole field.

6 Next steps and how to get involved in QNano

Year 2 will be an exciting one for QNano, with the first Transnational Access Users undertaking their research visits at the various partner facilities, with two additional transnational Access calls planned (opening 1st May 2012 and 1st November 2012). Additionally, there is an exciting schedule of theoretical and hands-on (laboratory) training events for young researchers, as well as events to engage with and assess the needs of industry with regards to nanomaterials safety assessment. Results will begin to emerge from many of the JRA WPs, and every effort will be made to disseminate this to the relevant stakeholder communities in an appropriate manner.

There are a number of ways that researchers and other stakeholders can get involved in QNano, including:

- TA for researchers (must be Transnational), which can also include industry researchers, as long as there is some published output from the funded research visit which acknowledges the funding from QNano;
- Via the Expert Resource Group which are external advisory groups supporting QNano in terms of activities. Expert groups on Toxicology, Eco-toxicology, Alternative methods (in vitro), Biological Methods, and to promote engagement within ongoing international efforts in Regulation, Standardisation and other International issues (e.g. databases);
- Via identification of training needs / support for development of training events to address gaps;
- Via RR processes on specific topics / protocol development / testing / nomination of nanomaterials etc.;
- Via participation in User Selection panels.

Members and stakeholders from the nanosafety and nanomedicine communities are invited to participate in these activities, and can express their interest in this via an initial email to the QNano project office (project-office@qnano-ri.eu) who will forward your details to the relevant persons depending on where you wish to contribute.



7 References

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8 Directory

Table 1 Directory of people involved in the QNano project as beneficiaries^{*1}.

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¹ Note there are also additional partners who are non-funded who are not included here.



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SANOWORK

Safe Nano Worker Exposure Scenarios



Contract Agreement: Under Negotiation Website: <http://www.sanowork.eu>

Coordinator: Anna Luisa Costa, ISTECCNR, Faenza, Italy

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4	Elmarco Sro	ELM	Czech Republic
5	GEA Process Engineering AS	NIRO	Denmark
6	Colorobbia Italia SPA	COL	Italy
7	Bayer Technology Services GmbH	BAYER	Germany
8	Institut National de l'Environnement Industriel et des Risques INERIS	INERIS	France
9	University of Lymerick	UL	Ireland
10	Università degli Studi di Parma	UNIPR	Italy
11	Università degli Studi di Pisa	UNIFI	Italy
12	Acondicionamento Tarrasense Asociacion	LEITAT	Spain
13	Istituto Nazionale Assicurazione Infortuni sul Lavoro INAIL	INAIL	Italy

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1 Summary

The main goal of Sanowork project is to identify a safe occupational exposure scenario by exposure assessment in real conditions and at all stages of nanomaterials (NM) production, use and disposal. In order to address this and more specifically the issues introduced by NMP.2011.1.3-2 call, we intend to: 1. contain hazard and worker exposure potential by developing exposure mitigation strategy based on "Prevention through Design" approach. 2. implement a rigorous exposure assessment in the workplace in order to evaluate the effectiveness of existing and

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Project Funding: 3.5 Mio. EUR

proposed exposure reduction strategies. 3. perform risk analysis off line and on site in order to identify substance product properties and operational condition that ensure a safer worker exposure scenario. 4. Assess COST/ EFFICIENCY of the proposed strategies on the basis of risk analysis results, materials/properties efficiency, risk transfer to insurance underwriter community. The Sanowork proposed risk remediation strategy will be applied to nanomaterial properties. The following "representative" pool of NM and nanoproducts have been selected: ZrO₂ (chemical or ceramic raw material); TiO₂ and Ag (ceramic or textile photocatalytic/antibacterial surfaces); CNTs (polymeric nanocomposites); organic/inorganic nanofibers (nanostructured membranes for water depuration system). The strategy is addressed to mitigate risk by decreasing adverse health hazard and emission potential of nanomaterials, setting back processes of transport to the point of entry. A sound balance between exposure and health hazards data, before and after the introduction of existing and proposed risk remediation strategies, will allow to evaluate the effectiveness of existing and proposed exposure reduction strategies. The cooperation with industrial key partners such as Plasmachem, Elmarco, GEA Niro, Colorobbia, Bayer will guarantee an accurate exposure assessment in the workplace.



2 Background

Strong proponents of nanotechnology, such as Lux Research, anticipate that “nanotechnology applications will affect nearly every type of manufactured good over the next ten years.” Nevertheless the promise by nanotechnology of a significant contribution in boosting the economy, living standards and improving the quality of life may be outweighed by the perceived occupational, environmental health and safety risks that it poses.

Due to the lack of quantitative risk assessment, underwriting such risk is particularly difficult and may compel the underwriter community to refuse to insure nanotechnology industry in the fear of potential bankruptcy. Small and Medium Enterprises (SMEs), which are in the driving seat of nanotechnology innovation, are particularly vulnerable to such conditions as they lack resources to put expensive preventive measures in place to safeguard their workers' health and safety.

Owing to increasing knowledge in the nanotechnological development and Occupational Health and Safety issues, there is a new awareness that safety options should also include “smart” nanoparticle (NP) design (i.e. safe by design) to ensure effectiveness of preventive measure.

Given the limited amount of information about the health risks associated with occupational exposure to NMs, the precautionary principle has suggested to take measures to minimize worker exposures. Research Institutions and Governmental Agencies have addressed specific efforts to clarify the nature and the extent of potential hazard raised by handling NMs and to provide a solid platform for nanotechnology occupational safety and health. Thus, a hierarchy of control measures as applied to inhalation and dermal risks, including elimination, substitution, process enclosure, engineering controls, procedural control, personal protective equipment (PPE) has been identified.

All these measures have been implemented on a voluntary basis by some industries worldwide, but the majority of companies does not foresee unintentional release of NMs throughout the life cycle.

Only recently, “Prevention through Design” (PtD) has been envisaged as a proactive tool to prevent possible exposure and risk. PtD is an approach (and in the U.S., a national initiative) to design out hazards rather than address them when there are exposures. Such an approach is particularly applicable to NMs at the molecular and process scales. At the molecular scale, there is potential for modification of molecules to retain commercial and scientific functionality while reducing toxicity. At the process scale, companies can look to the pharmaceutical industry for engineering controls that could be adopted for potentially hazardous NMs.

3 What is Sanowork

The SANOWORK project is built around the promotion, development and implementation of “Elimination/Substitution” control strategies and proposes to fill the gaps that already delay their diffusion. In order to address nanomanufacturing industries needs, SANOWORK project propose sustainable Risk Remediation Strategies with a balanced approach between design for manufacturing and design for safety.

The evaluation of proposed Risk Remediation Strategies will pass through a globally harmonized analysis and reporting of process/NMs performances, hazard data, emission/exposure collection, in relation to operational conditions and NMs physico-chemical properties. The process/NMs performances and risk specific evaluation will be performed off line (NMs as delivered, provided by companies or surface engineered during the project, exposure scenarios at lab scale level) and on-site (NMs collected on-site, exposure scenarios at pilot scale level). The process and NMs performances, as well as exposure and hazard profiles, will be assessed BEFORE and AFTER the introduction of risk control extra-steps.

We will develop and integrate NMs design options strategies within target processing lines as extra-steps for improving the efficiency of the process while preserving and/or increasing NMs performances. Health risk depending on the intrinsic hazard and exposure frequency/concentration level, will be evaluated according to NMs physico-chemical properties.

Five strategies will be proposed based on NMs surface engineering so that exposure can be reduced but if exposure has accidentally occurred the health hazard would still be decreased. Based on the knowledge of NMs dispersion behaviour, a combination of self-assembled monolayer coating and tailored aggregation processes will be developed in order to decrease the hazard and/or emission potential of target NMs (strategies: I, II, III, V). The described strategies will be accompanied by a process of immobilization of NMs with an expected exposure potential (strategies: IV). The SANOWORK proposed strategies are industry-driven and will therefore comply with the following criteria:

- satisfying the production requirements;
- cost-effectiveness and suitability for large-scale production;
- easy processing-line implementation for manufacturing nano-structured components;
- decreasing exposure potential and/or health impacts, while preserving nano-scale properties.

Manufacturing processes, relevant for different industrial sectors, were identified and the proposed ‘primary prevention’ of risk will be integrated within six processing lines and implemented at pilot scale level by companies involved in SANOWORK.

The final goal is to develop and demonstrate the efficacy of “design options” based Risk Remediation Strategies, providing practical tools for:

- ü developing potentially useful safe design features;
- ü preventing NMs related worker injuries;
- ü reducing the need of expensive risk management measures;
- ü implement safe manufacturing processes.

3.1 Summary of SANOWORK's key strengths

KEY CONCEPT

- Industrial driven strategies applied on-site to target processing lines.
- Product design options for safer nanotechnology.
- Decreased emission potential and/or human hazard, preserving the nano-scale properties.

- Practical risk assessment of NMs with a reasonable balance between health hazards and exposure data.

KEY BENEFITS

- Cost-effective tools for safer manufacturing processes.
- Primary prevention of potential risks that can occur during worker manufacturing or customer use.

4 Organisation of SANOWORK

The overall work plan is designed for 42 operational months and comprises 7 Work Packages (WPs). A schematic representation of the work plan is reported in Figure 1.

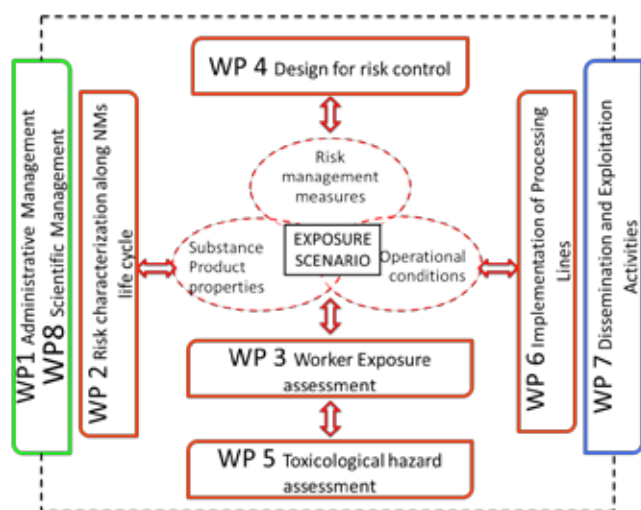


Figure 1: Sanowork WPs description (Pert chart).

