Compendium of Projects in the European NanoSafety Cluster

2011 Edition

February 2011

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 2011

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PREFACE

This is the second edition of the compendium. Since the first edition a number of important initiatives have been launched in the shape of large projects targeting integration of research infrastructure and new technology for toxicity studies and exposure monitoring.

The demand for research in the area of human health and environmental safety management of nanotechnologies is present since a decade and identified by several landmark reports and studies. Several guidance documents have been published. It is not the intention of this compendium to report on these as they are widely available.

It is also not the intention to publish scientific papers and research results as this task is covered by scientific conferences and the peer reviewed press.

The intention of the compendium is to bring together researchers, create synergy in their work, and establish links and communication between them mainly during the actual research phase before publication of results. Towards this purpose we find useful to give emphasis to communication of projects strategic aims, extensive coverage of specific work objectives and of methods used in research, strengthening human capacities and laboratories infrastructure, supporting collaboration for common goals and joint elaboration of future plans, without compromising scientific publication potential or IP Rights.

These targets are far from being achieved with the publication in its present shape. We shall continue working, though, and hope with the assistance of the research community to make significant progress. The publication will take the shape of a dynamic, frequently updated, web-based document available free of charge to all interested parties. Researchers in this domain are invited to join the effort, communicating the work being done.

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

ACKNOWLEDGMENTS

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

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 FP6 and FP7 Associated States. We gratefully acknowledge their continued support.

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QUOTE

Compendium of Projects in the European NanoSafety Cluster – 2011 edition

Michael Riediker and Georgios Katalagarianakis (Eds.)

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CONTENTS

Overview matrix : Research themes of the NanoSafety Cluster projects

Project Acronym	ENNSATOX	ENPRA	EURO-NanoTox	HINAMOX	InLiveTox	MARINA	ModNanoTox	NANEX	NANODEVICE	NanoFATE	NANOHOUSE	NanoImpactNet	NanoLyse	NANOMMUNE	NanoPolyTox	NanoReTox	NanoSafe2	NanoSustain	NanoTranskinetics	NanoValid	NEPHH	NeuroNano	NHECD	Qnano	SIIN
Start year	2009	2009	2007	2009	2009	2011	2011	2009	2009	2010	2010	2008	2010	2008	2010	2008	2005	2010	2011	2011	2009	2009	2008	2011	2011
End Year	2010	2012	open	2012	2013	2015	2015	2010	2013	2014	2013	2012	2012	2011	2013	2010	2009	2013	2014	2014	2012	2012	2012	2015	2014
Characterisation & measurement	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
Exposure assessment for humans and the environment		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
Human Exposure: Application of measurement and modelling methods on nanomaterials			X			X		X			X	X					X	X		X					X
Environmental Exposure Assessment						X		X		X	X	X						X		X	X				X
Interaction of nanomaterials with biological systems		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
Toxicokinetics	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
Predictive models	X	X		X		X				X	X	X		X		X		X	X	X					X
Long term monitoring and assessment			X	X								X								X		X			X
Human Health			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
Apply testing and assessment strategy		X	X	X		X				X		X						X		X		X			X
Coexposures / Mixture toxicology										X		X								X	X				X

Project Acronym	ENNSATOX	ENPRA	EURO-NanoTox	HINAMOX	InLiveTox	MARINA	ModNanoTox	NANEX	NANODEVICE	NanoFATE	NANOHOUSE	NanoImpactNet	NanoLyse	NANOMMUNE	NanoPolyTox	NanoReTox	NanoSafe2	NanoSustain	NanoTranskinetics	NanoValid	NEPHH	NeuroNano	NHECD	Qnano	SIIN
Ecotoxicology	X					X						X								X			X		X
Develop testing and assessment strategy	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					X
Develop & validate methods to evaluate control measures at workplaces			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
Control banding approach						X						X								X					X
Preliminary handling guidelines	X		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
Information transfer	X		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
Public dialogue	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
National and international collaboration	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
Testing	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
Standardisation			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

Foreword

Nanotechnology is referred to as the new "general purpose technology" of the 21st century, a springboard for long-term productivity increases, economic growth and a means of addressing grand challenges. It is expected that nanotechnology will play the role electronics played in the 20th century and metallurgy in the 19th. Manufactured nanomaterials are expected to yield significant innovation, hence providing a new competitive edge to European industry and strong benefits for the society in a very wide range of applications from medicine to agriculture, from biology to electronics.

Mindful of the safety aspects of these emerging technologies, the European Commission has actively promoted and supported, and continues to do so, research and development as well as innovation in this area. Ensuring the safe development of nanotechnologies, through a sound understanding of their potential impact on health or on the environment, and through the development of tools for risk assessment and risk management, is key factor to fully harvest the benefits from their deployment.

For several years now, the research community has responded by launching very valuable projects, marking significant technological progress both in the technology and in its safety management. Thirty projects are either completed or running and represent a total RTD investment of 112M€, from the NMP and other programmes, under FP6 (11 projects, 30M€) and FP7 (25 projects, 82M€). These projects together with a significant number of projects supported by government resources in the EU member states and the FP7 associated states, and other projects addressing safety as side objective, represent the valuable efforts of the scientific and industrial research community for progress.

Synergy among these projects, collaboration for maximising impact, policy elaboration, planning of future actions, and international cooperation are the main aims of the NANOSAFETY cluster (http://www.nanosafetycluster.eu), a projects and stakeholders open forum.

It is with great pleasure that we present this second edition of the compendium. Since last year, some projects have been finalised and other have reached mid-term. The excellent work and most relevant results are related in the following pages. We are also very pleased to announce new projects : *NanoValid* and *Marina*, which are targetting reference methods for managing the risk of engineered nanoparticles, *ModNanoTox* and *NanoTranskinetics*, strongly linked with US partners in the emerging field of computational nanotoxicology, *Qnano* for the integration of research infrastructures and ERANET-*SIINN* for collaboration of national programmes.

Nicolas Segebarth

George Katalagarianakis

ENNSATOX

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

Contract Agreement: NMP4-SL-2009-229244 Website: http://www.ennsatox.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	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

Contents

1 Overview

1.1 Project Title

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

1.2 Acronym

ENNSATOX

1.3 Start and End Dates

Start date: 1st July 2009

End date: 30th June 2012

Duration: 36 months

1.4 Size

€3,655,316 Total Budget

€2,816,500 EC Contribution

2 Abstract

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.

3 The ENNSATOX Project

3.1 *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"*, report, http://cordis.europa.eu/nanotechnology/home.html, *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/nn 49456/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/nn 49456/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 ecosytems. 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 (SiO_2) and titanium dioxide (TiO_2). 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 to 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. All the procedures for toxicity testing are 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 patented[1] 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.

3.2 *Scope & Objectives*

1) To source and comprehensively characterise a representative group of nanoparticles: initially ZnO and later SiO_2 and TiO_2 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.

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

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?
- 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?

3.3 *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 ($SiO_{2)}$, zinc oxide (ZnO) and titanium dioxide (TiO_2), 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 will be 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.

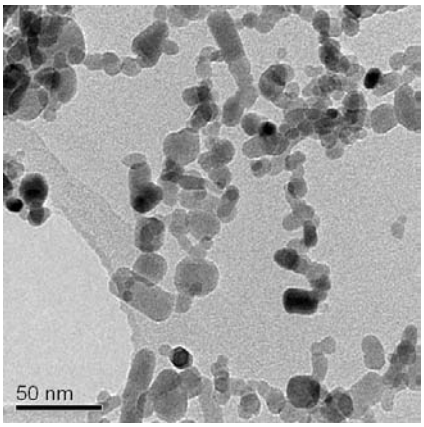


Figure 1: Shows TEM Bright field image of ZnO nanoparticles on a holey carbon film.

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).

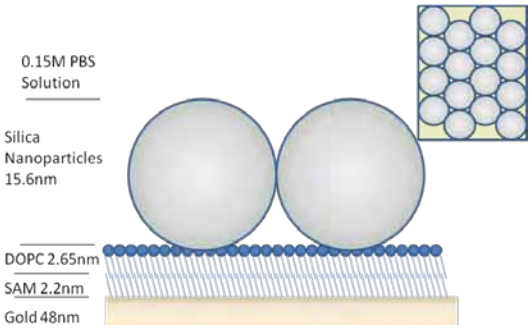


Figure 2: Shows schematic of nanoparticle adsorption on layers of lipid (DOPC), self-assembled monolayer (octadecanethiol) and gold surface. Inset: top-down view showing theoretical close-packed nanoparticle arrangement, with 0.6046 layer volume.

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

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 theory[2]. The form, structure and functionality of the particles 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 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 SiO_2 nanoparticles adsorbed on to a cyanobacterium membrane.

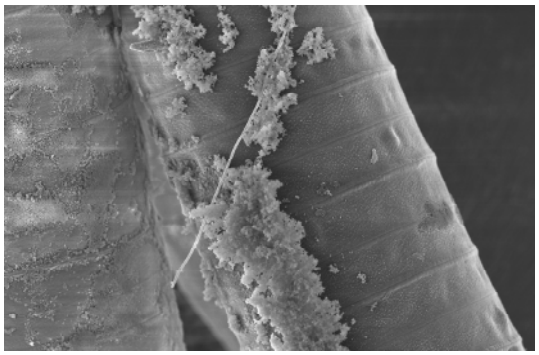
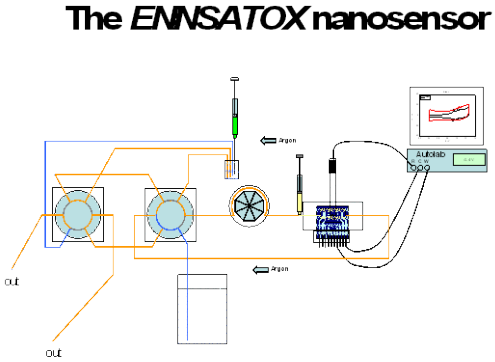


Figure 4: Shows Oscillatoria princeps incubated with SiO2. Cells separated by septa (arrowed). Right filament partially covered by SiO2, left filament completely covered.

To test the biomembrane activity of SiO2 nanoparticles a custom built high throughput sensor has been developed, which is displayed in Figure 5. 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 (see Figure 6).

[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.



Alex Vakourov, Patent filed 9 March 2010

Figure 5: Schematic of the ENNSATOX nansensor for high throughput assay of biomembrane activity of nanoparticle dispersions.

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. This is very clearly displayed in Figure 6.

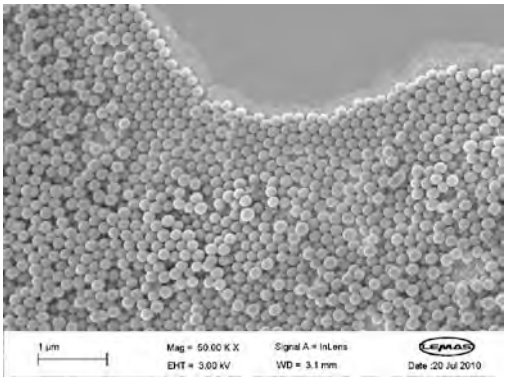


Figure 6: Scanning electron microscopy (SEM) images of phospholipid monolayer coated Pt/Hg film electrode after incubation with 175 nm AngstromSphere silica

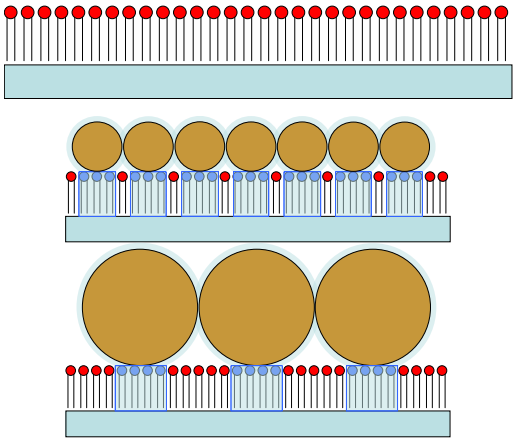


Figure 7 Schematic view of silica nanoparticles interaction with phospholipid domain. (TOP) phospholipid monolayer on mercury surface, (MIDDLE) small nanoparticles on phospholipid monolayer, (BOTTOM) large nanoparticles on phospholipid monolayer. Blue represents domains of DOPC with surface within interfacial layer and red represents domains of DOPC with surface outside interfacial layer.

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 (Figure 8).

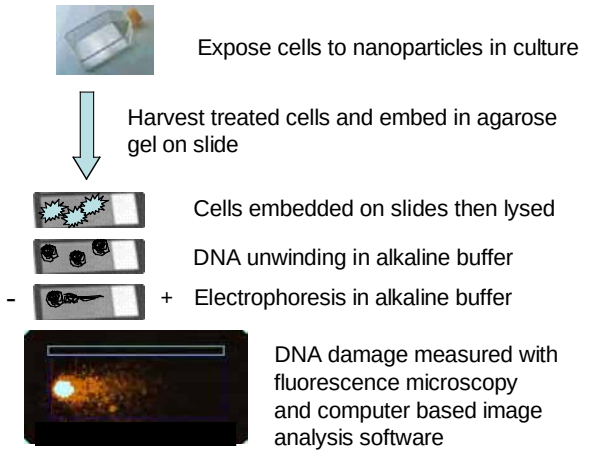


Figure 8: Shows the Comet assay protocol being used to assess nanoparticle dispersions' activity.

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. The effect of SiO2 nanoparticles on the development of ascidian larvae is shown in Figure 9.

Figure 9: Developmental assays. Ludox SM30 SiO_2 (Dialysed, DS left and undialysed, UD right) NPs diluted 1 in 100 in sterile filtered natural seawater ,1 x 10^{-6} sperm mL^{-1} added to media then after 5 minutes media added to eggs. Note the developmental problems with the NP exposed larvae.

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 will be 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. Figure 10 shows a dose response curve of the effect of SM30 SiO2 on a culture of freshwater algae.

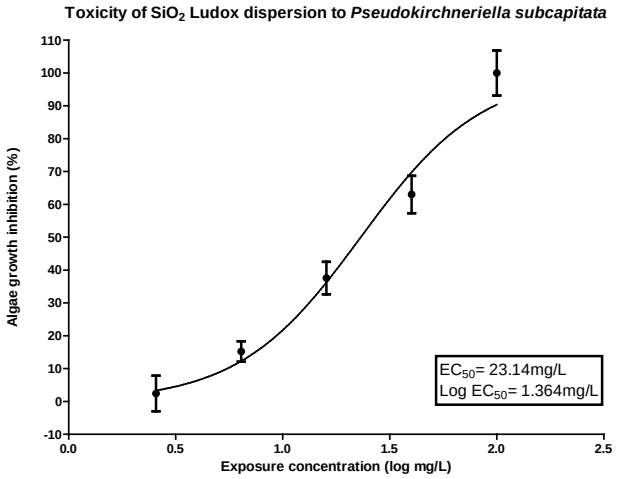


Figure 10: Toxicity of SiO_2 Ludox dispersion to the algae species Pseudokirchneriella subcapitata. On the X-axis the exposure concentration (2.56, 6.4, 16, 40, 100 mg L^{-1}) is indicated as a log-scale, whereas on the Y-axis the toxicity is indicated as the percentage of growth inhibition caused by the dispersion.

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 (see Figure 11) 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, total cell, cell organelles, 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 context[3]. The Coordinator has had extensive experience associated with constructing environmental models to predict the transport, behaviour and bioavailability of species when he worked at Plymouth Marine Laboratories, UK.

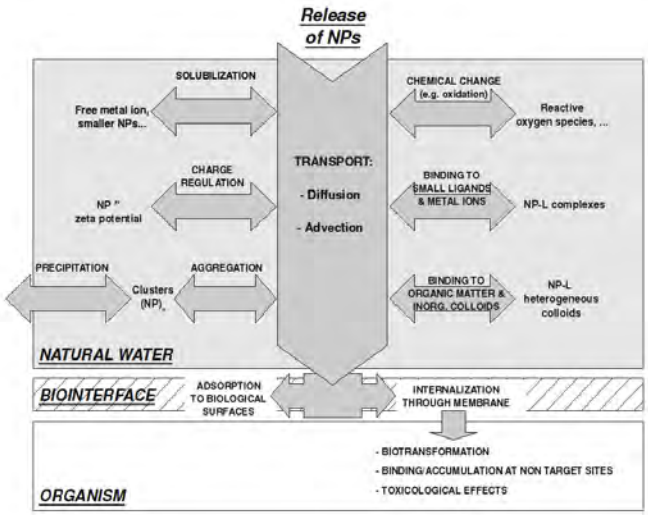


Figure 11: Shows physicochemical processes of relevance for the fate and behaviour of NPs in the environment used in the WP6 model.

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 co-ordinating 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 is shown in Figure 12.

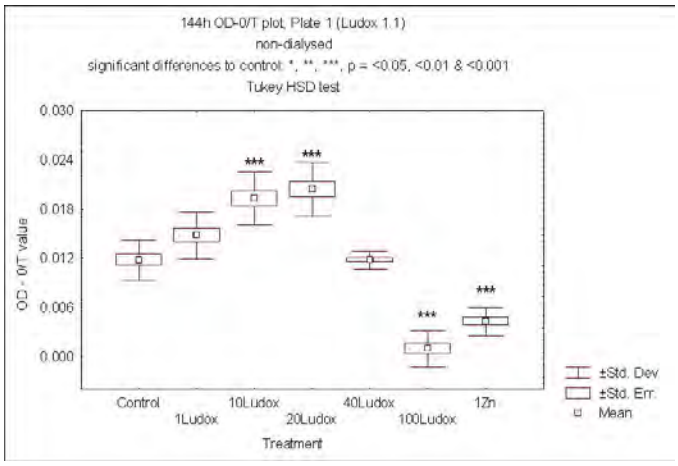


Figure 12. Isochrysis galbana. Growth (measured as absorbance at 440nm – absorbance at 750nm) after a 6 day period of exposure to SM30 as mg L-1 LUDOX, relative to controls (including positive control of 1 mg L-1 Zn++ as zinc sulphate).

WP8: Dissemination. Although this work package concerns dissemination it also feeds into WP7: Exploitation & Pre-validation. It identifies opportunities to publicise the achievements and 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 13 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.

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.

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

- 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 first half of the project:

- Development of nanoparticle characterisation protocols.
- Development of nanoparticle handling protocols prior to toxicity assays.
- Development of nanoparticle screening methods and structure: activity relationships.
- Cross calibration of results throughout the Consortium.
- Surface chemistry of nanoparticles in environmental media.
- Comprehensive understanding of the interaction of SiO_2 nanoparticles with phospholipid membranes and its relationship with toxicity to unicellular and multicellular organisms.
- Development of an on-line high-throughput device and corroborative algal toxicity test for rapid assay of nanoparticle biomembrane activity.

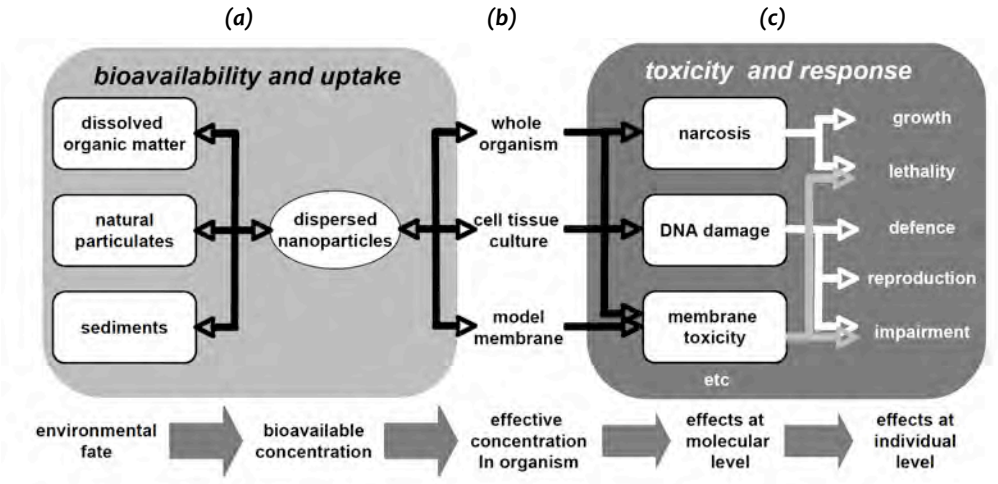


*Figure 13: Shows the environmental discharge and behaviour of the nanoparticles in the left hand compartment **(a)**; the impact of nanoparticles on the biological barriers in between the two compartments **(b)**; and, on the aquatic organisms in the right hand compartment **(c)**.*

3.4 *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:

1. Gel filtration is the most effective means of purifying SiO_2 dispersions.
2. Anodic Stripping Voltammetry (ASV) and Inductively Coupled Plasma Mass Spectrometry (ICPMS) have both been used to measure solubility of ZnO dispersions. Agreement between the two methods is generally good although ASV tends to give a lower result as expected.
3. Commercial ZnO dispersions have a tendency to aggregate with the exception of two dispersions which were stabilised with dispersant.

WP2:

1. SiO_2 particles adsorb on to phospholipid membranes and bacterial cell walls (see *Figures 4 and 6*).
2. Electrostatic forces bind the particles to the membrane and the influence of the particles on the phospholipid membrane's properties is related to the particles' packing which in turn is inversely related to the particle size.
3. DEGUSSA P-25 TiO_2 particles (50:50, anastase: rutile) do not show significant adsorption on to phospholipid membranes.
4. ZnO particles show an equivocal influence on the structure of phospholipid membranes and a release of soluble Zn^{2+} is observed.
5. ZnO particles which form a stable or metastable aqueous dispersion have a strong effect on the phospholipid membrane's properties.
6. An *on-line* method for measuring the biomembrane activity of nanoparticles has been developed, and was filed as a patent in March 2010.
7. The kinetics of the SiO_2 biomembrane activity is related to the particles' characteristics and form.
8. SiO_2 particles have been shown to be internalised by cultured cells.

WP3, WP4:

1. SM30 SiO_2 particles had cytotoxic and genotoxic effects but had no effect on membrane capacitance and ion channel activity on cell cultures at 10 mg L^{-1} concentration.

2. SM30 SiO_2 particles had a toxic effect on ascidian sperm and significantly affected the growth of both fresh water and marine algae in culture at between 20-40 mg L^{-1} concentration in the media. Interestingly at lower concentrations than 20 mg L^{-1} it had an *hormesis* effect on the growth of marine algae.
3. SM30 SiO_2 particles had no significant mortality effect on the marine crustacean *Artemia salina* and the freshwater crustacean *Daphnea magna*. Zebra fish were exposed to a concentration range of 0.01, 0.1, 1, 10, 100 mg L^{-1} of SM30 SiO_2 but no mortality occurred after 96h.

WP5:

1. The surface charge density and electrophoretic mobility of LUDOX SiO_2 LS30 particles are similar to results reported in the literature for other silica particles. Therefore, there is no measurable effect of the small size of these particles (radius 8 nm) on their electrochemical surface properties. The proton charge density as a function of pH and salt concentration follows the basic Stern double layer model. The iso-electric point of the SiO_2 NPs is at pH 2.
2. For large values of the ratio of Pb^{2+} ions to SiO_2 NPs, labile surface complexes are formed in the pH range around 6. For [Pb(II)]/[NP] ratios below unity, the metal binding characteristics differ between individual NPs. Such chemical heterogeneity at the level of one bound metal ion per particle is apparently significant for the NP regime.

WP6:

1. A comprehensive table of all the physical, chemical and toxicological characteristics of ZnO nanoparticles has been prepared.
2. A preliminary predictive model of the environmental toxicity and fate of a model nanoparticle dispersion has been developed.

WP7:

1. Evaluation and comparison of all Consortium tests has shown that SiO_2 toxicity is mediated at the biomembrane level.
2. This is mediated through a sensitivity of all single cells and single celled organisms to SiO_2 dispersions.
3. Multicellular organisms do not show an acute sensitivity to SiO_2 dispersions.

The scientific work plan for the next 18-months is as follows:

WP1:

- Water solubility of ZnO nanoparticles of varying size and functionality to be determined.
- Work is to be intensified sourcing and synthesising stable dispersions of ZnO nanoparticles, with provision of a set of well characterised single class of ZnO and TiO_2 nanoparticles to the Consortium.
- Characterisation of NPs after exposure to biological media and characterisation of NPs internalised by cells and organisms.

WP1/WP2:

- Full comprehensive relationship between the structure and functionality of SiO_2, ZnO and TiO_2 particles and their biomembrane activity and their *in vitro* and *in vivo* biological activity to be established.

WP2:

- Toxicity screens to be consolidated and inter-calibrated.
- Mechanisms of SiO_2 and ZnO particle interaction with biomembrane model to be 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 Pb^{2+}/SiO_2 nanoparticulate complexes by Eigen type reconstruction of the kinetic steps involved in the surface complex formation Surface characterisation of ZnO and TiO_2 NPs.
- Permeability of NPs in hydrogels/model cell walls. Physiochemical conditions on permeability rate established.
- Form, permeation of SiO_2 and TiO_2 determined. Rigorous kinetic analysis of permeation.

WP6:

- Advection/diffusion of NPs in aquatic environment studied.
- 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.

4 Directory

Table 1. Directory of people involved in ENNSATOX.

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5 Copyright

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)

No.	Beneficiary name	Short name	Country
	EU PARTICIPANTS		
1	Institute of Occupational Medicine	IOM	UK
2	Napier University	Napier	UK
3	University of Edinburgh	UEDIN	UK
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
8	Vrije Universiteit Brussel	VUB	BE
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

Contents

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; fibrogenecity; 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 technology 2. 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) fibrogenecity 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.

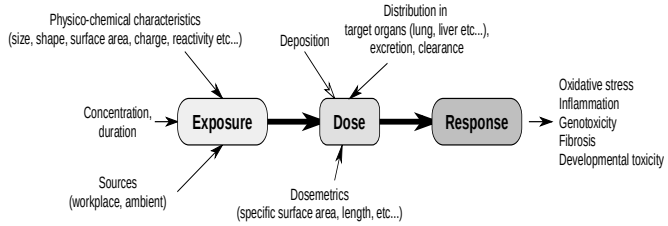

Fig 1. The Exposure-Dose-Response paradigm

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 Atlantics (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.

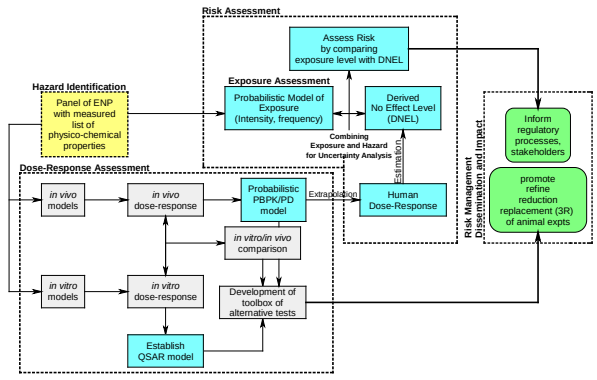

Fig 2. *The rationale of the ENPRA approach*

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.

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 (± 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

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 TiO_2), 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.

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 is being collected and currently analyzed.

The most notable results so far for WP5 are:

1. Results of kinetic study using TiO_2 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 has lead to a critical re-evaluation of the study set-up for the in vivo studies in mice with a pre-existing risk factor.

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.

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 period, ENPRA will

1. Identify the reason(s) behind the relative low toxicity of the ENP tested. Specifically, the mechanisms of toxicity of these ENP;
2. relate these facts to the physico-chemical characteristics of ENP in WP3
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.

