

Compendium of Projects in the European NanoSafety Cluster

March 2010

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

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PREFACE

The demand for research in the area of safety health and environmental management of nanotechnologies is present since a decade and identified by several landmark reports and studies. 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.

We would like to stress that this sector is under development and progressing very fast, which might make some of the statements outdated or even obsolete. Nevertheless it is intended to provide a basis for the necessary future developments.

The publication will take the shape of a dynamic, frequently updated, web-based document available free of charge to all interested parties.

Finally we would like to invite all researchers in this domain to join the effort, communicating the work being done and all readers to support the nano-safety community.

ACKNOWLEDGMENTS

The authors are well aware that without the help of the project managers this publication would not have been possible. Many thanks to all of them.

At the heart of the compendium the reader 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.

We also extend our special thanks to Caroline Delattre and Anne-Marie Cuesta.



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QUOTE

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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 $82.5M\epsilon$, from the NMP and other programmes, under FP6 (11 projects, $30M\epsilon$) and FP7 (19 projects, $52.5M\epsilon$). 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, a projects and stakeholders open forum.

It is a great honour and pleasure to present this compendium today. It will be a dynamic document, consisting of project summaries, research work posters, and research actors. We rely on your contribution for frequent updating and completion towards the aim of having a policy design tool, a reference platform, a benchmark, for an in-depth insight of the on-going work and the future response to the challenges ahead.

George Katalagarianakis

Nicolas Segebarth





CellNanoTox



Cellular Interaction and Toxicology with Engineered Nanoparticles

Contract Agreement: NMP-CT-2006-032731 Website: http://www.fp6-cellnanotox.net Coordinator: Prof. Rafi Korenstein, Tel Aviv University (IL)

No.	Beneficiary name	Short name	Country
01	Tel Aviv University	TAU	Israel
02	INSERM	INS	France
03	Joint Research Centre - Institute for Health and Consumer Protection	JRC	Italy
04	University of Muenster	UMUE	Germany
05	University of Mainz	JGU	Germany
06	BASF AG	BASF	Germany
07	Colorobia	COL	Italy
08	tp21 GmbH	TP21	Germany

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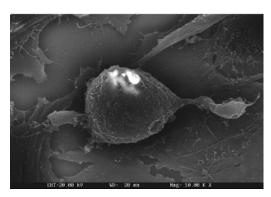
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1 Introduction : Scope and Objectives

CellNanoTox aimed at the development of innovative multidisciplinary sets of tests and indicators for toxicological profiling of nanoparticles (NPs) as well as unraveling the correlation between the physicochemical characteristics of NPs and their toxic potential on various organs of the human body.

For a comprehensive understanding of the complex data to be obtained on toxicology of NPs, based on in-vitro and ex-vivo studies, we have employed conventional toxicology combined with the methodologies of toxicogenomics, metabonomics, Knowledge Discovery from Data (KDD) and Data Mining (DM).



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Cobalt aggregates on immortalised mouse fibroblast (Balb/3T3 cell) by Scanning Electron Microscope (SEM-EDX).

This project was focused towards understanding the relation between the physicochemical properties of NPs and their toxicity in the different exposed organs of the human body. Since it was shown that the penetration of NPs into the human body proceeds principally through inhalation or orally, whereas penetration through healthy skin is restricted, we have chosen lung and intestine as the primary interacting tissues/organs with NPs, while liver, kidney and the immunological system have been selected to be the secondary major sites of interaction, following the penetration of NPs into the blood circulation. The interaction of the NPs with these different target organs has been studied by making use of alternative methods to animal experimentation by employing in-vitro cell model systems representing the different organs-systems as well as ex-vivo studies based on precision-cut slices of lung, liver and kidney.

The studies carried out within the CellNanoTox project address the needs of the European society for assessing the risk of occupational and general population exposure to industrially manufactured NPs. It is expected to generate new knowledge on potential health risk or the absence of it, providing objective arguments for recommendations and regulations.

2 Technical approach, Work Description, Achievements: High throughput toxicological screening of selected NPs

We have carried out toxicological screening of four family types of NPs consisting of cobalt NPs, gold NPs, cobalt-ferrites NPs and quantum dots, using in-vitro model systems of lung, intestine, liver, kidney and the immune system. Gold NPs, cobalt ferrite NPs, as well as corresponding radioactively labeled NPs were synthesized by the consortium. The screening was based on alveolar type II cells and lung-slices for lung, on Caco-2 cells for intestine, on MDCK and HEP-G2 cells for kidney and liver respectively and on murine primary dendritic cells for the immune system. Under the concentration range used, gold NPs and quantum dots were shown to be non-toxic whereas aggregates of cobalt-NPs and cobalt-ferrite NPs were shown to be toxic.

Dose-response curves of cobalt NPs aggregates were examined employing MTT, neutral red (NR) and Alamar blue assays. Since cobalt NPs undergo dissolution in aqueous media, we determined the dose-response curves for Co-ions, employing cobalt chloride for the same endpoints. The extent of cobalt NPs dissolution was determined to enable us to examine the indirect effect of cobalt NPs on the various cell-types due to dissolution as compared to the direct effect of aggregated cobalt NPs on the different cell models. Data analysis and modelling of the obtained data sets, for the toxicological doseresponse curves for cobalt NP aggregates, taking into account the dose-response curves of cobalt ions, was carried using the approach of Knowledge Discovery from Data (KDD). The first KDD goal was to discover rules for determining the toxicity of nanoparticles from the experimental results. The input data set included the consolidated experimental results, where each data record has the following attributes: (1) Cell type; (2) Particle



type (Co-ions or Co-NPs); (3) Concentration; (4) Exposure time; and (5) The extent of viability decrease.

The observed toxicity was modelled by a J48 decision tree classifier since such classifier model can be explained intuitively, in terms of simple if-then rules syntax, without any prior knowledge of data mining techniques. Since the model is based on information theory, we can infer that the concentrations of the Co-NPs and Co-ions are the most informative parameters for toxicity prediction. Thus, concentration is the most influential parameter (highest rank), as expected from the basic principles of toxicology. The second most influential parameter (second rank) is either the compound type (Co-ions or Co-NPs) or the cell type, depending on the concentration range. The third and the lowest rank in the model is that of the duration of exposure.

This model is pointing at the differential sensitivity towards toxicity of the different cell lines for cobalt ions and cobalt NPs. The hierarchy of cell sensitivity towards cobalt ions is given in the following sequence: A549 > MDCK > NCIH441 > Caco-2 > HepG2 > Dendritic cells, where A549 is the most sensitive cell line and primary dendritic cells are the least sensitive ones. However, a different hierarchy pattern emerges for Co-NPs: A549 = MDCK = NCIH441 = Caco-2 > Dendritic cells > HepG2. These hierarchies are an outcome of the dose-response curves, where the response is an average of viability determined by 2-3 different assay methods (MTT, NR and Alamar blue). It should be pointed out that when forming a sensitivity hierarchy based on EC_{50} data, a different pattern emerges. This difference is attributed to the different functional dependence of viability on concentration observed for the different cell lines. Therefore, the choice of the cut-off for toxicity may influence the observed hierarchy. Moreover, the modeling enables to assess the influence of exposure duration on the toxicological outcome. The comparison of the cytotoxic effects induced by Co-NPs aggregates with their respective Co-ions which leached into the medium shows higher toxicity for Co-NPs when using the Caco-2 cell model at concentrations ≤100µM both for 48h and 72 h duration. At the same concentration, A549 and NCIH441 lung cell models show higher toxicity for the Co-NPs only for the 48h exposure. The dendritic cells showed a higher toxicity for Co-NPs at 72h at concentrations 50μ M < C \leq 100 μ M and. In addition, at concentrations≤ 200µM the NPs were more toxic than Co ions for both exposure durations.