An exposure scenario as requested by REACH deals with the collection of substance/product properties, operational conditions, risk management measures to take the exposure of workers to NMs under hazardous level. The SANOWORK S/T methodology is driven by the REACH definition of exposure scenario and establishes a synergy between WPs 2-6, in order to take as much indications as possible for the development of a safe worker exposure scenario.

WP1 and WP8 are the Administrative and Scientific management workpackages, respectively. In this WP, we will implement a rigorous administrative management process to ensure the timely achievement of the project deliverables and the scientific excellence of each workpackage task.

Table 1 Workpackages (WP) of SANOWORK

WP	Title	Topic
1	Administrative Management	This workpackage is aimed to: <ul style="list-style-type: none"> - coordinate and manage the administrative, financial and legal-contractual activities of the project - ensure an accurate and on-time communication flow with the European Commission (EC) and the

WP2 is related to the assessment and analysis of the risks caused by the occupational exposure to SANOWORK target NMs, in different stages of their manufacturing life cycle. LEITAT will develop databases which will be versatile to include new data from other sources. The essential information on NMs characteristics, exposure potential and toxicity to be used for the risk assessment will be included from literature sources as well as from data generated in the project by WP3 and WP5 worker exposure and toxicological hazard characterization, respectively. The risk analysis will provide the inputs to: 1) evaluate the effectiveness of existing or proposed risk remediation strategies; 2) assist UL in transferring information to insurance companies.

An occupational exposure assessment strategy (WP3), will be developed by INERIS. INERIS will collect exposure data 1) off-line, by semi-quantitative approach on as-delivered NMs and on chronic or accidental scenarios, at the lab scale (data useful for the so called RISK 1 assessment); 2) on-site, by a quantitative approach on processing lines (data useful for the so called RISK 2 assessment). It will also apply a control banding approach to risk assessment and management, providing data useful for a comparison between existing and proposed risk remediation strategies.

The toxicological hazards (WP5) will be assessed by IOM, UNIPR and UNIFI.

ISTEC will develop "Design Options" based Risk Remediation Strategies and evaluate NMs functional properties and their performances in relation to specific steps of the process. UL will assist ISTEC, INERIS, UNIPR, UNIFI and IOM providing a physico-chemical characterization of NMs.

PCHEM, NIRO, ELM, COL and LEITAT will develop industrial demonstration platforms to prove the integration of proposed risk control extra steps in different pilot lines (WP6), and make the industrial scenarios available for exposure assessment on-site. ISTEC will supervise the implementation of target processing lines and will assist UL for COST/BENEFIT evaluation. The results of the risk analysis (WP2) merged with COST/BENEFIT evaluation (WP6) of the proposed strategies will represent the basis for the definition of the safe worker exposure scenarios, tailored for each of the target processing line.

In WP7 the results and other outputs of the project will be disseminated internally and externally, after appropriate intellectual property protections, as and when needed. The results of risk analysis and COST/BENEFIT evaluation will be disseminated and used to inform the industrial partners about the strategies to implement in their processes, in order to create a safer workplace environment. Sanowork is structured and organized around 4 technical Work Packages (WP2-6), beside project management and dissemination/exploitation of results (WP1, WP8 and WP7)



Project Consortium in order to track the project progress and meet its administrative objectives.

- | | | |
|---|---------------------------------|--|
| 2 | Risk Analysis | <p>The main objective of this work package is to assess the risks caused by the NMs studied in SANOWORK in all the stages of their life cycle. The risk assessment characterization will be done on the different NMs before and after applying the risk management strategies proposed by SANOWORK. The data obtained from the risk assessment will be used to inform the industrial partners about the strategies to implement in their processes to create a safer workplace environment.</p> <p>In this WP, we will develop databases for NMs along their life cycle which will be versatile to include new data from other sources. These databases will include description of materials and processes, their applications, their physico-chemical properties and their toxicological profile. These data will be generated in the project as well as included from literature sources and will be used in the risk characterization. The collected information and risk characterization transfer to insurance companies will be one of the relevant objectives of this WP2.</p> <p>Strictly connected with the sustainability assessment of the proposed strategies is the Cost / Benefit evaluation of the proposed strategies, that will be based on cost analysis and evaluation of process efficiency and NMs functional properties BEFORE and AFTER the introduction of Risk Remediation Strategies.</p> |
| 3 | Worker Exposure Assessment | <p>The aim of this work package is to assess risks associated with chronic and accidental worker exposure scenarios applied to Processing Lines 1-6 at pilot-scale levels. Therefore the following specific objectives are proposed:</p> <ul style="list-style-type: none"> • Devise a qualitative worker exposure assessment method based on design or control approaches; • Develop a control banding risk assessment methodology to evaluate risks at the pilot industrial scale level; • Develop a quantitative worker exposure methodology by performing on-site exposure measurements, to provide inputs to the control banding (CB) risk assessment methodology. |
| 4 | Design for Risk Control | <p>The main objective of this WP is the development of the following Risk Remediation Strategies and their integration within the target processing lines:</p> <p>Strategy I: Coating by organic or inorganic based additives.
 Strategy II: Control of colloidal forces and disintegration of NPs.
 Strategy III: Integration of spray-drying process.
 Strategy IV: Immobilization by organic film coating deposition.
 Strategy V: Wet milling.</p> <p>Target Processing Lines:</p> <p>Processing Line 1: ZrO₂ NPs as additives in the chemical industry .
 Processing Line 2: ZrO₂ NPs as additives in ceramic materials.
 Processing Line 3: Polyamide Nanofibers as membrane components for filter industry.
 Processing Line 4: TiO₂ Nanofibers as photocatalytic components in Solar Cells.
 Processing Line 5: TiO₂ NPs as photocatalytic additives and Ag NPs as antibacterial additives in the ceramic industry.
 Processing Line 6: MWCNTs as additives in plastic industry.</p> <p>Strictly connected with the above described objective is the assessment of process efficiency as well as of NMs functional properties. This objective aims to provide useful information for COST / BENEFIT evaluation performed by WP6.</p> |
| 5 | Toxicological hazard assessment | <p>In order to assess the performance and the validity of proposed Risk Remediation Strategies (WP4) the WP5 will:</p> <ol style="list-style-type: none"> 1) Identify the hazardous properties of NMs, by reviewing the available literature data and relying on the thorough physico-chemical characterization performed within WP4; 2) Assess the impact of pristine and surface modified NMs by using representative of exposure pathways in vitro models: a) primary human cells; b) established mammalian including human cell lines; 3) Determine how the cellular bio-reactivity of NMs relates to their physicochemical properties and give indications for the development of a safe and sustainable manufacturing of nanoproducts. <p>The following specific objectives will be pursued:</p> <ol style="list-style-type: none"> i) Review toxicological data on selected NMs and identify knowledge gaps in hazard which are expected for such newly synthesized NMs (e.g. nanofibers, new functionalized nanostructures); ii) Assess cellular responses to the pristine and surface modified NMs delivered by WP4 by developing a testing strategy based on in vitro tests, using human cell models representative of the pulmonary and cardio-vascular systems; iii) Implement an hazard characterization strategy in close collaborations with SMEs, by assess cellular |



		responses of nanoproducts collected by WP3, during exposure assessment of processing lines implemented by WP6.
6	Implementation of Processing Lines	The general objective of this WP is the implementation of target processing lines, BEFORE and AFTER the introduction of the Risk Remediation Strategies proposed in WP4.
7	Dissemination and exploitation activities	The activities of WP7 aim to disseminate information and results of the project within the partners and outside the Consortium to all relevant stakeholders: national, international and EU regulatory bodies, NGO and representatives of the Nanotechnology industry, as well as proposing exploitation transfer plans and managing the Intellectual Property Rights. Moreover, it aims to collaborate with existing EC funded activities such as NanoSafety Cluster.
8	Scientific Management	The general aim of WP8 is to coordinate the scientific and technical management of the project and maintain the project work plan and complete it within the agreed time schedule. Scientific coordination: <ul style="list-style-type: none">• Scientific review of the work performed by the partners including scientific deliverables and milestones.• Monitor annual project meetings and regular interim, mid-term and final reporting, as well as the scientific quality.• The supervision of project global critical path.

5 Expected Impact

To develop practical and cost effective strategies for reduction of worker exposure to NMs during all stages of NMs production, use and disposal

NMs design strategies developed by SANOWORK project will contribute effectively to reduce health hazard and exposure potential. In particular it will develop: 1) aggregation control strategy, especially addressed to decrease emission potential during production and use, also including accidental spills; 2) inorganic/organic coating especially addressed to decrease health impact and exposure during use and disposal inside and outside the workplace; 3) techniques for disintegration of NPs during washing, especially addressed to decrease emission potential of disposal treatment.

To establish synergy with QNano: the European Union-funded infrastructure for NM safety testing

The active participation of members of the SANOWORK consortium in the NanoSafety Cluster will strengthen the interaction with nano-safety Infrastructure project. Preliminary contacts established by other complementary projects that could be funded under the same call and whose partners are, as well, deeply involved in NanoSafety Cluster activities, will ensure a strong cooperation and synergy with EU projects dealing with nano-safety. Most importantly, SANOWORK is pledged to report its findings to the NANOFUTURES platform and CEFIC and therefore will promote the 'safe by design' approach to all the relevant stakeholders. SANOWORK possess strong synergy with the EU NanoSafety Cluster by providing industrial stakeholders' and 'the general public' appropriate knowledge of the risks of NMs for human health' (workers). SANOWORK achieves through a strong participation from SMEs and industrial partners who have

shown a high level commitment to the project by allowing exposure measurement field study. The SANOWORK project also implements a primary prevention of exposure of workers to NPs by intelligent design of NPs considering its processing pathways.