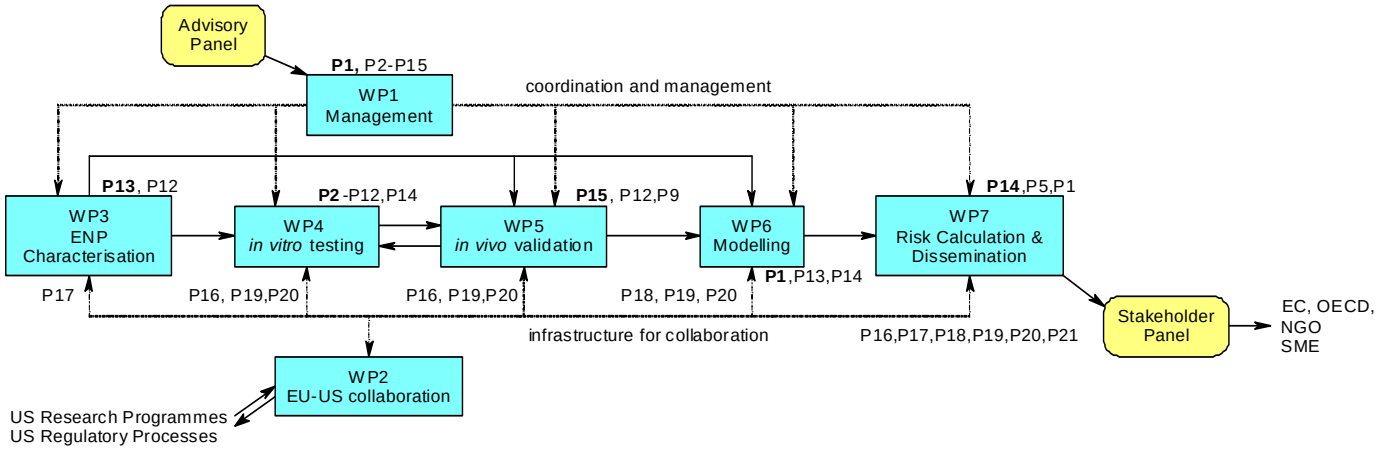

Fig 3. *Flow chart describing the information flow between different WP of ENPRA (solid lines) and the process of coordination, management and collaboration (dotted lines)*

3 Directory

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EURO-NanoTox

European Center for Nanotoxicology

Funded by: Federal Ministry of Science and Research Website: http://www.EURO-NanoTox.eu
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No.	Beneficiary name	Short name	Country
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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
7	Mondi Uncoated Kraft & Fine Papers GmbH, Department Research & Development	Mondi	Austria
8	University Salzburg, Department Molecular Biology	PLUS	Austria
9	Seibersdorf Laboratories, Department Toxicology	SL-Tox	Austria

Contents

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), nanostructered 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 nanostructered 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 nanostructered 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).

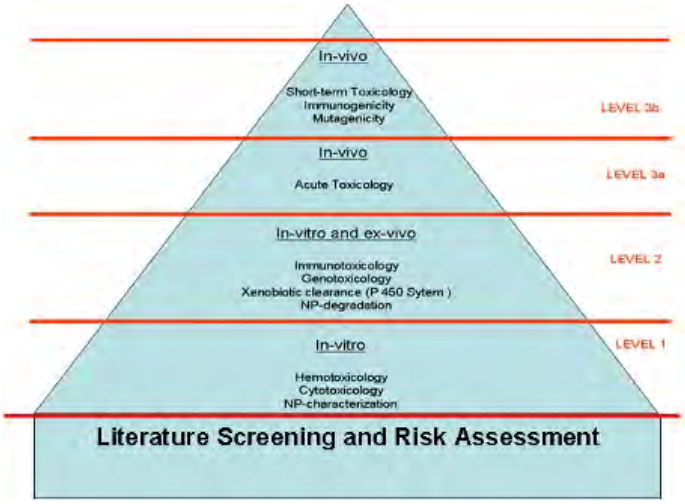

Figure 1: Risk assessment and development of a strategy for the determination of a nanotoxicological profile.

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 nanostructered materials (including workplace safety). This is absolutely necessary because without this basis of determining toxicity, no systemically 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. A first result is the catalogue of Austrian nanotoxicology expertise, which is available online (http://www.euro-nanotox.eu/images/stories/folder_euronanotox_webversion.pdf).

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 aspect as for example worker safety/workplace safety. EURO-Nanotox will provide valid scientific data and will perform validated scientific experiments to address the potential toxic profile of nano-structured materials.

Furthermore, EURO-NanoTox will help to set-up a European network of national hubs for nanotoxicology. This network will help to interchange recent developments and developed methods between different European member states and promote the development of European standards to help ensuring the successful development of nanotechnologies as a key for European growth.

8 Directory

Table 1 Directory of people involved in this project.

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9 Copyright

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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

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
5	Centro de Investigación en Química Aplicada	CIQA	Mexico
6	Zhejiang University	ZJU	China
7	PlasmaCHEM	PLASMACHEM	Germany
8	National Research Centre for the Working Environment	NRCWE	Denmark
9	Finish Institute of Occupational Health	FIOH	Finland

Contents

1 Summary

The HINAMOX project is concerned with the impact in human health and biological fate of metal oxide nanoparticles (NPs) like TiO_2, ZnO, Al_2O_3, CeO_2, etc. A key issue of HINAMOX is to set the basis for proper dose relation quantifications and distribution studies at cellular and body level. This task is paramount for the future definitions of nanosafety regulations, standards definitions and the assessment of the health effects of NPs. For "in vivo" studies we work on the development routes of fabrication and engineering of NPs and their radiolabelling, enabling the application of Positron Emission Tomography and Single Photon Emission Computed Tomography to a wide range of NPs.

HINAMOX works on establishing quantitative data and practical procedures to determine the concentration and distribution of NPs at cellular level applying Ion Beam Microscopy, Transmission Electron Microscopy, Confocal Raman Microscopy and Confocal Laser Scanning Microscopy. Also the cytological and pathological response to NPs are a major issue of study, considering aspects related to size, shape, and capping agents. We are presently studying the inflammatory response of the alveoli in primary cell cultures as a possible vehicle for the introduction of NPs in the body. The interaction of NPs with alveoli type II cells are followed in a primary culture environment simulating breath conditions. Detailed analysis of NP leaching and dissolution for the assessment of NP biodurability 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. 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. Throughout HINAMOX correlations between structure and chemistry of NPs and their toxicological endpoints and biological fate will be sought, being therefore the physical characterization and modelling important aspects of this project.

2 HINAMOX PROJECT

2.1 *Project description*

Understanding the safety, environmental and human health implications of nanomaterials and nanotechnology based materials and products, is an issue of paramount importance for Europe and the rest of the world. This understanding is required both for future assessments of the safety of nano-based products, and to achieve greater public acceptance of nanotechnology and public awareness of the overall benefits that nanotechnology can bring. This is a strong prerequisite for the future successful development and benefit of nanotechnology products.

The work of the HINAMOX consortium focuses 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. Some examples of these are TiO_2 and ZnO as catalysts and UV protectors, CuO in anti-fouling paints, Al_2O_3 as a surface protector, CeO_2 in polishing, indium-tin oxides forming anti-electrostatic coatings and various rare earth oxides in electronics manufacturing. The above mentioned industrial applications highlight the technological and economic importance of these NPs spanning the chemical, cosmetics, paint, electronics manufacturing and waste treatment industries.

Metal and metal oxide NPs may be toxic for two reasons:

i) They may possess increased catalytic activity due to nanoscale structure or chemical modification of their surface. These catalytic properties may interfere with numerous intracellular biochemical processes.

ii) The decomposition of NPs and subsequent ion leakage may result in a continuous formation of free radicals and metal ions, and in this way may heavily interfere with the intracellular free metal ion homeostasis, which is essential for cell metabolism and requires that metal ions are kept at extremely low levels in the cytoplasm.

Some examples of known health effects of free metal ions that result in the formation of reactive oxygen species in metallo-chemical reactions are neurodegenerative disorders, such as Alzheimer's disease, amyotrophic lateral sclerosis, prion disease, cataracts, mitochondrial disorders, and Parkinson's disease (Thompson et al., 2001). In addition to these observations, Elder et al. (2006) have shown that manganese oxide NPs can enter the olfactory bulb below the forebrain subsequent to inhalational exposure of rats. The pathway of the NPs to the brain is, in this case, by means of the axons of the olfactory nerves in the nasal cavity (Oberdörster et al., 2004). It is reasonable to assume that the oxidative homeostasis in sensitive cells might be affected by the presence of even a small number of metal and metal oxide NPs, both as catalytic entities and as the source of metal ions (Limback et al., 2007). Nature has provided a variety of protective mechanisms against the uncontrolled uptake of metal ions and compounds. Nevertheless, high acute and/or continuous exposure to these potentially dangerous agents might lead to deposition and/or systematic uptake of critical amounts of NPs through either defects in the skin, the digestive tract or the lung and bronchial tissues. In fact, perhaps the most striking impacts of NPs have been identified in the lungs where NPs of titanium dioxide have evoked inflammatory reactions (SCENIHR, 2007). In addition, carbonaceous NPs, notably carbon nanotubes, have been shown to induce pulmonary inflammation, granuloma formation, and even asbestosis like changes when introduced into the peritoneal cavity of mice (Poland et al., 2008).

Occupational production and handling of NPs involves a high risk of exposure to either repeated burst or long-term low-level exposures. Currently, producing and handling NPs does not require special regulation for protective equipment due to insufficient knowledge of exposure and NP hazard. Consumers may also be subject to acute high-level exposures during application of specific products and long-term exposure is evident during use of NP-based cosmetics and sun-creams. In concert with the increased implication of NPs in industry, the number of NP-based consumer products is expected to increase continuously over the next years to come. The economic importance and the presence of these NPs in everyday products like TiO_2 or ZnO in sun creams have led to concerted actions already within the 6th Framework Programme. For example, the aim of the Nanoderm project (http://www.uni-leipzig.de/~nanoderm/), which focused on the topical exposure of TiO_2, has been to determine to what extent these NPs can penetrate through the dermis and their effects on human health. In the 7th Framework Programme, there are on-going projects where some of the metal oxide and metal NPs are studied as part of other groups of NPs, for example in the NANODEVICE project coordinated by FIOH, a member of this consortium. HINAMOX addresses a more complete approach to the understanding of the effects of this class of NPs since the complex engineering of the NPs as well as the intracellular and organism level response to these NPs are considered. Moreover, several of the proposed oxides are chosen for the sponsorship program for safety testing of nanomaterials by the OECD Working Party on Manufactured NPs (www.oecd.org/sti/nano). The HINAMOX project follows a comprehensive approach to reach an in-depth understanding of different metal oxide NPs, such as their exposure risk, deposition and translocation, their interaction with physiological fluids and their stability, their systemic accumulation and cellular uptake, their in vivo inhalation effects on respiratory efficiency, and their inflammation and genotoxic effects.

Previous research and hypotheses 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 and occupational hygiene.

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 Al2O3 NPs, and AlumoSilb, a commercial additive for electroplating baths, which includes Al2O3 NPs in its formulation. PlasmaChem also provides surface modified Ti, Ce, Zn, Y and Fe oxides NPs with different surface functionalities. Examples of nanostructurated Ce and Zn oxides have also been purchased from Evonik, Germany. In this way, 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 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.

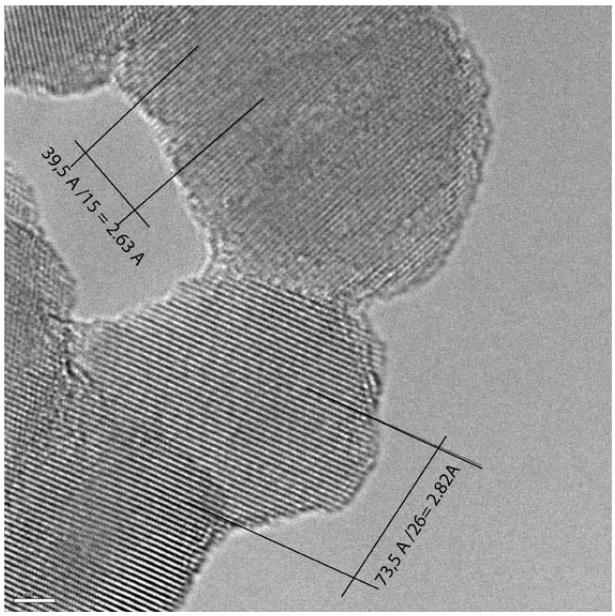

Fig.1 HRTEM image of ZnO NPs provided by Plasmachem

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.

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. 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 have not been accomplished so far, as a means to assess inflammatory effect and potential health risk. Only a few studies with PET/SPECT have been carried out in this area, but they show that the use of these techniques is, in principle, feasible for biodistribution studies. Almost all the publications are devoted to polymer NPs (Woodward et al., 2007; Pressly et al., 2007; Fukukawa et al., 2008; Matson et al., 2008). The feasibility of the approach is also demonstrated by the use of NPs as contrasting and therapeutic agents for magnetic resonance imaging (Neuwelt et al., 2007; Current Opinion Biotechnol., 2007). In the last two years, a relatively small amount of studies has been published addressing the potentially toxic effect of metal/metal oxide NPs. For example, ferrumoxtran-10, a dextran-coated magnetite-based NP for contrast enhancement, was found to be non-toxic to macrophages (Müller et al.2007). The toxicity of iron oxide NPs towards neurons (Pisanic et al., 2007) has been demonstrated. Several studies have focused on titanium dioxide and it is often used as a benchmark particle in recent nanotoxicological studies. TiO2 has been found to be toxic and inflammogenic (e.g, Grassian et al., 2007). Neurotoxicity related to oxidative stress has also been observed with commercial titanium dioxide NPs (Long et al., 2006). In this first year of HINAMOX radiolabelled NPs have been successfully prepared and will be used for biodistribution studies.

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

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 intracellularly 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).

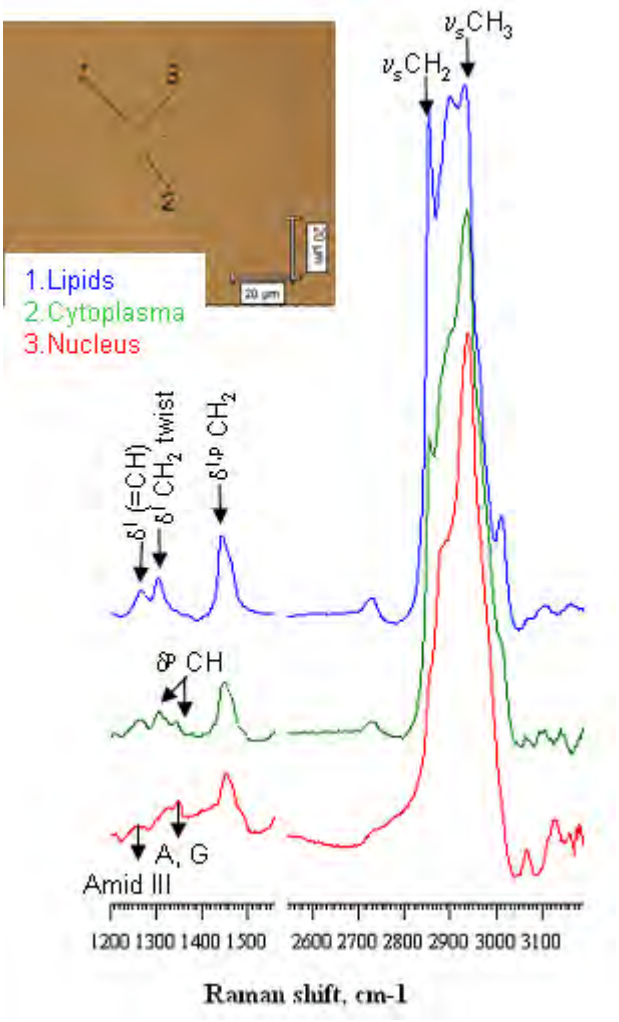

Fig. 2 Raman spectrum recorded at different positions within a cell from the HepG2 line (ν indicates streching and δ deformation vibration modes; l denote vibrations of lipids and p of protein). The inset corresponds to the image of the cell under studied. The numbers together with the color indicate the location in the cell at which the Raman Spectra were recorded

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) spectra of the cell components, and of the NPs incorporated in the cell. 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 will 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. As an alternative to CLSM Raman Confocal Microscopy has been applied for uptake studies. Confocal Raman Microscopy combines the recording of the spontaneous Raman emission with optical confocality. Confocal Raman has the advantage that it does not require the labelling of the nanoparticles, being the Raman emission a characteristic of the nanomaterial. Our work has based in the simultaneous recording of the Raman spectra of the cell components and that of the NPs incorporated in the cell. This allows us to determine in which environment in the cell the NPs are present.

3) 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 diffusion 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 will be investigated by biodissolution tests in an environmentally controlled, stirred batch reactor.

4) 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). 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.

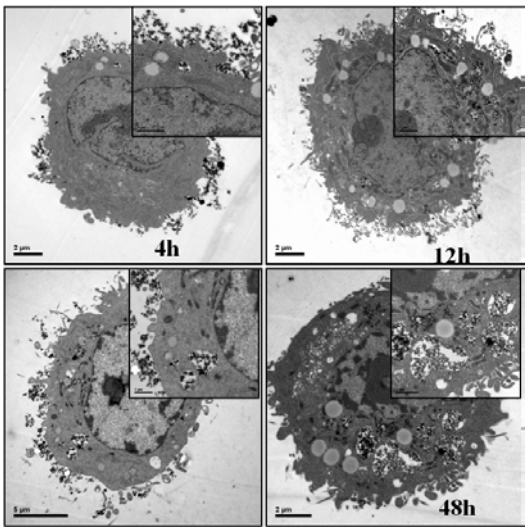

Fig. 3 Localization of metal oxide NPs within a H460 cell line visualized by TEM at 4, 12, 24 and 48 h.

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

Aspects such as the behaviour, fate, bio-persistence, bio-kinetics, exposure and behaviour of NPs will be addressed by HINAMOX, providing significant knowledge beyond the state of the art.

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 as well as exposure data generated in the NANOSH project, an FP6 founded project, 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. Attempts will also be made to use the mechanistic data together with the available exposure data to utilize approaches exploring possibilities to further develop the use of control banding in the management of risks associated with NPs. (see NIOSH draft document for comment, posted May 18, 2008, http://www.cdc.gov/niosh/topics/ctrlbanding/)

The project has an international dimension consolidated by the presence of scientific institutions from Mexico and China. The Chinese and Mexican participation will help to develop common safety standards within these countries, which is of particular importance for Europe for their economical significance and as a potential market for European nanotechnological products. The project will search for common dissemination activities and a fluid exchange of human resources with both Latin America and China.

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.

2.2 *Partners*

The HINAMOX consortium is formed by nine 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.

University of Vigo. The University of Vigo (UVIGO) is a recent University (15 years old); the Immunology Area was set up 10 years ago, and brought in researchers with experience in Medical Immunology. Since then, UVIGO has developed and trained scientists with experience in basic 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 cell lines and the production of oxidative stress.

University of Leipzig

Leipzig University 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

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 Nano-particles - 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)

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 Tecnolgia (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. Sate-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

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.

Finish Institute of Occupational Health

The Finnish 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.

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3 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

No.	Beneficiary name	Short name	Country
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3	Napier University of Edinburgh *	NU	United Kingdom
4	Saarland University	USAAR	Germany
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**

Contents

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 nanomaterials[1] 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.

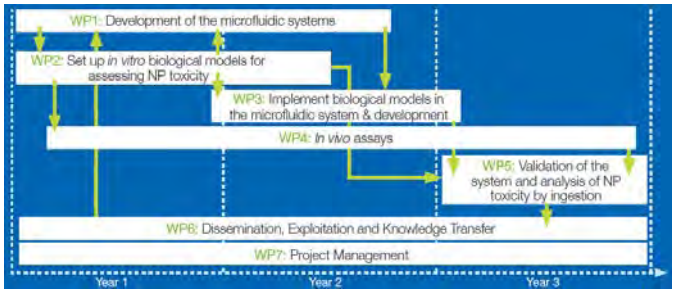
These objectives will be achieved by bringing together microfluidics technologies with cell culture models of human tissues to produce an *in vitro* test system that is more physiologically relevant. The microfluidics system will be flexible and modular so that the complexity of the system can be increased 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

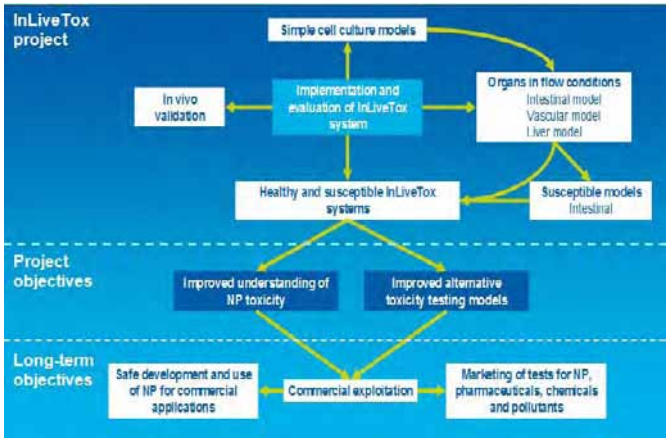
In a first phase of the project, in work package 1, a microfluidics device will be designed and fabricated for the simultaneous culture of different cell lines representing the intestinal epithelium, the vascular endothelium and the liver. In a second phase, feedback from the other work packages will 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 TiO_2 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. An essential part of this system is the newly-developed ILT1 bioreactor.

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 ILT1 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.

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8 Directory

Table 1 Directory of people involved in InLiveTox

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9 Copyright

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 àtomique (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'Environment Industriel et des Risques (INERIS)	France	Research Organisation
14	Instituto Nacional de Investigación y Tecnología Agragia 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

Contents

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 billion[1].

The market size for nanotechnology is expected to grow to over $3 trillion by 2015[2] 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 onitoring 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 unabled 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 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 projects[1]. 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 Association[2] and industrial key partners such as BASF and Nanocyl.
- we also recognised the geopolitical and economical 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

[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:.

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 activites 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 envrionment. 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 organ[5] , 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 charge[6]) or the formation of the protein or lipid corona[7] on the ENM surface. Inhaled ENM could be translocated to the brain via the olfactory bulb[8]. These ENM can also reach the terminal bronchial region and for high-aspect ratio ENM, they are likely to be further translocated to the pleura[9]. A small ENM fraction can reach the blood system where they can travel to secondary organs[5] like the heart, liver, kideny and beyond. The bio-distribution of internalised ENM follows a different pattern depending on the route of exposure: inhalation, ingestion or dermal[10]. For dermal exposure, there is some evidence that ENM can penetrate beyond the stratum corneum[11]. For oral exposure, TiO2 ENM have been shown to induce DNA damage and genetic instability in mice[12]. The paradigm for the adverse response is currently oxidative stress leading to inflammation[13]. Chronic inflammation could lead further to fibrosis, DNA damage and cancer[14]. ENM can also be genotoxic by direct by a totally new mechanism[15]. ENM driven oxidative stress also plays an important role in the immune response and apoptosis[16]. Cardio-vascular effects have been demonstrated in susceptible animal models exposed to ENM[17] 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 noted[18] . 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 level[19], including food chain effects. ENM uptake and tissue distribution within species are

mostly unknown, except for in a few larger species[20]. 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 al[21] 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 ENM[33] 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 suspensions[22]. Within the aquatic environment freshwater species, mostly pelagic have been tested[23]. The effects reported often differ depending on the method used to prepare the ENM for testing e.g. stirred ENM versus solvent carried ENM[24]. 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 ENM[25]. 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 disposal[26]. 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 NANODEVICE[39]), with measurements of as mass, particle size and size distribution[27]. Currently, there is no method for measuring the surface physico-chemical characteristics of the collected samples[28]. Also, dermal exposure from airborne ENM and secondary exposure to ENM from the environment are also not considered[29]. 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 NANEX[32] 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 compartments[33]. Some ENM may be persistent while others rapidly dissolve. Soils, sediments and surface waters are complex matrices with many possible different exposure and interaction scenarios[34]. 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 phase[32]. 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 strength [35-39] ; 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 unknown[42-46], 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 SG3[67] 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 charaterisation 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 las, industrial manufacturing, prcessing 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 Commitees, 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 - TiO_2, ZnO, SiO_2, CeO_2, 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 nanotechnologies 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.

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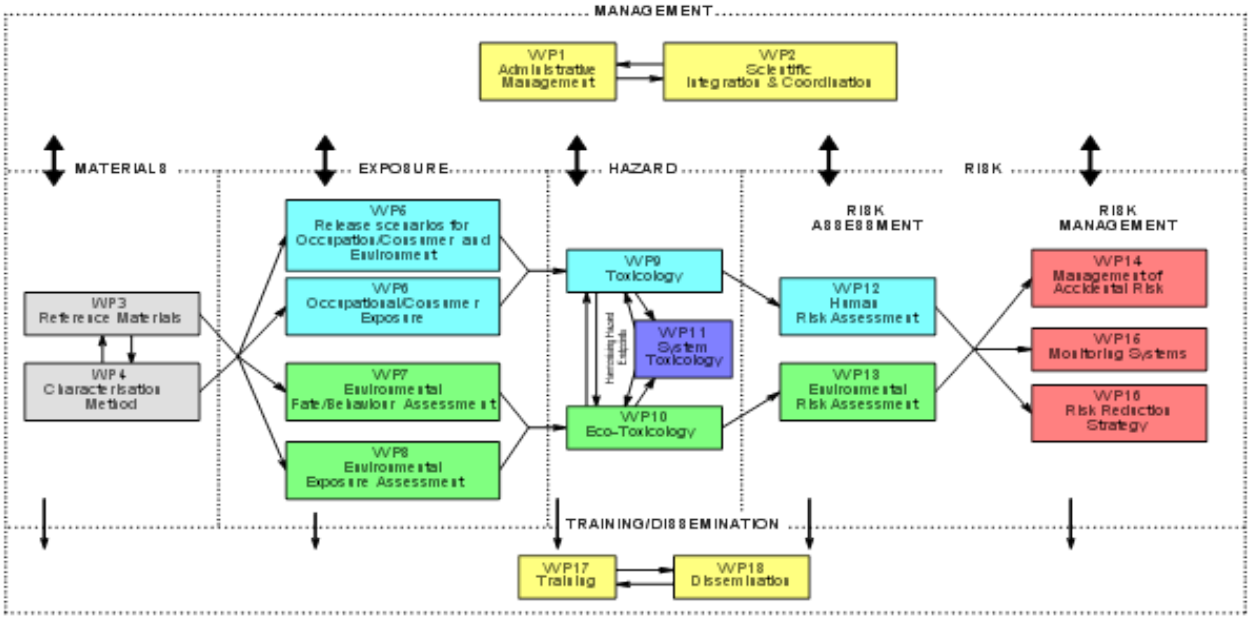


Figure 1 Flow chart of MARINA

7 Copyright

ModNanoTox

Modelling nanoparticle toxicity: principles, methods, novel approaches

Contract Agreement: under negotiation Website: N/A
Coordinator: Dr Eugenia Valsami-Jones, Natural History Museum, London, UK

No.	Beneficiary name	Short name	Country
1	Natural History Museum	NHM	United Kingdom
2	University of Birmingham	UB	United Kingdom
3	Roskilde Universitetscenter	RU	Denmark
4	Swiss Federal Institute of Aquatic Sciences and Technology	EAWAG	Switzerland
5	Eidgenössische Materialprüfungs- und Forschungsanstalt	EMPA	Switzerland
6	In-silico toxicology c.helma	IST	Switzerland
7	University of Nebraska Lincoln	UNL	USA

Contents

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 focussing 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 in research funding for nanotoxicology, as a result of which a substantial number of new projects are currently in progress generating nanosafety data. A significant proportion of these projects are within Europe, many of which funded by the EU. A significant research effort is also under way at 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. This may apply more to mechanistic than statistical models, and perhaps early on statistical models may prove more reliable. However, mechanistic models have the potential of higher sensitivity, as they respond to actual NP physicochemical properties and therefore are equally significant to pursue.

3 What is ModNanoTox

ModNanoTox will develop a number of well-documented 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 datasets. 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 (WP2) .

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).

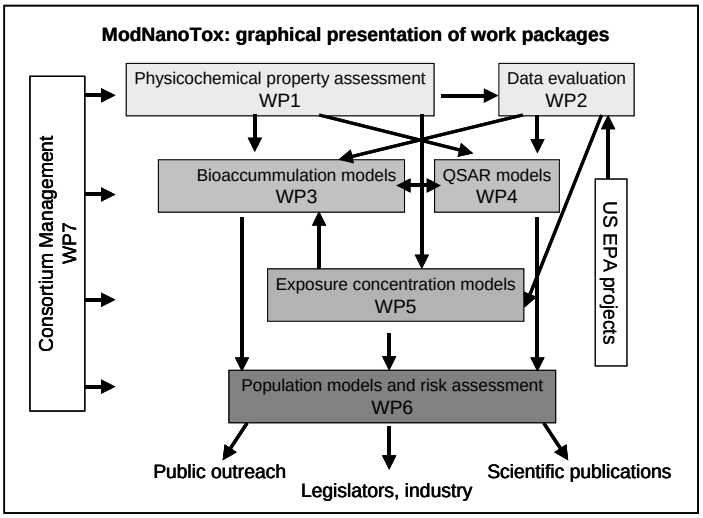
5. Toxic responses from cell culture studies and whole organisms can be modelled reliably by QSAR type approaches (WP4).