The toxicological effects of Co-Fe (CoFe₂O₄) NPs were examined using seven different cell lines representing lung (A549 and NCIH441 cell lines), liver (HepG2 cell line), kidney (MDCK cell line), intestine (Caco-2/TC7 cell line), and immune system cells (primary mouse dendritic cells and a human Blymphocyte cell line (TK6)). In addition, rat precision cut lung slices were examined. Dose-response curves were carried out in the concentration range of 0.05 -1.2 mM, employing MTT, neutral red and Alamar blue as viability endpoint assays following exposures of 24 and 72 h.

Data analysis and modelling of the obtained data sets was based on the decision tree model learned from the consolidated results after applying the KDD process. The concentration of the Co-Fe NPs emerged to be the most informative (first rank) parameter for toxicity prediction. The cell type turned out to be



the second rank parameter. The third and the lowest rank in the model was either the time of exposure or concentration depending on the cell type. This model suggests the following hierarchy of cell sensitivity towards the toxicological insult of Co-Fe NPs: TK6 > Lung slices > NCIH441 > Caco-2 = MDCK > A549 > HepG2 = Dentritic cells, where the two cellular models of the immune system consisting of B-lymphocytes (TK6) and primary dendritic cells turned out to possess the highest and the lowest sensitivity, respectively

3 Mechanistic aspects of interaction, uptake and recycling of selected NPs by the different cellular systems

We have selected quantum dots as the prime NP type and gold NPs as a secondary NP type for uptake studies and intracellular recycling studies. Studies based on radioactive labeled NPs was carried out employing gold and Co-Fe NPs. Optical monitoring by confocal microscopy of NP uptake was pursuit using carboxylated, amine and pegilated QDs, based on the far red emission of the NPs. The optical monitoring of gold NP uptake was demonstrated in dendritic cells, using reflection microscopy. The intracellular localization in membrane-bound vesicles of cobalt ferrite and gold NPs was also visualized by electron microscopy in alveolar cell types. Using radioactive labeled and unlabeled gold NPs, the uptake and the intracellular distribution of NPs in MDCK and HepG2 cells was investigated. The studies are being currently evaluated.

4 Exploration of toxicity mechanisms emerging following the interaction of NPs with the different *in-vitro* cell models

Preliminary experiments on the oxidative stress induced by cobalt ferrite NPs in Caco-2 demonstrate that they possess ROS generating potential, being able to decrease glutathione level (an important anti-oxidant of the cell) and to increase intracellular ROS measured by flow cytometry using dichlorodihydrofluorescein as an optical probe. Similar studies of ROS are being conducted using the other types of cell models. The possibility of NP- induced programmed cell death was initially examined in HepG2 and MDCK cells following their exposure to cobalt ferrite however, no significant apoptotic processes could be detected. Inflammation induced by Co-Fe NPs is being currently studied.

5 Exploration of NP-induced metabolic changes using precision-cut liver, kidney and lung slices by metabonomics

The metabonomic approach in tissue slices using carbon 13 NMR and mathematical modeling of metabolic pathways has been carried out. This task has been extended to the use of ¹³C-lactate in the liver, The task on the enzymatic measurements of the effects of commercially available and selected NPs on substrate removal and product formation by liver, kidney and lung slices has been completed for liver and kidney slices and is currently has only recently been initiated in the study of the lung tissue.

6 NP-induced activation and inflammatory response of the immune system

Experiments performed in the presence of carbon nanotubes (standard multiwall nanotubes of 20-50µm) indicate that for the tested concentrations there is no cytotoxicity and no activation of dendritic cells (DC). Tested QD were shown to be highly cytotoxic: they induced the death of DC, but did not activate DC since DC bearing a high amount of MHC class II molecules did not vary upon incubation in the presence of these NPs.

The influence of the NPs on the capacity of DC to secrete cytokines was tested after stimulation in the presence of various inductors. It was shown that most of the NPs, when used at high concentrations, reduced the secretions of IL12, but this activity could be restored to its normal level if the strength of activation was increased.

Analysis of the database obtained throughout the project by Knowledge Discovery from Data (KDD) and Data Mining (DM)

A central activity of the consortium was to introduce the domain of Knowledge Discovery from Data (KDD) and Data Mining (DM) for integration and modeling of the obtained results. Parts of this activity were demonstrated in the analysis and modeling of toxicity induced by Co-NPs, Co-ions and Co-Fe NPs.

7 CellNanoTox Partners





Toxicology

Ecology

Germany

Italy

BASF Product Safety -

Dr. Robert Landsiedel &

Dr. Giovanni Baldi & Dr. Daniele Bonacchi

Regulations,

Ludwigshafen,

Dr. Susanne Boehn

CERICOL Vinci,

and

Tel viv University

Department of Physiology and Pharmacology of the Faculty of Medicine Tel Aviv, Israel Prof. Dr. Rafi Korenstein → Mechanistic aspects on adsorption and uptake of NPs by cells

Department of Industrial Engineering of the Faculty of Engineering Tel Aviv, Israel Prof. Dr. Oded Maimon

→ Data Mining and Knowledge Discovery



INSERM

Institut National de la Santé et de la Recherche Médicale U 820: Métabolomique et Maladies Métaboliques Lyon, France Prof. Dr. Gabriel Baverel

 $\rightarrow\,$ Cellular Metabonomics, NMR spectroscopy, NPs interaction with liver, kidney and lung

U 823: Immunologie Analytique des Pathologies Chroniques Grenoble, France Dr. Patrice Marche & Dr. Christian Villiers

 \rightarrow $% \left({{\rm Immune \ Response \ on \ NPs, \ dendritic \ cell \ biology, \ NPs \ interaction \ with \ biological \ fluids \ } \right)$



ightarrow NPs analysis by electron microscopy and mass spectroscopy



Institute of Pathology Mainz, Germany Prof. Dr. C. J. Kirkpatrick & Dr. Chiara Uboldi

Johannes Gutenberg University of Mainz

 \rightarrow In-vitro study on NPs-cell/biomaterial interaction, effect on alveolar and endothelial cells



BASF SE

→ NPs interaction with lung



Colorobbia Italy Spa

 \rightarrow NPs synthesis, functionalisation, modification

tp21

RTD			м	anag	gement
Saarbruecken,				G	ermany
Dr.	Petra	Zalud	&	Dr.	Hanno
Wittig					

tp21 GmbH

→ Project Management, Public Relations

8 Exploitation of results

CellNanoTox is a knowledge driven project. The studies carried out within the project address the needs of the European society for assessing the risk of occupational and general population exposure to industrially manufactured NPs. It is expected to generate new knowledge on potential health risk or the absence of it, providing objective arguments for recommendations and regulations.

On long term view, one major outcome for sustainable use is the Data Mining Know-how to support a better understanding of the impact of engineered nanoparticles on health and the environment. On medium and short term view, the gained NP synthesis Know-how as well as new assays methods to measure the interaction of nanomaterials with living structures are major exploitable results of CellNanoTox (see below). Cellular metabolomics applied to the prediction of the safety and efficacy of test compounds is offered as contract service by a CellNanoTox-partner's spin-off.

Result:	'Data Mining and Knowledge Discovery Know-how'
Exploitation potential:	New FP7 project: NHECD: Creation of a critical and commented database on the health, safety and environmental impact of nanoparticles



The NHECD approach will be based on the integration of the following features:

- Innovative text mining tools designed specifically to extract information from scientific research papers in the nanoparticles domain
- Automated content extraction and review processes designed to handle very large number of electronically published scientific research papers keeping quality results
- Leading toxicology domain knowledge provided by the NHECD partners with links to data-bases inside and outside Europe
- Web site as a front-end for hosting facilities as well as a web based user interface for the data base application
- Effective public relations operation to expose the repository to various audiences such as scientific community, regulatory bodies and the general public, dealing with generating white paper and production of summaries for interaction with stakeholders and dissemination to Industry and public.

It is expected that the combined features of the suggested database will lead to a synergistic outcome enabling:

- Better understanding of the impact of nanoparticles on health and the environment, and definition of future actions;
- Safe and responsible development and use of nanotechnology;
- Support to research and regulation;
- Support to regulatory measures and implementation of legislation;
- Implementation of the European Commission's Action Plan for Nanotechnology;
- Support to good governance in nanotechnology.