To contribute to the advancement of the EU NanoSafety Cluster goals and agenda by:

- providing data on toxicological effects of target NPs (pristine and modified ZrO₂ NPs, polyamide and TiO₂ nanofibers, TiO₂ and Ag NPs and CNTs) to facilitate the formation of 'a consensus on nanotoxicology in Europe';

- Creating Life Cycle Database on physico-chemical, and toxicological properties of these materials based on FP6 and FP7 research. This database will be made available to NanoSafety Cluster 'to avoid duplicating work and improve efficiency';

- Identifying worker exposure scenarios and implementing the prevention/intervention measures to minimise Risk;

- Participating in meetings and workshops organised by NanoSafety Cluster for 'discussion, problem solving and planning R&D activities in Europe';

- Preparing and implementing a communication strategy to inform the identified stakeholders: industries, insurance companies, general public on risk remediation tools that prevent risks during production, use and disposal of NMs.

To facilitate research cohesion and integration in this area

The cohesion and integration of the results generated by SANOWORK will be facilitated by the active participation of the project members in the NanoSafety Cluster meetings and by dissemination activities of the project. More specifically, the



SANOWORK partners will integrate with the activities within the NanoSafety Cluster through activities in the following areas:

- pristine and modified ZrO₂ NPs, polyamide and TiO₂ nanofibers, TiO₂ and Ag NPs and CNTs and their physicochemical (chemistry, crystal structure, size, shape, coating, surface charge etc.);
- results on sound toxicological endpoints: 1) cell viability, including apoptosis, and mitochondrial activity; 2) induction/inhibition of pro-inflammatory cytokines and chemokines; 3) oxidative stress; 4) effects on unique cell function or cell –specific endpoints (e.g.

6 Exploitation and dissemination strategy

The dissemination of the results of SANOWORK will be promoted at all levels. Dissemination activities will be defined by the Management Committee and implemented at the Scientific Committee level. The website will be created at the beginning of the project. In fact, it is assumed that the results will not only be interesting to the scientific community but also to a great number of SMEs that work with Nanotechnology and Material Recycling/Recovery solutions. The dedicated website will produce an extensive record of all publications and communications originated on the course of the project. It will consist of a public area, a registration area and a private area. Public area will contain general information on the project, useful links to the EC services, nanotechnology portals, etc., a download area for downloading project brochures, newsletter, and other useful material such as public deliverables, news for communication of events, workshops, conferences related to the project, and contacts to allow the website visitors to have a direct link to the Consortium. The registration area will allow every public user to upload personal data (name, affiliation, area of interest and email address) in order to participate in the project's mailing list. The private area will be exclusively used by the Consortium for exchanging and sharing electronic data. The partners will be able to access the private area by using their own username and password. Finally, the interactions with relevant technology (e.g. nanotechnological and advanced materials) an environmental management platforms will allow the Consortium to widen the potential applications and the dissemination of the results. There are several major areas of Sanowork research activity, which are expected to generate new technology and may therefore generate new intellectual property opportunities and regard:

- Design for safe and manufacturing NMs
- Hazard characterization

immune function, barrier properties, etc...); 5) particle uptake and translocation; 6) fibrogenicity; 7) genotoxicity and cell transformation;

- results of worker exposure assessment plan;
- COST/BENEFIT evaluation of proposed risk remediation tools;
- insurance risk quantification of identified worker exposure scenarios.

- Exposure characterization

Industry has a vital interest that products are safe within current and future legislation. In particular the strategies proposed by SANOWORK project are integrated within specific industrial processing lines, all relevant and strategic for the companies involved. It is so evident that companies will be strongly interested to exploit the results of proposed design for safety and manufacturing strategies by promoting safe and sustainable NMs and processes. The new surface modified NMs and improved processes will be COST/BENEFIT evaluated and the technological break-through easily converted in commercial products, in accordance to IP rights. Other data produced by the project will be used as product documentation in order to provide to industrial stakeholders (customers, insurance companies, chemical industry association) the necessary scientific and technological information in terms of product characteristics as well as toxicological and exposure features.

Another big exploitable advantage that industries can take by the project is given by the promotion of safe occupational exposure scenarios as requested by REACH, giving free resources to safeguard their workers' health and safety. This expertise should be used internal in the Companies as well as a service in the external market for some Companies.

The interests of academics/public/private bodies involved in NMs Occupational Health and Safety issues relies on the attempt to overcome the lack of information about novel formulations that could pose an immediate problem for hazard and risk assessment, considered in the early stages of a product development.

In particular the development of proposed risk reduction tools beyond common state-of-the-art will be relevant for the scientific community as well as a framework for interdisciplinary developments of the design for safety strategies. Naturally, the dissemination of results by publishing in high impact factor journals and through the participation to the most important events promoted by NanoSafety Cluster will be another relevant exploitation interest of such organizations.



7 Directory

Table 2 Directory of people involved in this project.

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Scaffold

Innovative strategies, methods and tools for occupational risks management of manufactured nanomaterials (MNM) in the construction industry

Contract Agreement: Under Negotiation Website: Under Construction
Coordinator: Jesús M. López de Ipiña, Tecnalia Research and Innovation, Miñano-Alava, Spain

No.	Beneficiary name	Short name	Country
1	Fundación TECNALIA Research and Innovation	TECNALIA	Spain
2	Commissariat à l'Énergie Atomique et aux Énergies Alternatives	CEA	France
3	National Centre for Scientific Research "DEMOKRITOS"	DEMOKRITOS	Greece
4	Centralny Instytut Ochrony Pracy - Państwowy Instytut Badawczy	CIOP-PIB	Poland
5	Acciona Infraestructuras S.A.	ACCIONA	Spain
6	Asociación Española de Normalización y Certificación	AENOR	Spain
7	Mostostal Warszawa S.A.	MOSTOSTAL	Poland
8	ROSSAL SRL	ROSSAL	Romania
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10	NETCOMPOSITES Limited	NETCOMPOSITES	UK
11	Institutul de Cercetari Pentru Echipamente si Tehnologii in Constructii	ICECON	Romania
12	European Virtual Institute for Integrated Risk Management	EU-VRI	Germany
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14	Regents of University of Minnesota	UMN-PTL	United States
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1 Summary

Project Duration: Three years

Project Funding: 2,5 Mio. EUR

Manufactured nanomaterials (MNM) and nanocomposites are being considered for various uses in the construction industry and

related infrastructure industries, not only to enhance material properties and functions but also in the context of energy conservation. Despite the current relatively high cost of nano-enabled products, their use in construction materials is likely to increase because of highly valuable properties imparted at



relatively low additive ratios, rapid development of new applications and decreasing cost base MNMs as they are produced in larger quantities.

Thus the use of nano-products in the construction industry is a reality and can be expected to grow in the near future. Consequently, there is a general uncertainty with respect to health and safety risks and how to properly manage them in order to protect workers and be in compliance with OHS legislation.

SCAFFOLD is an industrial oriented idea specifically addressed to provide practical, robust, easy-to-use and cost effective solutions for the European construction industry, regarding current uncertainties about occupational exposure to MNMs. This will be achieved by introducing a new paradigm to improve workers protection against MNMs in construction, based on a novel holistic Risk Management approach (RMM).

The aim of SCAFFOLD is to develop, test, validate and disseminate a new holistic, consistent and cost effective Risk Management Model (RMM) to manage occupational exposure to MNMs in the construction sector. This will be done by integrating of a set of innovative strategies, methods and tools developed within the project into consistent state-of-the-art safety management systems (OHSAS 18001 + ISO31000).

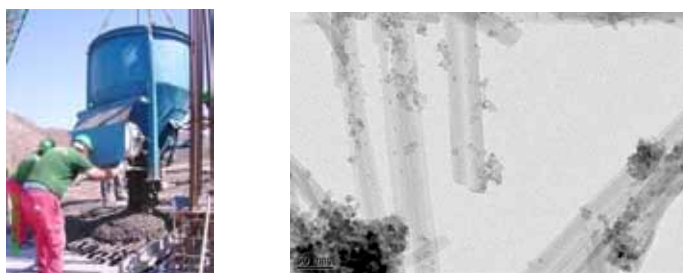


Figure 1 a) Handling self-compacting concrete with nanosilica additive for structural applications; b) TEM image of TiO₂ supported on modified sepiolite, as a decontaminant additive for construction materials.

2 Background

Manufactured nanomaterials and nanocomposites are being considered for various uses in the construction industry and related infrastructure industries, not only for enhancing material properties and functions but also in the context of energy conservation (Figure 1). Despite the current relatively high cost of nano-enabled products, their use in construction materials is likely to increase because of highly valuable properties imparted at relatively low additive ratios, rapid development of new applications and decreasing cost base MNMs as they are produced in larger quantities.

Recent studies suggest that workers handling nano products have mostly worked with cement or concrete products, coatings or insulation materials. Other types of products, including road-pavement products, flame retardant materials or textiles, were only indicated by some. However, a survey developed by FIEC and EFBWW (2009) shows that the majority of workers and their employers in the construction sector (~75%) are not aware that they work with nano-products.

Occupational exposure to these emerging risks may be accidentally or incidentally produced at different stages of the construction industry life cycle (Figure 2). Due to the novelty, these same nano-products might pose new health and safety risks to the worker onsite, which scientists are just starting to understand. Detailed information about the product composition and their possible nano-specific health and safety issues though, is generally lacking and the information available for the raw material manufacturer is often lost while stepping down the user chain. As a consequence, for the average construction company it is very difficult to conduct a proper risk assessment and organize a safe workplace for its employees.

Despite the potential risks, the use of nano-products in the construction industry is a reality and can be expected to grow in the near future. Consequently, there is a general uncertainty with respect to health and safety risks and how to properly manage them to protect workers and be in compliance with OHS legislation. This calls for a new approach for dealing with uncertainties, providing construction companies with new strategies, methods and tools to appropriately manage these emerging risks.