6. Exposure concentrations can be assessed reliably and incorporated in appropriate models (WP5).

7. Ecological effect models can be developed by extrapolation from (eco)toxicological observations and can be built into risk assessment models (WP6).

4 Organisation of ModNanoTo x

ModNanoTox consists of six RTD and one management workpackage. The workpackages and their interdependence are shown schematically below :



5 Directory

Table 1 Directory of people involved in this project.

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NANEX

Development of Exposure Scenarios for Manufactured Nanomaterials

Contract Agreement: NMP4-SA--2010-247794 Website: http://www.nanex-project.eu/
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2	Commissariat à l'Énergie Atomique	CEA	France
3	Swiss Federal Laboratories for Materials Science and Technology	EMPA	Switzerland
4	European Research Services GmbH	ERS	Germany
5	Institut universitaire romand de Santé au Travail [Institute for Work and Health]	IST	Switzerland
6	Fundación LEIA CDT	LEIA	Spain
7	Naneum Limited	NANEUM	UK
8	Nanocyl S.A	NANOCYL	Belgium
9	Nanotechnology Industries Association	NIA	Belgium
10	Netherlands Organisation for Applied Scientific Research	TNO	The Netherlands
11	JRC – Joint Research Centre – European Commission	JRC	Transnational (EU)
12	National Centre for Scientific Research "Demokritos" -	DEMOKRITOS	Greece

Contents

1 Summary

Nanotechnology is a fast growing industry producing a wide variety of manufactured nanomaterials (MNMs) and numerous potential applications. Consequently, the potential for exposure to humans and the environment is likely to increase. Human exposure to MNMs and environmental release of these materials can occur during all life cycle stages of these materials. For each stage of the life cycle of an MNM, exposure scenarios will need to be developed that effectively describe how exposure to humans and the environment occur and what measures are required to control the exposure. The NANEX project was a 12 month project, completed in November 2010, which aimed to develop a catalogue of occupational, consumer and environmental release exposure scenarios for MNMs taking account of the entire lifecycle of these materials. NANEX collected and reviewed available exposure information, focussing on three very relevant MNMs: (1) carbon nanotubes; (2) nano-sized titanium dioxide (nano-TiO2); and (3) nano-sized silver (nano-Ag). In total, 62 exposure scenarios, of which 57 were related to occupational exposure and 5 to consumer exposure, were developed using publicly available data and data collected in sampling campaigns run by NANEX partners,. Due to the wide differences in information available on consumer and occupational exposure, the approach to building the consumer and occupational exposure scenarios was quite different. With nearly no empirical information available on which to base exposure scenarios, the consumer exposure scenarios were based on existing exposure estimation models and default or worst case assumptions. In contrast, all of the occupational exposure scenarios were developed using information and measurement data from the literature or from sampling campaigns. No environmental contributing exposure scenarios were developed for any of the occupational and consumer exposure scenarios. Information on environmental release was not available for the activities described in the occupational exposure scenarios, and the information available on consumer use of MNM-containing products was deemed too limited to be of use for running environmental release models. In parallel, NANEX gathered 'case illustrations' (or case studies) to check the applicability of the generic exposure scenarios. The case studies developed in partnership with industries using or manufacturing MNMs, collected and reviewed available exposure information and developed a set of exposure scenarios for CNTs, nano-Ag, and nano-TiO_2. Based on the information collected during the project an analysis of key data gaps and research needs was carried out. A list of minimum data needs was created outlining categories of information that should be included when describing results from exposure studies of MNMs, and includes both nano-specific information and generic information. The list of research needs span both the short- and long-term research priorities. The most urgent short-term research needs include harmonization of data collection/reporting in terms of choices of metrics and verification of effectiveness of risk management measures. Longer term research needs focus on developing more and better data over the life cycle of MNMs and the subsequent development of a more advanced understanding of MNM exposure. This includes a better understanding of the determinants of MNM exposure and development of MNM-specific exposure estimation models.

2 Background

Nanotechnology is a fast growing industry producing a wide variety of manufactured nanomaterials (MNMs) and numerous potential applications. Since the publication in 2004 of the Royal Society and Royal Academy of Engineering review of the opportunities and uncertainties of nanotechnology[1] there have been numerous reviews published considering the potential risk from exposure to nanoparticles. The reviews have been remarkably consistent and some of their findings can be summarised as follows:

- There is a potential risk to health and the environment from the manufacture and use of nanoparticles;
- There is a lack of knowledge about what these risks are and how to deal with them; and
- The lack of data makes it difficult for manufacturers, suppliers and users to have effective risk management procedures and comply with regulatory duties.

Many nanoparticles and other MNMs are currently only produced on a bench-scale, in small quantities and with relatively few exposed workers. However, other MNMs are mass produced and some industrial sectors make use of nanoparticles in significant quantities, such as in paints and coatings, cosmetics, catalysts and polymer composites.[2] In addition, MNMs will vary widely in their potential to cause health effects in humans following exposure. Total production of MNMs is likely to grow rapidly as is the diversity of MNMs and their applications. Consequently, the potential for exposure to humans and the environment will also increase.

Human exposure to and environmental release of MNMs can occur during all the life cycle stages of these materials. The main life cycle stages for MNMs are shown in Figure 1 and can be summarised as: i) manufacturing of nanoparticles, ii) formulation of nanomaterials and nanoproducts, iii) industrial use of nanomaterials or products; iv) professional and consumer uses of nanoproducts; v) service life of nanoproducts; and vi) waste life stage nanoproducts.

[1] The Royal Society and The Royal Academy of Engineering, 2004
[2] Boxall et al, 2007

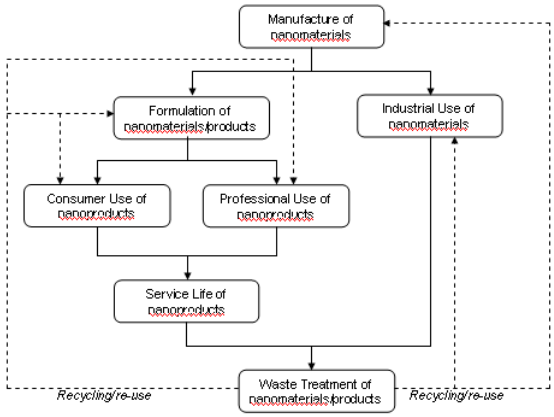

Figure 1: Simplified overview of life cycle stages of manufactured nanomaterials

To make an assessment of the potential for exposure to MNMs information is required on:

i) the mechanism of release of nanoparticles from a wide range of production processes, formulations and use;
ii) the effectiveness of risk management measures;
iii) the range of exposure levels (human and environmental) experienced during the life cycle stages of the nanomaterials; and
iv) the availability and applicability of tools to assess exposure, including measurement methods and models.

The limited information that is currently available on the human exposure to MNMs and release of MNMs to the environment during the life cycle of these materials has generally been collected during relatively small, experimental studies, and hence not always collected in a consistent manner. With respect to occupational exposure to MNMs, a very limited number of workplace air monitoring studies have been published so far[3]. The situations described vary widely: from bench scale production to processing of MNMs in large-scale commercial production in dedicated production facilities and downstream use of MNMs in conventional types of industry. Most studies have focussed on carbonaceous nanomaterials, e.g. carbon black[4], fullerenes[5], carbon fibres[6], and carbon nanotubes[7], or metal(oxide)s[8]. Industrial stewardship programs and ongoing (inter)nationally sponsored projects have generated and will generate new data on exposure issues, however, most data are not (yet) publicly available. Moreover, the potential release from matrix embedded nanomaterials or by processing or machining of nanomaterial products has rarely been studied.

The performance of currently available models for estimating occupational and consumer exposure is unknown as they have not been specifically calibrated and validated for these materials. Within the REACH guidance[9], so called first tier models are recommended for assessment of both inhalation and dermal exposure, e.g. EASE, ECETOC-TRA, Stoffenmanager[10] (inhalation) and RiskofDerm (dermal). For some use scenarios specific models have been developed, e.g. for dermal exposure during spraying[11], and dermal exposure estimates for (biocide) use-scenarios. Currently, an advanced REACH tool (ART) for exposure assessment is being developed by a consortium which includes TNO and IOM.[12] ART utilises a calibrated mechanistic model and analogous exposure measurement data to provide reliable and accurate exposure estimates. However, this model has not been calibrated for exposure to MNMs and it is likely that the mechanistic model will require some modifications for MNMs.

REACH exposure scenarios are defined as sets of information describing the conditions under which the risk associated with the identified use(s) of a substance can be controlled, including operational conditions and risk management measures. Exposure scenarios are the basis for quantitative exposure assessment but are also used as a communication tool in the supply chain. There are currently no provisions in REACH referring specifically to MNMs. However, as REACH deals with chemical substances, in whatever size, shape or physical state, it follows that MNMs are implicitly covered by REACH.

The core information of an exposure scenario consists of substance characteristics, process and products, risk management measures, environment/surrounding, duration/frequency of use, and number of people exposed. However, in the case of MNMs it is also important to know in what forms the MNMs can be released[13], whether as (1) free nanoparticles, (2) aggregated and agglomerated nanoparticles, or (3) integrated in a nanomaterial or within a micrometer sized particle.

Due to the lack of information on the toxicology of MNMs and the paucity of quantitative (personal) exposure data, it will not be possible to develop exposure scenarios (as defined under the REACH regulations) that ensure that exposures are sufficiently controlled to prevent risk to human health and to the environment. However, it may be feasible to develop "exposure scenarios" for specific data rich applications of certain MNMs.

3 What is NANEX

The aim of the NANEX project was to develop a catalogue of exposure scenarios for MNMs relevant for human exposure taking account of the entire lifecycle of these materials. Data collected

[3] Brouwer et al. (2009) submitted to J Nano Part R
[4] Kuhlbush et al., J.Occup.Environ.Hyg. 1 (2004); J.Occup.Environ.Hyg .3(2006)
[5] Yeganeh et al., Environ.Sci.Technol.42 (2008); Fujtani et al., J.Occup.Environ.Hyg.5 (2008)
[6] Methner et al., J.Occup.Environ.Hyg.4 (2007)
[7] Maynard et al., J.Toxicol. Environ.Health A 67 (2004); Han et al., Inhal.Toxicol. 20 (2008); Bello et al. Carbon 266 (2008); J Nanopart Res 11 (2008)
[8] Demou et al., Ann Occup Hyg (2008) Methner J.Occup.Environ.Hyg. 5 (2008), Tsai et al. J Nanopart Res 11 (2008A); Tsai et al., Aerosol Air Qual Res (2008B), Peters et al., J.Occup.Environ.Hyg. 6(2009)

[9] ECHA, (2008)
[10] Marquart et al 2008; Marquart et al (2007)
[11] Brouwer et al , Semple et al
[12] Tielemans et al., 2007
[13] Koehler and Som 2008

included both quantitative (measurement results if available) and qualitative, contextual exposure information (risk management measures). The project reviewed the applicability of existing models for estimating occupational and consumer exposure as well as available models for estimating environmental release and human exposure through the environment. In addition, a small number of specific case illustrations were carried out, covering occupational, consumer and environmental release/exposure scenarios. Finally, a gap analyses was performed on the available knowledge and data and research priorities were defined.

The NANEX project had the following specific objectives:

1. To describe generic requirements for the development of exposure scenarios for MNMs.
2. To collect and review exposure data, exposure metrics, risk management measures and existing models for the development of *occupational exposure* scenarios for CNTs, nano-TiO_2 and nano-Ag.
3. To collect and review exposure data, exposure metrics, risk management measures, and existing models for the development of *consumer exposure* scenarios for CNTs, nano-TiO_2 and nano-Ag.
4. To collect and review data on environmental release, risk management measures, and existing models for estimating *environmental release and exposure* during the various life cycle stages of MNMs for CNTs, nano-TiO_2 and nano-Ag.
5. To carry out a number of case illustrations collecting, in the context of REACH, detailed exposure information for occupational, consumer and environmental release/exposure scenarios for CNTs, nano-TiO_2 and nano-Ag.
6. To develop a catalogue database containing generic and specific exposure scenarios based on information collected during the project.
7. To identify gaps in knowledge/regulation/standardisation with respect to development of exposure scenarios for REACH, exposure assessment and risk management measures and define research needs for occupational, consumer and environmental release/exposure scenarios.
8. To disseminate information to stakeholders.

3.1 Summary of NANEX's key strengths

The NANEX consortium brought together leading experts in the field of human and environmental exposure and risk assessment for nanomaterials specifically and chemical agents in general. The various partners had complementary areas of expertise including exposure assessment and modelling (occupational, consumer and environment), chemical risk assessment and REACH, and exposure and risk assessment of nano technology.

4 Organisation of NANEX

The various activities within NANEX were carried out in 9 complementary workpackages (Table 1).

Table 1 Workpackages (WP) of NANEX

WP	Title	Topic
1	Management	This WP implemented a tried and tested management structure ensuring the timely and efficient implementation of the work plan.
2	Development of generic exposure scenario description	This WP principally served two functions; firstly, to provide the format for describing occupational and consumer exposure scenarios; and, secondly to develop the library of exposure scenarios (together with WP7). In addition, WP2 facilitated other work packages by providing lists with references and information from other EU projects and provided WP7 with a list of realistic exposure scenario titles for the three candidate manufactured MNMs and their downstream usage.
3	Occupational exposure	This WP reviewed the open literature with respect to use for exposure scenario building requirements. It also compiled data generated by two major measurement campaigns (NANOSH, NanoINNOV). Based on these data exposure scenarios were developed. In addition, a performance check of existing exposure estimating models was performed by comparison of modelled exposure scenario exposure estimates with actual data from the data campaigns mentioned above.
4	Consumer exposure	This WP reviewed the open literature in relation to nanomaterials and consumer exposure and assessed the applicability of existing exposure models for estimating nanomaterial exposure. Based on these activities it was attempted to build consumer exposure scenarios for the MNMs in focus in the project.
5	Environmental release	This WP reviewed the literature on environmental releases of MNM. The availability and applicability of existing i) models, ii) experimental settings and iii) analytics for assessing MNM environmental release were discussed. Hence, the objective of this report was to answer the questions: a) what is the relevant knowledge regarding MNM release to the environment, b) what is known, has been modelled or measured, which methods are available, c) what can these models and measurements deliver, d) in what way is the REACH approach suited for MNM.

6	Case illustrations	This WP aimed to provide real-life data to i) evaluate relevant and representative exposure scenarios (in collaboration with WPs 3-5) and ii) test and illustrate the applicability of the generic exposure scenarios developed by WP2.
7	Integration and gap analyses	This WP integrated the information compiled over the course of the project, identified gaps in existing information, developed recommendations and described research needs.
8	Dissemination	This WP coordinated the dissemination activities, including the development of a website, and organised a final dissemination session at the NANOSAFE 2 Conference in Grenoble in November 2010
9	Scientific management	This WP coordinated the scientific activities of the project, monitored progress of the work packages, performed scientific review and prepared the scientific part of reports for the European Commission.

5 Results

The following section provides an overview of the findings obtained from the various workpackages.

5.1 *WP2 Development of generic exposure scenario description*

The format for describing exposure scenarios provided in the REACH guidance was adopted and used to develop an MS Access database to collate occupational and consumer exposure scenarios by WP3 and WP4. Information was obtained from the literature and by searching information from other current and recent projects funded by the European Commission and other organisations; these details were forwarded to the work package leaders. A workshop was organised in Lausanne on 8 and 9 March to discuss data requirement, sources of information and models for occupational, environmental and consumer exposure. In addition, the workshop discussed the data requirements for gap analyses and for defining research needs.

A list of generic exposure scenario titles was developed for the three candidate MNMs. This was used by WP7 to identify major gaps in current knowledge of exposure within these scenarios.

The initial intention of NANEX was to develop an extensive web-based library of occupational and consumer exposure scenarios that would be publicly available. However, following review of the available exposure scenarios, it was clear that the many of the scenarios were not of sufficient quality to be included in a web-based library. It was therefore decided to only provide a number of examples of occupational and consumer exposure scenarios, for which contextual information was made available. One note of caution is that the exposure scenarios developed within the NANEX project do not describe safe use of the MNMs (as defined under REACH), but rather describe the exposure situation (i.e., no risk assessment was carried out). We believe with more measurement data and contextual information becoming available over time (e.g., from EU and other projects), that the expansion of the exposure scenario library would provide a useful source of information for risk assessment and other purposes. However, rigorous quality assurance procedures will need to be developed and applied to ensure that the exposure scenarios are of sufficiently high quality.

5.2 *WP3 Occupational exposure*

Based on 40 literature references 22 ESs were developed, 14 ESs for CNT, 5 for nano-TiO_2 and 2 for nano-Ag. Most of the scenarios related to production/synthesis of the nanoparticles, although some were also developed for downstream use. In total 35 ESs were derived from the data sets of the two large measurement campaigns. Most ESs were for CNTs (n=14), which predominantly related to research-scale activities. Most nano-TiO_2 scenarios (n=8) were on commercial-scale manufacturing and formulation. Only 2 ESs could be built for nano-Ag, whereas 11 ESs were built other substances, including other metal oxides.

Based on the process of developing these ESs the following main conclusions could be drawn. Most studies either reported in the literature or as part of the measurement campaigns had an explorative character and were focused on exposure analysis. Therefore, the reports of these studies did not usually include sufficient information to build ESs. For example, the amount of MNMs used, basic characterisation of products, operational conditions, and frequency of activities was often not available. Most importantly, there was a lack of harmonization of the measurement strategy and the analysis and reporting of measurement data.

Following a review of existing exposure models, it was concluded that the basic concepts of existing worker exposure estimation models may be suitable MNMs. However, none of the models reviewed are currently suitable for providing estimates for nanoparticles and model estimate will be inaccurate and possibly overestimate the (mass) concentration levels. Furthermore, existing models provide exposure estimates in mass concentrations, which may not be appropriate for expressing exposure to nanomaterials.

5.3 *WP4 Consumer exposure*

Despite the fact that MNMs are increasingly utilised in consumer products, there is a paucity of exposure data for consumers. Many consumer products contain MNMs in solid matrices, which may significantly reduce consumer exposure. On the other hand, MNMs

also occur in consumer liquid products and thus the potential for dermal exposure and not the least inhalation following spray applications needs attention. The consumer exposure scenarios developed within the NANEX project were based on very limited information. Limited measurement data were available and hence, all most exposure estimates were based on models using many conservative assumptions, leading to unrealistic exposure estimates.

5.4 *WP5 Environmental release*

Following the review of the literature is was clear that there is a lack of reliable data on MNM production and application amounts and empirical information on release for all life cycle stages of MNM-containing products and environmental compartments. To improve and validate environmental release assessments more information on MNM production amounts is urgently needed. Also quantitative indications on the allocation of the produced volume to the relevant product categories (e.g. cosmetics, plastics, etc.) that contain the MNM are needed. Empirical (experimental/analytical) release information for the main release sources during all MNM life stages is needed as well (MNM production and nanoproducts' manufacture processes, MNM products consumption and disposal).

5.5 *WP6 Case illustrations*

In total, four industrial case studies were carried out during the NANEX project. These case studies were carried out in the context of REACH, which requires detailed exposure information for occupational, consumer and environmental release/exposure scenarios for specific MNMs. Available information on four MNMs was collated and reviewed:

1. nano-TiO_2
2. nano-TiO_2 (Mn-doped)
3. nano-Ag
4. Multi-walled Carbon Nanotubes (MWCNTs)

During the discussion of the exposure scenario case studies, it became clear that the case studies could serve as MNM product-specific examples only and that no generalisation with regard to practices within an entire MNM type-specific branch could be based on these individual exposure scenario case studies.

5.6 *WP7 Integration and gap analyses*

During the final stage of the NANEX project, work package 7 integrated the information compiled over the course of the project, identified gaps in existing information, and developed recommendations. The process of building exposure scenarios for MNMs highlighted many challenges in translating experimental data and modelled estimates, which are often developed for research purposes, into a more applied format. However, this process also revealed several areas where researchers could improve data collection to meet the needs of exposure and risk assessors. This WP identified gaps in the information needed for characterizing exposure to MNMs, prioritized research needs in exposure science, and provided project-wide conclusions.

For the aims of WP7, it was necessary to note basic characteristics and appropriateness of the methodologies employed in the references used to develop the exposure scenarios as well as the completeness of the catalogue of exposure scenarios. To reach these aims in a systematic and transparent fashion, two linked databases/libraries were created in partnership with WP2. The first database was a reference database that was used to document information on the publicly available information sources that were reviewed. The second database stored the exposure scenarios themselves in a format based on that in the ECHA guidelines for REACH compliance (ECHA 2010).

Overall, 63 distinct references were entered into the reference database. Thirty-three of these were relevant to occupational exposure, sixteen to consumer exposure, thirteen to environmental release, and three contained general information. (Two references were considered relevant to both consumer exposure and environmental release). Very few of the references that were associated with consumer exposure contained original data on exposure or release. Over 75% of the occupational reference entries described exposure during activities associated with primary manufacturing of MNMs, which were often at the small/pilot scale in research facilities. Although experimental results demonstrate potential for worker exposure to MNMs, the exposure estimates provided in these occupational studies were not suitable for estimating environmental release.

In total, 62 exposure scenarios were entered into the exposure scenario database, of which 57 were related to occupational exposure and 5 to consumer exposure. Due to the wide differences in information available on consumer and occupational exposure, the corresponding two work packages took a necessarily different approach to building the exposure scenarios. The consumer work package, with nearly no empirical information available on which to base exposure scenarios, relied on existing exposure estimation models and default or worst case assumptions to build exposure scenarios for MNMs. In contrast, all of the occupational exposure scenarios were developed using information and measurement data from the literature or from sampling campaigns. No environmental contributing exposure scenarios were developed for any of the occupational and consumer exposure scenarios. Information on environmental release was not available for the activities described in the occupational exposure scenarios, and the information available on consumer use of MNM-containing products was deemed too limited to be of use for running environmental release models.

In parallel, NANEX gathered 'case illustrations' (or case studies) to check the applicability of the generic exposure scenarios. The case studies, conducted within WP6 in partnership with industries using or manufacturing MNMs, collected and reviewed available exposure information and developed a set of exposure scenarios for CNTs, nano-Ag, and nano-TiO_2.

The results of the database analysis, combined with the contents and conclusions of the other WP reports, were critically reviewed and used to identify initial gaps and create discussion points for a NANEX gap analysis meeting, held in at National Centre for Scientific Research 'Demokritos', Athens, Greece on 21 and 22 October 2010. At this meeting, partners discussed the shortcomings in existing information and methods and identified research needs. The conclusions reached during the meeting were used to develop the final conclusions and recommendations of the NANEX project.

The most significant challenges to building exposure scenarios were related to limitations in available data. There is very limited quantitative information on MNM exposure and release over the life cycle (i.e., occupational manufacture and use, consumer use, and environmental release). Often, studies do not contain adequate description of the form of MNMs released. Also notable, particularly among occupational exposure studies, was the lack of standardization for collecting, reporting and interpreting data. The studies that were reviewed had widespread differences in sampling strategies, metrics and/or other parameters used to describe exposure, and level of detail provided.

It is often not feasible to measure human or environmental exposure and hence, exposure estimation models are necessary and important tools for assessing both human and environmental exposure. The existing human exposure estimation models were not designed to accommodate nano-specific features and have limited to no applicability to MNMs. Environmental release models for MNMs are characterized by high parameter uncertainty due to the lack of data on volumes of MNMs used and released by industry. Currently, there are not enough data available to build or validate exposure estimation models for MNMs.

The inconsistencies in how measurements are collected and reported will be a significant barrier to risk assessment for MNMs in the future if actions for harmonization are not taken. It is currently not possible to pool or combine data or to make meaningful comparisons between studies, which will limit efforts to make MNM specific models or epidemiology studies. Further, it is not possible to assess the relevance of a study to other situations, which creates a high burden of data generation for companies trying to assess MNM exposure in their own facilities.

A list of minimum data needs for exposure studies was developed as well as a list of research priorities. The list of minimum data needs outlines categories of information that can be easily included or noted in exposure studies and provides an immediate starting point for improving exposure assessment of MNMs, and includes both nano-specific information (description of physical and chemical form of the MNMs and released nanoparticles, other sources of ultrafine and nanoparticles, and the use of multiple metrics to characterise and quantify exposure) and generic information (information on process, site, risk management measures, environmental release information, sampling and analytical strategy). The list of research needs span both the short term and long term, the latter building upon the results of the short term efforts. The most urgent short term research needs are harmonization of data collection/ reporting in terms of choices of metrics and verification of effectiveness of risk management measures. Longer term research needs focus on developing more and better data over the life cycle of MNMs and the subsequent development of a more advanced understanding of MNM exposure, such as understanding the factors that are most important for determining MNM exposure and development of MNM-specific exposure estimation models.

6 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: FP7-211464-2 Website: http://www.nano-device.eu

Coordinator: Professor Kai Savolainen, Finnish Institute of Occupational Health

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7	INSTITUTE FÜR ARBEITSSCHUTZ DER DEUTSCHEN GESETZLICHE UNFALLVERSICHERUNG	DGUV-IFA	Germany
8	INSTITUT NATIONAL DE RECHERCHE ET DE SECURITE	INRS	France
9	NATIONAL RESEARCH CENTRE FOR THE WORKING ENVIRONMENT	NFA	Denmark
10	STOCKHOLMS UNIVERSITET	SU	Sweden
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Content

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 the safe production and us 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 the 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 Intentions for use and impact

The potential of the NANODEVICE project and the Consortium executing the project are multi-focal. The expected impacts at different levels of activities are briefly listed below:

1. The project is expected to have a major impact on the way that these and other types of devices will be developed in the future, i.e. by creating multi- and truly interdisciplinary consortia capable of cross-fertilizing their competencies in solving challenging research-based problems. This means that problem solving may be carried out by the adoption of, and adaptation to, novel research challenges in an innovative and a flexible approach.

2. The systematic, strategy-driven research effort of this project can be expected to become a benchmark approach in types of endeavours in which a considerable amount of expertise and talent is required for simultaneous and interdisciplinary problem solving.

3. The multitude of dissemination and data acquisition approaches will dramatically increase the impact of the project among the end-users of ENP in workplaces using these materials and among civil authorities responsible for workplace measurements.

4. Promotion of global dialogue under the auspices of the NANODEVICE project will have a major impact on the implementation of standards for ENP, such as the selection of metrics of ENP to be used for the assessment of safety and exposure levels.

5. The devices produced will allow industrial enterprises, workplaces, governmental and other research institutions to generate large amounts of reliable data about exposure levels to a large variety of ENP and thereby to produce large datasets and databases on levels on different ENP in workplace air, for the first time in a user-friendly manner and, comparable and affordable way.

6. The data produced here and other data on associations between characteristics (metrics) of ENP and their effects will provide regulators with a firm foundation for development of means for reliable protection of workers at the workplace.

7. The NANODEVICE project also paves the way for the next generation ENP monitors that may utilize living cells in the workplace monitoring devices to directly assess the biological significance, magnitude and risk of a given exposure. The knowledge for such innovative approach is not yet available, and thus an important next step will be through novel innovative technological solutions to come up with devices that can capture the essential information of exposure to ENP. This knowledge can then be used for the development of the next generation devices, ideas for which are already being generated within the NANODEVICE Consortium.

8. Together these impacts of the NANODEVICE project can be envisaged to have positive societal impact and promote the social wellbeing and health among the working population and beyond.

9. It goes without saying that the results of the NANODEVICE project will also support the Lisbon strategy goals by supporting technological progress and innovation within European Union Member states, thereby paving its way to become the most competitive knowledge-based society of the world. Thus the overall goals of the NANODEVICE project, while being purely scientific, also promote and support the social and workplace policies of the European Union, and provide targeted support to the European Union strategies related to nanotechnologies and nanosciences.