<u>Contact:</u> Oded Maimon, Tel Aviv University, E-mail: <u>maimon@eng.tau.ac.il</u>

Result:

'Assay protocols on inflammation'

Exploitation potential: Inflammation assay for risk assessment for industry in the frame of NP production

See also: Analysis of the toxicity of gold nanoparticles on the immune system: effect on dendritic cell functions.

Villiers C., Freitas H., Couderc R., Villiers M.-B., Marche P.

Journal of Nanoparticle Research **12**, 1 (2010) 55-60 - http://www.hal.inserm.fr/inserm-00458282/en/

<u>**Contact:**</u> Christian Villiers, INSERM U823, Grenoble, E-mail: christian.villiers@ujf-grenoble.fr

Result: 'Contract service based on cellular metabolomics'

Exploitation potential: Metabolys spin off: founded Nov. 2008 Grenoble (F)

Metabolys is a company with 8 employees, founded on the 20th November 2008 at the Laennec Faculty of Medicine (Lyon 8) by Gabriel Baverel (CellNanoTox member), University Professor and Hospital Practitioner, former Director of the INSERM Research unit 820 (Metabolomics and Metabolic Diseases) and Head of the Department of Renal Function Exploration at the Edouard Herriot Hospital. Metabolys is a spin-off from the Claude Bernard-Lyon 1 University and the INSERM.

Metabolys has 2 complementary sectors of activity: on the one hand, it offers contract services for various industries e.g. cellular metabolomics applied to the prediction of the safety and efficacy of test compounds (drug candidates, biologics, chemicals, cosmetic and agrofood products) and, on the other hand, it has the ambition to discover new antidiabetics at the preclinical development stage.

Proof of concept for the use of novel NPs.

Contact: METABOLYS, Lyon, E-mail: baverel@sante.univ-lyon1.fr

Result: 'Nanoparticle synthesis know-how'

Exploitation potential: New multifunctional nanomaterials with more than one chemical/physical function at the same time, making them compatible with widely differing applications in a range of fields, including glass, ceramics, textiles, biomedical, pharmaceuticals, building, agro-food and cultural heritage conservation.

<u>Contact</u>: Colorobbia Nanomaterial c/o Centro Ricerche Colorobbia

SOVIGLIANA VINCI (Firenze), E-mail: infocericol@colorobbia.it

Dissemination

CellNanoTox project has been actively promoted towards the scientific community at the occasion of European and international conferences.

9 List of publications

Ponti, J., Colognato, R., Franchini, F., Gioria, S., Simonelli, F., Abbas, K., Uboldi, C., Kirkpatrick J.C. and Rossi, F. **A quantitative in vitro approach to study the intracellular fate of gold nanoparticles: From synthesis to cytotoxicity**. *Nanotoxicology* 3 (4), pp. 296-306 (2009)

Uboldi, C., Bonacchi, D., Lorenzi, G., Iris, M.I., Pohl, C., Baldi, G., Unger, R.E., Kirkpatrick C.J. Gold nanoparticles induce cytotoxicity in the alveolar type-II cell lines A549 and NCIH441 *Particle and Fibre Toxicology* 6, art. no. 18 (2009)

Villiers C., Freitas H., Couderc R., Villiers M.-B., Marche P. Analysis of the toxicity of gold nano particles on the immune system: effect on dendritic cell functions. Journal of Nanoparticle Research 12 (1) 55-60 (2010)



Limor Horev-Azaria, James C. Kirkpatrick, Rafi Korenstein, Patrice N. Marche, Oded Maimon, Jessica Ponti, Roni Romano, Francois Rossi, Ute Golla-Schindler, Dieter Sommer, Chiara Uboldi, Ronald E. Unger, Christian Villiers. Predictive toxicology of cobalt nanoparticle aggregates and cobalt ions: comparative in-vitro study of different cellular models using methods of knowledge discovery from data (submitted)

Limor Horev-Azaria, Giovanni Baldi, Delila Beno, Susanne Boehn, Daniel Bonacchi, James C. Kirkpatrick, Rafi Korenstein, Robert Landsiedel, Oded Maimon, Patrice N. Marche, Jessica Ponti, Roni Romano, Francois Rossi, Ute Golla-Schindler, Dieter Sommer, Chiara Uboldi, Ronald E. Unger, Christian Villiers. Predictive toxicology of cobalt ferrite nanoparticles: comparative in-vitro study of different cellular models using methods of knowledge discovery from data (Submitted)

10 Links to information

General information on CellNanoTox is presented via the project website under http://www.fp6-cellnanotox.net . The website presents links to all major nanomaterial related funded national and EU-projects

An eight pages **CellNanoTox brochure** is distributed on national and international events on nanotechnology. The booklet is available and can be ordered via the project website.

11 Directory

Table 1 Directory of people involved in this project.

First Name	Last Name	Affiliation	Address	e-mail
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Gabriel	Baverel	INSERM	U 820: Métabolomique et Maladies Métaboliques Lyon, France	baverel@sante.univ-lyon1.fr
Francois	Rossi	European Commission - Joint Research Centre Institute for Health and Consumer Protection JRC-IHCP	BMS Unit, TP203 21020, via E. Fermi, 1 Ispra (VA) ITALY	Francois.Rossi@jrc.it
Ute	Golla-Schindler	Westfälische Wilhelms University of Muenster	Institute of Mineralogy Corrensstrasse 24 48149 Muenster	golla@nwz.uni-muenster.de
James	Kirkpatrick	Johannes Gutenberg University	Institute of Pathology, Bldg 402, Langenbeckstrasse 1, D - 55101 Mainz	kirkpatrick@pathologie.klinik.uni mainz.de
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Giovanni	Baldi	CERICOL- Colorobbia	Via Pietramarina 123 50053 Sovigliana- Vinci, Italy	baldig@colorobbia.it
Petra	Zalud	TP21	tp21 GmbH Tuerkenstrasse 4 D-66111 Saarbruecken, Germany	zalud@tp21.com



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CellNanoTox is a Specific Targeted Research Project under the European Commission's 6th Framework Programme.

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DIPNA

Development of an Integrated Platform for Nanoparticle Analysis to verify their possible toxicity and the eco-toxicity



Contract Agreement: STRP 032131 DIPNA Website: http://www.dipna.eu Coordinator: Antonietta M. Gatti, CNISM - University of Modena & Reggio Emilia, Italy

No.	Beneficiary name	Short name	Country
1	Consorzio Nazionale Interuniversitario Sviluppo Materiali - University of Modena and Reggio Emilia	CNISM- UNIMO	ITALY
2	University of Salzburg	USALZ	AUSTRIA
3	Fraunhofer Institute of Biomedical Engineering	Fraunhofer	GERMANY
4	Consiglio Nazionale delle Ricerche	CNR	ITALY
5	Università della Magna Graecia	BioNEM	ITALY
6	Grimm Aerosol	Grimm	GERMANY
7	Flemish Institute for Technological Research	VITO NV	BELGIUM
8	CSEM SA	CSEM	SWITZERLAND
9	Institut Català de Nanotecnologia	ICN	SPAIN
10	Commission of the European Communities - Directorate General -Joint Research Centre Ispra – European Center Validation Alternative Methods	COMC-IHC	ITALY

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1 Summary

The project aimed at creating and validating new instruments and assays to assess the possible toxicity of nanoparticles and to detect nanopollution at occupational sites, in order to promote safe NP manufacturing and handling.

The impact of Cobalt, Gold, Cerium, and Iron Oxide NPs in liquid suspension and in dry state on different types of human defence cells was investigated *in vitro* to identify biomarkers of nanotoxicity and design assays. Upon acute exposure most of the NPs tested did not cause any relevant toxic effect nor could affect selected inflammatory parameters in human leukocytes, and lung or gut mucosal epithelial cells. Cell growth inhibition and production of reactive oxygen species was observed only for cobalt NPs, and was probably due to cobalt ion release rather than NP-cell interaction. Also the expression of inflammation-related genes was not modulated by NPs when given in a chronic or cumulative fashion over 15 days of culture. Using genome-wide transcriptomics, novel immune-related gene markers induced by Cobalt and Cerium NPs were identified.