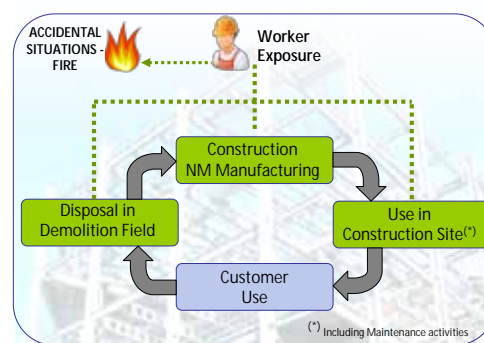


Figure 2 General overview of the Life Cycle of the MNMs in the construction sector

3 What is SCAFFOLD

SCAFFOLD is an industry-oriented idea specifically focussed on providing practical, robust, easy-to-use and cost effective solutions to the European construction industry, regarding current uncertainties about occupational exposure to MNMs. This will be achieved by introducing a new paradigm to improve workers protection against MNMs in construction, based on a novel holistic Risk Management approach (RMM).

The core of the project is the integration of three basic elements into the new paradigm (Figure 3):

- § Existing relevant strategies and methods for risk management coming from ongoing or relevant research that has concluded (e.g. FP7 - EU NanoSafety Cluster projects such as NANOHOUSE, NANOSAFE, NANEX, ...; projects from the EU-OSHA and other relevant projects).
- § Innovative strategies, methods and tools produced from focused research undertaken by SCAFFOLD to cover selected gaps.



- § Consistent state-of-the-art OHS management models (OHSAS 18001 + ISO 31000)

The integration of these three inputs will allow the construction of a novel, consistent and cost-effective SCAFFOLD RMM, particularly addressed to SMEs. SCAFFOLD will focus research in construction. Thus, industrial implementation of the new paradigm in such a very OHS complex sector will allow generalizations face other industrial sectors without too much additional efforts in research. In addition, SCAFFOLD will integrate the NANOHOUSE approach in order to cover the global life cycle of the construction industry.

SCAFFOLD RMM will be constructed on the basis of consistent and effective OHS / Risk models (element 3), applicable to any organisation from all types of business sectors, activities and sizes of which implementation in industry, and specifically in construction, is quickly growing. According to that, SCAFFOLD RMM will be supported by the continuous improvement concept. Thus RMM will be able to incorporate any relevant future input produced by ongoing MNMs research, assuring RMM sustainability. In addition, compatibility of the RMM with other management systems (e.g. quality - ISO 9001, EFQM, environmental - ISO 14001, EMAS), will facilitate its integration by organizations, should they wish to do so.

4 Objectives

The aim of the SCAFFOLD project is to develop, test, validate in real conditions and disseminate a **new holistic, consistent and cost effective Risk Management Model (RMM) to manage occupational exposure to MNMs in the construction sector**. This will be done by integration of a set of innovative strategies, methods and tools developed by the project into consistent state-of-the-art safety management systems.

The SCAFFOLD project has the following specific S&T objectives:

1. To assess effectiveness of existing risk reduction strategies, methods and equipments (confinement of processes, PPEs, filtration, etc) in construction scenarios.
2. To develop novel methods leading to the formation of less risk-posing MNMs: safer concentrated dispersions of metal oxide nanoparticles for concrete manufacturing.
3. To propose safer process alternatives for nanocomposite / coatings production jointly with safer nanocomposites and coatings formulations (minimising emissions in machining / spraying tasks or in case of fire).
4. To produce novel strategies and methods for exposure assessment (inhalation and dermal) and modelling adapted to the real sector scenarios, jointly with exposure data in the sector and a decision making strategy for risk assessment.
5. To develop novel risk protection strategies for the sector, including a proposed method for ISO standardization and a decision making strategy for PPEs selection.
6. To adapt the Control Banding approach to the sector, and to test it.
7. To construct a robust and cost-effective model (RMM) for risk management of occupational exposure to MNMs along the life cycle. This will include a set of innovative tools (Toolkit) to

support implementation and customized applications for SMEs of the construction sector.

8. To test and validate the RMM and associated tools in construction industry (Industrial Use Cases in Large companies and SMEs).
9. To deploy a strategy to promote implementation of the SCAFFOLD approach in the European construction industry.
10. To coordinate dissemination actions with the *European Nanocluster* to maximize the project's impact.
11. To strengthen synergies among MNMs research groups in Europe, Canada and the USA.
12. To produce pre-standardization and pre-regulation documentation addressed to standardization Technical Committees and regulators regarding construction nano-products, PPEs and OHS management issues.

5 The SCAFFOLD approach and organisation

SCAFFOLD will collect, review and analyse relevant quantitative and qualitative information and data on current strategies, methods and tools for workers protection against MNMs (WP1), in order to identify needs and gaps for proper risk management. Four main topics will be analysed: *Risk prevention* (MNMs safe design, safe design of manufacturing processes, etc), *Risk assessment* (occupational exposure and toxicology, measurement equipment and procedures, exposure limit values, etc), *Risk protection and control* (filtration, PPEs, etc) and finally *Risk management* (safety management models, tools, implementation level, work procedures, "good practices", *risk communication*, etc).

A complementary and focused research in the above mentioned four fields (WP2 to WP5) will be developed to fill the identified gaps selected and provide innovative input on strategies, methods and tools to construct an integrated RMM and a set of advanced software tools (Toolkit). In order to assure RMM & Toolkit robustness, soundness and cost-effectiveness, testing and validation activities will be carried out in real conditions in a sample of large companies and SMEs involved in the project Consortium (WP6). As a key factor for the success of the project, relevant dissemination and exploitation activities will be carried out to promote implementation of SCAFFOLD approach in the European Construction industry, particularly in SMEs (WP7).

To guarantee a feasible approach and a cost effective project, an initial project roadmap has been developed by the Consortium. For its development, the SCAFFOLD Consortium analyzed the potential applications of MNMs in the construction sector in the areas of 1) Cement, 2) Pavements and 3) Advanced Materials. Fifteen MNM applications, MNMs involved in each case, as well as potential exposure scenarios along the life cycle were initially identified. The occupational safety research priorities were determined within this preliminary set of applications by using a 3x3 matrix with two decision criteria: Level of risk and Innovation rate. The results of this preliminary analysis were encapsulated in the SCAFFOLD initial roadmap, where the following priorities were selected for the project (A review of all of them will be done in WP1):

- § Six applications of MNMs in construction: 1) Depollutant mortars, 2) Self-compacting concretes, 3) Stabilised,



Bituminous road-surface, 4) Self-cleaning external coatings, 5) Fire-resistant panels and 6) Insulations.

- § Five MNMs: TiO₂, SiO₂, Cellulose Nanofibres, Carbon Nanofibres and Nanoclays.
- § Six categories of exposure scenarios (integrating 26 individual exposure scenarios): 1) Manufacturing MNMs, 2) Manufacturing product containing MNMs, 3) Preparation, mixing, and application on site, 4) Assembly and machining, 5) Demolition and disposal and 6) Accidental fires (Combustion of MNMs).
- § Five European Industrial Use Cases covering life cycle steps of MNMs,

Within the SCAFFOLD Consortium five industrial partners will carry out the demonstration activities through real-life Industrial Use Cases (IUC). IUC will focus on three stages of the MNMs Life Cycle in the construction sector: 1) MNMs manufacturing (Raw MNMs and construction products containing MNMs); 2) Use in construction sites (Building construction and Civil works, including potential maintenance activities) and 3) Disposal in demolition field. The demonstration of SCAFFOLD results will aim to:

1. Test the SCAFFOLD RMM into industrial construction companies in real-life situations to demonstrate their validity and use for effective management of MNMs occupational

exposure along Life Cycle in the European Construction Sector.

2. Focus research activities on some specific and priority industrial applications, scenarios and MNMs of the European Construction industry.
3. Focus the project research tasks in the IUC (industrial demonstration) from the very beginning of the project.
4. Develop demonstration activities (IUC) across Europe considering different safe-cultures and awareness levels as well as company scales (large and SMEs).

With the aim of achieving the S&T Objectives set in the project, the Consortium will tackle multidisciplinary research activities that have been grouped into eight Workpackages (WPs): WP1-5 as RTD activities, WP6 as Demonstration activities, WP7 as Other activities (Dissemination and exploitation) and WP8 as Management. The Figure 1.5 shows the project approach and strategy according to the organization of WPs.