10. The goals of the NANODEVICE project are complex, multiple and multi-focal and require close collaboration between several industries (e.g. developers and producers of nanomonitors, end-users of ENP, aerosol and nanotechnological scientific community) as well as many scientific disciplines involved in exploring the safety of ENP including occupational hygiene and toxicology. None of the European Union Member States can provide these competencies and talents alone, and therefore multinational, European Union wide research capable of providing the required impact, becomes a necessity.

11. The NANODEVICE Consortium does not work and exist in a vacuum, instead it is a proactive project that seeks collaboration from around the globe and has already created close collaboration with a number of ongoing EU-funded research projects working on the safety of ENP and NT. Moreover, close contacts have been developed with a multitude of research organizations and players, not only in Europe, but also in the USA and Asia.

4 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.

5 Specific objectives of the research project:

1. To identify relevant physical and chemical properties for specific measurement of engineered airborne ENP, and to develop reference materials for ENP aerosols.
2. To investigate the relationships between physical and chemical properties of ENP and their potential toxicity or bioactivity.
3. To analyze the ENP emitted from industrial processes during the production and handling of ENP and to assess levels of ENP in workplaces in order to define performance requirements.
4. To develop technologies that enable utilization of new concepts in miniaturized and field-worthy specific monitors for ENP.
5. To develop methods for calibration and testing of the newly developed concepts and methods and devices in simulated and real life exposure settings.
6. To effectively disseminate the results of the research, to promote the safety of ENP by guidance and standard development, to provide training and guidelines education, so that ENP can be safely produced and handled, and by promoting collaboration of all those concerned with the safety aspects of ENP. The main goal is to assure that the impact of the project on the society can be assured.

6 Work carried out during the course of the project

The work has been divided into several work packages (21) collected into six larger subprojects (SP) on 1) characterization; 2) association between ENM features and toxicity; 3) exposure scenarios in industrial processes associated with nanotechnologies; 4) development of instruments; 5) calibration and testing of the novel instruments; and 6) assuring impact of the results of the project. Management of the project aims at assuring effective and fluent flow of the work within the project and appropriate interactions between work packages and subprojects, and leadership, of the undertaking. In the characterization subproject, work has been carried out to identify and characterize reference and model ENM aerosols and to build a nano-metal oxide reactor. Emphasis has also been on the production of well characterized carbon nanotubes (CNT). A part of the project has focused on exploring on the characteristics of different ENM by using visual technologies such as electron microscopy, and characterizing of ENM through using non-imaging techniques to delineate physical-chemical properties of ENM. Association between ENM characteristics and their association with toxicity has been explored with a large variety of methods. This subproject has also provided a list of test aerosols to be used for the performance testing of the instruments to be developed within the project. One part of the project has been generating performance requirements to set specifications of the easy-to-use devices and portable devices to assess ENM -based evaluation of the limitations of presently available static devices. A part of this goal is to evaluate existing and future work place exposure scenarios. Furthermore the subproject has created sampling strategies for appropriate assessment of exposure to ENM. A key-part of the project has focused on the development of novel on-line ENM measurement instruments with the capability to capture essential ENM features, as well as to produce innovative and new measurement principles aiming at discovering novel technologies capable of revealing essential ENM features with an association of toxic properties of given ENM. Several approaches have been chosen and work has been carried out to develop such instruments including the following principles: a) on-line, inexpensive, portable, on-line surface monitor; b) size discriminating number and surface area aerosol monitor and sampler; c) wide-range size-resolving aerosol monitoring and sampling system based on a personal size-resolved ENM selective sampler and an on-line monitor; d) high-sensitivity optical sensor for single particle number and mass allowing particle measurements at a 50-80 nm size range; e) high-sensitivity MEMS-based sensor for single-particle number and mass down to 10 nm; f) catalytic and surface-chemical aerosol monitor based on novel device concepts for new metrics proving information in a highly sensitive fashion and near real-time monitoring of catalytic activity and surface chemical groups of ENM; and g) nanofiber monitor that is a robust, portable, easy-to-use quasi real-time device to monitor airborne fibers in workplace environments.. A part of the project focusing on testing and calibration of the instruments has carried out work to establish the facilities and processes for testing and calibration of the instruments to be developed within the part of the project aiming at development of monitoring instruments both in laboratory conditions and in the field in work places A part of the project has aimed at maximizing the dissemination of the results of the project to a wide variety of target audiences, and finding ways to assure an impact of the project to promote safety of ENM and nanotechnologies. Hence, tailored training courses and good practice guidelines have been prepared. A data-base supporting risk assessment of ENM based on the project results is under development by several partners and in collaboration with other EU-funded projects. Also information leaflets, posters and a project newsletter have been developed. Establishment of a strategy for the European and international standardization of all project results is under intensive preparation with different EU-funded projects and European Union stakeholders in this area. An advisory forum - 'Annual Forum for Nanosafety' - with the representation of leading experts and most qualified organizations from five continents has been supporting the project. Organizing an international congress on safety of engineered ENM and nanotechnologies is under preparation, and discussions are ongoing to publish a state-of-the-art nanosafety handbook based on the project. An ongoing internal mobility program has been established, and increased the internal cohesion of the project.

7 The expected final results and their potential impact on safe nanotechnologies

The project results will have a marked societal impact especially on workplace safety in small and medium size enterprises and microenterprises through bringing into market affordable on-

line measuring devices providing reliable exposure information on engineered nanomaterials. This will not only greatly increase our knowledge and understanding on the true workplace exposure to these materials. It will also support regulators and decision makers in making evidence based conclusions and actions on necessary measures to protect the workers from excessive exposure, especially the setting of occupational exposure limits for different types of engineered nanomaterials. One can expect remarkable savings to the industry through reliable exposure information at low or affordable expenses. These outcomes of the project will also enable safe use of these materials, and promote safe production of engineered nanomaterials, and thereby increase the competitive edge of nanotechnologies through emphasizing safety aspects in all areas of nanotechnology applications. The project has successfully moved toward its impact objectives as demonstrated by the successful progress of the technical and impact work. Stakeholder contacts will assure a marked further impact through making the project results more visible among ETUC and Business Europe partners, occupational safety and health and research community, among governments, and the Commission

8 Conclusions

NANODEVICE will provide new information on the physico-chemical properties of engineered nanoparticles (ENP) and information about their toxicology. Also a novel measuring device will be developed to assess the exposure to ENP´s from workplace air. The purpose of the project is also 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.

9 Directory

Table 1 Directory of people involved in this project.

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NanoFATE

Nanoparticle Fate Assessment and Toxicity in the Environment

Contract Agreement: NMP4-SL-2010-247739 Website: http:// www.nanofate.eu
Coordinator: Dr Claus Svendsen (csv@ceh.ac.uk), NERC - Centre for Ecology and Hydrology, Wallingford, UK

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3	Oxford University	UOXF.DJ	United Kingdom
4	University of Aveiro	UAVR	Portugal
5	Faust & Backhaus	F+B	Germany
6	NanoTrade	NT	Czech Republic
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8	Institute of High Pressure Physics, Polish Academy of Sciences	IHPP	Poland
9	Cardiff University	CU	United Kingdom
10	Amepox	AXME	Poland
11	Gothenburg University	UGOT	Sweden
12	SYMLOG France	SYMLOG	France

Contents

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 CeO_2, 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 (Haughton et al. 2003; Heard et al. 2003), where environmental impacts rather that 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 CeO_2 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 CeO_2. Initial predictions will be generated based on worst case conditions.

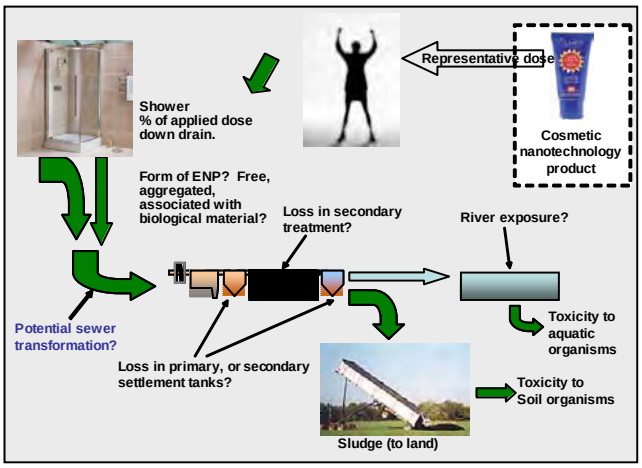

Fig. 1. Schematic illustrating key issues concerning the disposal, fate and environmental release pathways of an example "down the drain" nanotechnology product (e.g. ZnO ENP containing sunscreen).

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 pathways 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 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:

3. 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);

4. 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);

5. 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)

6. Matrix Assisted Laser Desorption/Ionization (MALDI)-Imaging mass spectrometry for detection of surface interactions of ENP with particulate matter and possibly also imagine of tissues for metal ENPs inclusions (UOXF.DJ, UNIPMN).

7. 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.

8. 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).

9. 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).

10. 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.

11. 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.

12. 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).

13. 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).

14. 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 CeO_2 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 CeO_2 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.

15. 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).

16. 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).

17. Quantify additional toxicity (if any) beyond that predicted to be caused by the free metal ion.

18. 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).

19. 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).

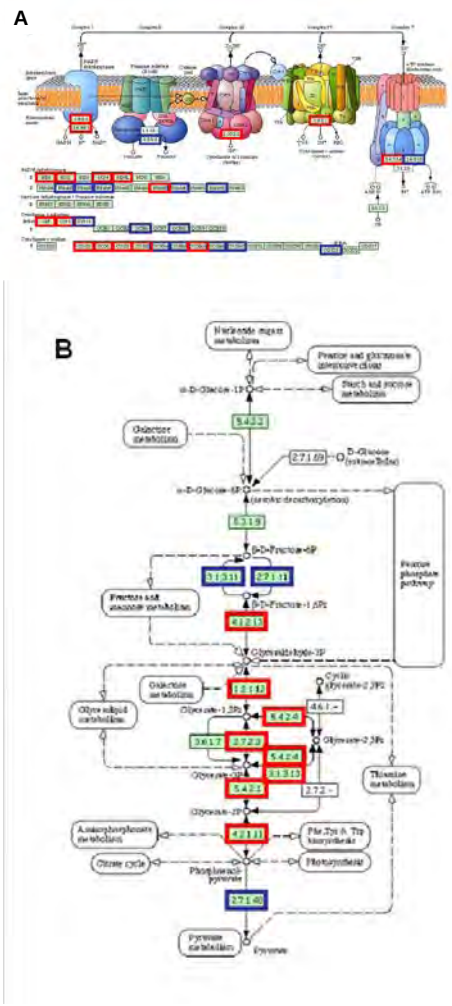

Fig. 2. Example analysis of the impact of ionic copper on oxidative phosphorylation (A) and on glycolysis/gluconeogenesis (B) of earthworms in a pathway based visualisation of the mechanism of toxicity. Transcripts outlined in bold are represented on the

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:

20. 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).

21. 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).

22. 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).

23. 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).

24. 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.

25. 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).

26. 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).

27. 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 CeO_2 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.

28. 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.

29. 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.

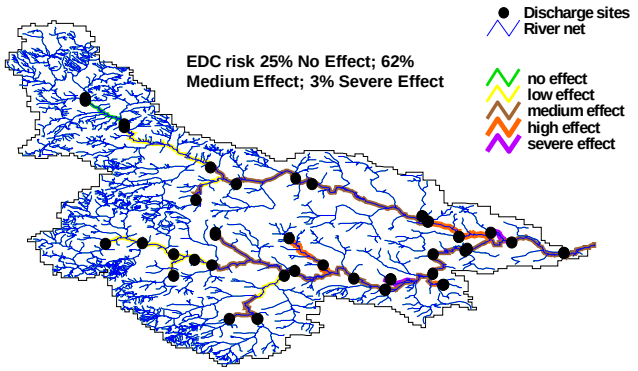

Fig 3. Catchment risk map of predicted endocrine disruption of fish from effects of oestrogenic chemicals for the Aire and Calder rivers, Yorkshire, UK.

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.

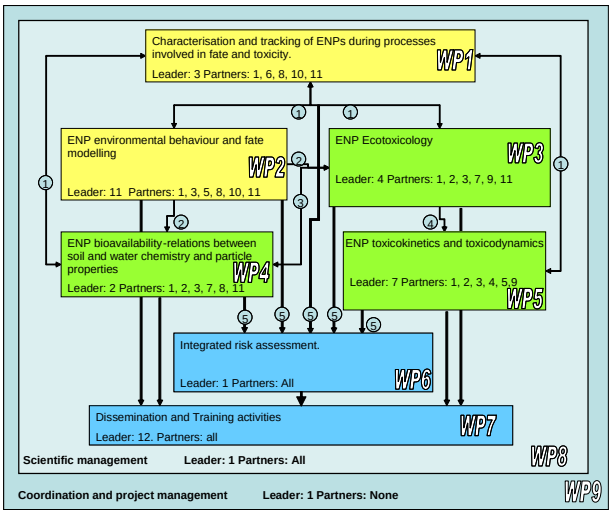

Fig 4. NanoFATE PERT diagram showing flow of data and products between the seven workpackages within particle chemistry and fate component (yellow), ecotoxicology and bioavailability component (green) and the risk assessment and communication component (blue). The flow of knowledge, technology and data 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.

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2	Eidgenössische Materialprüfungs- und Forschungsanstalt	EMPA	CH
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4	Katholieke Universiteit Leuven	KULeuven	BE
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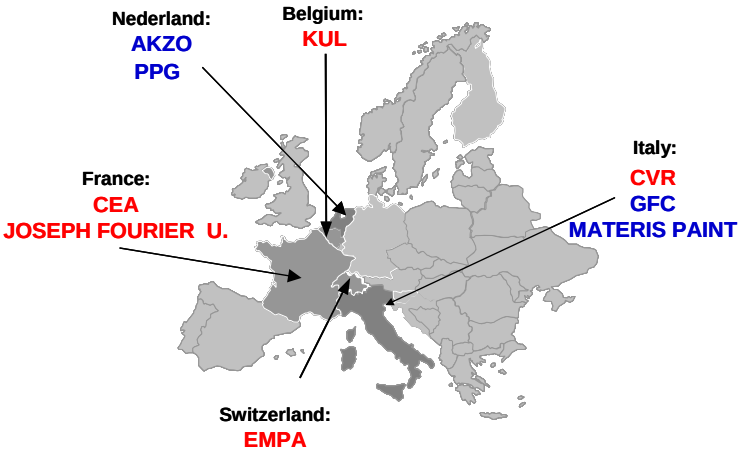
6	MATERIS PAINTS ITALIA	MATERIS	IT
7	GFC CHIMICA	GFC	IT
8	AKZO NOBEL COATINGS S.A.	AKZO	NL
9	PPG Europe BV	PPG	NL

Contents



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.

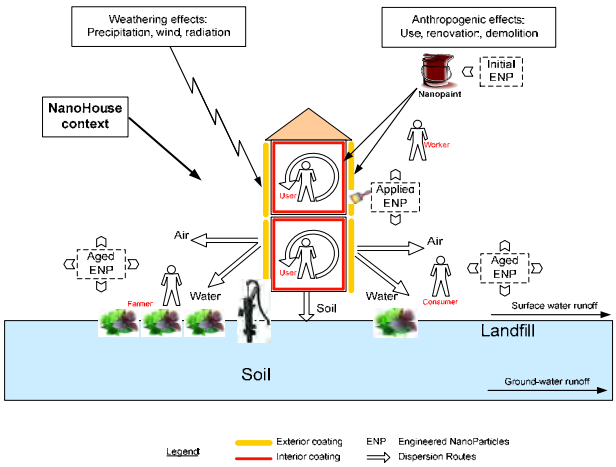
This project started January 2010 for a duration of 42 months (until 06/2013) and a total budget of 3.1 M€.

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-TiO_2 and nano-SiO_2 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-TiO_2 and nano-SiO_2 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.

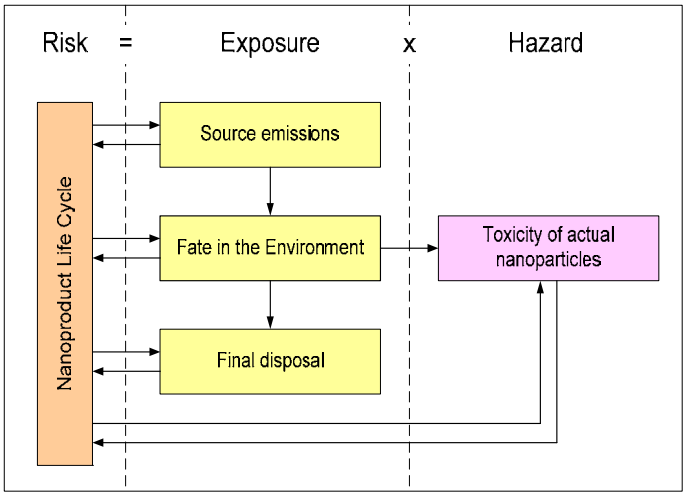


Table 1 Workpackages (WP) of NanoHouse

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 Nanosmile[1] 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*

Nanoproducts and associated nanoparticles for the study have been selected.

An overview on the types of ENPs used in paint industry has been provided, as well as a first analysis of life cycle stages of façade coatings. Paint panels were fabricated and provided to involved partners in WP2 in order to be able to identify sources of ENP release. A detailed literature research concerning leaching test for paints and NPs release into the environment was conducted.

Collected information and data were used to lead a definition of a specific protocol for leaching tests for the nano-based products considered in the NanoHouse project. Panels for UV exposure and Taber for leaching test have been done. Estimation of ENPs release via the wet route is in progress. The Taber standard method for investigating wear resistance to abrasion by "dry route" is chosen. The abrasion Taber device was started and coupled to an aerosol measurement system in order to measure dust released by the abrasion process. Estimation of ENPs release via the dry route is started. First measurements of dust released from paints were performed.

An aged-like process was defined in order to supply "aged" ENP's (TiO_2, Ag,SiO_2) in high quantities (few grams) for environmental fate experiments (WP3) and toxicological tests (both *in vivo* and *in vitro*) (WP4). Preliminary millings tests were performed on as received free film paints and on UV exposed free film paints. The size and distribution of the particles/agglomerates obtained were evaluated through SEM, DLS and Laser Granulometer.

Radio labelled Ag-nanoparticles and TiO_2 fluorescent nanoparticles have been synthesized.

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-12 meeting

The Month-12 meeting was held in St Gallen Swizerland the 29th-30th November 2010.

5.2.2 Other workshops

The NanoHOUSE project has been represented in Lausanne: 03/2010 (EMPA); Ispra: 04/2010 (B. Nowak, EMPA); Edinburgh:

06/2010 (P. Hoet, KULeuven). And in the "Nanosafety Cluster" in Prague 12/2010 (S. Zuin CVR).

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 will send samples and Dario Cervellati (GFC) will take care of their weathering.

6 Directory

Table 2 Directory of people involved in this project.

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7 Copyright

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

No.	Beneficiary name	Short name	Country
1	Institut universitaire romand de Santé au Travail [Institute for Work and Health]	IST	Switzerland
(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)
12	Wageningen University and Research Centre / Stichting DLO-RIKILT	RIKILT	The Netherlands
13	Eidgenössiche Materialprüfungs- und Forschungsanstalt	EMPA	Switzerland
14	National Centre for Scientific Research "Demokritos", Agia Paraskevi - Athens	Demokritos	Greece
15	National Institute for Public Health and the Environment	RIVM	The Netherlands
16	University of Bern	UBERN	Switzerland
17	Commissariat à l'Énergie Atomique	CEA	France
18	Dublin Institute of Technology	DIT	Ireland
19	Health and Safety Laboratory	HSE-HSL	United Kingdom
20	Slovak Medical University	SMU	Slovakia
21	Finnish Institute of Occupational Health	FIOH	Finland
22	University of Copenhagen	UCPH	Denmark
23	Stichting Dienst Landbouwkundig Onderzoek	Alterra	The Netherlands
24	University of Plymouth	UoP	United Kingdom
25	St. Mary University College [1]	SMUC	United Kingdom
26	Heriot-Watt University [2]	HW	United Kingdom

[1] *Professor changed from partner 11-UNIS to 25-SMUC (11 discontinued).*
[2] *Professor changed from partner 2-NU to 26- HW (2 discontinued).*

Contents

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 which are different from those of larger entities of the same materials and from molecular forms of the materials (where these exist). Nanoparticles can pass through body barriers, although the detailed mechanisms are not yet understood. This is interesting for medical applications, but it 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 nanoparticles 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

'Nanotechnology' and the term 'nanomaterials' under its most commonly used definition refers to materials that have at least one structural dimension at the nanoscale, i.e. between 1 to 100 nanometers. Nanomaterials often have chemical, physical and bioactive characteristics that are different from those of larger entities of material with the same chemical composition, and from molecular forms where these exist. Nanotechnology 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 the novel characteristics specific to nanomaterials have lead 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 nanoparticles 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 nanoparticles 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 nanoparticles. 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 nanoparticles 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

During the project's first three years, NanoImpactNet organized a series of events and training schools, and produced several reports described below. These illustrate the wide range of questions addressed within NanoImpactNet.

5.1 Events

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 nanoparticles.
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 TiO_2, 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 nanoparticles 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.

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.

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.

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 nanoparticles 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, 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.

The 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 hopsitals, 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 reserarch; 4. Nanotechnological tools for impact assessment; 5. From research to policy) and featured over 40 presentations from leading experts providing insight into the latest nanotsafety 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 Organisastions) gave their views. This was followed by a lively debate including questions for conference delegates.

A short report of this stakeholder session is available and its conclusions are also going 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 communicaton 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 again welcomed 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."* The programme was 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?"* The programme was 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.

Reports with results and conclusions of these training schools are currently in preparation.

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 examplee. 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. A technical report of the workshop is in production.

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.

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 personel 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.

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

Call identifier FP7-KBBE-2009-3, Grant Agreement: 245162 Website: http://www.nanolyse.eu
Coordinator: Stefan Weigel, RIKILT- Institute of Food Safety; Stichting Dienst Landbouwkundig onderzoek part of Wageningen UR, The Netherlands

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5	The Secretary of State for Environment, Food and Rural Affairs	FERA	United Kingdom
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9	Agencia Estatal Consejo Superior de Investigaciones Cientificas	CSIC	Spain
10	Vysoka skola chemicko technologicka v Praze	VSCHT	Czech Republic

Contents

1 Summary

The NanoLyse project will focus on the development of validated methods and reference materials for the analysis of engineered nano-particles (ENP) in food and beverages. The developed methods will cover all relevant classes of ENP with reported or expected food and food contact material applications, i.e. metal, metal oxide/silicate, surface functionalised and organic encapsulate (colloidal/micelle type) 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, an organically surface modified NP and organic nano-encapsulates. Priority will be given to methods which can be implemented in existing food analysis laboratories. A dual approach will be followed. Rapid imaging and screening methods will allow the distinction between samples which contain ENP and those that do not. These methods will be 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 will be 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 36 months (2010 – 2012).

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 will address 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:

(i) imaging and screening methods for a rapid decision on the presence of ENP in food samples and

(ii) 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 workpackages (fig. 1). All WPs are closely linked with each other to ensure maximum synergies. WP1 will supply all method developers with characterised ENP dispersions and test and reference materials for method development and validation. All method development WPs (2-4) will especially in the first phase of the project collaborate very closely on the sample preparation via the respective inter-WP working group (WG). In the same way WP 3 and WP4 will collaborate on the analytical separation techniques. All RTD WPs (1-4) will contribute to the dissemination and training activities which are organised by WP5. WP6 will supply all information necessary for the successful execution of the project to all WPs and collect all data needed for the regular reporting and the monitoring of the progress of work.

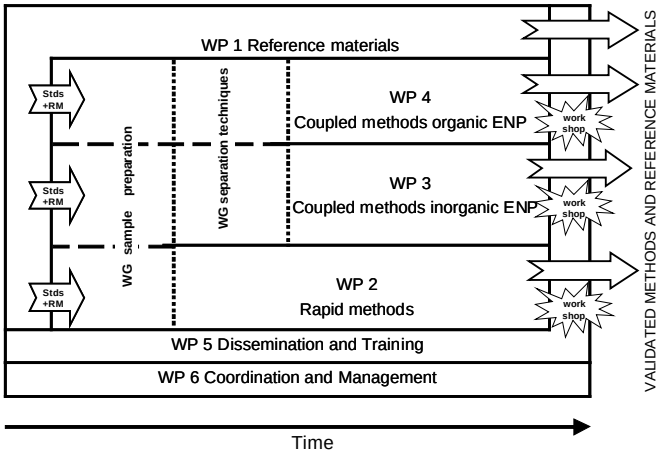

Figure 1: Interrelations between Workpackages (WP) of NanoLyse

4 Project tasks

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 will be 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, surface-modified nanoparticles and organic nanoparticles. These particles will be sourced and, in the first stage of the project, aqueous suspensions of these four types of particles will be prepared. These materials can then be used by the other project partners for spiking experiments during method development. The aqueous suspensions will be 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.

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 workpackage will explore a range of engineered nanoparticle types that are relevant to food such as: metal-based (Ag), metal oxides (TiO_2, SiO_2), and surface modified engineered nanoparticles.

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 workpackage 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 immuno- 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.

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

To quantify them 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 (SPLITT) 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.

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, sugars). 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.

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 will address 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 will be 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 will be distributed actively to stakeholders and other interested parties. The newsletter will inform on the scientific progress in the analysis of engineered nanoparticles in food and beverages within and outside 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" will be 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, Guillaime 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:

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 *Lectures and posters*

LECTURES:

Stefan Weigel: Nanoparticles in food: emerging analytical task. 4th International Symposium on Recent Advances in Food Analysis (RAFA 2009), 4-6 November 2009, Prague, Czech Republic.

Stefan Weigel: Detection of nanoparticles in food – an analytical challenge. IFT International Food Nanoscience Conference; July 17, 2010; Chicago, IL.

Stefan Weigel: Detection and characterization of nanoparticles in food. Max Rubner – Conference 2010: Nanotechnology in the Food Sector, October 10 – 12, 2010: Karlsruhe, Germany.

Stefan Weigel, Hans Bouwmeester, Hans Marvin, Ruud Peters: Nanoparticles in food – analytical methods for detection and characterisation. International Conference on Safe production and use of nanomaterials, Nanosafe 2010; November 16 – 18, 2010 Grenoble, France.

POSTERS:

Stefan Weigel, Ruud Peters, Hans Bouwmeester: Nanoparticles in Food - Analytical methods for detection and characterisation Poster presented at 2nd NanoImpactNet Conference, 9-12 March 2010, Lausanne, Switzerland.

Agnieszka Dudkiewicz, Karen Tiede, Kristian Mølhave, Alistair BA Boxall: Imaging techniques for the detection and characterization of inorganic nanoparticles in food. Poster presented on 26th October in University of York Jeol Nanocentre on occasion of The 2010 Cantor Nanoscience Lecture presented by Prof. Sir H. Kroto, Nobel Laureate in Chemistry.

Agnieszka Dudkiewicz, Karen Tiede, Kristian Mølhave, Alistair BA Boxall : Imaging techniques for the detection and characterization of inorganic nanoparticles in food. International symposium on nanotechnology in the food chain, Brussels, Belgium, 24th November 2010.

Stefan Weigel, Ruud Peters, Hans Bouwmeester: NanoLyse: Nanoparticles in Food Analytical methods for detection and characterisation. International symposium on nanotechnology in the food chain, Brussels, Belgium, 24th November 2010.

Stefan Weigel, Ruud Peters, Hans Bouwmeester: Nanoparticles in Food - Analytical methods for detection and characterisation Poster presented at 3rd NanoImpactNet Conference, 14-17 Feb. 2011, Lausanne, Switzerland.

5.3 *Open days*

A NanoLyse Open day is scheduled on occasion of the 5th International Symposium on Recent Advances in Food Analysis (RAFA 2011), 1-4 November 2011, Prague, Czech Republic.

5.4 *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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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

No.	Beneficiary name	Short name	Country
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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

Contents

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

1.1 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, a recent and 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.

1.2 *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.