Different systems were constructed to simulate singlet NP-cell interaction with built-in sensors to evaluate NP effects. These included chips to monitor membrane changes by single-cell impedance spectroscopy, a nanodispenser for depositing NPs in array format on cell culture supports, and microinjection techniques and Raman spectroscopy combined with principal component analysis. The singlet NP-cell interactions did not



cause any measurable effect. An automated breadboard system, consisting of a controlled incubator, fluidic system, and optical detection units, was constructed for nanotoxicological analysis in the field. Finally, a system for repeated spraying of dry NPs in air was developed.

The results of DIPNA provide novel information on *in vitro* assays for the detection of NP effects on human cells, instruments and methods for environmental NP detection, and recommendations for nanotoxicologists and researchers.

2 Project execution

The aim of the DIPNA project was to provide knowledge about the impact of four different NPs (Cobalt, Gold, Cerium and Iron Oxide) in wet suspension and in dry conditions on different types of cells (human immortalised THP-1, Jurkat, HepG2, CaCo-2 and A549 cell lines, murine 3T3, human CD34⁺-derived dendritic cells, and primary human monocytes) to identify novel biomarkers and develop assays for the evaluation of nanotoxicity. The project also developed technological solutions to set up singlet NP-cell interactions with built-in sensors to evaluate the effects, a device for automatic evaluation of the toxicological impact of nanoparticles at occupational sites, and a system for repeated spraying of dry NPs in air.

The project was divided into 6 Workpackages:

WP1: Fabrication and characterisation of selected NPs

- WP2: Nanotoxicology: NP-induced alterations of human defence cell physiology
- WP3: Single particle impact on cells
- WP4: Integrated platform for monitoring NP effects upon chronic and repeated exposure
- WP5: Field validation and development platform
- WP6: Coordination, management, training and public awareness

2.1 Fabrication and characterisation of selected NPs

Within WP1, NPs of different composition (Au, Co, Fe₃O₄ and CeO₂), size (from 4 to 50 nm), shape (spheres, rods and disks), and surface state (organic or biological coatings, positive or negative surface charges) have been synthesized by Partner ICN and routinely distributed to the partners. Additionally, Ag NPs have been synthesized, characterised and distributed as reference material due to their well-known germicidal effects and toxicity for mammalian cells. In order to obtain NPs that are stable in aqueous solutions, two controlled (composition, size, shape, surface characteristics) synthesis routes based on wet chemistry methods have been performed: synthesis in aqueous phase, and synthesis in organic phase and further phase transfer to water. During the third year of the project, fluorescencelabelled Au NPs, and Au and Ag NPs with self-assembled monolayers conferring positive or negative surface charge, have also been synthesized, characterised and distributed. All these kinds of NPs have also been prepared in powder form by precipitation of the solid phase (the NPs) from the colloid and subsequent drying process. Only small amounts of powder can

be obtained for Au NPs, whereas other NPs can be obtained in larger quantities.

Dry NPs of the same chemical composition were also bought, aliquoted, physically and chemically characterised, sterilised, depyrogenised and sent to all partners for the *in vitro* studies.

2.2 NP-induced alterations of human defence cell physiology

The objectives of WP2 were :

- 1. Set-up of common protocols for analysis of NP effects on human defence cells
- 2. NP impact on T cells
- 3. NP impact on epithelial cells
- 4. NP impact on monocytes, macrophages and dendritic cells
- 5. NP biokinetical and bio-cumulative effects on human immune cells
- 6. NP toxicology, genotoxicity and carcinogenicity

Common protocols were devised and implemented to verify the impact of NPs on different types of human defence cells: T cells, epithelial cells, monocytes, macrophages, and dendritic cells. NPs genotoxicity and carcinogenicity were also assessed in murine Balb/3T3.

Partner USALZ has studied the reactivity of human T lymphocytes and lung epithelial cells to NPs, by using a panel of Jurkat T cell lymphoma and A549 human alveolar carcinoma cell lines stably transfected with the reporter genes Luciferase or GFP under the control of IL-8, TGF- β , IFN- γ promoters or the NF- κ B binding sequence. Control assays were run on human primary peripheral blood mononuclear cells, on the human acute monocytic leukemia cell line THP-1, and on human primary bronchial epithelial cells.

The reporter cell lines resulted suitable for detecting cellular stress and immunotoxicity effects in NP preparations. The Luciferase reporter gene used for most assays is highly sensitive and its optical readout, based on luminescence, did not exhibit any interference by the metal NPs tested here. In addition, a new GFP reporter cell line under control of the IL-8 promoter (marker for cell stress and inflammation) was created and successfully used for bulk tests, as well as for single-cell assays (together with Partner Fraunhoder).

Particles of known toxicity (Co and Ag NPs) were identified by the reporter cell setup to be toxic as well. The reporter gene assay, which is highly sensitive, technically easy-to-use, cheap, reproducible, robust and suitable for medium-to-high throughput screening, can have functions regarding several applications:

- use within a device to measure airborne particle pollution that combines physical information (NP size distribution and number) with information on their biological effects. To pursue this approach, Partners USALZ and Grimm have founded a start-up company (Cell-Mobile GmbH) based in Salzburg.
- aid the development of safe nano-based drugs and diagnostics.
- processing of large sample numbers, *e.g.*, for screening purposes.



• assess mixture toxicity, which may include NPs as well as any other particulate, chemical, or biological entities.

It should be mentioned that Partners USALZ and ICN have shown that residual solvent from synthesis can have a more significant effect on cell activation than the NPs under study. Particles were produced specifically for the project under highly reproducible conditions and by a dedicated research laboratory (Partner ICN). They were directly shipped to Partners and used by all Partners under strict protocols. Only the adherence to these strict protocols made it possible to uncover the source of the main effects in some assays, which were, from a strictly nanotoxicological point of view, an artifact. The objective of "Setup of common protocols" was for the DIPNA project a genuinely important accomplishment which contributes to the ongoing development of standard procedures in the emerging science of nanotoxicology.

Partner CNR studied the effects of acute exposure to NPs (1, 4, and 24 hours) on human primary blood monocytes and differentiated human CaCo-2 gut epithelial cells. The two cell professional and non-professional types represent innate/inflammatory defence cells, the endpoints of activation were chosen among innate/inflammatory genes and cytokines, in particular cytokines of the IL-1/IL-18 family (IL-1β, IL-18), their receptors (IL-18Rα, IL-18Rβ, IL-1RI, IL-1RAcP) and their regulators (IL-18BP, IL-1Ra, TIR8, caspase-1). Real-time PCR was used to assess gene expression, while ELISA, protein arrays and cytofluorimetry were used for protein detection. Particular care was taken in using only endotoxin-free NP preparations to avoid unintentional activation of responses by contaminants. Overall, no significant effects were observed with any of the NPs provided by Partner ICN in either cell type. A positive control (i.e., able to activate cellular responses) was represented by commercial large Co NPs (allegedly > 200 nm, which however were aggregated in larger particles upon depyrogenation and addition to culture medium). It was confirmed, besides the general lack of activity by NPs, that solvents tend to have some activity, a finding that calls for particular caution in evaluating results obtained with NPs. Additional caution is in order when interpreting the results obtained with test using an optical readout (e.g., ELISA), as it is important to either eliminate NPs before the assay (ultracentrifugation) or make sure that NPs do not interfere with readings and generate false results.

Partner VITO has investigated the *in vitro* maturation of human CD34-derived dendritic cells (DC) to IL-1 β plus TNF- α , in the presence of wet NPs. Maturation was assessed by cytofluorimetric evaluation of maturation markers. No significant activating effects were observed (Nelissen *et al.*, manuscript in preparation).