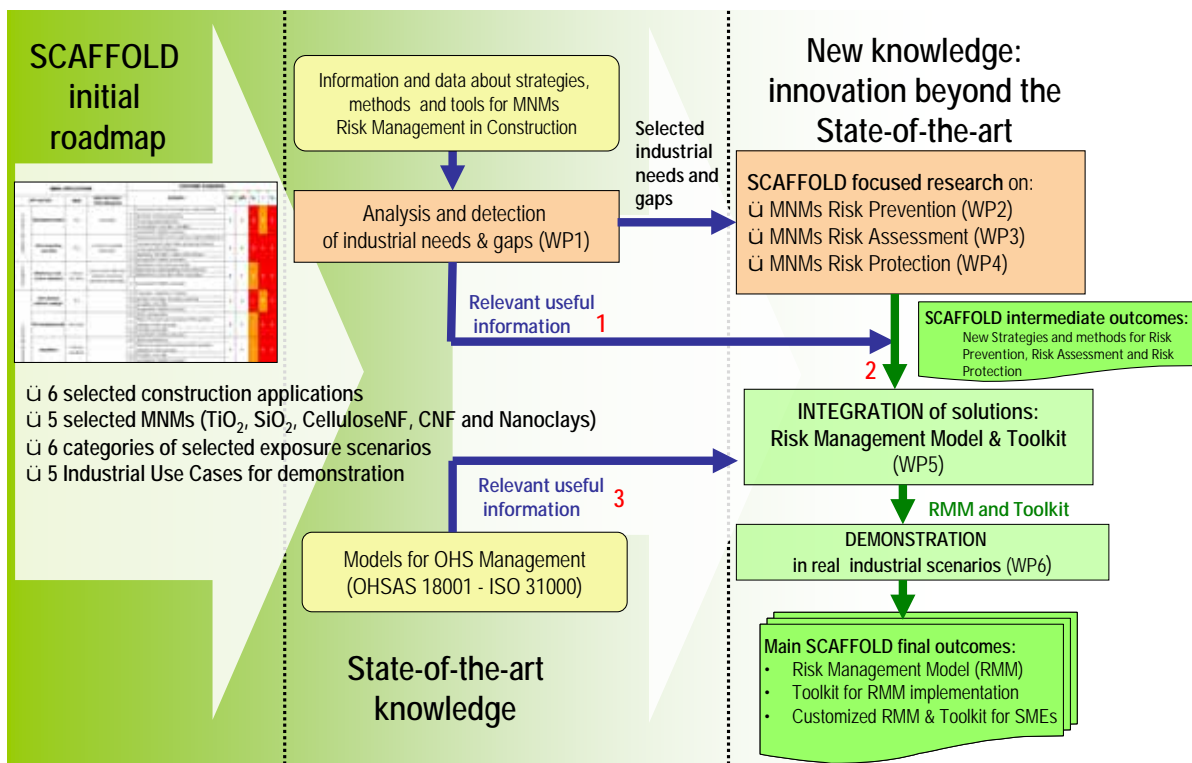


Figure 3 The SCAFFOLD approach



Table 1 Workpackages (WP) of Scaffold

WP	Title	Topic
1	<i>Profiling the European construction industry that face MNMs occupational exposure</i>	WP1 will aim to develop a profile of the European construction industry, in order to face the occupational exposure to MNMs and to provide relevant available information - strategies, methods and tools - to construct the RMM & Toolkit (WP5).
2	<i>MNMs Risk Prevention</i>	WP2 will aim to develop innovative strategies and methods for Risk Prevention of the occupational exposure to MNMs by safe product design.
3	<i>MNMs Risk Assessment</i>	WP3 will aim to develop innovative strategies and methods for Risk Assessment of the occupational exposure to MNMs in scenarios of the construction sector.
4	<i>MNMs Risk Protection and control</i>	WP4 will aim to design and develop innovative strategies and methods for Risk Protection and control against the occupational exposure to MNMs in construction.
5	<i>MNMs Risk Management: Integration of solutions</i>	WP5 will aim to integrate relevant available results from WP1 and innovative results produced in WP2to WP4 to construct a Risk Management Model (RMM) - ISO 18001 & ISO 31000 based – and a set of innovative tools (Toolkit - Software) to effectively manage the occupational exposure to MNMs in construction.
6	<i>Testing and validating the RMM & Toolkit in construction industry</i>	WP6 will aim to test and validate the RMM & Toolkit in real conditions - Five Industrial Use Cases (IUC).
7	<i>Dissemination and exploitation</i>	WP7 will deal with raising public awareness, disseminating project results and defining & managing dissemination and exploitation plans.
8	<i>Project management</i>	WP8 will deal with coordinating and managing the project by covering technical, administrative, legal and financial issues of the project and the relation with the EC.

6 Progress beyond the state-of-the-art

The SCAFFOLD Consortium has identified the main technologies that are involved in the S&T Objectives and grouped them in four areas related to the occupational exposure to MNMs: Risk prevention by MNMs design, Risk assessment, Risk protection & control and Risk management. In this respect, the Consortium has conducted a thorough analysis of the state-of-the-art in those four technology areas, identifying the technological challenges associated with the project. The aspects of SCAFFOLD which go beyond the state-of-the-art will be:

1. Risk Prevention by design of MNMs: New intrinsically safe MNMs formulations; new specific fire retardant nanocomposite formulations with minimum risk to health & safety and new specific strategies for safe nano-filled concrete, bituminous pavements, coatings and insulation.
2. Risk Assessment: New methods - screening/advanced - to measure worker exposure to MNMs and to measure exposure to nanoparticles and toxic fumes in case of accidental situations (selected construction products); an exposure measurement database: inhalation & dermal, at lab scale, pilot and real scale (selected scenarios) and accidental situations (fire); numerical models to obtain exposure data at the breathing zone, deposition in the

Human Respiratory Tract and dermal deposition and Proposals on OELs for selected MNMs.

3. Risk Protection and Control: New method for evaluation of the efficiency of collective protection in complex form, in real conditions of used ventilation systems; new methods for characterization of PPE efficiency (proposed to ISO standardization procedure); a database collecting the determination of effectiveness of main collective protection alternatives operated in different conditions; a novel methodology for the assessment and selection of PPEs against MNMs in the construction sector; new methods and devices for risk protection; Control Banding Approach adapted, tested and customized to the construction sector; new guidance and exposure register model for monitoring the health of workers exposed to MNMs.
4. Risk Management: A consistent and cost-effective model for risk management during the complete life cycle (RMM), tested and validated in real industrial conditions (Industrial Use Cases), OHSAS 18001 and ISO 31000 based and fully compatible with ISO 9001 and ISO 14001; a set of innovative tools (Toolkit) to support implementation, including customized applications for SMEs.



7. Impact

European Industry and Society

Employment in nanotechnology will grow to reach a predicted 10 million jobs worldwide in 2014. This will account for 11% of the employment in the manufacturing sector. If the population and the occupational structure in the EU remain unchanged, it would mean that almost six million people will be working in Europe's nanotechnology sector by 2014. As it has been already mentioned, the construction industry is the biggest industrial employer in EU27 (3 million enterprises and 14,9 million people employed). In parallel, the construction industry has one of the worst occupational health and safety records in Europe.

In this context, the implementation of SCAFFOLD's practical and cost effective strategies, methods and tools for reduction of worker exposure to MNMs will produce a very important impact on companies and workers, in terms of OHS improvement. A proper management of MNMs based on the SCAFFOLD approach will preventively contribute to avoiding potential chemical accidents and diseases at work, contributing to reaching the overall objective of the Community Strategy aimed at reducing the total incidence rate of accidents at work per 100 000 workers in the EU 27 by 25%. This preventive approach will produce parallel benefits by reducing non-safety bills both in construction companies and in the whole European industry.

The presence of SMEs is a highly relevant issue in the construction industry, representing 99.9% of all enterprises in the sector. In total, there are 13.1 million people employed in SMEs of the construction industry (88%). SMEs are more vulnerable to occupational risks and in particular those companies working in dangerous sectors like construction. SMEs account for 82% of all occupational injuries and about 90% of fatal accidents. In total, SMEs account for around 80% of all occupational diseases caused by chemical agents. In addition, SMEs in the construction industry are generally present *through a subcontractor*, which represents a relatively high share (45%) compared to other sectors.

This picture means that the implementation of the SCAFFOLD framework in SMEs will have a very relevant impact on the European construction industry. To take this significant issue into account, SCAFFOLD will produce a specific approach for SMEs by customizing both the RMM and Toolkit. Subcontracting, identified as an emerging risk in the sector, will be a significant issue with which to be dealt. It will allow the generation of a practical, easy-to-use and cost effective framework to properly manage the occupational risks associated to MNMs.

A Spanish study shows that a relatively large, and increasing percentage of SME subcontractors (23 %) recognise the obligation to fulfil a safety management standard (e.g. OHSAS 18001) imposed by their clients. Increasing awareness on the part of the contractors about occupational risks of MNMs will bring new requirements for SMEs. The implementation of SCAFFOLD RMM will provide SMEs with a competitive advantage, impacting positively on the sector. Finally, an additional benefit for SMEs integrated in the SCAFFOLD Consortium will be the participation in research programmes.

Market

Freedonia has recently forecasted that the annual US demand for nanocomposites will more than double by 2011, reaching 130,000 tonnes. By 2025 it is expected that nanocomposites will be a 6.5

billion € market, with volumes approaching 2.2 million tonnes. The authors note that construction will emerge as a significant market. Related sources suggest that 360 million pounds of nano-additives will be required by 2020 at a value of 1.4 billion €, over half of which will be used to purchase CNTs. Regarding nano-oxides, the global market was 166 million Euros in 2006, and is estimated to reach 760 million Euro by 2011.

A critical issue regarding the success of any method for MNM production is its cost effectiveness. But a second market requirement is to guarantee safety of the product during the complete life cycle. In this sense, SCAFFOLD results are very promising since they allow the use of reasonably low cost raw materials and, furthermore, this production method results in important increase of product safety and savings in energy and cost. Therefore, it is imperative that the HSE issues (safe product design, safe manufacturing, OHS risk management, compliance with OHS regulations, etc) surrounding these materials and sectors in particular are adequately solved and risk prevention strategies put in place rapidly. SCAFFOLD may play an important role in this sense.

Regarding the market of safety management systems, the OSHAS 18001 is the worldwide reference in occupational health and safety management. The number of certified companies has increased dramatically since 2003. In three years (from 2007 to 2009) the number increased by 73% in the world. A recent report shows how the penetration of this standard is higher amongst SMEs; in fact, the study shows that 81% of the companies were SMEs, 52% between 50 and 250 workers and 29% with less than 50 employees. Concerning the construction sector, it is leading the implementation of OHSAS (37%), which shows the commitment of the construction companies, especially SMEs, to the improvement of labour safety in Europe. Certification market for OSHAS 18001 will be twice its current size in two years (in 2012 more than 100.000 companies will be certified). If the tendency continues, 80% of those companies will be SMEs. It is easy, from a very conservative perspective, to estimate that market will be doubled again by 2020, which would easily represent a 2,000 M€ market only for OSHAS 18001. Considering the current evolution of nanoparticles and nanomaterials, and also considering the expected evolution of OHSAS 18001, the creation and commercialization of the RMM model could have a great impact in the market, especially for SMEs.