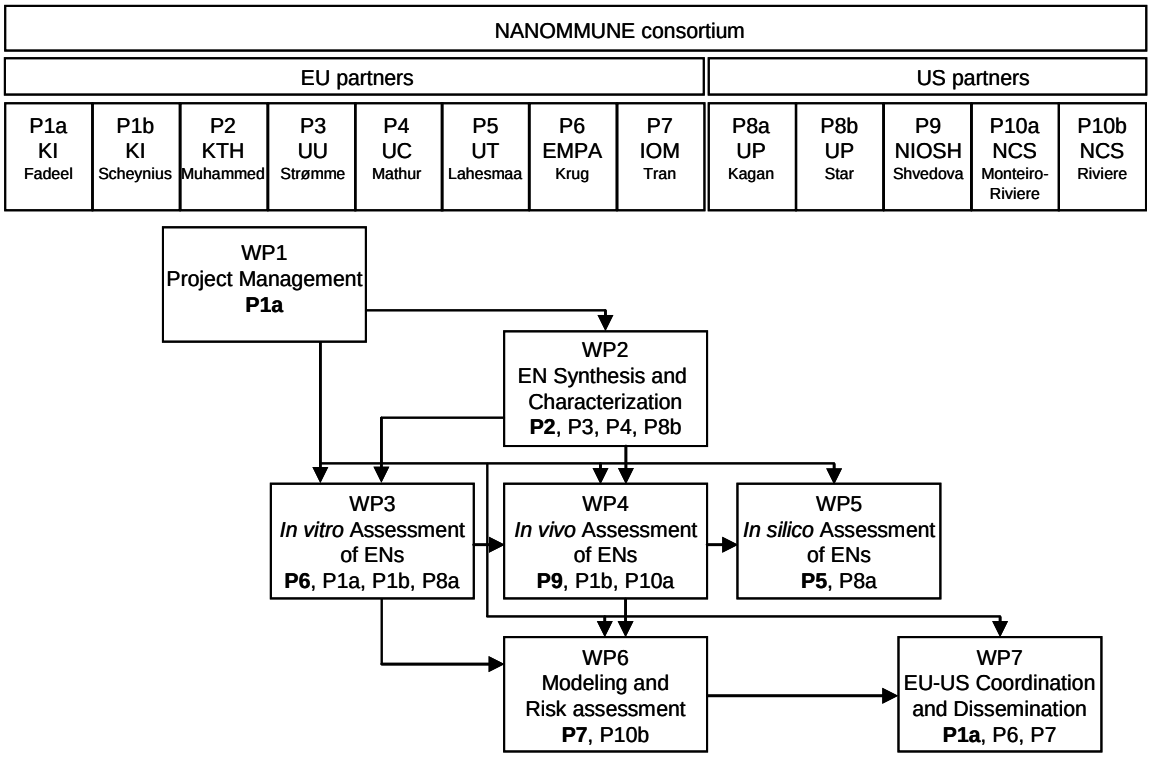

Figure 1. Schematic representation of the consortium and work packages.

1.3 *Technical approach, work description*

1.3.1 Synthesis and characterization of nanomaterials

Our studies are focused on three categories of nanomaterials of technological and/or biomedical importance: a) metallic nanoparticles, such as gold; b) oxide nanoparticles; iron, silica, titania, cerium, etc; 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. TiO_2 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.

1.3.2 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 (Dobrovolskaia 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 engineered nanomaterials 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.

1.3.3 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. Another important portal of entry for ENs is the skin, the primary physical barrier that protects our body against the outside world. Members of our consortium have established several important model systems for the biological assessment of skin effects of ENs, including *in vivo* assessment of ENs primarily in pig, because porcine skin is similar anatomically, physiologically, and biochemically to human skin. The skin is also an immune organ insofar as immune-competent cells, including Langerhans dendritic cells, are present in the skin and can process and present foreign antigen that enters through the skin barrier.

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 two major routes of exposure: inhalation/aspiration and topical/dermal. The project will also study the effects of ENs on host immune responses towards infectious agents, using real-time *in vivo* monitoring in live animals.

1.3.4 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.

1.3.5 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-Pharmaco-Kinetics (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. So far, no (Q)SAR model has been developed 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 has a strong international dimension as it is comprised of several leading European and US institutes.

1.4 *Achievements: a progress report*

At the time of writing, the NANOMMUNE project is in its third and final year. The project will conclude in 2011. In the following sections, some important research results generated by our consortium are highlighted (and see list below of selected publications from our consortium).

1.4.1 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 phagocytozing 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). Furthermore, aspiration of PS-coated SWCNT in mice stimulates their uptake by alveolar macrophages. These studies also demonstrated that PS-coated SWCNT triggered less pro-inflammatory cytokine secretion than non-coated nanotubes. Finally, PS-coated nanotubes enabled the targeted delivery of a pro-apoptotic factor (cytochrome c) into macrophages followed by activation of caspase-dependent apoptosis. Thus PS functionalization can be utilized for regulation of toxicity and targeted delivery of SWCNT with specified cargoes (regulators, inhibitors) into professional phagocytes.

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. These studies, co-funded by US sources (NIH and other funding agencies) and the EC, represent one of the most significant achievements to date of the NANOMMUNE consortium.

1.4.2 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 TiO_2 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.

1.5 Conclusion, continuation plans

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 will perform 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 project will conclude on August 31, 2011, at which time a summary report will be prepared. The consortium will also collate Standard Operating Procedures (SOPs) in a NANOMMUNE Quality Handbook which we plan to make freely available to all interested parties.

1.6 Dissemination of results

Results of the NANOMMUNE consortium are disseminated according to the following strategies:

- A public-access website has been developed as a principal portal of dissemination of our results (at the time of writing, 2745 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.
- 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); and 2nd Nobel Forum Mini-Symposium on Nanotoxicology, Stockholm, Sweden, 2010 (Dr. Bengt Fadeel). The latter event was an official part of the Karolinska Institutet Bicentennial celebration. We will organize a NANOMMUNE Closing Workshop in June 2011 in Stockholm to highlight key results of the consortium and to interact with other European researchers in the field of nano-immunosafety.
- 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.
- 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 are also considered, whenever appropriate. For instance, the project coordinator, Dr. Bengt Fadeel has presented the NANOMMUNE project to a broad assembly of journalists at press briefings hosted by the European Commission at the EURONANOFORUM in Prague, Czech Republic (2009); and at the 6th World Conference of Science Journalists, London, United Kingdom (2009).

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2 Directory

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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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6	DHI Water & Environnent	DHI	Denmark
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8	Lati Themoplastic Industries S.p.A.	LATI	Italy

Contents

Summary

NanoPolyTox main objective will consist 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 nanoproducts, 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 (one selected type), metal oxide nanoparticles (three types of nanoparticles) and nanoclays (two types). The selection has been 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 work package, 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).

One of the main issues in these studies is the control over the purity of the raw nanomaterials in order to obtain exploitable physical and chemical data upon the evolution of the nanomaterials along their life cycle. Moreover, the purity of the nanomaterial will ensure the reproducibility of their toxicological and ecotoxicological profile in all the steps of the study (from synthesis to recycling/disposal) leading to a coherent evolution of all the samples. This control over the nanomaterials is of high importance to determine the environmental impact of released nanomaterials, monitoring their properties (structure, chemical composition and toxicity) at the different stages of their life cycle.

The studies carried out in this work package will include the characterization full characterization of the nanomaterials synthesized. The physical characterization will consist of 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 includes 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. Physical and chemical data of the different nanomaterials will be described in 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 this work package is to generate polymeric nanocomposites including the nanomaterials obtained in WP1 using the typical industrial processes such as extrusion and injection techniques. The first step to achieve this goal will be the selection of the polymeric matrices. The selection will be based mainly on the polymer industrial uses and in their chemical nature, covering several fields of applications and presenting different chemical properties.

Then, the synthesized and fully characterized nanomaterials obtained in WP1 will be included in the selected polymeric matrices by double screws extrusion processes. 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 concentration of nanofillers after the injection processing will be measured in order to detect possible migration and release during the transformation processes. The distribution of the nanomaterials in the polymeric matrix will be determined by Microscopy analysis (SEM), quantification of the nanomaterials in the matrices will be verified by ICP-MS and UV-vis in the particular case of MWCNT. Furthermore, the physical properties of the nanocomposites will be evaluated and compared with the properties of the unmodified polymer. These findings will explain how the inclusion of nanomaterials into polymeric matrices influences their properties. Consequently, novel properties and new fields of application could arise for these materials.

2.3 *Weathering of polymer nanocomposites (WP3)*

In WP3 the main objective is to simulate the outdoor use of polymeric nanocomposites, which is one of the main uses of these materials. These studies will provide important information on the degradability of the polymers under external conditions (Sun-light and climate simulations). Consequently, the migration and/or release of nanomaterials from the host polymers can be determined. The nanocomposites will be weathered under normalized conditions (DIN 75220) leading to an accelerated aging of the materials under aggressive outdoor conditions. Nanomaterials and unmodified polymers will be aged together with the nanocomposites to obtain comparative data. All these materials will be submitted to different weathering cycles that could then be extrapolated to real aging time if the studies in real time conditions are done in parallel. The materials will be aged in climatic chambers specifically tailored for the collection of any releases during weathering. The materials will be submitted to the following weathering cycles: 12 weathering cycles, 60 weathering cycles, 120 weathering cycles.

After weathering of the materials, the nanocomposites and the unmodified polymers will be characterized to evaluate the degree of physical degradability and the preservation of their properties.

The physical characterization will be compare with the data obtained in WP2 for the un-weathered nanocomposites and polymers. The weathered nanomaterials will be characterized using the same techniques as in WP1 and then these data will be compared with that obtained in WP1. The degradation material collected during the aging process will be characterized by ICP-MS and in the particular case of MWCNT-containing products by UV-vis in order to quantify the release of nanomaterials.

2.4 *Development of non-destructive separation techniques: Proof of concept of NM recycling or immobilization techniques (WP4)*

The recycling of polymers is usually done by 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.

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.

The colloidal solution will be then filtered to separate the nanomaterials form the polymeric matrices. In a first approach, the nanomaterials will be separated from the polymeric matrices by optimized methods based on existing technologies, such as centrifugation and membrane nanofiltration. In a second approach, novel organic/inorganic filters based on nanofibers will be generated using electrospinning technology. The development of nanofiber-based filters will include the fabrication of tailored mineral nanofibers of different shapes and morphologies on metallic grid supports. Additionally, nanofiber surface functionalization will be investigated to modify surface properties improving nanomaterials recovery. The filtration properties of these novel filters will be compared with the conventional filters used for nanofiltration.

The efficiency of the separating processes (centrifugation, membrane nanofiltration or filtration with nanofiber-based filters) will be evaluated measuring the presence of nanomaterials in the filtered solution using classical analytical methods such as ICP-MS spectrometry and UV-vis spectroscopy in the case of MWCNT. The two techniques will be evaluated in terms of price and industrial viability.

The nanocomposites generated in WP2 (nanocomposites after processing), WP3 (nanocomposites after aging) and WP7 (nanocomposites after mechanical recycling) will be dissolved by non-destructive methods and then the nanomaterials will be separated from the polymeric matrix by the optimized method in this work package. 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 will not 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 evaluation of human health impact of nanomaterials will be first done by *in vitro* testing. The objective of the *in vitro* studies will be to evaluate the toxicological profile of nanomaterial under different cellular context and to identify underlying hazardous mechanism. A set of 15 cell lines was selected in order to cover all the putative portals of entry of the nanomaterial. The rational to select this set of cell lines 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.

Based on the data collected on previous *in vitro* assays, toxic nanomaterials will be selected to be analyzed by *in vivo* assays. The nanomaterials will be administered intravenously setting up at least three treatment groups and a control group. The body weight will be monitored along the assay to follow the evolution of the animals under the treatment with nanomaterials. Nanomaterials will be identified in the target organs using either ICP-MS analysis or TEM imaging of the target organs to locate and, if feasible, quantify the nanomaterials in the following organs: liver, spleen, kidney, GI tract, brain, lungs and heart. Additionally, a kinetic study will be performed collecting blood samples in several time points.

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 and the risk assessments 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. The analysis will consist of evaluating the influence of the different processes through which nanomaterials are passing at each stage of their life cycle as nanofillers in different polymeric hosts. The different stages of the life cycle under evaluation will be the processing, the aging, and the recycling processes. The influence of these processes in the physico-chemical properties of nanomaterials will be studied and correlated with the toxicological data obtained at each life cycle stage. 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 developed in accordance with the ISO standards, which establishes four interrelated basic stages for LCA studies: the goal and scope, the inventory analysis, the impact assessment, and the interpretation. A comprehensive framework describing the impact and risks 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 three strategies for the recycling and 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 in order assess the risk of migration during this process.
- 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.

  Migration of the nanomaterials in the glass composites will be characterized using weathering conditions under the appropriate normative. Potential applications of the new glass composites will be considered at the end of the project, for example in the construction sector.

2.8 *Dissemination and exploitation activities (WP8)*

Dissemination activities will be defined by the Management Committee and implemented at the Scientific Committee level. 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 member 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 9 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 analytical techniques described in WP1. The data collected have been 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. In some cases, functionalized MWCNT and metal oxide NP (SiO_2, TiO_2 and ZnO) were previously (before extrusion) tested at roller scale to confirm their compatibilization with the polymeric matrices. The compatibilization and dispersibility of the nanomaterials in the polymeric matrices were evaluated by microscopy analysis (SEM). Physical characterization of the polymeric nanocomposite demonstrators provides information on the added value conferred by the inclusion of nanomaterials and it will be reflected in the technical card generated in WP1.

3.3 Weathering of polymer nanocomposites

The conditioning of climatic chambers for the weathering tests that will be done within the Nanopolytox project is in progress. The demonstrators developed in WP2 and the raw nanomaterials, including unmodified polymers will be submitted to the selected aging conditions and the nanomaterials released from the polymeric matrices will be collected and quantified.

3.4 Toxicological and ecotoxicological evaluation of NM at different stages of their life cycle

The starting point of the toxicological studies is 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 H_2O. 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. Toxicological evaluation in vitro of the dispersed raw nanomaterials will be carried out in the coming months.

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

Contract Agreement: CP-FP 214478-2 Website: http://www.nanoretox.eu
Coordinator: Dr Eugenia Valsami-Jones, Natural History Museum, London, UK

No.	Beneficiary name	Short name	Country
1	Natural History Museum	NHM	United Kingdom
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
12	Intrinsiq Materials Ltd	IML	United Kingdom

Contents

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.nanoretox.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 cardiovasculature 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 foetus1. 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 TiO_2, ZnO, SiO_2, 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.

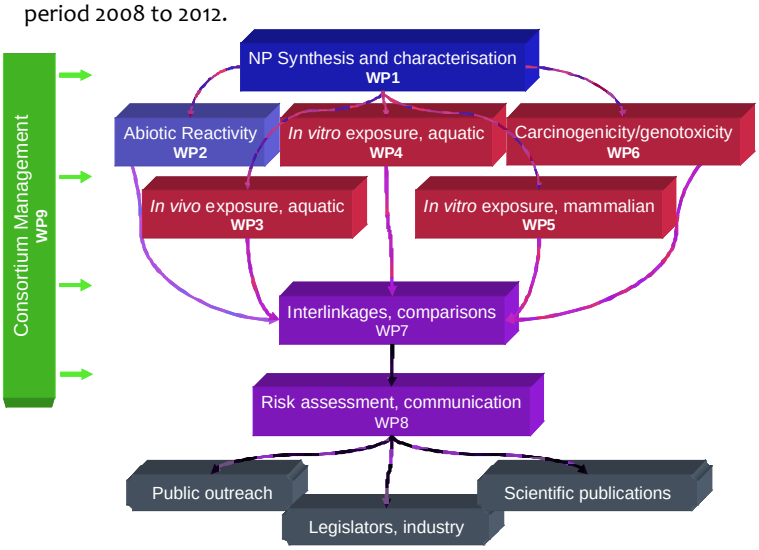
Finally, we will use the extensive experience within our team, and a specific work package devoted to risk assessment and risk communication to develop appropriate criteria, considering factors that have, historically, generated surprising risks in the past.

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 devided 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 and plasma 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: TiO_2, SiO_2, ZnO, CuO, CdS, Ag 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.

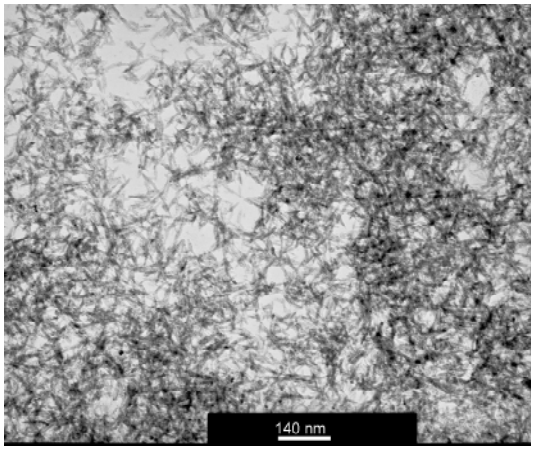


Figure 1. TEM image of in-house synthesised TiO_2 nanorods.

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.

Bioavailabilty 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.

Figure 2. Test organism: Platynereis dumerilii.

These experiments will accompany studies of adverse responses. Partners will experiment with different organisms 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, Corbula amruensis, Macoma balthica*). Freshwater and marine snails (*Lymnea stagnalis, Hydrobia ventrose*) that ingest plant material where nanoparticles might deposit will be compared to animals that ingest sediments (polychaetes *Nereis diversicolor* and *Playnereis dumerilii* (Figure 2) 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 assure internal uptake of nanomaterials. Oxidative stress, metallothionein induction, lysosomal membrane destabilization and histopathology 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 internal nanomaterials, evaluation of internal dissolution and manipulation of experimental design is used to determine if responses are due to internal dissolution of the metal oxide particle or due to disruption by the particle itself.

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, TiO_2, SiO_2, 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 sulphydryl 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. 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 arealso studied in mussels, where haemic or haemocytic neoplasia and gonadal neoplasia have been reported.

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 mis-communication 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 mangement 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 two years, NanoReTox has produced the following key results:

5.1 *Results from WP1 and WP2*

Well-characterised sets of nanoparticles have been developed, some provided by the industry partners and some tailored to show properties that vary systematically, so that robust links to toxicity can be made.

In order to transfer existing knowledge and skills from previous projects to this multi-disciplinary team, a large batch of industrially produced (plasma synthesis) CuO nanoparticles served as the method development standard. These CuO NPs were used to set-up toxicological and ecotoxicological protocols and compare and harmonise methodologies between groups. Following this initial step, a number of other "model" nanoparticles, were produced. Some particles were tailor-made for toxicity studies with well-controlled size amongst other properties. The achievements here were two fold: (1) particles were produced with properties showing regular variation (e.g. same composition and shape, but different sizes, same size, different surface functionalisation) and (2) particles were made available in a form suitable for (eco)toxicity experiments (e.g. excluding toxic surfactants). Along with these, industrially produced (plasma synthesis and milling) particles were also made available, as well as "standard" particles where appropriate and bulk analogues. All particles delivered to (eco)toxicology partners were thoroughly characterized.

5.2 *Results from WP3 and WP4*

The main objective of these WPs during the first part of the project were to test if engineered nanoparticles are available for uptake by whole organisms and if the nature of the particle and/or the biological trait of the organism affect that uptake. Also, the stress responses of selected aquatic species to nanoparticles accumulation were investigated.

A key achievement here was to set-up and harmonize protocols and to cross-reference the data generation for future comparison between different studies. A number of particles from a variety of sources have already been tested (CuO, Ag, Au, TiO_2) and produced the following findings: (1) a set of preliminary indications of ecotoxicity, as a function of nanoparticle properties, (2) demonstration of bioaccumulation by some organisms, even of aggregated NPs in seawater; (3) demonstration that metal containing NPs yield bioavailable metals, more so than bulk particles; (4) establishing of interspecies differences in bioaccumulation.

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.

As for previous WPs, the important challenge here was to set-up protocols to harmonize the data generation for future possible comparison between different studies. Also, the CuO that was made available early was used for preliminary experiments to cross-reference work and fine-tune methodologies. The greatest achievements here were: (1) demonstration of a size effect on toxicity of NPs, with nanosized particles being more toxic than bulk analogues; (2) demonstration of toxicity being NP specific, but also cell specific (e.g. in the case of Au NPs).

6 Directory

Table 1 Directory of people involved in this project.

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Nanosafe 2

Safe Production and use of nanomaterials

Integrated Project

Contract Agreement: NMP2-CT-2005-515843 Website: http://www.nanosafe.org
Coordinator: Frederic Schuster, Commissariat à l'Energie Atomique et aux énergies alternatives, France

No.	Beneficiary name	Short name	Country
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2	Procter & Gamble	P&G	Belgium
3	BASF	BASF	Germany
4	ARKEMA	ARKEMA	France
5	INTRINSIQ	INTRINSIQ	United Kingdom
6	NANOGATE	NANOGATE	Germany
7	OXONICA	OXONICA	United Kingdom
8	Katholic University of Leuven	KUL	Belgium
9	CSEM	CSEM	Switzerland
10	CAESAR	CAESAR	Germany
(11)	Oxford University	OXFORD	United Kingdom
12	University of Glasgow	GLASGOW	United Kingdom
13	Institut National de la Santé et de la Recherche Médicale	INSERM	France
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15	Helmoltz Zentrum München	HZM	Germany
16	Josef Stefan Institut	JSI	Slovenia
17	University College London	UCL	United Kingdom
18	Institut National des Risques Industriels	INERIS	France
19	Health and Safety Laboratory	HSE-HSL	United Kingdom
20	SWISSI	SWISSI	Switzerland
21	DGUV-BIA	DGUV-BIA	Germany
22	DEKATI	DEKATI	Finland
23	UMICORE	UMICORE	Belgium

Contents

1 Summary

The Nanosafe 2 integrated project is the follow up of a first study supported by the European Commission from 2003 to mid 2004: Nanosafe "Risk assessment in the production and use of nanoparticles with the development of preventive measures and practice codes". This study has assembled and reviewed available information on improvement but also possible hazards involving the production of industrial nanoparticles, to assess the risk to workers, consumers and the environment. It has concluded to a lack of data in three main areas: impact on health and environment, emission paths and volumes, measurement techniques. Nanosafe 2 has been built to address these issues.

2 Background

The ***NANOSAFE 2*** project does not claim to solve all the problems related to nanoparticle safety, but intends to treat thoroughly a limited number of reference nanoparticles and situations in order to bring about the first effective industrial solutions.

This project intends to develop innovative detection, traceability and characterization techniques for engineered nanoparticles. It will supply the "nanotoxicologists" with a methodology, based on an innovative generic approach for analyzing the toxicity of nanoparticles. ***NANOSAFE 2*** also develops safe and cost-effective measures to minimize the exposure of workers, consumers and the environment to manufactured nano-scale entities. It also supports a wide range of studies to evaluate current and future projected levels of exposure, the adequacy of current approaches to control exposure and propose measures and recommendations. ***NANOSAFE 2*** fits with the strategy of the action plan for nanosciences and nanotechnologies proposed by the European Commission. This strategy aims to responsibly integrate risk assessment related to human health, the environment, consumer and workers at all stages of the life cycle of nanotechnology, starting at the point of conception and including R&D, manufacturing, distribution, use and disposal or recycling.

NANOSAFE 2 helps to exploit the whole potential of nanomaterials to improve properties and revolutionary applications in the fields of energy, environment, medicine, pharmacy, agriculture, textile etc. in a sustainable and responsible manner.

3 What is Nanosafe 2

Sub-project 1: Detection, monitoring and characterization

In ***NANOSAFE 2***, SP1 was devoted to the detection and the characterization of engineered nanoparticles which represent the first step toward both the control of the potential risks and the associated social acceptance linked to the use of nanoparticles.

- First, the requirements and constraints of the "nano" industry in terms of nanoparticle measurements were identified on the basis of actual levels of nanoparticles measured at industrial sites and knowledge of the configurations of working places.
- Then, both on-line and off-line tools for particle detection were developed with different approaches: **optimization of the existing particle counting techniques and development of new methods** less mature but presenting much lower background noises e.g. detection using the specific physical properties of the nanoparticles and detection by tracing methods.
- New **traceability labels** specifically adapted to nanomaterials were setup using phosphor nanotags and oligonucleotides.
- Finally, methods and tools have been developed to measure the dense phase properties of nanoparticles for the **explosion risk**, the characterization of nanoparticles **in biological media**, etc.

↳ ***Industry requirements for nanoparticle monitoring***

First, the different nanoparticle measurement equipments available on the market such as Scanning Mobility Particle Sizers (SMPS), Condensation Particle Counters (CPC), Electrostatic Low Pressure Impactors (ELPI), etc. were tested and compared by the partners in terms of actual performances, accuracy and reliability. It was found that for equipments using different physical principles (sometimes even for 2 strictly identical equipments), results can differ in a quite large extend. This highlighted **the necessity to develop an easy-to-use and portable calibration tool in the project**.

Nanoparticle measurement tools in liquids where also tested and compared: Differential Centrifuge and Dynamic Light Scattering equipments.

Both long term exposure measurement campaigns and shorter campaigns were conducted at very different laboratory and industrial sites producing nanoparticles such as: a plasma production workshop, a wet processing workshop, a Chemical Vapor Deposition (CVD) production plant, clean rooms, grey corridors, etc.

Numerous information on the actual levels and behavior of the nanoparticles at working places were obtained: important variations of the background levels of nanoparticles from 0 to 100 000 /ml and temporal instability from minutes to minutes due to many parasitic sources of non engineered nanoparticles: window opening, vacuum cleaner, etc. Taking into account the reality of the background levels, a **total monitoring strategy** of exposure measurement was proposed including pre-helium tracing steps, parallel measurements, etc. These results also highlighted the necessity to develop new specific measurement tools less sensitive to background fluctuations.

↳ ***On line particle detection***

New on-line aerosol measurement equipments were developed in the frame of ***NANOSAFE 2*** project such as: a new CPC and 2 cheap equipments specifically designed for continuous monitoring of early events: an electrostatic device without clogging effect and a device based on a smoke detector.

A transportable equipment using **Light Induced Back Scattering (LIBS)** technique was also developed as a technique specific of the engineered nanoparticles. The response of conventional CPC and SMPS equipments to Carbon Nanotubes was determined as well and practical conversion tables were proposed.

On line detection techniques of nanoparticles in liquids were also investigated without success: one using PCS (Photon Correlation Spectroscopy) but rapidly saturated, the other one using peptides to recognize selectively the engineered nanocolloids and measurement of the mass shift by microbalance.

A **portable calibration tool** for nanoparticle measurement equipment using TiO_2 nanoaerosols has been set up, addressing both the particle size and the concentration calibrations.

A new approach using **tracers** for the selective detection of nanoparticles has been proposed and tested in industrial context. It consisted in adding a cheap tracer of fluorescein nanoparticules just after the production reactor. Another approach consisting in generating directly luminescent silicon quantum dots in a reactor has to be pursued.

When the nanoparticles need to be ultra pure for microelectronic applications for example, the new "post it" strategy has been successfully tested. It consists in using an organic tracer e.g. $C_8H_{13}Cl$ presenting enough affinity for the nanoparticles to be a permanent tracer but able to desorbs after a simple thermal treatment before processing.

- ***Off line particle detection***

For off line particle detection, different collection technologies for sampling were evaluated and qualified. Conventional impingers (bubling the aerosols in liquid solutions) were proved to be not efficient for nanoparticles. So, **2 new different liquid samplers were developed**. The first one uses the same principle as the CPC: growth of the nanoparticles in hot supersaturated vapor before impinger. The second grows the nanoparticles by condensation and accelerate the nanoparticles electrostatically toward a film of water enabling to concentrate the content of many m^3 of air into some ml of water only.

Collection of nanoparticles on **ultra pure filters** before detection by elemental analysis was investigated. In this case the collection efficiency is 100%. Work focused on how to purify ultimately the filters in terms of metallic contamination. **This technique was used to set up a first demonstrator of nanobadge**. In this case the filter is analyzed by Inductive Coupled Plasma Mass Spectrometer. It was shown that this selective analysis enable one to improve the low limit of detection obtain with particle counters (about 1E5 part/ml at 10 nm due to the background noise counts) to 1 or 3 orders of magnitude according to the elements for a 24 hours sampling e.g. TiO_2 nanoparticles detected by the presence of Ti. In order to simplify the fastidious step of chemical mineralization before ICPMS measurement, physical detection on ultra pure filters by LIBS, X-Ray Fluorescence (XRF) and Total reflection X-Ray Fluorescence (TXRF) were optimized. It was shown that XRF and LIBS are very convenient for that purpose and more sensitive than conventional counters (even for CNT detected by the presence of the residual catalyst), but are still a bit less sensitive that ICPMS.