Using whole-human genome transcriptomics, insight was gained into immune-related genes and molecular processes that are induced in the bronchial BEAS-2B and alveolar A549 epithelial cell lines, and the CaCo-2 gut epithelial cell line, exposed to Cobalt and Cerium Oxide NPs (3, 6, 10 and 24 hours) at non-cytotoxic concentrations (Verstraelen *et al.*, manuscripts in preparation). By comparison of the gene expression profiles between the two lung epithelial cell lines a number of overlapping immune-related gene markers were identified that showed significant alterations in their expression levels in response to NP exposure as compared to solvent treatment (>1.5-fold and p<0.05), and were independent of the particle

type. These genes were related to the biological functions of antigen-processing and –presentation (e.g. *IFNG*, *HLA-DRB*3) and (cell surface receptor-linked) signal transduction (e.g. *PTPRC*, *FASLG*). Among the immune response markers that showed significantly altered gene expression following interaction of the studied NPs with the gut epithelial cells, about 20 markers were observed to overlap between the two particles, and two of them were in common with the lung cell models (*PTPRC*, *HLA-DRB*3).

In BEAS-2B cells the potential of NPs to induce cytotoxicity, production of reactive oxygen species, IL-8 protein secretion, and the mRNA expression of three oxidative stress markers (nfr2, HO-1 and NQO1) was investigated (Nelissen *et al.*, manuscript in preparation). Some of the tested NPs significantly induced cellular toxicity, oxidative stress, and inflammation compared to their respective solvent, and these reponses were observed to be dependent on the chemical composition (*e.g.*, cobalt NPs) or surface coating (*e.g.*, chitosan-coated silver NPs). Difficulties in the interpretation of the results was experienced due to the lack of positive control NPs, and several factors interfering with the test results were detected, such as solvent toxicity.

Partner COMC-IHC tested the toxic effects of NPs in standardised validated assays. During the course of the project, the Consortium asked Partner COMC-IHC to adopt the genotoxicity test (micronucleus) on human peripheral blood leukocytes, as this is much closer to the activity assays on human leukocytes run by other partners, rather than the initially proposed assay of Balb/c 3T3 (mouse fibroblasts). Results show that none of the wet NP preparations generated by Partner ICN, at any concentration, had a significant genotoxic effect. Assay of dry NP preparations was hampered by their clustering and stable aggregation when resuspended in liquid medium, which did not allow the visual evaluation of micronuclei on the slides. NPs were also radiolabelled and used for following their interaction with different types of cells (Balb/c 3T3, BEAS-2B, CaCo-2). All NPs were found to interact, with small differences depending on the type of NPs, their concentration, and the cell type. The standardised protocol thus shows that NP-cell interaction occurs and that this can be due to either uptake and internalisation, or to strong interaction with the cell membrane.

2.3 Single particle impact on cells

The overall objective of WP3 was the development of a technological platform to allow the investigation of small defined amount of NPs on a small defined number of cells (down to the single cell-single NP interaction) in order to get insights on the threshold of NP-induced cellular alterations.

Partner Fraunhofer developed and evaluated protocols for cell assays in miniaturised cell culture chambers to enable a sensitive determination of NP threshold causing toxic effects on single cells. Inflammatory effects of NPs on the lung epithelial cell line A549 were assessed to enable:

- a. implementation of microculture-based multi-parameter cell assays under reproducible conditions with statistically relevant numbers of tests, and
- b. integration of novel test protocols into laboratory work flow.

A microculture chamber array was designed, microfabricated and evaluated for the response to NPs of the IL-8-GFP A549 reporter cell line developed by USALZ. The chip-supported method resulted more sensitive and reproducible than assays performed in standard culture chambers. An image acquisition software was adapted and used for the quantitative evaluation of fluorescent cells, For single cell analysis, confocal laser scanning microscopy (CLSM) allowed to investigate the internalization of Gold NPs in single cells, and simultaneous immunohistological fluorescence analysis was performed to identify the cell activation associated to intracellular location of NPs.

The correlation between NP association to individual cells and single cell activation was studied upon exposure to Au NPs. However, no correlation was found, as NPs could be detected (by scanning electron microscopy) also in cells which did not show any GFP expression. The microcavity array also allowed evaluation of NP effects on cell membranes by single-cell impedance spectroscopy. A system for non-perturbing injection of NP into cells, using a gentle ultra slow cell manipulation system, was also developed. Barrier function of CaCo-2 cells was evaluated upon acute or chronic exposure to Ag and Au NPs using a transwell system (CellZScope) and measuring the transepithelial electrical resistance (TEER) of the cell layers under physiological conditions. No effect on the tight junctions was detected.

Partner CSEM developed a system for the deposition of NPs in array format on cell culture support, while Partner BioNEM evaluated the NP effects on cells physiology through microinjection techniques and Raman spectroscopy combined with principal component analysis (PCA). Microinjection of small numbers of NPs was performed, but no significant effects were detected. Raman spectroscopy combined with PCA analysis proved however to be an excellent and very sensitive tool for evaluating biochemical changes in selected areas of single cells.

2.4 Integrated platform for monitoring NP effects upon chronic and repeated exposure

Objectives of WPs were: 1. Field NP bio-detection systems, 2. Field NP selection systems, 3. Field flow chambers for NP-cell interaction, and 4. Optical sensors for detecting NP-cell interaction. USALZ and CNR selected an in vitro model based on differentiated CaCo-2 cells for evaluating the NP effects upon chronic and repeated exposure. The model on monocytes was considered less feasible because of the problems of sensitivity to biological contaminants of the NP batches (e.g., endotoxin) and for the fact that monocytes spontaneously and rapidly differentiate in culture and acquire different functional characteristics. On the other hand, the model based on differentiated CaCo-2 cells resulted a robust and reliable assay for assessing chronic and cumulative exposure to NPs in terms of regulation of inflammation-related genes and proteins. However, ideally the exposure schedule should be limited to 15 days. With this system, data of gene expression and protein production showed that NPs have no significant effect. It must be noted that solvents can have some effects that would obliterate or make uninterpretable the NP data.

In order to mimick a massive exposure to NPs, Partner CNISM-UNIMO has used a lipofection technique to obtain a significant



and homogeneous entrance of NPs in HepG2 or 3T3 cells (confirmed by ESEM and STEM observations). All wet and dry NP preparations, as well as and radiolabeled NPs, were used, and cell viability and proliferation were measured. A toxic effect was evident only with Cobalt NPs. Morphological observations confirmed the presence of NPs within cells. In the case of lipofection with Co and Ce NPs, the presence of nanosized precipitates of cobalt phosphate and cerium phosphate suggested NP corrosion with subsequent release of unstable ions readily combing with phosphorus and oxygen to create new chemical species. The presence of these new species is an important observation since similar entities were identified *in vivo* in human tumours and in rat tumours developing after implantation of metallic NPs.

An important effort was deployed by Partner Grimm to develop an exposure chamber to deposit airborne agglomerates of NPs on cell cultures. The system was equipped with a 5-stage preimpactor in order to reduce the concentrations of larger agglomerates (> 1 μ m) by more than two orders of magnitude obtaining a cutoff approximately at 400 nm.

A system was developed by Partner CSEM to assess the inflammatory effect of NPs on cells in culture, by quantifying the concentration of cytokines in the cell culture supernatant. Detection is based on a sandwich immunoassay on a surface, combined with the optical detection device WIOS. The WIOS chip was designed to allow detection of three analytes in one assay, by immobilisation of different capture antibodies. The A549 cells were used, upon stimulation with TNF α or NPs. An ELISA test for IL-8 detection was implemented as reference method and control. Production of IL-8 in the culture supernatant was analysed with the optical system and ELISA with excellent correlation. Three sandwich assays were implemented on the system, for IL-8, IL-6, MCP-1. The sensor surface for IL-8 was optimized for regeneration and can be used several times without loss of performances.

Highlights and Recommendations

It is important to assess the final state of NPs after interaction with cells. FEG-ESEM and SEM observations can show if NPs have been internalised or are on the cell surface. The features of the NP-cell interaction is important for correlating biological effects with NP presence. A warning should be issued on the high risk of data misinterpretation, due to the significant nonspecific interference of NP samples (solvents, unwanted contaminants, physical interference with optically-based assay readouts).