European policies, regulations and standards

The Community Strategy on Health and Safety at work for the period of 2007 – 2012 includes nanotechnology as an important topic to be developed in the context of the identification of new and emerging risks. In addition to the previously mentioned Strategy, other specific objectives tangibly impacted by SCAFFOLD will be: 1) the development of new methods to identify and evaluate new potential risks and 2) to support SMEs and the high-risk sectors - like construction - in the implementation of the legislation in force and its adaptation to changes in the workplace. On the other hand, SCAFFOLD will positively contribute to 1) getting some of the objectives defined by the Action Plan for Construction of the Lead Market Initiative (LMI), 2) supporting nanosafety issues of the European policy on nanotechnology, currently in revision, 3) supporting the Innovation Strategy for Europe by introducing innovation in the core of the construction sector, contributing to enhance Europe's global economic competitiveness in a global market.



SCAFFOLD will contribute positively to European Regulations by providing new information to elaborate better regulations about issues related to safety of MNMs and support compliance with current legislation requirements. The two main topics to be addressed will be: a) Health and Safety at work in the sector and the future strategy for managing the occupational exposure to MNMs in the construction industry –SCAFFOLD deploys a specific task to build a proposal in this field – and b) the Safety of products (PPEs, construction products).

Standardisation is a highly relevant issue in this sector. In the European Committee for Standardisation (CEN), the construction sector covers around 3,000 work items on product standards and test methods. Of these, about 500 standards will be harmonised under the Construction Products Directive (89/106/EEC). Pre-standardization activities of the SCAFFOLD project will positively contribute to developing European standards that aim to reduce potential barriers that might cause an increase of the time to place in the market of new construction nano-products (nanocomposites, nano-coatings, etc) and PPEs. In addition, the SCAFFOLD project will also provide innovative inputs for new/improved OHS standards in the field of OHS management and workers protection against MNMs.

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9. Directory

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SIINN

Safe Implementation of Innovative Nanoscience and Nanotechnology

Contract Agreement: NMP4-2010-265799 Website: <http://www.siinn.eu>
Coordinator: Rainer Hagenbeck, Forschungszentrum Jülich GmbH, Germany

No.	Beneficiary name	Short name	Country
1	Forschungszentrum Jülich GmbH	JÜLICH	Germany
2	TEMAS AG	TEMAS	Switzerland
3	Bundesministerium für Verkehr, Innovation und Technologie	BMVIT	Austria
4	Executive Agency for Higher Education, Research, Development and Innovation Funding	UEFISCDI	Romania
5	Service Public de Wallonie	SPW-DGO6	Belgium
6	Fundacion madri+d para el Conocimiento	madri+d	Spain
7	National Funding Agency for Research	ANR	France
8	National Hellenic Research Foundation	NHRF	Greece
9	Technology Strategy Board	TSB	United Kingdom
10	Federal Ministry of Education and Research	BMBF	Germany
11	Ministerio de Ciencia e Innovación	MICINN	Spain
12	Veneto Region	RVE	Italy
13	Chief Scientist Office, Ministry of Health	CSO-MOH	Israel
14	Science Foundation Ireland	SFI	Ireland
15	Federal Office of Public Health	FOPH	Switzerland
16	Stichting voor de Technische Wetenschappen	STW	The Netherlands
17	Veneto Nanotech	VN	Italy
18	Commissariat à l' énergie atomique et aux énergies alternatives	CEA	France
19	Fundação para a Ciência e a Tecnologia	FCT	Portugal

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1 Summary

Project Duration: 1 August 2011 – 31 July 2014

Project Funding: 1.5 Mio. EUR

The primary aim of the "SIINN" ERA-NET is to help create an optimum environment within Europe with which to promote the safe, rapid transfer of innovative nanoscience and nanotechnology (N&N) research and development into industrial application.

Starting in 2011, this will be achieved by pooling together appropriate national and regional resources in order to create a sustainable, coordinated, transnational programme of N&N-related RTD work across Europe which is based upon utilising the synergies to be gained from the national or regional programmes and the genuine desire of their owners to cooperate. Thus, in addition to strengthening the European Research Area itself, SIINN will create an effective network of ministries, funding agencies and academic and industrial institutions active in the N&N fields, which, together with further stakeholders such as industry, other N&N networks and organisations, standardisation bodies, etc., will create Europe's first sustainable transnational programme of applied N&N research.

The commercial application of products containing nanomaterials (NMs) is increasing rapidly, but one important question, the potential risks of NMs for the environment and human health and safety (EHS), remains a substantial barrier to their wide innovative use.

Therefore as a first priority, SIINN will initially focus on developing a consolidated framework with which to address and manage nanosafety issues i.e. nano-related EHS risks.

National activities in Europe in N&N EHS remain largely uncoordinated and fragmented, resulting in the sub-optimal use of available resources, such as human resources, research funding and research infrastructures. Furthermore, the data used for EHS assessments world-wide is often based on toxicological studies of nanomaterials which are scant, unreliable or even contradictory; the data is often gathered for nano-systems which are either ill-defined or not clearly defined at all.

The SIINN Project will thus focus on ways of remedying this unsatisfactory situation and will, in its first three-year phase, of necessity concentrate on obtaining sound toxicological data for NMs for nanosafety and EHS risk assessment and management.

SIINN's activities will be undertaken in close cooperation with various national and international networks, organisations and groupings, including the NanoSafetyCluster, the QNano infrastructure, the OECD Working Party on Nanomaterials (WPNM), the NANOfutures ETIP and NanoImpactNet.

Two transnational calls will be prepared during the lifetime of the SIINN ERA-NET allowing the scientists from the participating countries and regions to perform joint research activities. The intention is to provide a basis for long term joint activities of funding organizations coordinating their programmes in the area of nanosafety and nanotoxicology in close contact with the research community and the industry.

2 Background and Goals

Nanosciences and Nanotechnologies (N&N) are two of the fastest growing research areas of the last decade and currently more than 1000 nano-enabled products are currently available on the market in more than 20 different countries, whereby the total global market for nanoproducts is expected to exceed € 3000 billion by the year 2015).

However, the most serious handicaps for nanomaterials to enter the market are EHS-related issues or to be more precise, the lack of accurate and reliable data on which to base a detailed assessment of the EHS behaviour of such man-made or "engineered" nanomaterials.

The primary aim of the SIINN ERA-NET is therefore to create an optimal, sustainable environment within Europe in order to promote the safe, rapid transfer of innovative N&N research and development into industrial application.

The uncertainties associated with the safe use of engineered nanomaterials presently hinder the creation of a new, globally highly competitive, nano-based industry within the EU. Therefore as a first priority, SIINN will focus on developing a consolidated framework with which to address and manage nano-related EHS risks. This will include the development of a joint, transnational R&D programme looking not only into EHS risk assessment but also of necessity the toxicological behaviour of nanomaterials and, if required, addressing also the very basics of nanotoxicology. The framework will be developed based on existing, verified information and knowledge, complemented with calls for actual research projects to close identified data gaps. By utilising this framework, the foundation will be laid for the rapid market uptake of safe, nano-based technologies and products and this will strengthen the development of high added-value products as the basis of a new, globally competitive industry in Europe.

Other parameters such as production engineering, quality control or the protection of intellectual property rights are also very relevant for stimulating technology transfer in the nanomaterials sector. However, along with the EU Commission and industry itself, the governmental bodies (or their representatives) united in the SIINN Consortium are convinced that at this stage it is nanosafety and nanotoxicology which should be the immediate focus of their resources within the first three-year phase of this ERA-NET.

Responding to the apparent increasing knowledge gap between the development of N&N and our understanding of how nanomaterials interact with the environment and the human body, many research and technological development studies now also address nano-specific aspects of product safety. Because of the complexities of nanomaterial-containing systems, however, where the physical and biological impacts of these nanomaterials are highly dependent upon the system themselves, the problem of the reliability of current physical and biological data for nanomaterials is both real and large. The large number of studies regarding engineered nanomaterials also poses problems in terms of data



management and reliability, especially as data are often shown to be even contradictory.

Thus, studies focusing on the behaviour of nanoparticles systems in biological settings are being carried out in many areas of the world but their results are not always transferable or directly applicable.

The SIINN ERA-NET has therefore been devised in order to also overcome this problem by setting the conditions through joint, transnational cooperation at government or regional level within Europe which will enable science and society to be provided with reliable data which can be implemented for the safe use of engineered nanomaterials.

A common database platform which will allow entry and searching from a unique starting point in the various existing nanosafety data sources (verified by SIINN) will therefore be developed and implemented as a tool to aid programme owners and implementers in deciding on future research themes. This tool will, as a spin-off of the SIINN ERA-NET, be made available to all interested stakeholders (government, industry, education, research, standardisation bodies) via the SIINN website.

Europe maintains a strong nanotechnology research base, heavily supported by public funding in nanotechnology research at both European Union and national levels. SIINN will launch at least two joint transnational research calls in the field of nanosafety, nanotoxicology and risk assessment during its initial three-year life. This joint effort could ultimately lead to joint RTD programmes being developed between the EU Member and FP7/FP8 Associated States involved. In the mid-to-long term, joint activities with key countries outside of Europe (e.g. the USA or Japan) are also feasible.

Today, current nanosafety research is concentrating on the characterisation of occupationally relevant airborne nanoparticles, the definition of an appropriate consensus on the chemical and physical parameters to be considered in EHS risk assessment and the development of appropriate standardised test methods to be employed. SIINN will augment these by including deliverables that are aimed at establishing "recommended practices" for the safe handling of nanoscale materials.

At the end of the project's initial three years, SIINN will have established a coordinated, transnational programme of nanosafety and nanotoxicology-related activities across Europe which will address the potential environmental, health and safety implications of nanoscale materials, and which will include the development of standards for environmental and toxicological studies of nanoparticles and a metrology infrastructure supporting these standards.