A compact sampling device was designed **to collect nanoparticles on TEM grid** using anti thermophoresis and diffusion processes. For colloidal suspension, a new electrostatic collection process was set up using positively and negatively charged polymers layers in the "electrolyte filter".

- ***Tracing-ability of nanoparticles***

Two types of **nano labels** carrying information such as manufacturer name, batch production number, toxicity, physical properties, etc. were developed during the project. The first one uses different mineral or organic dyes embedded in SiO_2 in order to constitute an **optical bare code**. The demonstration of the robustness of the process was performed on an industrial batch of TiO_2 nanoparticles. The associated portable electronic of detection was set up as well.

The second developed approach used **small oligonucleotides** to carry information. A demonstration of the sticking and then readability was performed on a batch of industrial CNT. The associated PCR and lab on cheap devices were investigated as well.

- ***Nanoparticle characterization***

A large number of SEM, TEM characterizations of nanoaerosols of TiO_2, CNT, carbon black and aluminum nanoparticles were performed without the need of specific adaptation requested for nanoparticles. New methods for measuring the surface charges and the size of nanoparticles in biological media were set up. The wetting properties of nanoparticles were measured by micro calorimeter.

A very large work was performed in the frame of the **explosion behavior of nanopowders**. Thus, **new measuring equipments were designed** in order to improve the accuracy of the measurements performed at nanoscale powders and for very small available quantities as well as in order to improve the safety aspects during the measurements. Many interesting results where obtained on the thermal parameter of different powders such as Al and CNT: minimum ignition energy, explosion parameters, etc

Sub-project 2: Health and hazard assessment

In a first task of SP2, the literature concerning health and toxicity of nanomaterials was critically evaluated and discussed. Since the start of ***NANOSAFE* 2** project, the number of papers concerning nanotoxicology increased significantly every year to more than 800 publications in Medline Pubmed in 2008. Most important categories of materials examined are metals, metal oxides and carbon containing particles. In an attempt to increase the visibility (and the importance of the availability) of nanotoxicology we started in the second phase of the project to announce the papers of the month (POM). We have, every month, since May 2007 produced a short review (total of 23) of papers we found to be "outstanding" in one or another way (scientific, subject studied, technique used…).

Also a general overview of the most important data published during the run of the project was summarised and discussed in the final report. The focus was mainly on toxicological effects of the non-soluble nanoparticles, and on the assays used to screen the effects.

The second task of SP2 was concentrated on the **toxicity and the body distribution of nanomaterials** (more specific the reference materials). Firstly the **cytotoxicity** of the materials was assessed, using different existing systems. We showed that the cytotoxicity of nanomaterials was influenced by several confounding factors and therefore several controls have to be included to produce conclusive data. Also subtoxic conditions were evaluated in order

to study translocation in vitro and in vivo. In vitro, we showed that, at low concentrations, carbon nanoparticles or TiO_2 do not reach the central nervous system. In vivo we found, in rats, no translocations to the body after inhalation. (From these observational experiments we can not speculate on the interaction of the material on the barrier functions). These and other translocations studies were used to feed **the development of generic numerical physiological toxicokinetic / toxicodynamic (PBPK) model, predictive of nanoparticles internal exposure and potential health effects in humans**. Finally, the developed PBPK model was based on human exposed, via inhalations, to carbon nanoparticles.

The third task in SP2 was the **development of a system that will facilitate the screening of nanoparticles for toxic effects in humans**. Translocation of nanoparticles by the in vitro model implies an increased risk of toxicity because "internal" organs will also be exposed to nanomaterials. In particular, a system that detects whether nanoparticles are likely to cross the lung-blood barrier and enter the human body would be very usefull. The system developed here would, however, be a significant improvement on current test procedure as it would include miniaturisation to reduce material costs of the tests as well as automation to: reduce labour costs of the tests; improve test reproducibility and to increase the number of tests (construct a high throughput system). The concept was setup as a **microfabricated device ("chip")** together with a biological test system and associated instrumentation and consumables. The system consists of two major parts which can easily joint together: microfluidics and microfabricated culture plate. This allows a larger custom dependent flexibility. In the cell culture plate electrodes for electrical measurements of the resistance are integrated. The external instrumentation includes pumps for the microfluidics, a system to measure the electrical resistance of the cell layers and a detection of the nanoparticles that cross the cell membrane.

Sub-project 3: development of secure industrial production system and safe applications

SP3 was also a very successful project partly because the highlights are exceeding the lowlights by far. After some re-focussing in the middle of the project duration, evidence was produced in these aspects:

- There is a way to handle potential harmful nanoparticles in industrial practise, based on scientific results.
- It could be shown that filters, masks, gloves and other currently available personal protection means are very well suited for secure handling.
- Transportation of nanoparticles can be carried out in a safe way. Even for CNT UN container only need slight modification to serve as storage or transportation means.
- For explosive nanoparticles new evidences showed that formerly suspicious filling and re-filling procedures can securely been carried out if proper flow conditions are obeyed.
- Very remarkably is the fact that the usage of CPC, SMPS and LIBS measuring tools lead to testing and surveillance methods which proofed their ability to be used in industrial processes.
- Exhaust systems do not need to be highly sophisticated in order to safely carry away high nanoparticle concentrations, but have to be present wherever free following nanoparticles are emitted.
- In exhaust systems no significant accumulations of nanoparticles could be found.
- High nanoparticle concentrations can be found also in process which are widely used in coating industry (e.g. spray pyrolysis).
- Most important and in the centre of the focus are the strategies the nanoparticle producers established to handle nanoparticles in a secure way. Although not all industrial partners contributed to the same extend, it became visible what precautions can and have to be taken into consideration at least for laser-pyrolysis and plasma-pyrolysis technologies covering a big portion of currently used production process for nanoparticles.

On the other hand it has clearly to be stated that the external political discussion and the implying economical consequences significantly bothered several SP3 partners to contribute in a more open way. Some partners had to face severe problems in setting up the commercialisation of their products due to public pressure, often due to improper knowledge, what led to sluggish and only half-hearted project reporting, stop of cooperation or even economical disasters. Considering this situation it has to be critically considered, if the targets of this SP3 were not over-committed already at begin of the project, reflecting the choice of the consortium. In this context, it is even more remarkable that quite a transparent image with high value for nanoparticle producers and handlers could be derived. **It could clearly be shown by evidence that there are ways to produce and handle nanoparticles securely in an industrial environment using currently available tools and means**. However, it also became clear that there has to be a new way to classify harmful nanoparticles, reflecting their crystallography, habitus, size, chemical surface chemistry and physical properties. The formerly used mass concentration rules are absolutely inappropriate and will lead to incorrect results.

Sub-project 4: Environmental and societal aspects

SP4 consisted in four main workpackages, namely one for the legislation and standardisation, a second for the Risks assessment and Life Cycle Analysis, a third one for Training and Education, and the fourth one for Dissemination.

↳ ***Legislation and standardisation***

A thorough review of main EU legislation and standards was performed and led to a position paper presented at Nanosafe 2008 conference, and published in Journal of Physics Conference Series.

SP4 members, being participants in the development of new European standards, were instrumental to the specific development of the following ISO and CEN documents:

- Report ISO/TR 27628 "Workplace Atmospheres - Ultrafine, nanoparticle and nano-structured aerosols - Exposure characterization and assessment 'Workplace atmospheres – Characterization of ultrafine aerosols/nanoaerosols – Part 1: Determining the size distribution and number concentration using differential electrical mobility analyzing systems'.

- PG5/WG3 (Guidance on physico-chemical characterisation of engineered nanoscale materials for toxicological assessment) (ISO TC229).
- PG10/WG2 "General Framework for determining nanoparticle content in nanomaterials by generation of aerosols" (e.g. dustiness)) (ISO TC 229).
- TR ISO TR 27628 "Ultrafine, nanoparticle and nano-structured aerosols - Inhalation exposure characterization and assessment".
- "Guide of nanoparticle measurement methods and their limitations (CEN/TC352).

In addition, SP4 members served the role liaison facilitators between various EU standards committees.

➧ ***Risk analysis and Life Cycle Analysis***

In the Risk analysis and Life Cycle Analysis workpackage, **a risk analysis methodology** taking into account both the chronic and accidental risks, integrating scenarios of nanopowder explosion was developed. An illustration of the risk analysis methodology was given for the nanoparticle production line at Intrinsiq facility (UK).

A predictive **epidemiological study on the emission of nanoparticles from the use of tyres or laser printers**, using specific allometric scaling models, indicated that, depending on the metrics used (number of particle or mass of particles), a potential of up to 0.03 to 3 deaths per million inhabitants in Germany for the former and of 6-34 for the latter could be predicted. In this regard, a particular attention should be given to the proper ventilation of laser printer rooms. Together with this study, a life cycle analysis on tyre filled with nanomaterials (carbon blacks) showed that the emission impact due to tyre use is relatively low. However, should this filler be Carbon nanotubes, this impact could be much greater. It is noted that a complete life cycle analysis is difficult to perform because of lack of either emission data or process data or disposal data.

➧ ***Training***

Nanosafety courses targeting engineer and technician audiences have been dispensed in France, in the UK and in Germany. A portail NANOSMILE has been constructed to help bridge the societal gap between research & industry and the public. This portail, originally written in French, has been translated into English. This portal, free of charge, provide about 8 hours of material consultation. This portail received about more 16000 visits, while the private commercial use of a similar portail created at Oxford University did not receive as many.

5 Nanosafe 2 Events and Reports

To provide visibility of the project, a web portail nanosafe.org was created. During the project, a project description leaflet, three newsletters, and seven dissemination reports were provided to a targeted audience identified by VDI. A first international

4 Organisation of Nanosafe 2

The overall aim of NANOSAFE2 is to develop risk assessment and management for secure industrial production of nanoparticles. As the world of nanoparticles is already very wide today, only a finite number of reference cases of nanoparticles will be treated. These cases will be representative of main particle characteristics, main production processes and related risks.

NANOSAFE2 starts from the paradigm of risk assessment and risk management, which is used in risk analysis worldwide. In NANOSAFE2 the two different types of risks will be accessed: explosion during manufacturing processes and human health due to nanomaterial exposure. The risk assessments in the project will estimate whether and/or how much damage or injury can be expected from exposures to a given risk agent, and to assist in determining whether these effects are significant enough to require action (modification of production systems, regulations, etc.).

NANOSAFE2 goes beyond classic risk assessment by having a pro-active risk management strategy: choices to be made in order to solve the most vexing problems will be formulated and decision makers, safety regulators and other stakeholders will be involved to formulate preventive measures and guidelines for production processes and safe use.

The different technical subprojects of NANOSAFE2 address the whole items of both risk assessment and risk management.

- SP1 mainly dedicated to nanoparticle detection addresses exposure assessment and control.
- SP2 devoted to toxicology allows determining dose-response criteria and identifying hazard.
- SP3 dealing with safe production technologies gives input to economical factors.
- SP4 dedicated to social aspects and regulation brings data to legal considerations and societal factors.

Conference on Nanosafety was programmed in Grenoble (2008-France) with more than 73 oral presentations, 39 posters and 10 industrial products exhibits. Papers from this conference were published in Journal of Nanoparticle Research, and Journal of Physics. A new Nanosafety Conference has been organized in 2010 in Grenoble.

6 Directory

Directory of people involved in this project.

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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

Content

1 Summary

Objective of the NanoSustain project is to develop innovative solutions for the sustainable use, recycling and final treatment of nanotechnology-based products. This will be achieved by performing a comprehensive data collection and generation of relevant missing data as well as their evaluation and validation for specific Engineered Nanomaterials (ENMs), products or product groups in relation to human health and environmental hazards and possible impacts that may occur in particular during after-production stages. Although production of nanomaterials is rapidly increasing, our knowledge about possible health and environmental effects associated with these materials is still rather poor. This lack of knowledge calls for more research. Due to their small size, nanoparticles behave different than the same bulk chemicals. They can be taken up more easily and in a unique way

with possible adverse effects in man and organisms. Assessing their hazard is complex and needs new approaches and a close international cooperation. NanoSustain will address the questions, (1) how and to what degree society and the environment will be exposed to nanomaterials and associated products in particular during after production stages, and (2) where do these particles finally end up? Expected results will improve our present knowledge on the impact and fate of these particles after entering economic and natural elemental cycles. NanoSustain has mobilized the critical mass of expertise, skills and resources to tackle this complex issue. Based on results from hazard characterization, impact assessment and LCA, lab-scale experiments will explore new technical solutions for the innovative design of nanomaterials and associated products and their sustainable use, re-use, recycling, final treatment and/or disposal. As the concerned industry is actively participating in the planned project, NanoSustain will set the base for the development of new sustainable nanoproducts and industrial applications, which in turn will help to strengthen competitiveness of the European nanotechnology industry.

2 Scientific / industry needs and problems addressed

The behavior and properties of nanomaterials can be quite different to macro-materials, a fact that drives considerable international research and development activities towards exploitation 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 particles and associated materials and products might also have negative impacts on human and environmental health. However, we still do not know how exactly different nanoparticles (inter)act in the human body or in the environment, to what extent these materials may be released or leach from products, or how they are transported, transformed, emitted and accumulate in living organisms or environmental systems, like soils or waters, e.g. when used directly, or 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, data on the (eco)toxicity of nanomaterials is still limited, although first studies prove that there are toxic effects on wildlife and a potential for bioaccumulation in various organisms.

The increasing amount of nanomaterials produced world-wide 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, why they may accumulate in waters or soils, may aggregate or disperse, which in turn will change their properties compared to single nanoparticles to an extend we still do not know. Also for this reason, existing regulations based on concentration data alone may not be appropriate to quantify the true exposure to nanoparticles in solution or suspension, but need more accurate measurement of nano-specific parameters, like surface area, degree of dispersion or aggregation.

More reliable scientific data is needed on properties, 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 in the environment, to develop more accurate impact, exposure and risk assessment models, and to find efficient solutions for product design that in turn may favour 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 characterize and assess their risk and hazard. As a result, there is an urgent need for preliminary assessments at an early stage of product innovation and to validate and further develop current methods to accurately detect, characterize and measure these new materials in various matrices and compartments, like air, water, soil, and products, the media in which men and ecosystems are exposed to, as well as in cells, body fluids or tissues.

Although existing regulatory frameworks (like REACH) that are triggered by mass (in tons) may be adequate for areas, where only small amounts of nanomaterials are used (like in research laboratories), they may not be applicable for the industrial mass production of nanomaterials, where the number and/or shape of these materials could be of higher relevance than for the same bulk chemicals. We need to test and if necessary adapt or modify and validate the applicability of current standardized methods, like those given in the OECD-Guidelines for measuring and testing of hazardous substances. For this reason, NanoSustain will evaluate the extent to which existing regulatory and associated risk assessment frameworks (strategies, methodologies and tools) can be applied to after-production stages of selected nanomaterials, in particular to their recycling, final treatment and disposal.

Looking beyond the potential technical risks associated with nanomaterials, there is a particular need to address potential impacts along the whole lifecycle of a product, from manufacture through disposal ("cradle to grave"), and to consider this 'life cycle view' not only when assessing possible toxicological effects during different stages of a material/product life cycle, but also regarding the use or consumption of required energy and materials.

3 Scope and objectives

NanoSustain is based on the concept of "sustainability" and "scarce resources", which means that the use of new 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 has to 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.

Distinct processes for a 'preliminary assessment' are elaborated that can deliver reliable 'preliminary information' as the base of precautionary measures that may build upon information about chemical and/or physical properties, on quantitative structure activity relations (QSAR), or on the probability of exposure (e.g. because of extreme mobility and/or persistence) to finally avoid or reduce exposure or establish principles for a safe and sustainable design of nanomaterials and products.

NanoSustain considers four main aspects of the life-cycle of specific nanomaterials, i. e. their selection and design, manufacture, application and disposal/recycling. Although most work still focuses 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 with the implementation of a nanotechnology. For this reason, the ultimate project goal is to develop new technical solutions for the sustainable design, use, recycling and final treatment or disposal of nanotechnology-based products.

Specific objectives are:

- to assess the hazard of representative groups of 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;
- to assess the impact of selected products by LCA (in relation to material and energy flows);
- to assess the impact of these materials in relation to toxicology, eco-toxicology, exposure, environmental and biological fate, transport, transformation, and destiny; and
- to explore the feasibility and sustainability of new technical solutions for end-of-life processes, such as reuse/recycling, final treatment and/or disposal.

The following specific organic and inorganic nanomaterials have been selected and are investigated in detail within the project:

- nanocellulose based materials and products;
- CNT and associated products;
- nano ZnO based composites, and
- nano TiO_2 and associated products

4 Technical approach and work description

A variety of available advanced analytical, characterization and biological test methods are integrated, tested, validated and applied, including spectrometric and image giving analysis, molecular biology and biochemistry, to deepen our present understanding of the impact that follows after possible release and intake of nanoparticles, and to identify possible health risks. Various laboratory and modelling approaches are used to assess hazardous properties of the selected materials and products, to improve existing monitoring systems to control distribution, transport and final destiny of nanomaterials, and to explore appropriate technical solutions for safe handling, use, recycling and final treatment.

Different test strategies and in vitro tests are examined, to assess possible effects under real conditions, but also to reduce the amount of animal testing. Results will be used to build up a project-specific nanomaterial database, to further develop and validate preliminary and established risk assessment methods, and to allow for a more careful design and use of products, and of sustainable solutions for recycling and final treatment.

To identify potential impacts arising from production, application/use up to final recycling, treatment and disposal, existing LCA-methods and exposure models for LCIA are tested and used, and if needed adapted/modified and further developed, to generate data on prospective environmental concentrations and to define criteria and guidelines for a more 'precautionary design' that may improve recyclability of selected nanomaterials.

NanoSustain is structured and organized around 4 technical Work Packages (WP2-5), beside project management and dissemination/exploitation of results (WP1 and WP6):

WP1: Project management and scientific-technical coordination

WP2: Data gathering, generation, evaluation and validation

WP3: Hazard characterization and impact assessment

WP4: Life Cycle Assessment (LCA) and Prospective Technology Assessment

WP5: Exploration of new technical solutions for reuse/recycling, final treatment and disposal

WP6: Dissemination and exploitation of results

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 Workpackages (WP) of NanoSustain

WP	Title	Topic
1	Project management and scientific coordination	Work package 1 organizes and coordinates 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 is 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	Data gathering, generation, evaluation and validation	WP2 continuously collects and evaluates all available and relevant data for the selected types and groups of nanomaterials and products and provides the processed data to all other WPs in an appropriate form. A materials and literature database is built up to help to identify the real scope of the work and of additional work needed to reach objectives, deliverables and milestones in particular with regard to hazard characterization and impact assessment (WP3), LCA performance (WP4), and the exploration and development of new technical solutions for the sustainable use, recycling and safe disposal of selected nanoproducts (WP5).
3	Hazard characterization and impact assessment	WP3 generates representative test samples for consequent measuring and testing the source strengths from handling and reworking dust emissions, the toxicological and eco-toxicological relevant physicochemical properties and concentrations of nanoparticles from pristine material, in after-production materials and in the environment. Standardized protocols for nanoparticle characterization will be developed to (1) assure validity and consistency across participating laboratories, (2) produce new reliable data on human exposure to nanoparticles during handling of after-production materials, (3) establish dose-response relationships of critical end-points for health effects for nanoparticle containing after-production dusts in mice, and comparison with pristine materials, (4) evaluate suitability of current eco-toxicology test methods for environmental risk assessment of selected nanomaterials, (5) identify gaps in the manufactured nanomaterials lifecycle where adequate risk assessment methods are not available, (6) develop techniques to fill these gaps, (7) develop and test accurate and repeatable analytical methods to detect, characterize and quantify nanoparticles in environmental matrices, and (8) apply these methods to real samples.
4	Life-Cycle Assessment (LCA) and Prospective Technology Assessment	Based on results from WP2 and WP3, WP4 develops (1) a specific process model for the application end use phase, including all relevant material flows of selected nanoproducts and associated materials, (2) a specific model for the end-of-life and recycling phases (re-use, recycling and/or final treatment and disposal) of nanoproducts, including a substance flow model for possible impurities and tramp elements in recyclates, (3) a specific exposure model of engineered nanoparticles, and (4) implements a Life Cycle Assessment of the selected nanomaterials, including associated products for life cycle stages, with special focus on materials use, re-use, recycling and/or final treatment and disposal, including all relevant environmental impacts. Based on steps (1) - (4), criteria and guiding principles for the precautionary design of engineered nanomaterials are developed, tested and improved as well as design guidelines for improved recyclability and precautionary design applied to the newly developed technical solutions in WP5.
5	Exploration of new technical solutions for reuse/recycling, final treatment and disposal	Specific nanocellulose based materials and associated end-products are produced for various recycling experiments, supplied in different qualities and optimized during the course of the project to meet requirements of end-products with regard to their sustainable recycling. Laboratory studies explore the suitability of composting of these organic nanomaterials (organic recycling) by standardized composting tests and evaluate the influence of these materials and products on the final quality and safety of compost end-products. Also new solutions for final treatment of various MWCNT composite materials are tested by waste incineration and characterization by using various detection and characterization techniques, including also gaseous and particulate emissions from incineration of selected products by standard techniques such as CPC, SMPS, ELPI, BLPI and FTIR. Established glass recycling processes (e. g. melting) are investigated for recycling of nano-ZnO in glass by measuring the nanoparticle emissions to air during the melting process and by elemental analysis of emitted particles. Also final land-filling of selected nanomaterials is tested under conditions mimicking landfill leachates and appropriate test methods and measures developed to estimate, model and minimise transport and release of nanoparticles from landfills and discharges to the environment or leachate treatment plants.
6	Dissemination and exploitation of results	WP 6 provides the project results in a suitable format for the wider community to access and use; through regular news and information streams, reports, 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.

5 Expected Impact

NanoSustain helps to improve our current insufficient knowledge on the hazard, impact and sustainability of nanomaterials and products, in particular in relation to end-of-life stages, like reuse/recycling, final treatment and disposal. It substantially contributes to update and validate existing material databases and methodologies required to make current LCA and risk assessment more accurate and reliable. As almost nothing is known about the release, fate and impact of used nanomaterials during end-of-life processes, the project generates solid scientific data on potential risks and their probability of occurring during reuse/recycling and final treatment or disposal. For the first time new and innovative technical solutions are explored and developed on a lab-scale for (1) recycling of nanomaterials from waste products, 2) for incineration of nanowaste as safe final treatment, and for 3) land-filling of nanoparticle containing products. It is expected that this will bring about a step change in the control of these still unresolved major barriers towards a sustainable development of nanomaterials and associated applications, which in turn will improve their environmental performance.

6 Exploitation and dissemination strategy

NanoSustain will provide the wider community with access to project results and events to inform and support the uptake of developed knowledge and methodology. Information on new project developments in LCA, hazard and risk assessment for the selected nanomaterials that are planned or in production will be disseminated globally by means of Cordis, Alpha Galileo, Nanoforum and the participating Institute of Nanotechnology, to reach most experts in nanotechnology, nanotoxicology and related fields. A dedicated project website (www.nanosustain.eu) enables users to download project results and to access discussion fora allowing users to contribute and widen our understanding of approaches in LCA, risk and hazard analysis and new data being produced. This website is linked with other information sources such as NanoArchive (www.nanoarchive.org), NanoImpactNet (www.nanoimpactnet.eu), observatoryNANO (www.observatory-nano.eu) and the Nanosafety cluster (www.nanosafetycluster.eu) and will provide presentations and proceedings from events and meetings planned during the project. Users are required to register online, providing basic information on their organization and activities within the area of nanomaterials production, use and/or analysis, which in turn will provide the consortium with links to the wider community and ensure disseminating project findings effectively and promoting other activities such as workshops.

Also a quarterly newsletter is produced and sent to all registered users to inform the community about project developments and also be used to promote other relevant activities, such as events.

A major dissemination event will be organized at the end of the first year to present the initial findings from WP2 (analysis of published literature), and successively three workshops to present the outcomes from WP3, WP4 and WP5, and the presentation material will be made available online as a training resource. To ensure maximum exposure and uptake by industry, all events will be held with industrial organizations and associations demonstrating the functionality and applicability of the knowledge generated within the project to the processes employed within these organizations.

7 Status of the project

7.1 *Project management (WP1)*

NanoSustain was launched by a kick-off meeting held 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.

A second project meeting took place on 16-18 November 2010 in Ispra, Italy, where partners met to discuss the work and results obtained during the first 6 months, and to prepare the next steps according to the work plan.

First technical results achieved during the first 6 months are summarized in the following:

7.2 *Build up of a technical and literature database (WP2)*

With the help of the industrial partners involved in the project, a preliminary product database has been designed and established for all 4 selected nanomaterials, and a 1st version of the database uploaded on the intranet of the project website. New data generated during the project will be continuously fed into this technical database, after careful validation and quality check.

In addition, a scientific literature database with an advanced search tool has been drafted and a 1st version also uploaded on the project intranet. Only papers with a documented pc characterization of nanoparticles before further testing have been considered and included into the database. Newly published relevant papers are continuously added, to keep project partners up-to-date concerning latest advancements on EHS aspects of nanomaterials.

NanoSustain has also constituted an Internal Project Committee to ensure an ongoing scientific evaluation and successive validation of project results. This group of internal experts will also decide on necessary validation measures, including inter-laboratory cross checking of the results, where appropriate.

In close cooperation with other EU FP7 projects, NanoSustain will undertake all efforts to contribute to the harmonization of material and literature databases created by individual projects for various purposes, and their integration within the Nanosafety cluster, towards the creation of a common EU database. For this reason, the project is strongly engaged in Working Group 4 which focuses on the development of a central research database within the Nanosafety cluster.

7.3 *Hazard characterization and human health & environmental impact assessment (WP3)*

Most of the work done in WP3 during the first 6 months has been to 1) decide on the type of nanomaterials and life-cycle-scenarios

to be tested and studied, 2) order, prepare and distribute samples to be tested by partners involved in physicochemical characterization, 3) obtain appropriate life-cycle products, 4) plan the experiments to be done in WP3 (and WP5), and to 5) review the relevant literature and design the toxicological and eco-toxicological studies and exposure assessment. First results on the characterization of selected test materials have been obtained. In this context, mutual cooperation agreements have been concluded with the EU FP7 NanoHouse project on weathering testing, and with the Danish Paint and Lacquer industry and the "Teknologisk Institut", Denmark, on the production of painted boards and CNT-containing epoxy plates. Appropriate information, such as available material data sheets and other relevant information, has been uploaded on the Project intranet.

7.4 *Life cycle assessment (WP4)*

During the first half year, work in WP4 was focused on a critical evaluation of the available published literature and data on life cycle analysis (LCA) of nanomaterials and on nanotechnology based applications. More than 35 publications have been carefully analyzed and evaluated so far focusing on CNT, metallic oxides etc. However, no suitable publications on LCA aspects of nanomaterials, and on nanocellulose, have been found. Also a questionnaire on manufacturing processes of nanomaterials has been prepared, sent to participating manufacturers and partly analyzed. Current work in WP4 is now addressing the:

• development of specific process models for the application and use phase, including all relevant material flows of selected nanoproducts, and

• development of specific models for the end-of-life and recycling phases (reuse, recycling and/or final treatment and disposal) of nanoproducts.

A mutual cooperation agreement has been concluded between NanoSustain and the EU FP7 NEPHH project concerning relevant LC issues.

7.5 *Development of technical solutions for use, recycling & final treatment (WP5)*

During the first 6 months, WP5 has realized the production and characterization of nanocellulose. Also planning of the experiments and construction of the facilities for the experiments has been completed. The necessary consideration and discussion of safety issues associated with the performance of the planned CNT experiments (incineration) have delayed the qualification work required to set up the aerosol measurement instrumentation to characterize CNT in the gas phase. The laboratory set up of modifications needed for the planned glass melting experiments and demonstration has been performed.