2.5 Field validation and development platform

Objectives of the WP5 were: 1. Integrating several sensing methods for determination of NP toxicity on a common platform, 2. Recommendations for a redesign of the integrated platform.

Partner CNISM-UNIMO took care of field analysis of occupational nanotoxicity in nanotechnological industrial settings. Ten industries allowed on-site analysis in working places, at chimney outlets, and in their immediate surroundings, which were performed using non expensive NP collectors (gravimetric sensors based on adhesive carbon discs, or active air pumps with NP trapping cellulose filters). ESEM and FEG-



ESEM observations identified the particles, their morphology, size, and chemical composition by means of an X-ray microprobe of an Energy Dispersive X-ray Spectroscopic System. Semiguantitative analysis of the elements present in the spectra was performed. Collected data were used to create a databank. NPs synthetized in a laboratory could be found also in nearby areas, probably transported by the operators. A personal active sensor of nanopollution was constructed by Pollution srl and used over several days. Sensor filters can be examined under a FEG-ESEM equipped with a special software (GSR) that provides the size of entrapped particles, their total number and chemical composition. This "NP exposimeter" can be activated as a service by a private laboratory already contacted. A small remote-controlled aircraft ("DIPNA nanodustfinder") was constructed for the detection and collection of the pollution out of the chimneys of nanotechnological industries.

Partner CSEM finished the breadboard system for the automatic detection of the nanotoxic potential of NPs.



Figure .1. The developed DIPNA breadboard system

The main DIPNA products that will be disseminated are:

1. Protocol for the handling and storage for NPs suspended in liquid; 2. Protocol for the handling and storage for dry NPs; 3. Protocol for cell preparation for the ESEM observations; 4. Protocol to assess the real uptake of NPs in 3T3 and HEP-G2 cells; 5. Protocol for cell preparation for the STEM observations; 6. Protocol to simulate a chronic exposure in cells to NPs; 7. Disperser to transfer dry NP powders in airborne state; 8. Preimpactor to select well defined size ranges from airborne nanomaterial: 9-Exposure chamber to deposit airborne nanomaterial onto cell cultures; 10. Platform technologies to allow sensitive and quantitative biological tests of NPs that include: a. cavity chip-based system for the determination of single cell response, b. interface for multi-cavity arrays to ELISA reader, c. bioreactor for testing the effect of NPs on cells dependeing on exposure profiles, d. optimised planar electrode chip for testing the effect on barrier function of cell layers, e. dynamic channel bioreactor in combination with a system for visualisation of NPs, f. chip-based determination of cell response of living single cells, g. gentle micro-injection of NPs in cells, h. bioreactor for testing the effect of different profiles of NP exposure on cell response, i. interface for multi micro cavity arrays to ELISA reader and microscope.

Other products obtained beyond the objective of DIPNA are :

1. Collectors for the passive personal exposure in working place.

- 2. Active sensor to detect the nanopollution in working place.
- 3. Aeroplane model equipped with an air pump to detect the air nanopollution.
- 4. Identification of reference zero standard of nanopollution.
- 5. Creation of a data bank of occupational micro and nanopollution.

The result exploitation is the following:

1. Partners Grimm and USALZ have founded in 2008 the startup company CellMobile GmbH with the purpose of designing, testing and placing on the market a cell-based assay that can be used to routinely monitor working-place safety. This business development is a direct consequence of work performed within (http://www.cellmobile.at/). Cell-Mobile develops DIPNA biological measurement procedures for monitoring health relevant parameters for the safety on the working places. The procedure is based on the use of stable Reporter Cells, developed by Partner USALZ and adapted for NP assessment during DIPNA. These cells present the following advantages: robust, reproducible method, reasonably priced, suitable for routine work, measurements on site are possible, results are immediately to see, no need for a special labor, no need for a special trained personnel. In combination of existing physical measurement methods with biological procedures, a new quality in the monitoring from the inhaled air is the result. CellMobile is starting the development of devices suitable for monitoring the safety on work places, environment and general

safety monitoring of the inhaled air in public buildings.

2. Partner CSEM has founded the start-up company Dynetix **AG** to construct and commercialise the prototypical instrument for the automatic evaluation of NP toxicity. The company is located in Switzerland. Also in this case, internal resources were found. CSEM developed a system able to perform nanotoxicological analysis at the working place. Starting from the work performed in WP4 on the application of the WIOS system for the detection of IL-8 in cell culture medium, a breadboard set-up was realised for the automated detection of IL-8 and other cytokines (IL-6 and MCP1) in the cell culture medium of two flasks (one is used as reference, the other as the test culture). This breadboard includes a miniaturised incubator with controlled CO₂, temperature and humidity. A fluidic system allows sampling a few ml of the cell culture medium for analysis. Two types of analyses can be done: one using a WIOS platform functionalised with antibodies was implemented for the detection of cytokines in the cell culture medium, the other is based on light absorption and Alamar Blue for the measurement of cell proliferation. A software written in LabView was developed to control the fluidic system and the two types of measurements. The system can monitor the cell culture during several days measuring about every hour the cytokine in cell culture medium and the cell proliferation at the end. This system can be used not only to look at the effect of NPs on cells, but also for the detection of the effects of contaminants (chemicals) on cell cultures in an automatic way.



2.6 Coordination, management, training and public awareness

The project was coordinated by Dr. Antonietta M. Gatti of the CNISM of the University of Modena and Reggio Emilia, and contributed improving the knowledge of nanotoxicity.

The educational and training activities were basically the following: 1. recruitment of students and young researchers for the DIPNA experimental activities; 2. training-by-research; 3. participation to congresses; 4. participation to scientific publications.

The DIPNA contribution to policies is represented by Consensus Paper that summarizes the implications of the DIPNA results and provides suggestions and tools for new safety standards. The results of the DIPNA project mostly contribute to EU policies with:

- recommendations for the NP handlings in nanotechnological laboratories. They can be translated to the nanotechnological industries, but an adaptation is necessary case by case, when a preliminary investigation in the occupational sites identifies nanopollution and a possible risk for nanoworkers.
- 2. reference protocols for nanotoxicity tests (acute exposure with transfected A549 cells, chronic and cumulative exposure with differentiated CaCo-2 cells, massive acute exposure with lipofected HepG2 cells). Each of such protocols, after appropriate validation, could be adopted as standard test.

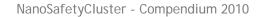
- 3.a prototype for an automatic test to detect the possible toxicity of NPs according to biological parameters, a nanodispenser to be used in other nanotoxicological tests, and sprayers of NPs in air. They can represent tools for standardised tests.
- 4. passive and active collectors of NPs in occupational sites to be used in nanotechnological enterprises.
- 5. software to identify and quantify NPs in an air filter that can represent the basis for the identification of threshold concentrations of nanopollution and therefore give standard limits in occupational sites.
- 6.Creation of a databank for occupational micro and nanopollution that can be used for traceability of human exposure.
- 7. recommendations for workers' safety in occupational sites and for nanotoxicologists.

The web page of DIPNA (www.dipna.eu) contains the most important project results and is (and will be) a reference point for other scientists and common people. It is divided in a public and personal area. In the public area there is a summary of the results that will be updated as soon as the Officer gives his approval to the final DIPNA report. It already contains the recommendations for NP handling for scientists and nanoworkers in occupational sites ("Protocol for the handling and storage for NPs suspended in liquid" and "Protocol for the handling and storage for dry NPs").

3 Directory

Table 1 Directory of people involved in this project.

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ENNSATOX

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



Contract Agreement: NMP4-SL-2009-229244

Website: http://www.ennsatox.eu

Coordinator: Dr 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

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1 Overview

1.1 Start and End Dates

Start date:	1 st July 2009
End date:	30 th June 2012
Duration:	36 months

1.2 Size

€3,655,316 Total Budget €2,816,500 EC Contribution

2 Summary

The use of engineered nanoparticles 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 a) between 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; and, 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/cae/servlet/contentblob/717964/publication File/48609/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), to be administered by the Department of Environment, Food and Rural Affairs (DEFRA). On 17 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. on 20 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 focuses 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/nanocoderecommendation-peo894co8424en.pdf for the purpose of future regulation by the EU (REACH Directive) and Member states.