3 Current Status of SIINN ERA-NET

The SIINN ERA-NET started its work on August 1, 2011. The kick-off-meeting on September 19 and 20, 2011 in Berlin established the structures and confirmed the readiness of the partners to cooperate on establishment of joint research activities. The meeting of the SIINN Steering Committee on December 12, 2011 in Brussels has reviewed the progress. Decisions were taken towards the preparation for the first joint SIINN call in March 2012.

At the end of 2011, after five months, the six Work Packages (WP) have reached the following major results:

Within WP1, the important criteria and terms in the area of NM toxicology were defined and the health and safety relevant information which is currently available from, and to Europe were examined. Based on the analysis of this information, WP1 identified important knowledge gaps with respect to occurrence and toxicity of manufactured nanomaterials.

Within WP2, an inventory of directed liaisons, initiatives and actions with respect to the national activities of the SIINN partner countries and regions was started. Contacts to the major international organizations active in the field N&N safety have been initiated. The preparation of a common database platform has been started and is ongoing.

WP3 started an inventory of existing characterization methods and a data collection with respect to evidence-based EHS risk assessment for human health and environmental safety progresses.

WP4 has established preliminary parameters and procedures for the first joint call for proposals. The first call is intended to be launched in March 2012. The boundary conditions for the call and a draft of the Memorandum of Understanding have been prepared. Approximately 10 funding organization from different countries/regions have expressed their principal interest in participation.

WP5 has established the SIINN webpage (www.siinn.eu). The PR and communication plan has been accepted by the partners. An information campaign in the context of the first call for proposals is under preparation.

WP6 has established the management structures and bodies and is assuring their smooth functioning. The contractual arrangements have been adapted to the present actual status.

4 Summary of SIINN's Key Expected Impacts

- Strengthening of the European Research Area in nanoscience and nanotechnology,
- Decrease in RTD fragmentation and improvement in the coordination and exploitation of synergies between the owners of national funding programmes, other authorities related to N&N and nanosafety, the corresponding research community and industry, including an enhanced interaction with the EU Framework Programme, Generation of a programme of transnational RTD, initially for nanosafety and EHS assessment,
- Generation of a carefully examined set of data which will allow reliable guidelines for the development of legal frameworks (e.g. precautionary measures and steps towards regulations) to be developed to increase safety and reduce risks through all stages of a product's life-cycle, from R&D to disposal and recycling,



- The efficient identification of knowledge gaps from this data set, helping to clearly and efficiently specify goals for current and future transnational research programmes so that they may be developed in a concerted way,
- Efficient use and leverage of resources (such as knowledge, capital and investment at European level) through common calls, thereby avoiding duplicity in projects (unless specifically required) and enhancing the common use of knowledge, capital and investment at European level,
- The possibility of the rapid assessment and management of potential risks is a crucial success factor for industry to enable the more rapid adoption of N&N for the development of safe products,
- A higher standard of safety and confidence for the population and the environment which will help promote acceptance for applications of nanotechnology.

establish the reliability of the available data. Noting any irregularities or deficiencies, this WP will set down a list of research objectives which will be used as input to WP4, the WP charged with establishing the joint transnational research programme and carrying out tenders for R&D projects to assess these deficiencies.

For the first time in the nanomaterials sector in Europe, joint transnational calls will be carried out in WP4 to overcome identified deficiencies in current nanosafety knowledge for assessing the risks of NM and NM-containing products. All the stages associated with the carrying out of a research call will be undertaken in this WP, which will also have a “fast-track” mechanism which will allow for some small projects to be quickly undertaken in order to obtain critical results rapidly (rather than under normal project funding conditions).

WP5 will be responsible for the dissemination of information both within the project itself as well as to external recipients and stakeholders such as government bodies, industry, research organisations, standardisation bodies and importantly, the public at large.

Finally, WP6 will oversee the complete work of the SIINN project and ensure that the tasks and deliverables are undertaken according to timetable and within the scopes required for the success of the project. WP6 will thus undertake the technical and administrative management of the project and will also include any horizontal issues such as Quality Management.

5 Organisation of SIINN

The project is strategically organized into six workpackages:

Workpackage 1 *Identification of sources and inventory of available information*

Workpackage 2 *Liaison with European and global initiatives; networking and information management and exchange; roadmapping*

Workpackage 3 *Validation of Existing Characterisation and EHS Assessment Methods (Including Life-Cycle Validation) and Identification of Knowledge Deficiencies*

Workpackage 4 *Contractual Framework and Implementation of Joint Calls*

Workpackage 5 *Dissemination, Exploitation and Sustainability*

Workpackage 6 *SIINN Coordination and Management*

The overall strategic concept of SIINN when applied to the example of nanosafety is to first catalogue which information is available to researchers and what state is it in (e.g. is the available data specified for a particular, defined NM in a defined system?). This is the central task of Workpackage 1 (WP1).

Parallel to this, Workpackage 2 (WP2) will establish close liaisons with organisations working in the EHS risk assessment of NMs, both within Europe and elsewhere, in order to form a close network and to exchange information. This cooperation will also include various strategically important tasks such as the identification of best practices, synergy potentials and the elaboration of recommendations for future collaborations on the strategic and operational level addressing NM EHS including precautionary measures, pre-normative work, steps towards regulations, common actions and projects. Together with this information, WP2 will also develop a roadmap to describe these future activities necessary in the risk assessment of NM.

Starting a little later than the first two WPs, WP3 will closely look at the EHS risk assessment of NM and (following on from WP1) will

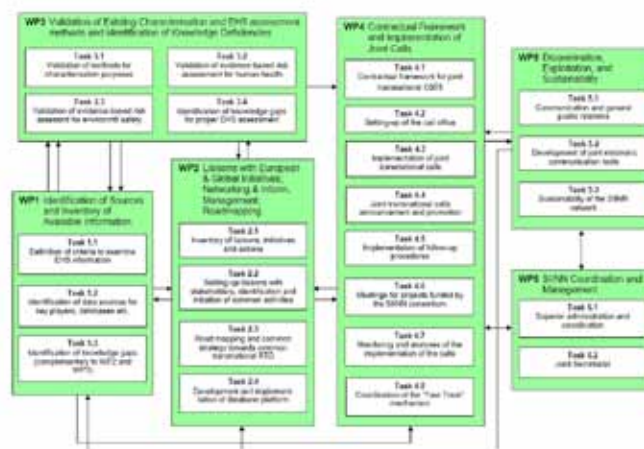


Figure 1 Interplay of SIINN Workpackages (WPs)

The SIINN management structure has been defined to allow for clear responsibilities and rapid decision making whilst still maintaining the flexibility required with respect to its membership structure and to participation in the transnational calls and other planned activities. This structure is based on experience gathered in similar large international projects and in other ERA-NETs.

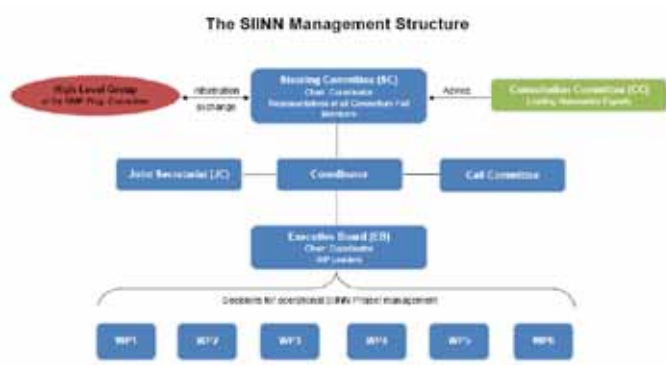


Figure 2 Management Structure of the SIINN Project

Although the full Consortium will be responsible for the overall policy of the SIINN network through the network's most senior body, the Steering Committee, the operational management will be delegated to a formally constituted executive body, the

Executive Board, which will be composed of the Coordinator and Workpackage Leaders and Task Leaders. Past experience has demonstrated that such a body is paramount if strategy is to be speedily and efficiently implemented. The Executive Board will prepare all recommendations on policy and strategy issues which will be required to be addressed and decided upon by the Steering Committee.

In addition, a nanomaterials technology and innovation advisory group, the Consultation Committee, will be formed, whose members will assist with the strategic and operational needs of the ERA-NETs activities and programmes. At least one member of the COM's NMP Programme Committee and one member of the NMP High-Level Group on Nanotechnology will serve in the Consultation Committee.

6 Directory

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SMART-NANO

Sensitive MeAsuRemenT, detection, and identification of engineered NANOparticles in complex matrices

Contract Agreement: NMP4-SE-2012-280779 Website: TBA
Coordinator: Stéphane Follonier, CSEM SA, Bahnhofstrasse 1, 7302 Landquart, Switzerland

No.	Beneficiary name	Short name	Country
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2	JRC – Joint Research Centre – European Commission	JRC	Belgium
3	FeyeCon Development & Implementation BV	FEY	Netherlands
4	Postnova Analytics GmbH	PNA	Germany
5	Avid Nano Ltd	AVN	United Kingdom
6	AHAVA Dead Sea Laboratories Ltd	AHA	Israel
7	Ruđer Bošković Institute	RBI	Croatia
8	ABICH Srl	ABI	Italy

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1 Summary

Project Duration: 1 June 2012 – 31 May 2014

Project Funding: 3.5 Mio. EUR

The project SMART-NANO is aimed at developing an innovative, cost-effective technology platform that provides a total solution “from sample-to-result” for the detection, identification, and measurement of engineered nanoparticles (ENPs) in a wide range of matrices. The outcome of the project will be a miniaturized, modular, cartridge-based technology platform integrating all analytical steps needed for separation, detection, and quantification of ENPs in complex matrices. Parallel to the development of the technology platform, analytical methods and protocols will be developed and tested on the field, resulting in ready-to-use, cost-effective cartridges, for immediate, widespread use in real-life applications.

The SMART-NANO consortium is formed by 8 partners (3 academic and 5 SME's) from 8 different countries. The balance between industrial and academic partners is one of the key strengths of the project.