7.6 *Dissemination and exploitation of results (WP6)*

During the first six months of the project one major achievement of WP6 was the creation of a user-friendly and attractive project website incorporating a partner intranet and database. In addition a promotional project flyer and the 1st quarterly project Newsletter have been designed for dissemination by all project partners.

7.7 *Cooperation*

Since project start, NanoSustain is actively participating in the rapidly growing European Nanosafety cluster and is represented in most Working Groups (WG).

In addition, cooperation agreements have been prepared during the last months with the EU FP7 NanoHouse project on the performance of weathering tests of NanoSustain samples, and with the EU FP7 NEPHH project (Nanomaterials related Environmental Pollution and Health Hazards Throughout their Life Cycle) on LCA-methodology and on the collection of relevant LCA data.

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.

8 Directory

Table 2 Directory of people involved in this project.

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9 Copyright

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
1	National University of Ireland, Dublin / University College Dublin	NUID UCD	Ireland
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 will also cooperate with ongoing projects in Duke University and University of North Carolina, as well as with the EU modelling cluster (initially ModNanotox coordinated by Natural History Museum).

Contents

1 Summary

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 currently 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.

2 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 physiochemical

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]

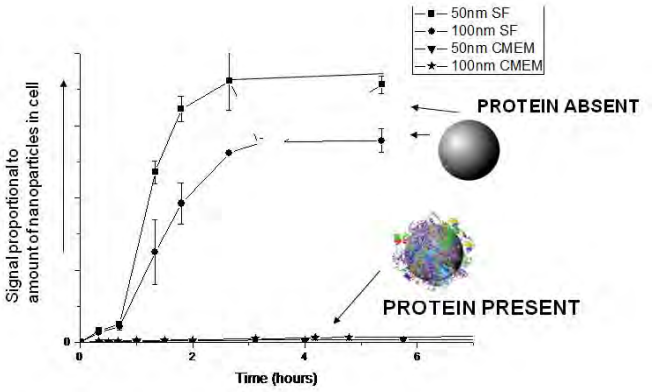

Figure 1. *Comparison of endocytosis of 50nm and 100nm Sio_2 nanoparticles at 100ug/ml in the presence (complete MEM) and absence (Serum free Media) of serum proteins. Note the very significant particle uptake in the absence of serum, compared to the much lower uptake in the presence of serum. It has 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.

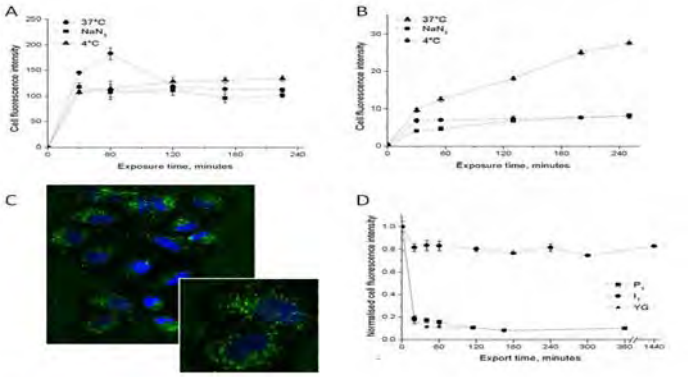

Figure 2. A. Uptake of green fluorescent dye (molecular) by A549 cells – no effect of energy depletion. B. Effect of cellular energy depletion on uptake of 50nm nanoparticles of similar composition to the dye. C. Confocal image showing the localisation of those 50nm nanoparticles in the lysosomes of A549 cells. D. Lack of export of those 50nm nanoparticles from A549 following removal of the particle source (I1), compared to rapid release of molecular dye (YG). All data from Partner 1.5

3 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.

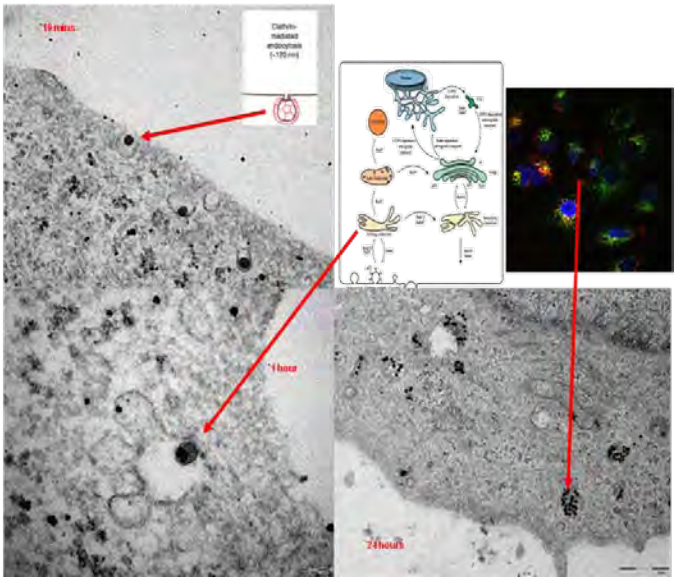

Figure 3. *Electron Microscopy images after 10 minutes, 1 hour and 24 hours of exposure to 25 µg/mL 50nm red-fluorescently labelled SiO_2 nanoparticles. From these images the localisation of the nanoparticles on the surface (some in clathrin and caveolin receptors), early endosomes and finally lysosomes 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 (discussed in the proposal) 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 will be 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 gives us much assurance. However, the design and modularity of the project, with the capacity of exposure to the critical evaluation of experimentalists ***at 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. In addition, the EU's Nanosafety Cluster allows for communication between all existing FP6 and FP7 research teams that have been funded by the EU. Within that we believe that a realistic approach for various programs will be successful from the current call, and other ongoing activities nationally funded will also be of use. Key collaborations already exist between the program and US partners. However, key collaborations also exist with other major US and Japanese centres, and these are being 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.

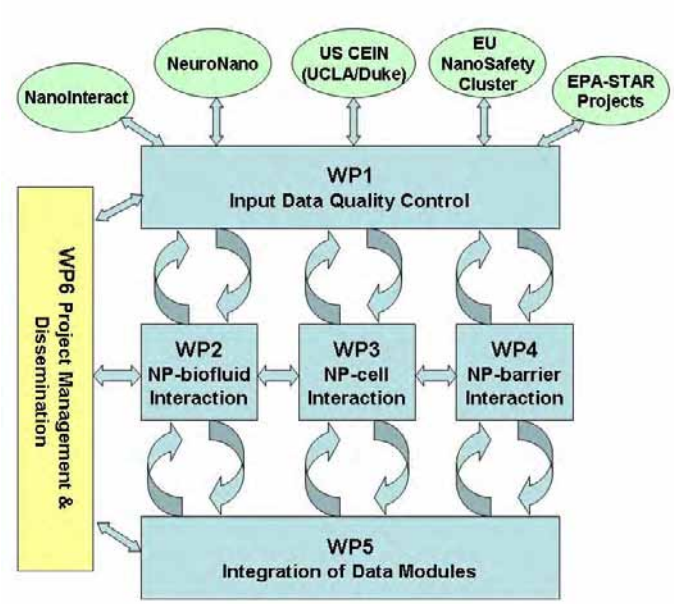

Figure 4. *NanoTransKinetics Pert chart.*

4 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 will be 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 to be 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 will be 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, will enable 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 will allow 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:

Data from several successful EU FP6 and FP7 projects will be 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 are 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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6 Directory

Table 1 Directory of people involved in the QNano project as beneficiaries.*

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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
1	NordMiljö AB [Environmental Assessments]	NOMI	Sweden
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 Tampare	UTA	Finland
5	University of Salzburg	PLUS	Austria
6	University of Zaragoza	UNIZAR	Spain
7	University of Namur	FUNDP	Belgium
8	University of Ljubliana	UNILJ	Slovenia
9	Natural History Museum	NHM	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	Alberta University	AU	Canada
24	Veneto Nanotech	VN	Italy
25	Nanologica AB	NLAB	Sweden
26	StratiCell	STC	Belgium
27	Grimm Aerosol Technologies	GAT	Germany
28	QUANTIS	QUANTIS	Switzerland
29	Centro Ricerche FIAT	CRF	Italy
30	Philips Research Europe	PEN	The Netherlands
31	ARKEMA	ARKEMA	France
32	University of Massachusetts Lowell	UMASS	Unites States
33	Northeastern University	NEU	United States
	USEPA represented by Maureen Gween: no full member of the NanoValid consortium but associated by a LOI to the project	USEPA	United States

Contents

1 Summary

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 will be 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) the 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 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 eco-toxicology.

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:

Priority 1 test materials: metal oxides (SiO_2, TiO_2, ZnO, CuO), metals (Ag, Au and Pd), CNTs (SWCNTs and MWCNTs) and fullerenes.

Priority 2 test materials: quantum dots (CdSe, CdS, CeO_2), 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:

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.

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.

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 TiO_2 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 currently 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) labelling 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 will be selected and tested, and new nanomaterials 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 (p c) 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 p c methods derived from the extensive intercalibration and intercomparison 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 p c 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 up a comprehensive knowledge 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 (DIN) and international (OECD) 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 will be organized in five technical Work Packages (WP), and two non-technical (management and dissemination) WPs, as follows:

WP1 Project management and scientific coordination

WP2 Fabrication of test materials and selection of test methods

WP3 Validation of p c methods, *in vitro*, *in vivo* and computational methods (*in silico*)

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

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 address the complexity and multidisciplinarity 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 Table1 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 Workpackages (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 nanomaterial 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 p c methods, *in vitro, in vivo* and computational methods (*in silico*)	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	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

	to risk (RA) and life cycle assessment (LCA)	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.

5 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 eco-toxicology 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.

6 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:

- ✓ standardized test materials that can be used by different industries to validate the physicochemical properties of ENMs they manufacture or purchase;
- ✓ novel ENP samplers, e.g. for hot gases;
- ✓ 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;
- ✓ a multi-compartment cell barrier model to mimic physiological systems and provide a more realistic evaluation of ENP fate and effect in living organisms;
- ✓ 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 will provide a dedicated project website 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 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.

7 Directory

Table 2 Directory of people involved in this project.

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8 Copyright

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.

No.	Beneficiary name	Short name	Country
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3	Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine	IBC	UA
4	Cracow University of Technology	CUT	PL
5	Tomsk Polytechnic University	TPU	RU
6	Fundación Tecnalia Research and Innovation	TECNALIA	ES
7	Centre national de la recherche Scientifique CNRS – ECCOREV – CEREGE - LEMIRE	CNRS	FR
8	Grado Zero Espace Srl	GZE	IT
9	Asociación para la Prevención de Accidentes	APA	ES
10	Laviosa Chimica Mineraria	LAVIOSA	IT

Content

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.**

1.1. Introduction to NEPHH

While nanosciences and nanotechnologies (N&N) offer a number of beneficial applications, the potential impact on the 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.

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.

1.2. 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 nano-structures 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 (SiO_2), 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 nanoproducts 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.

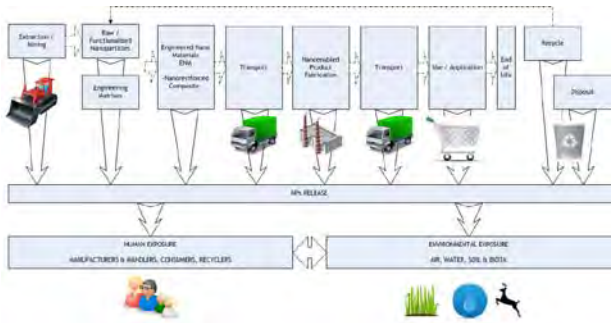

Fig 1: Diagram representing different stages of Nanotechnology based Applications / Products' Life Cycle.

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).

1.3. 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 (SiO_2), 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.

Afterwards, developed polymer nanocomposites have been used to fabricate macro-scale structural specimens to be physically processed (WP3). Compression moulding technique has been used in the case of Polyamides and Polypropylenes whereas chemical synthesis was used in the case of Polyurethane.

Proper characterisation of the nanostructured materials by microscopic, X-ray and spectroscopic methods has been performed in order to ascertain 'structure-property' relationships and assess the influence of nanoparticles on the selected properties of the laboratory and industrial nanomaterials.

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.

During the first year of NEPHH nanoparticles have been generated by impacting polyurethane nanocomposites with different nanofillers (Montmorillonite, Nanosilica, Glass fibres, Foam Glass Crystal) via low velocity impact testing. The 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.

Results on nanoparticles generated by impacting polyurethane/montmorillonite nanocomposites –PU/MMT- via low velocity impact testing shed new light on fundamental understanding on nanoreinforced products. The work done to date clearly showed that the nanoparticle which was integrated in the polymeric matrix could be re-found in the fracture of the nanofoam, but furthermore a hybrid particle of PU/MMT could be detected. These results illustrate new insight into nanoparticle behaviour and advice on a new dimension for nanomaterial risk assessment.

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 nanotechnologycal products.

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.

In present period raw 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.

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.

In present period 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 human health impacts in samples originated in WP3 has been reached, with the main target of all project partners evaluating the same (nano)objects.

WP6, to be accomplished on latter stages of NEPHH, 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 intended.

1.4. NEPHH Project Dissemination

The list of articles released within the first 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, Accepted, 2010.
- Adeel Irfan, Sophia Sachse, James Njuguna, Huijun Zhu*, Ainhoa Egizabal, Krzysztof Pielichowski, María Blázquez. *Are engineered nanomaterials safe? Focusing on polymer-nanosilica composites throughout their life cycle*. Accepted, 2010.

Other Dissemination Materials for NEPHH Include:

- Six Montlhy Newsletter.
- Six Montlhy 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).

1.5. 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. Validated testing strategies for novel materials are envisaged.

The project contributes to the objectives of the Work programme. This contribution is in line with the research needs in the Strategic Research Agenda of the European Technology Platform Industrial Safety. NEPHH Project will also contribute to other European strategies such as The Nanotechnology Action Plan, as it assures that all applications and use of Silicon based nanomaterials comply with a high level of public health, safety, consumers and workers protection, and environmental protection.

Moreover, the 'Code of Conduct for Responsible Nanosciences and Nanotechnologies Research' will also benefit from NEPHH Project, since it intends to ensure that nanotechnologies are developed in a safe manner. This important public consultation will make it very simple to address the legitimate concerns that can arise regarding nanotechnologies. This objective aligns with the EC aims at reinforcing nanotechnology and, at the same time, boosting support for collaborative R&D into the potential impact of nanotechnology on human health and the environment via toxicological and ecotoxicological studies.

Finally, NEPHH contributes to the acceptance of the nanotechnology to the wide public, thus assuring its sustainable introduction into market.

2 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: NNP4-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

Contents

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 run for 36 months. NeuroNano draws 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, for it 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.

- 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 are being addressed within the NeuroNano project are directed towards understanding the implications on each 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 is being 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 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 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, any nanoparticles in contact with tissue induce oxidative stress,(Brown 2007; Brown 2001; Duffin 2007; Xia 2006) 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.

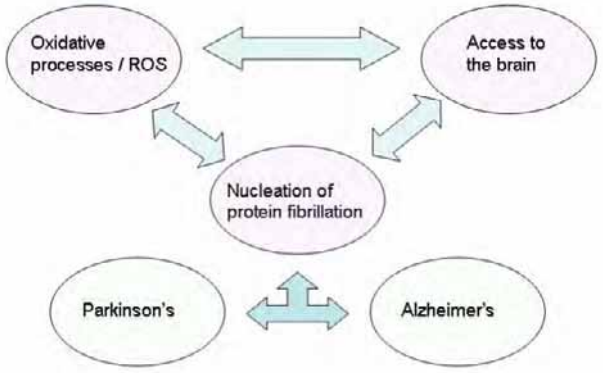

Figure 1. *Overview of the interplay between various factors regarding nanoparticle interactions with living systems that could pose a risk for the development of neurodegenerative diseases.*

This research program is deeply challenging, and entails the gathering of entirely new knowledge in a field (neuronanotoxicology) that would itself be born within NeuroNano. It requires the marshalling of unique expertise, methodologies, techniques and materials, many themselves completely new, and the whole never before brought together in the required combination.

Thus, the overall science and technology objective of this program is to determine if engineered nanoparticles could constitute a significant neuro-toxicological risk to humans for two diseases endpoints, Alzheimer's and Parkinson's diseases. *We also consider it important that the program does not presume neurotoxic hazard.* Thus, a major focus will be the critical evaluation of the entire chain of reasoning leading to the present concerns. This will be achieved by the detailed determination of cellular and molecular mechanisms involved along the 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 will be 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 1/1000th 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 will be ranked according to these three parameters, and mapped onto the FIBROS map (Figure 2).

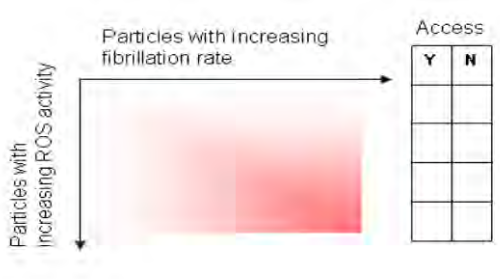

Figure 2. *The FIBROS map as a potential screening assessment for nanoparticle risk in terms of neurotoxicity. Engineered nanoparticles will be tested for their oxidative effects, fibrillation effects and access to the brain.*

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 an outcome would represent a durable contribution to science, and a highly significant contribution to society at large.

3 Project Description and Organisation

The NeuroNano project is organised into 5 scientific Work Packages and 1 management Work Package, 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 is 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 is 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 is 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 is essentially no literature on the role of nanoparticles in neurodegenerative disease.

The three main strands of the project are 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 multi-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, we have included the relevant and most-appropriately skilled partners with expertise at cell-level, at animal level, and (where appropriate) at human and disease level.

The tiered approach to the three stands 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 wide range of imaging techniques, offers the unique possibility to bridge the cell, tissue and animal studies.

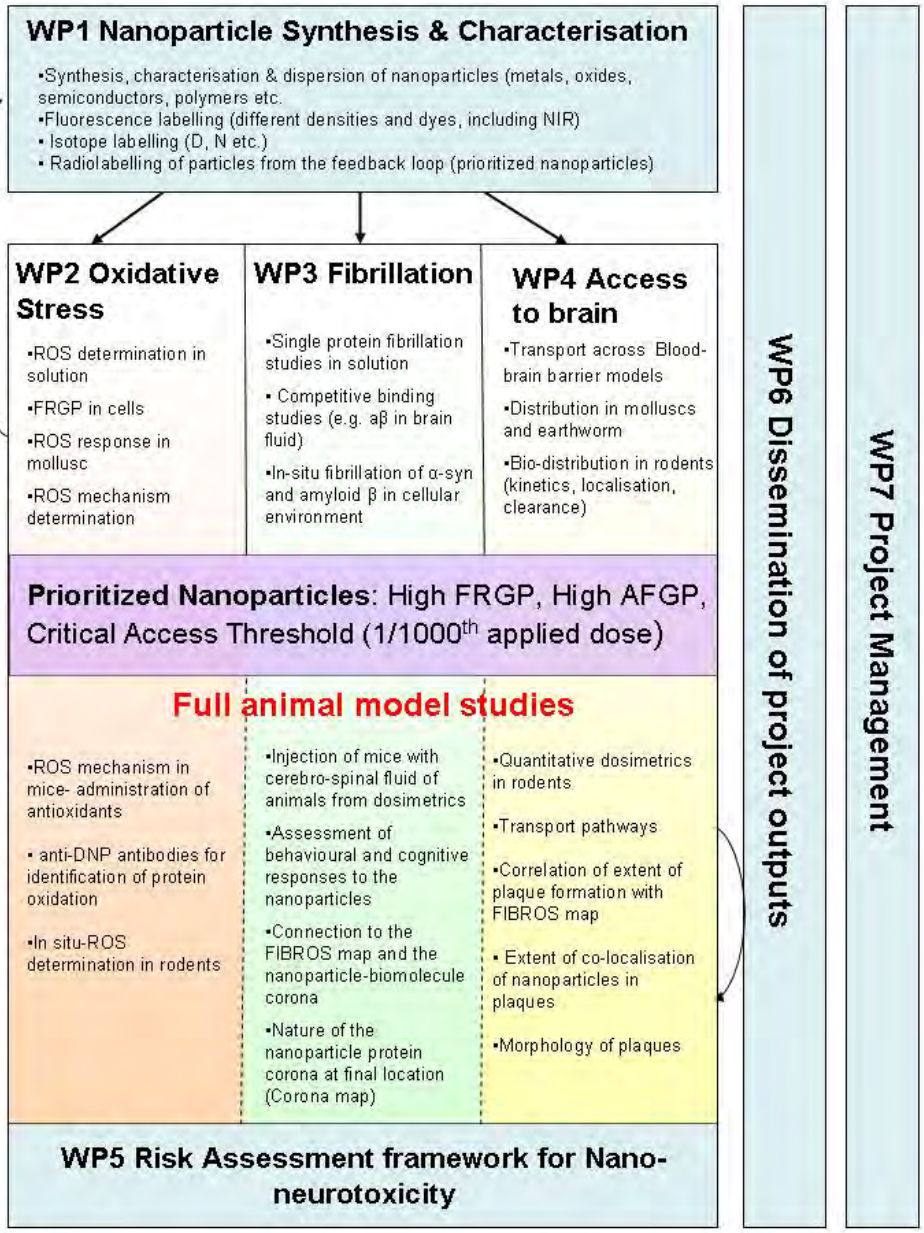

Figure 3. *Workflow and ideological layout of the work packages and the information flow within the NeuroNano project.*

4 Progress to date

Very significant progress has been made already, and the project has recently passed mid-term, and will enter its final year in February 2011. All workpackages are progressing well, and the emerging data is very promising. The first set of candidate nanoparticles chosen for the programme included titanium dioxide (several different sources and crystal phases), cerium oxide, copper oxide, silicon dioxide and polystyrene nanoparticles, for a range of different reasons/end-points. The silica and polystyrene nanoparticles were chosen as they are available as high quality fluorescently-labelled particles in a range of sizes, and their dispersion, cellular interaction and uptake are already well characterized via the NanoInteract (FP6 STReP project). Titania and ceria were chosen as candidates for radio-labelling to enable quantitative *in vivo* dosimetrics studies to be performed, as suitable strategies for labelling are available, obviously with some significant research element to optimize the labelling efficiency.

The radio-labelling procedure developed is such that the resultant particles can be re-dispersed back into solution as nanoscale entities, rather than as fused / aggregated clusters of particles. Importantly, the chosen nanomaterials are on the OECD list of priority nanomaterials (OECD, 2008).

Novel dispersion strategies based on biological and biocompatible molecules have been developed, and we believe that we now have mastery over dispersion of titania nanoparticles down to almost the primary particle size. We are currently investigating the exchange kinetics for the biological dispersants once the particles come into contact with a biological fluid, such as plasma.

The logistical aspects of materials shipping between partners and the nanoparticles database and tracking website have been established, beta tested and further refined based on partner feedback. Improvements to the shipping protocols, including addition of temperature monitors to the shipment packages, to report on any significant variations in the temperatures to which particles were exposed, which may affect their dispersion stability /

quality have been implemented, and have significantly decreased the amount of repeat shipments needed, and reduced the likelihood of poor quality experimental data resulting from poor quality dispersions being presented to cells / animals. Examples of the distribution of NeuroNano nanoparticles across the programme and their use in the various research elements of NeuroNano are shown in Table 1.

Quantitative dosimetry studies have produced some very interesting results, suggesting a critical role for the biomolecule corona in determining the fate and distribution of nanoparticles, as different delivery methods (instillation, inhalation, inter-tracheal, inter-osophogeal etc.) which result in different initial biomolecule coatings, result in different particle uptake and distribution behaviors. Work is ongoing to try to identify the specific biomolecules involved in directing nanoparticles to different target organs. For the particles tested to date, only very low levels of nanoparticles are being detected in the brains of the animals tested. Again, the correlation with the biomolecule coatings is underway and will shed key light on this question.

Nanoparticle labelling and dispersion

Industrial TiO_2 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 TiO_2 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 TiO_2 NP samples followed by subsequent full dispersion could not easily be achieved. While this goal remains under study, it was decided to concentrate on radio-labelling of industrially produced ST-01 TiO_2 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.

Several test irradiations on different types of TiO_2 were carried out, and XRD structural damage assessment was performed on P25 and ST-01 TiO_2 NPs after proton irradiation. The XRD results indicated that no significant damage was caused, even at activity levels as high as 1MBq/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 TiO_2 NPs. This included several steps of dispersion, leaching, centrifugation, "washing" and filtration. In late 2009 and early 2010, three sets of activated ST-01 TiO_2 NP samples were delivered to HELMUC for *in vivo* biokinetics studies. The suspension consisted of NPs of approximately 100nm hydrodynamic size (DLS performed at HELMUC), that were stably labelled to an activity level of more than 1MBq/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 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.

Work to radiolabel ceria NPs is underway: The neutron capture cross sections for stable cerium isotopes are all very low which means that ion-beam activation has to be used for radio-labelling 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. This means that thermal damage to the NPs might be a problem. This still needs to be assessed through XRD and dispersion studies at the activities required for subsequent *in vivo* tracing applications. Deuteron activation tests have been performed on CeO_2, indicating a yield of 0.05 MBq/mg with an irradiation of 5 hours at 2μA. 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 will be tested in the near future, with a factor of 10-20 higher being the target for the *in vivo* studies, given the reasonably long half-life of ^{141}Ce.

The first tests have been carried out to assess the feasibility of radio-labelling carbon-based (carbon black, MWCNT etc) nanoparticles. The initial results indicated that ^{7}Be is created via the $^{12}C(p,3p3n)^{7}Be$ reaction in reasonable quantities, with a half-life of 53 days. A high incident proton energy is required for this reaction, and combined with the fact that the ^{12}C mass is low compared with other activation elements of interest to NeuroNano, there might be a severe problem associated with momentum transfer to the target nucleus. This momentum transfer happens probably in all ion-induced reactions, but recoil range is limited with lower incident ion energies and higher target masses. The recoil problem, as well as the stability of ^{7}Be in carbon NP matrices, needs to be thoroughly studied, though initial studies indicated that ^{7}Be remained well attached to activated carbon black NPs in water. The activation yield measured on carbon black has been improved to 5 kBq/(μA.h.mg), meaning an irradiation of 15 hours at 10 μA would give 0.75 MBq/mg, easily enough for in vivo tracing studies given the relatively long half-life of the ^{7}Be radiotracer.

Recently an irradiation has also been carried out on MWNCTs at 9MeV energy to induce radiation damage without causing ^{7}Be production. This allowed subsequent XRD analysis of the structure and samples will be sent for TEM analysis. The initial XRD results showed no major changes to the MWCNT structure, apart from a (possible) minor increase in point defect concentration. This is very encouraging, but TEM will be required to be fully sure that the MWCNTs can be activated without morphological changes to their structure. Such studies will be performed in the near future, as well as more sophisticated leaching studies of ^{7}Be from high-energy activated MWCNTs.

Many efforts have been made to try to disperse titania nanoparticles in different media, most of them unsuccessful, particularly when trying to disperse titania from dry powders in water. The most successful strategies involve steric stabilisation using oligomers. These small polymeric chains get adsorbed to the surface of the nanoparticle forming a layer that completely coats the surface of the nanoparticle, hence changing its behaviour. Moreover, these stabilising agents tend to have high levels of toxicity.

Within NeuroNano, a novel strategy to disperse titania 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 1 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 65 nm were successfully achieved, as shown in Figure 4. The effect of temperature, sonication strategy, wetting process and pH on the quality of the dispersion was also investigated, including in relevant biological media, such as that used for the in vitro Blood-Brain barrier studies.

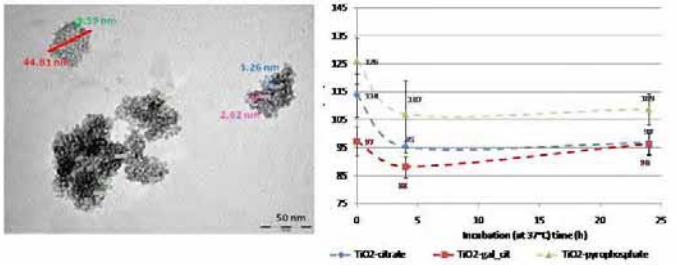

Figure 4. Left: TEM images of TiO2 NP dispersion prepared sing the NeuroNano dispersion strategy which utilises biological dispersing agents as "capping agents" which increase the solubility in water, and are then replaced by proteins once the nanoparticles are introduced into a biological fluid such as tissue culture medium. Right: Time stability of the TiO2 NP dispersions stabilised by different biomolecules (citrate, gallic acid, pyrophosphate) upon dispersion in tissue culture medium contain 3% foetal calm serum. As shown, the dispersions are very stable over the time-course of typical in vitro uptake and biodistribution experiments.