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

The philosophy of ENNSATOX's work plan is to initially produce and thoroughly characterise different morphologies and sizes of a model nanoparticle, such as zinc oxide (ZnO), using the most advanced state-of-the-art methods in physical chemistry and microscopy. This will be extended to additional classes of nanoparticles in particular silicon dioxide (SiO₂) and titanium dioxide (TiO₂). At the same time the programme will look at the



nanoparticles' activity towards a series of biological models of increasing complexity and organisation. Next, the behaviour of the nanoparticles in environmentally relevant aquatic systems will be examined 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 patented¹ as a generalised toxicity testing procedure which can be applied to investigate the activity of nanoparticles. We see a major outcome of this study as the delivery of calibrated and accredited toxicity testing protocols for nanoparticle biological activity. A very recent SETAC World Congress in Sydney (August 2008) had an extensive session on nanomaterials and it was apparent that there were many issues to be addressed concerning how the materials should be tested for biological activity and the mechanism of toxicity. ENNSATOX therefore has a great opportunity to make advances which could be a significant asset commercially.

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.

In order to accomplish the challenge ENNSATOX has assembled a group of RTD performers of unprecedented excellence from across Europe:

- 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).

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



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, biopersistence 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? and,
- 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 will be 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 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) to follow their behaviour and fate in the respective systems. Figure 1 shows a characteristic image from this study of ZnO nanoparticles.

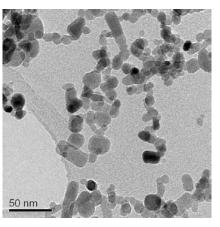
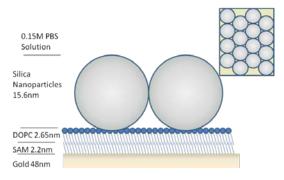
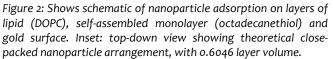
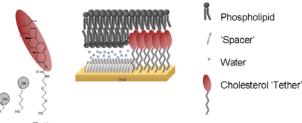


Figure 1: Shows TEM Bright field image of ZnO nanoparticles on a holey carbon film.

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).







Spacers Tether

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

Wageningen has considerable expertise in surface and colloid chemistry and extensive expertise modelling membrane interactions, and are responsible therefore for correlating the model membrane-nanoparticle interactions with theoretical



mathematical models using self consistent mean field theory². The form, structure and functionality of the particles will be related to their activity towards the model membrane systems. Anton Dohrn will examine 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 SiO₂ nanoparticles adsorbed on to a cyanobacterium membrane.

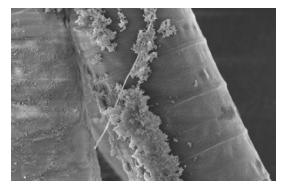


Figure 4: Shows Oscillatoria princeps incubated with SiO2. Cells separated by septa (arrowed). Right filament partially covered by SiO2, left filament completely covered.

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 5).



Expose cells to nanoparticles in culture

Harvest treated cells and embed in agarose gel on slide

Cells embedded on slides then lysed

DNA unwinding in alkaline buffer

Electrophoresis in alkaline buffer



DNA damage measured with fluorescence microscopy and computer based image analysis software

Figure 5: 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 available to the project, with considerable world expertise in electrophysiology.

WP4: Interactions with *in vivo* models. The *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 will be 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.

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 has extensive experience in the relationship between the chemical speciation of dissolved and particulate material in 'natural' waters and its bioavailability. They will study 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.

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, predictions as to the impact of the nanoparticles on the aquatic environment can be made. The model (see Figure 6) 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 'natural' waters, biointerface (the cell membrane, cell organelles, total cell, and tissue) and model aquatic organisms. The model based on ECoS3 (developed by Plymouth Marine Laboratory) allows the set-up and integration

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.



of sets of advection–diffusion equations representing multiple constituents interacting in a spatial context³.

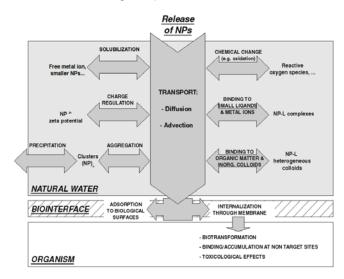


Figure 6: Shows physicochemical processes of relevance for the fate and behaviour of NPs in the environment used in the WP6 model.

WP7: Exploitation and pre-validation. The Marine Biological Association (MBA) 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 will include considering all the above issues as well as developing accredited toxicity tests and assays for these particles in the aquatic situation. An important output will be aiding environmental legislation on these materials. Another important outcome will be guidance as to effective means of calibrating and accrediting the toxicity testing procedures being developed.

WP8: Dissemination. Although this work package concerns dissemination it will also feed into WP7: Exploitation & Prevalidation. It will identify 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 will therefore also serve 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 dayto-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 7 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.
- 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.

With the following having been achieved within the first six months of the project:

- Development of nanoparticle characterisation protocols.
- Development of nanoparticle handling protocols prior to toxicity assays.
- Development of initial nanoparticle screening methods and initial structure: activity relationships.

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



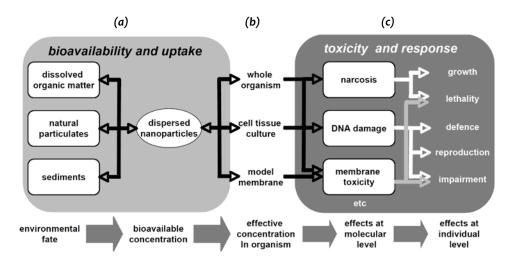


Figure 7: Shows the environmental discharge and behaviour of the nanoparticles in the left hand compartment (a); the impact of nanoparticles on the biological barriers in between the two compartments (b); and, on the aquatic organisms in the right hand compartment (c).

3.4 Conclusion To-Date & Plans for the Future

Initial conclusions from the first six months' work associated with relevant work package are outlined below:

WP1: In order to ensure stability of SiO_2 and TiO_2 particle dispersions it is necessary to purify the dispersions, which can be done by dialysis or gel filtration. SiO_2 dispersions change their properties over time. Cleaning SiO_2 particles using gel filtration ensures a more stable dispersion with constant particle size. Methods for determining ZnO nanoparticle solubility using Anodic Stripping Voltammetry (ASV) in a flow cell have been developed.

WP2: LUDOX SiO₂ particles particle diameter ~30 nm adsorb on to phospholipid membranes and bacterial cell walls (see Figure 4). DEGUSSA P-25 TiO₂ particles (50:50, anastase:rutile) do not show significant adsorption on to phospholipid membranes. ZnO particles show no apparent effect on the structure of phospholipid membranes although a release of soluble Zn^{2+} is observed. SiO₂ particles from different batches gave distinctly different responses on phospholipid membranes. An online method for measuring the biomembrane activity of nanoparticles has been perfected. Measurements of the biomembrane activity of six groups of purified and well characterised SiO₂ nanoparticles have been carried out. The kinetics of the SiO₂ biomembrane activity has been measured and is related to the particles' characteristics and form.

The scientific work plan for the next 6-months is as follows:

WP1: Water solubility of ZnO nanoparticles of varying size and functionality to be determined. Provision of a set of well characterised single class of nanoparticles.

WP1/WP2: Full comprehensive relationship between the structure and functionality of SiO₂ and ZnO particles and their biomembrane activity and their *in vitro* and *in vivo* biological activity to be developed.

WP2: Initial toxicity screens to be developed.

WP3: Detailed assessment and preliminary feedback of *in vitro* test results to **WPs 2** and **4**.

WP4: Measurement of acute toxicity of nanoparticles to representative aquatic organisms and effect on acute species sensitivity distributions.

WP5: Quantitative relationship between the charge of NP and pH of the medium, NP binding isotherms for cationic and anionic counter-ions.

WP6: Compilation of tables of characteristic parameters in the studied systems.