2 Background

Nanotechnology is having a large impact in many industrial sectors and the use of nanoparticles is continuously increasing in many fields, from paints, automotive components, consumer products (such as cosmetic sunscreens and anti-odorant) up to biology and medicine. It is estimated that 3-4 new products containing some form of nanomaterial enter the market every week and that by 2014 1.6 trillion Euros of manufactured goods will be based on nanotechnology.

The recent introduction of legislation on the labelling requirements of cosmetic products containing nanoparticles and the on-going activities in the legislative arena at EU level (see for example the “Commission Recommendation on the definition of nanomaterial” –L 2011/696/EU-) on the labelling of ENP-containing consumer products clearly call for cost-effective, practically implementable, robust, highly sensitive, and specific methods for the detection and measurement of nanoparticles, all these attributes being required for their widespread use and acceptance as standards in the community.



3 Scientific and technological challenges

The detection, quantification and identification of ENPs is complicated by the sheer variety of complex matrices in which engineered nanoparticles (ENPs) are embedded, ranging from food, to clothing and cosmetic products. For a proper assessment of the fate and potential safety risks associated with nanoparticles, ENPs also need to be detected downstream in matrices as different as waste water sludge, biological systems and soils. The biggest challenge that researchers face today is thus to develop a technology platform that can be used in a wide range of application scenarios (in terms of embedding matrices and wide range of concentrations) with just minimal adjustments and optimizations for new applications.

As of today, there are technologies that partially address the three areas of detection, identification and quantification, but usually they have not been optimized for the detection of ENPs nor have they been tightly integrated with each other. An example of required technology innovation is size measurement of nanoparticles in the single digit nanometer range (1-10nm), which still suffers from a severe lack of technological progress. More importantly, there is a lack of integration between the different technologies with respect to sample preparation, ENPs isolation, pre-concentration and separation as well as their measurement and identification. These shortcomings render the routine use of current analytical methods difficult, particularly in the case of samples containing nanoparticles of different size and/or chemical composition. Moreover, the many required process steps are time consuming and introduce a large variability of the results obtained. The lack of methods for detection and characterization of nanoparticles in complex matrices has been recently highlighted as the main factor holding up the successful transfer of nanotechnology to nanomedicine.

The SMART-NANO project aims at developing a key technology platform able to address the measurement requirements of the most common applications with ready-to-use application-specific "from sample-to-result" (i.e. integrated) cartridges and protocols. The technology platform is based following a modular and scalable approach, which gives the flexibility to target new applications with minimal optimization.

4 Objectives

The SMART-NANO consortium has selected four key technological advances that will yield innovative, practically implementable, and cost-effective measurement approaches for the identification, detection and quantification of ENPs. These advances are:

- To increase the sensitivity, specificity and versatility of nanoparticle detection instruments using newly developed and optimized pre-separation and separation steps.
- To increase the sensitivity and quantification of nanoparticle detection by next generation dynamic light scattering.
- To provide robust, highly sensitive, miniaturized, and application-specific separation and detection cartridges.

- To validate the SMART-NANO detection platform and demonstrate its application for cost-effective measurements of nanoparticles in complex matrices such as biological systems, consumers products and in the environment.

The integration of all of these advances into a single technology platform delivering all steps "from sample-to-results" (i.e. sample preparation, ENP isolation, pre-concentration, separation, quantification and identification) represents the key breakthrough targeted by SMART-NANO.

5 Organisation

In order to successfully achieve the ambitious objectives of the project, an efficient, well-defined but flexible management structure was defined, shown in Fig 1. The management structure also ensures an effective communication with other ENP-related activities at the EC level.

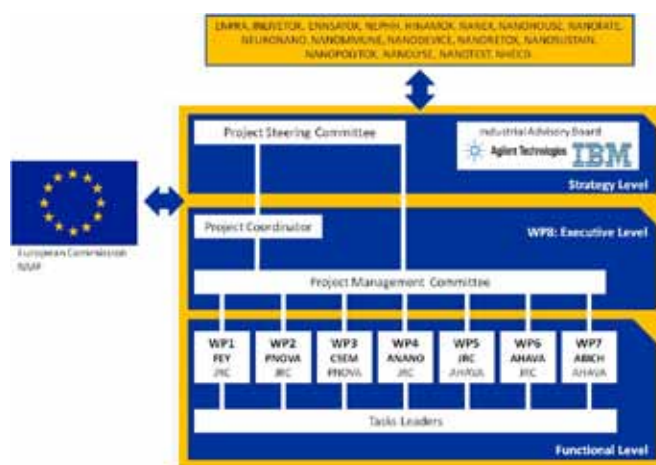


Figure 1. The management structure of SMART-NANO

The SMART-NANO project is organized in 8 Work Packages (WPs), which are strongly interlinked as shown below.

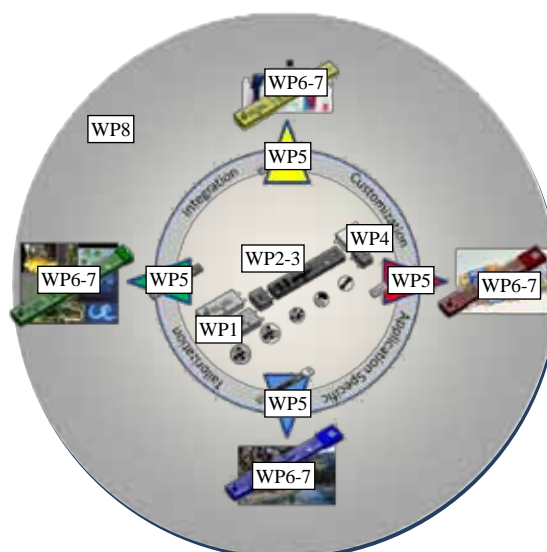


Figure 2. The 8 Work Packages of SMART-NANO and their interrelationship



The four first Work Packages (WP1-4) target the key technological advances described in the previous section. These four innovation driven WPs yield modules that are integrated to build the SMART-NANO application-specific “from sample-to-results” cartridges. In order to maximize progress and accomplishments within the given schedule and the available resources, these four WPs are designed to progress simultaneously and are interlinked with each other only at the microfluidics interface level. This will reduce the project risk while maintaining a high level of resilience and flexibility in the proposed platform. Integration of the different cartridge modules

into the SMART-NANO platform is accomplished in WP5, while WP6 is dedicated to the design and optimization of workflow and protocols for specific applications. WP7 and WP8 are focused on impact and dissemination activities and project management and will run for the whole duration of the project.

The various Work Packages and their respective goals are described in more detail in the following table.

Table 1 Workpackages (WP) of SMART-NANO

WP	Title	Objective
1	Ecologically-sound ENPs Isolation Cartridge Modules	Establishment, testing, and benchmarking of an ecologically-sound technology for the sample preparation for the analysis of ENPs in complex materials and in highly diluted samples. Two approaches will be followed: CO ₂ -based isolation and chemical isolation.
2	Innovative Cost-effective ENP Separation Cartridge Module	Development of macroscopic (first) and microscopic (later) separation modules combining two known flow field-flow fractionation sub-technologies (SPLITT and asymmetrical AF4). Upgrade of the standard instrumentation environment to allow the integration of the microscopic separation module.
3	On-chip in-line ENP Detection, Measurement and Quantification Cartridge Module	Integration of UV and light scattering detection with the microfluidic-based cartridges. This integration will lead to an increased sensitivity of the system, a reduced dilution of the sample, and a lower cost of the whole instrumentation. In addition, the development and testing of calibrating solutions containing ENP of different sizes will allow the size measurement of the most common NP based on their elution time.
4	Innovative ENP Hyper-Sensitive Size Measurement and Identification Modules	Development of a method for the highly sensitive size measurement and identification of ENPs. The hypersensitive size detection will be achieved by homodyne optical fiber dynamic light scattering (HDLS), and the hypersensitive identification by single nanoparticle ICP-MS.
5	Cartridge & Instrument Integration	Develop and test the integration of the different modules that have been developed in WP 1-4 at the cartridge and instrument levels.
6	Applications Specific Cartridges Protocol Optimization	Optimize protocols for ENPs detection and identification to meet future industry needs. Analyse the level of applicability of the technology platform for a successful implementation in the industry, provide added value to the end user, and meet relevant regulatory demands.
7	Exploitation, Impact and Dissemination Activities	Activities for promotion and dissemination of the scientific and technological results achieved during the project, in and beyond the industrial reference European market.
8	Project Management and Consortium Coordination	Ensure that the project is executed successfully, i.e., that the objectives of the project are reached within the project's scope, schedule and resources, and ensure maximum impact of SME-related activities.



6 Expected Impact

SMART-NANO is expected to have a strong impact in different areas:

Scientific and technical impact

The innovative, cost-effective technology platform providing a total solution for the detection, identification and measurement of ENPs in complex matrices, will certainly generate high scientific and technical impact and innovations, some of which will be patentable inventions. It is critical, therefore, to provide a strong link between the dissemination activities and the intellectual property strategy plan to protect the results appropriately for commercial exploitation.

Commercial impact

There will be a strong commercial impact from SMART-NANO as the outcome of the project will be a validated platform for exploitation and distribution by the SME partners. Moreover, Agilent Technologies has expressed strong interest in the outcome of the project as this could potentially enable smaller and more affordable ICP-MS systems.

Policy impact

We expect the scientific evidence gathered during the application phase of the project will be translated into guidelines and recommendations for control of ENPs in consumer products, food, environment, in situ in biota. Our dissemination strategy views the policy impact as a key factor in the subsequent success of raising public awareness and commercial development of the outputs from the project. We will engage with the policy makers to inform governmental environment, food and health agencies at an EU-wide and national level.

Societal impact

The information generated by SMART-NANO will have a significant impact on the safety of the European population as analyses from a broad range of ENPs in consumer products, food, environment and in biota. To enable this, awareness of the uses and benefits of the technology has to be created amongst the industrial and governmental stakeholders. Developing a good relationship with the accredited laboratories end-users will also provide a valuable feedback opportunity during the commercial development of the technology platform.

7 Directory

Table 2 Directory of people involved in this project.

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