Nanoparticle access to the brain

By careful application of a robust protocol, it has been possible to obtain quantitatively reproducible results across two teams regarding the transport of a simple macromolecule (FD4 kDa), a known brain transporter protein (ApoE) through an *in vitro* BBB model based on hCMEC/D3 cells which form a monolayer and tight junctions when grown on transwell filters coated with collagen/fibronectin. This internal benchmarking validation of the *in vitro* BBB model confirmed the formation of the *in vitro* BBB and the appropriate functioning of a receptor-mediated transport mechanism, based on the transport of the positive control ApoE shown by each team.

Having validated the hCMEC/D3 BBB model, preliminary studies using fluorescently labelled 50nm SiO_2 NPs were performed by the two teams, again with excellent reproducibility and agreement (see Figure 5). However, these studies, whilst showing promise (in particular in their reproducibility) nanoparticle (50nm SiO_2) transport remains less compelling, in particular due to limitations of pore-particle interactions. In all cases, the pore size of the transwell filter was 0.4 µm, which although significantly larger than the nanoparticles, did impact on the transport across the filter, and the effect from the filter was quite large relative to the impact of the hCMEC/D3 cell monolayer. However, EM imaging confirms the NP transport is via a transcellular process. Time-resolved mechanistic work is underway, and will be reported separately. We emphasize that if indeed the particles are passing through the cells, then this is significant. It suggests that such models do seem to enable a true transcytosis-based mechanism for nanoparticle BBB crossing, and justifies further investment in them in future.

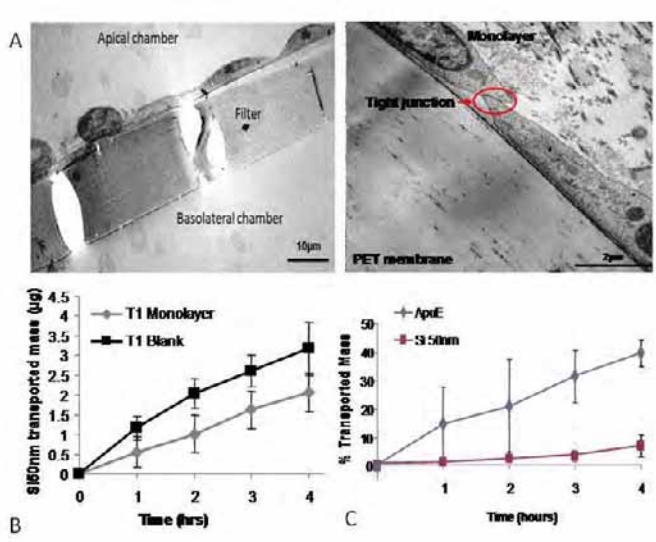

***Figure 5**. A: Electron microscope image of the hCMEC/D3 monolayer grown on a collagen/fibronectin coated 0.4µm membrane. The EM image on the left clearly illustrates the growth of a confluent monolayer. On the right, an electron dense tight junction between two adjacent hCMEC/D3 cells can be seen. B: Transport assay of SiO_2 50nm NPs across the in vitro BBB over 4 hours. (Data are mean ± std, 3≤n≤6. Two-Way ANOVA showed no significant differences of 50nm SiO_2 transport upon comparison of T1 versus T2 monolayer or blank values over time). C: Percentage transported mass of 50nm SiO_2 NPs and ApoE transcytosis control. A higher percentage of ApoE was found to cross the BBB monolayer compared to 50nm SiO_2 NPs (3 ≤ n ≤ 6).*

More recent work in our laboratory has been carried out, repeating this work using a transwell filter with 3 µm pores. After considerable efforts the cell has been shown to develop its function, and resulted in a significant alteration in the amount of nanoparticles crossing the barrier in the presence of the hCMEC/D3 cell monolayer, relative to the amount of nanoparticles crossing the blank filter alone.

From an *in vivo* viewpoint, nanoparticles may reach the brain either by neural transport from nasal epithelia to the olfactory bulb or by blood circulation. Within NeuroNano, we focus on the latter and consider translocation of nanoparticles either from the lung epithelia, or gut epithelia or direct intravenous application into blood circulation. In estimating the fraction of well characterized nanoparticles from blood circulation to the brain, two approaches were carried out:

1. Data analysis of existing quantitative biokinetic data of previous studies of our lab on (a) inhaled 20 nm nanoparticles aerosols of iridium or elemental carbon and (b) gold nanoparticles ranging in size from 1.4 nm to 200 nm and different surface charges administered as

suspensions either intratracheally to lungs, intravenously to blood or intra-oesophageally to the gastro-intestinal tract.

2. New experimental studies on quantitative biokinetics and specifically the accumulation in the brain after inhalation and after intranasal instillation of ^{48}V radio-labelled 20 nm TiO_2 anatase nanoparticle aerosols. In addition, administration of ^{48}V radio-labelled TiO_2 ST01 anatase nanoparticles suspensions either intratracheally to lungs, intravenously to blood or intra-oesophageally to the gastro-intestinal tract.

An additional approach being developed is the formation of conjugates of radio-labelled gold nanoparticles with Apolipoprotein E for quantitative biokinetics studies.

Nanoarticle type	Synthesized / Modified by	Used by	Assays performed
Radiolabelled TiO_2	JRC (radiolabelled)	HELMUC	Biokinetics / biodistribution
Radiolabelled Au (incl. spark)	JRC (radiolabelled)	HELMUC	Biokinetics / biodistribution
Dispersible TiO_2 - several ligands, several sources	Commercial; UCD (Dispersed)	UCD UU	Protein corona Biosistribution / behavioural changes
Fluorescent PS – COOH, NH_2- etc.	Commercial UCD (fluorescently-labelled)	UCD UCD UCC UEdin UCD	Apoptosis studies Gene arrays Redox proteomics ROS potential Uptake & localisation BBB uptake
HSA and Trp modified PS/ SiO_2	UCD (protein functionalised)	UCD	Uptake & localisation BBB uptake

Table 1. Examples of NeuroNano particles, their modification/functionalisation within NeuroNano, and their study within the Work Packages.

Redox effects – proteomics and transcriptomics impacts of nanoparticles

Cationic nanoparticles (NPs) have been shown to induce apoptosis in various cell types. Using cationic NPs to target diseased cells and selectively induce apoptosis could be a promising tool for nanomedicine. For this purpose, a full understanding of the apoptotic mechanism triggered by these NPs is required, in order to elucidate which pathway is activated and to determine the timing of the events along the apoptotic cascades induced by NPs.

Functional assays and imaging techniques were used to study apoptosis induced by 50 nm amine-modified polystyrene NPs in an astrocytoma cell line - 1321N1. YoPro 1/ Propidium iodide staining, western blot of PARP cleavage and activation of different caspases have previously confirmed that the cell death occurs mainly by apoptosis. Electron microscopy images showed damage in lysosomes, mitochondria and endoplasmic reticulum. Both flow cytometry and live cell imaging were used to follow lysosomal rupture, ROS generation, mitochondria membrane potential collapse and intracellular and mitochondria calcium flux changes. Moreover, fluorescently labelled cationic NPs were used to visualise the uptake and sub-cellular distribution at different times by confocal microscopy.

All the evidence shows that stimulating 1321N1 cells using 50 nm amine-modified polystyrene NPs results in disruption of both lysosomes and mitochondria, as shown in Figure 5. The key markers for this toxicological outcome involve caspase activations, and PARP cleavage, in a pathway that has now been elucidated. The time resolved intensity of these signals have also been identified, opening the way to couple the key parameters to fraction of cell death. In Figure 6 some of the time resolved data are given for illustrative purposes. Further studies will be focused on the stress in the endoplasmic reticulum and other

organelles to complete the map of the apoptotic signalling pathway activated by cationic NPs.

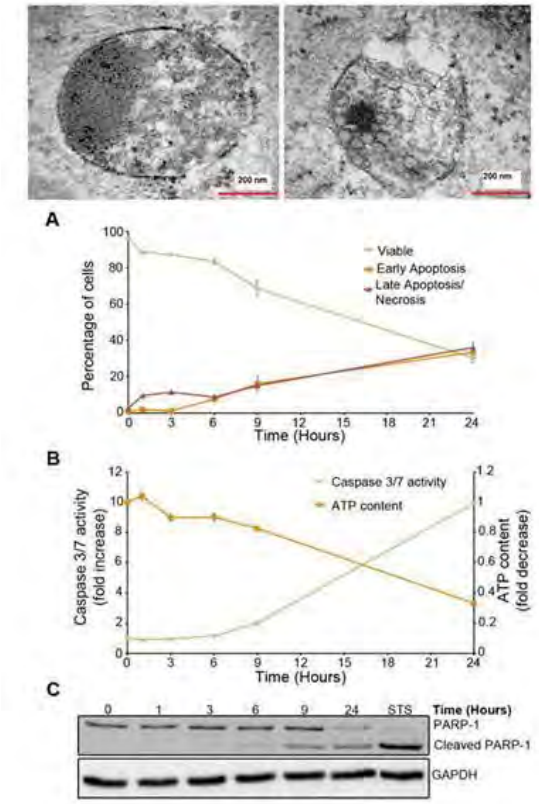

Figure 6. *Top: TEM images of untreated lysosome (Left) and lysosome treated with 50µg/ml 50nm amine-modified polystyrene nanoparticles for 6 hours (Right). Data from P1. NH_2-modified polystyrene nanoparticles induce caspase 3 and 7 activity and PARP-1 cleavage in 1321N1 cells. (A) YoPro-1/PI staining of 1321N1 incubated with 50 µg/ml of 50nm NH_2-modified nanoparticles shows an increase in the population of dead cells (early apoptotic and late apoptotic/necrotic) and a decrease in the viable cell population over a 24 hour period. (B) Analysis of the apoptosis-specific activity of caspases 3 and 7 reveals an increase over time, indicating that apoptosis is one of the mechanisms involved in cellular death induced by 50nm NH_2-modified nanoparticles. Analysis of cellular ATP content indicates that there is also energy depletion in the cells. Results shown are representative of 3 different experiments, each with 3 different replicas. Error bars represent standard deviations between three replicas. (C) Western-blot for the apoptosis-specific cleavage of Poly (ADP-ribose) Polymerase (PARP)-1 (116kDa) shows an increase of the cleaved protein (89kDa) over time in the presence of 50µg/ml of nanoparticles. Incubation with staurosporine (STS) for 6 hours was used as positive control for the experiment and GAPDH was used as the loading control. Data from Bexiga et al.*

A key finding from this work is that apoptosis and autophagy are intimately linked – in fact they are the same thing expressed in different ways – dose dependence of both. By changing the dose of nanoparticles presented to the cells we can also switch into completely other processes – e.g. cell cycle regulation. It is also worth noting that the observed effects are cell type-dependent – brain cells are more susceptible to the presence of nanomaterials. Detailed work is now underway to link the time-resolved apoptosis data to the impacts on protein expression and protein lesions, as well as to changes in gene expression in cells in response to the presence of the nanoparticles. For example, LC3 is marker of autophagy – several mechanisms – some induced by protein unfolding / degradation, which could be indicative of a role for protein unfolding. Within NeuroNano, we are now trying to make a fingerprint of the real numbers of protein lesions, changes in protein expression etc., not just individual cell – mapping out the insult processes, in order to clarify the different interactions induced by different NP types. This may be an approach to fingerprinting oxidative stress in the future - relating the nature of the NP to the profile and to the gene arrays, and from this profile to *in vivo*.

An evaluation of the changes in gene expression in response to the presence of cationic nanoparticles has produced some apoptosis and cell cycle is obvious – manifested at cellular level also even from cellular studies. Our experimental data was correlated with gene array data from patients with Alzheimer's disease. The first analysis shows evidence of clusters of monotonic expression changes and early up/down regulation. Interestingly, an unfolded protein response was observed, which is the same issue as occurs in protein fibrillation. It is likely that the observed protein unfolding response the not due to direct interaction of the proteins with the NPs, as these have been shown to be constrained in the lysosomes (see Figure 5).

Impact of nanoparticles on protein folding

Many proteins are capable of forming aggregates under certain appropriate conditions. This aggregation of proteins is closely linked with the onset of a number of diseases, such as Alzheimer's disease, Parkinson's disease, Haemodialysis related Amyloidosis etc. The fibrillation of amyloidogenic proteins is a nucleation process with a lag phase corresponding to the length of time it takes for critical nuclei to form. After nucleation occurs, small aggregates elongate into long fibrils. Many reports conclude that the small aggregated proteins are the root cause of the onset of such 'fibrillar' diseases rather than the fibrillar form, as was previously believed.

In previous studies it has been seen that, in the case of the amyloid β protein, polymeric nanoparticles can inhibit the formation of fibrils (Cabaleiro-Lago et al., 2008). However, very little is known as to the exact mechanism by which this effect occurs. Within NeuroNano we are using Fluorescence Correlation Spectroscopy to analyse the effect of nanoparticles on the protein fibrillation process with a view to elucidating the onset of fibrillation. By using different fluorescent dyes for the nanoparticles and proteins, we can follow the interaction and potentially the fibrillation process by analysing the cross-correlation function, studying the binding of the proteins in progressive states of aggregation to the surface of the nanoparticles. We have used a modified Aβ that has a fluorophore attached to the N terminal of the protein. This inherent dye allowed us to monitor how the nanoparticles affect aggregation right from monomeric stage, thus allowing greater elucidation of the overall mechanism.

We have also begun work using a cybrid cell line that inherently produces the Lewy Bodies that are associated with Parkinson's disease (PD). Different nanoparticles have been tested on control and PD cybrids with a view to examining if they could alter protein fibrillation in a biological context. However, due to the inherent "sickness" of these cells, we were unable to usefully determine any significant impacts from nanomaterials, and thus, the decision was made at the mid-term review to discontinue this line of research (cybrids) and to instead focus on the potential of transcriptomics to indicate impacts from nanoparticles related to protein folding.

Behavioural studies

We have completed a preliminary short-term behavioural study in rats following challenge with intra cerebro-ventricular (ICV) injection of titania nanoparticles (NP), with a variety of stabilising agents.

To determine the effects of nanoparticles on learning and memory in normal animals, rats were trained in an operant conditioning task to respond under an alternating-lever cyclic-ratio schedule (ALCR). Subjects capable of carrying out a complete schedule were subsequently implanted with a permanent ICV cannula, allowed to recover for 7 days and dosed with titania (3-9 nm) stabilised with either citric acid, gallic acid or pyrophosphate. Rats were then subject to further ALCR schedule and the number of lever switching errors, incorrect lever perseverations and response rate recorded.

ICV injected nanoparticles affected the ability of the rats to complete the ALCR schedule. A significant overall increase in the mean number of switching errors and mean number of incorrect lever perseverations was observed with gallic acid stabilised particles (Fig. 3B and D; $p<0.05$) when compared to control. Furthermore, a significant increase (Fig. 3A; $p<0.05$) in switching errors was observed on day 2 of the schedule in titania-gallic acid and day 3 in titania-pyrophosphate dosed rats when compared to control.

Further studies, including a longer term exposure are underway. Additionally, Histological investigation of brains from short-term rat behavioural studies are underway, including immuno-cytochemical staining for: GFAP for activated astrocytes, iba1 for activated microglial cells, anti-8-oxoguanine and super oxide dismutase-1 (SOD1) for oxidative stress detection are being performed. If nanoparticles induce any inflammatory or oxidative stress response, this will be apparent. The results will be correlated with the behavioural results and nanoparticle type.

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6 Directory

Table 1 Directory of people involved in the NeuroNano project

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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 of 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	Upsalla University	UU	Sweden
15	Institut Català de Nanotecnologia - Consejo Superior de Investigaciones Científicas	ICN	Spain
16	Stichting Dienslandbouwkundig 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

Contents

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 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.

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.

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]

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.

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.

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.
- **NA5** - Expert Resource Groups to advise on issues related to but not directly covered by the IA

Transnational Access:

- Provision of access to the nanomaterials processing, characterisation and exposure assessment facilities of the 15 TA Participants via a single application and evaluation process.

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 $8m^2$ 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, lead 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 inter-disciplinary 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.

5 QNano activities

QNano will have three functionally distinct elements that will 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 will not operate in isolation, but will be 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 will 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): 25 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 to contribute to the infrastructure of the field. Several of these are outstanding scientists in particularly relevant research functions.

Networking Activity (NA): Most of the partners will contribute in several (some in all three) activities. However, besides contributing to JRA, some have central roles in Networking. Of these, several partners have been chosen because in addition to having adopted the principles and practices of reproducibility and characterisation in their own sites early on, they represent excellent links to the different User constituencies, and have key management and networking functions facing those constituencies

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 four horizontal themes, 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).

The QNano objectives can be encapsulated as follows:

Provision of over 3,000 User days to some 400 Users, allocated over 6 six-monthly calls, with the first deadline being 12 months after the start of the project. These User days will be fully supported with both technical expertise in the particular state-of-the-art equipment being accessed, and also with technical expertise in nanomaterials sample preparation / dispersion to ensure validity of experimental data obtained.

Establishment of the Nanomaterials Hub and its mirror site (the nanomaterials specimen collection). This will involve two distinct elements, as follows:

Development of positive control nanomaterials for selected biological end-points (e.g. apoptosis).

Provision of additional test nanomaterials, the nature of which will be determined in consultation with stakeholders.

Provision to Users of 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;

Establishment of the Nanotraining Hub, building on the foundations laid by NanoImpactNet, and utilising the expertise of the Expert Resource Groups in Toxicology, Ecotoxicology, Modelling, Biologics, Occupational Exposure, and Ethical issues, which will function as a virtual training resource, a life-long learning materials repository, and an expert panel resource to address key educational and outreach issues, as well as having an active calendar of open access training events.

Establishment of a core infrastructure to address the critical issues currently hampering the industrial deployment of nanotechnologies across a range on industry sectors.

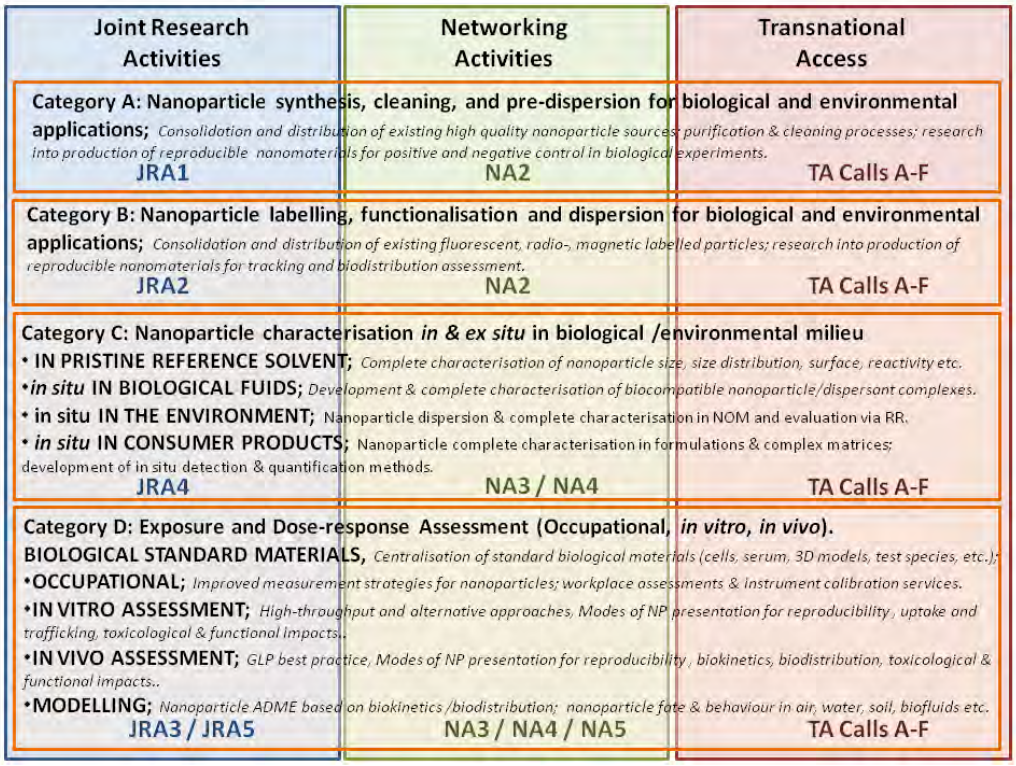

Figure 1 Conceptual overview of QNano and distribution of the operational activities (JRA, NA, TA).

Development of a sustainability plan to ensure longevity of the research infrastructure beyond the initial 4 years of funding, which will include addressing issues related to nanomedicine and nanodiagnostics, which will ensure a role for the infrastructure beyond the current urgency for clarity in the nanosafety issue.

Development of beyond the state of the art approaches for nanomaterials processing, labelling and identification and characterisation *in situ.*

Development of novel analytical approaches and tools where most urgently needed (e.g. characterisation of nanomaterials *in situ* in complex biological milieu) to enhance understanding of health and safety issues in nanotechnology.

Creation of 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.

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. These interlinkages are further illustrated in the Pert Chart in Figure 2.

Integrating NA with TA

One of the principle challenges facing nanomaterials research is the current lack of uniformity in the materials and protocols that researchers are using to conduct their studies. Therefore the reproducibility of subsequent results is jeopardised. The NAs will work closely with the TA to ensure that all particles and protocols provided for researchers via TA and NA (Nanoparticle hub) have been thoroughly quality-tested in NA before being delivered to their recipients. Additionally, the training elements provided by the Knowledge Hub will ensure that best practice is implemented both by the TA facilities (TA) and by the Users, which they can then implement in their own institutions also.

Integrating JRA with TA

Part of the work of JRA will be the development of new nanomaterials and new tools for assessment of nanomaterials in complex milieu such as are experienced in biology, in the environment and in consumer products such as food. In time this will lead to more and vastly improved standard nanomaterials being offered under TA in the final 24 months of this project.

As part of the RR validation processes for the standard nanomaterials and novel tools and protocols development, an internal bench-marking process will be established, and TA facilities will be able to compare and rank themselves alongside the key laboratories. This same process will also be used to bench-mark potential new Participants for inclusion in an expanded QNano project.

Integrating JRA with NA

All successful JRA nanomaterials, tools and protocols will be adopted as best practice, and will be promoted to all Participants and Users to facilitate the rapid development of the field. Hands-on training courses will be provided in implementation of the protocols, and a living, web-based handbook of best practice will be developed and continuously updated.

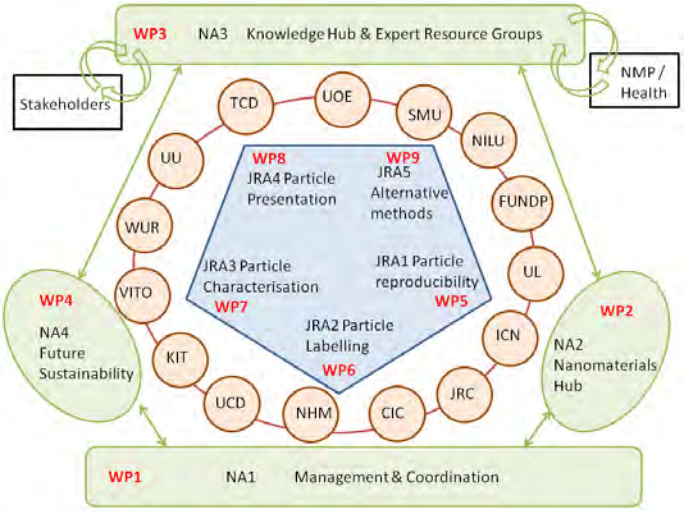

Figure 2 *Pert chart showing the interlinkages and interdependencies between the work packages in QNano.*

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7 Directory

Table 1 Directory of people involved in the QNano project as beneficiaries.*

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*** Note there are also Additional partners who are non-funded who are not included here.**

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SIINN

Safe Implementation of Innovative Nanoscience and Nanotechnology

Contract Agreement: Project not yet started Website: will be announced later
Scientific Coordinator: Anthony R. Flambard, Forschungszentrum Jülich GmbH, Berlin Bureau, Germany
Administrative 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	Technical 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	The Secretary of State for environment, food and rural affairs	DEFRA	United Kingdom
15	Science Foundation Ireland	SFI	Ireland
16	Austrian Institute of Technology	AIT	Austria
17	Federal Office of Public Health	FOPH	Switzerland
18	Stichting voor de Technische Wetenschappen	STW	The Netherlands
19	Fundação para a Ciência e a Tecnologia	FCT	Portugal

Contents

1 Summary

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.

European activities 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 NanoSafety Cluster, the QNano infrastructure, the OECD Working Party on Nanomaterials (WPNM), the NANOfutures ETIP and NanoImpactNet.

After defining the criteria important for NM toxicology (in accordance with the OECD WPMN) and for nanosafety risk assessment, the EHS information currently available to Europe will be examined and strategic liaisons with European and global stakeholders will be established. Following the identification of the major knowledge gaps important for NM EHS assessment, at least two joint, transnational calls will be organised in order to fill these gaps. The topics of life-cycle assessment and standardisation will also be addressed.

Recent technological advances allow the targeted production of objects and materials in the nanoscale (smaller than 100 nm). Nanomaterials have chemical, physical and bioactive characteristics, which are different from those of larger entities of the same materials and from molecular forms of the materials (where these exist). Nanoparticles can pass through body barriers, although the detailed mechanisms are not yet understood. This is interesting for medical applications, but it raises concerns about their health and environmental impact. The objective of NanoImpactNet is to create a scientific basis to ensure the safe and responsible development of engineered nanoparticles and nanotechnology-based materials and products, and to support the definition of regulatory measures and implementation of appropriate legislation in Europe. It includes a 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 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 bio. 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 state 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 future 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

As of February 2011, SIINN is entering its final negotiation phase with the Commission Services with the aim of getting the project off the ground during the second quarter of 2011.

A "kick-off" meeting bringing together all partners and stakeholders is being planned for June 2011 in Berlin.

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.

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 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.

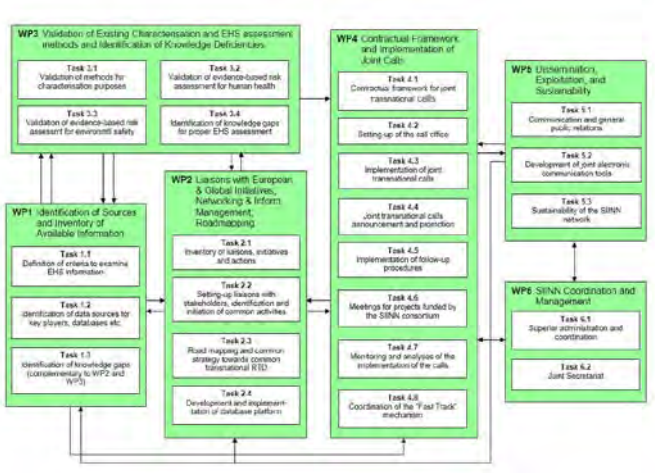

Figure 1 Interplay of SIINN Workpackages (WPs)

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.

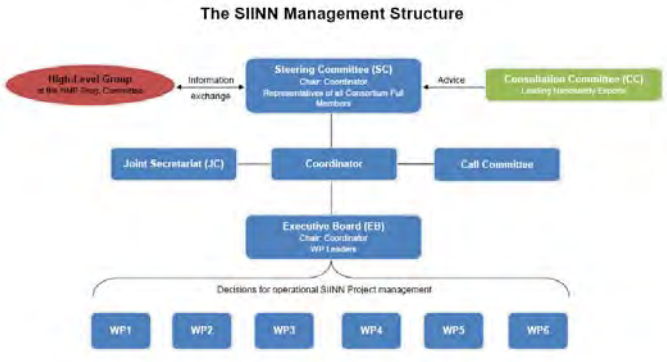

Figure 2 Management Structure of the SIINN Project

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

Table 1 Directory of people involved in this project.

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