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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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	ЮМ	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 will be linked to the project by a Memorandum of Understanding signed by their legal representatives		
18	US Environment Protection Agency	EPA	US
19	National Institute of Occupational Safety & Health	NIOSH	US
20	National Institutes of Health - National Institute of Environmental Health Sciences	NIH-NIEHS	US
21	The Woodrow Wilson Center	WWC	US

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:	2.1 Aim and Objectives		

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 wellknown 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 exposuredose-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:

USTOURN P

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 exposuredose-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.

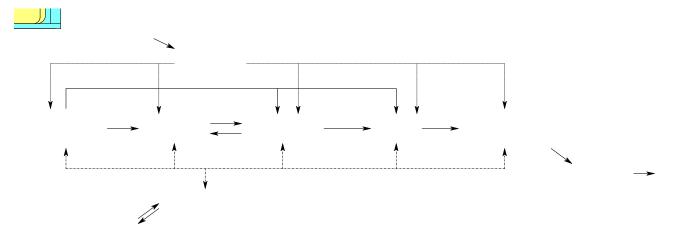


Fig 3. Flow chart describing the information flow between different WP of ENPRA (solid lines) and the process of coordination, management and collaboration (dotted lines)

3 Directory

 Table 1 Directory of people involved in this project.

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ENRHES

Engineered Nanoparticles: Review of Health and Environmental Safety

Contract Agreement: CSA-SA 218433 ENRHES Website: http://nmi.jrc.ec.europa.eu/project/ENRHES.htm Coordinator: Professor Vicki Stone, Edinburgh Napier University. Scientific Coordinator: Dr Steve Hankin, Institute of Occupational Medicine.

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3	Technical University of Denmark	DTU	Denmark
4	European Commission Joint Research Centre (Institute for Health and Consumer Protection)	IHCP-JRC	Italy
5	Institute of Nanotechnology	ION	United Kingdom

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1 Summary

The Engineered Nanoparticles: Review of Health and Environmental Safety (ENRHES) Project was a 12 month project which commenced in August 2008, with the final report published in January 2010. ENRHES presents a comprehensive and critical scientific review of the health and environmental safety of four classes of nanomaterials: fullerenes, carbon nanotubes (CNT), metals and metal oxides. The review considers sources, pathways of exposure the health and environmental outcomes of concern, followed by a risk assessment based upon this information. The report includes an illustration of the state-of-the-art as well as on-going work, while identifying knowledge gaps in the field. Prioritised recommendations have been developed and set in the context of informing policy makers in the development of methods to address exposure as it relates to the potential hazards posed by engineered nanoparticles, and in the development of appropriate regulation.

The review's findings strongly support the further development of thorough characterisation (including proper considerations of agglomeration/aggregation) of the nanoparticles in exposure media when conducting exposure assessment, as well as in the generation of data for determining exposure to both humans and the environment as well as assessing hazardous properties. This is a crucial prerequisite for carrying out a meaningful assessment of the risks. Further testing strategies are required to be established to cover all relevant endpoints needed for a risk assessment. At present, carrying out risk assessment of nanoparticles can only sensibly be done on a case-by-case basis. Only when more data becomes available may it be possible to group nanomaterials according to their physical, chemical and/or biological properties or mode of action, so that testing could be done for representatives of each group.

2 ENRHES Overview

2.1 Background

Nanotechnology is a new and fast emerging field that involves the design, production and use of structures at the nano-scale i.e. 1 to 100 nanometres (nm)¹. Nanotechnology is a sector of the material manufacturing industry that has already created a multibillion \$US market, and is widely expected to grow to 1 trillion \$US by 2015. Nanoparticles are defined as particles with all three external dimensions in the nanoscale, while nanoobjects are discrete pieces of material with one or more external dimensions in the nanoscale, such as nanotubes. These definitions are provided by the British Standards Institute (BSI)². To put the size of nanoparticles into perspective, a human hair is typically 80000 nm wide, a red blood cell has a diameter of 7-8000 nm, while virus particles are similar in size to many nanoparticles, with maximum dimensions of 10 to 100 nm.

Due to their small size, nanoparticles exhibit novel properties that are often vastly different from their bulk counterparts (larger sized particles with the same chemical composition), such as high tensile strength, low weight, high electrical and thermal conductivity, and unique electronic properties, the discovery of which has led to widespread interest in their potential commercial and industrial applications. Nanoparticles tend to be more reactive than the corresponding conventional forms due to two main properties: i) per unit mass, nanoparticles have a much higher surface area and thus a greater proportion of constituent atoms exposed to the environment on the surface; and ii) quantum effects appear to become more important at the nano-scale, particularly for nanoparticles at sizes of less than 10 nm, resulting in constrained bonds which are more likely to be disrupted.

Many applications of nanotechnology involve the use of both nanoparticles and nano-objects. In fact, numerous nanoparticles are already on the market, in products such as paints, sunscreens, cosmetics, nanomedicines, self-cleaning glass, industrial lubricants, advanced tyres, semiconductors and food. This proliferation of nanotechnology has prompted concerns over the safety of engineered nanoparticles where exposure to humans and/or the environment occurs intentionally or accidentally.

In 2004, the Royal Society and the Royal Academy of Engineering published, at the request of the UK government, a major review of the opportunities and uncertainties of nanotechnologies3. This was one of the first reports to highlight the potential risks to health and the environment that



may arise from exposure to nanomaterials, especially nanoparticles (which included nano-objects such as nanotubes). Since then, more than 50 national and international reviews carried out by government departments, industry associations, insurance organisations and researchers have considered nanoparticle risk issues. These reviews have provided a remarkably consistent view about the nature and the potential risks of nanoparticles, which may be summarised as follows:

There are potential risks to human health and the environment from the manufacture and use of nanoparticles;

There is a lack of knowledge about what these potential risks might be and how to deal with them;

The lack of data makes it difficult for manufacturers, suppliers and users to have effective risk management processes and to comply with their regulatory duties;

All of the stakeholders (regulators, companies) need to start to address these potential risks now.

Since publication of the joint Royal Society and Royal Academy of Engineering report, there has been a significant increase in research activity in the UK and internationally, intended to fill these gaps. A great deal of emphasis has recently been placed on the need for research in the field of nanoparticle risk assessment, particularly evident in the European Union through the Sixth and Seventh Framework Programme calls, and initiatives elsewhere around the world. Outputs from this research can further contribute to the field's evidence base through the conduct of timely and comprehensive reviews that assimilate the wealth of scientific data on health and environmental implications of manufactured nanomaterials alongside knowledge of materials' production, application and resulting potential new exposure pathways.

2.2 Aims and Objectives

The overall aim of the ENRHES project was to perform a comprehensive and critical scientific review of the health and environmental safety of four classes of nanomaterials: fullerenes, carbon nanotubes (CNT), metals and metal oxides. The review considers sources, pathways of exposure, the health and environmental outcomes of concern, illustrating the state-of-the-art and identifying knowledge gaps in the field, in order to coalesce the evidence which has emerged to date and inform regulators of the potential risks of engineered nanoparticles in these specific classes.

The specific objectives of the ENRHES project were to review information on:

- production, use and exposure to the target engineered nanomaterials;
- persistence, bioaccumulation, toxicity (i.e. PBT) and interactions of the engineered nanoparticles in living and environmental systems;
- differences in toxicity posed by variations in physicochemical characteristics.

¹ BSI. 2007, Terminology for nanomaterials, PAS 136:2007, British Standards Institute, UK.

² BSI. 2007, Terminology for nanomaterials, PAS 136:2007, British Standards Institute, UK.

³ RS/RAE. 2004, Nanoscience and nanotechnologies: Opportunities and uncertainties, The Royal Society and The Royal Academy of Engineering, London, UK.



The final objective of the project was to perform a coherent evaluation of the feasibility of conducting a regulatory risk assessment for each material type and perform basic risk assessments to the extent possible based on the information presented within the review.

The final report provides an overview of the current state of knowledge concerning exposure to nanoparticles and ongoing work in the area. Prioritised recommendations have been developed and set in the context of informing policy makers in the development of methods to address exposure as it relates to the potential hazards posed by engineered nanoparticles, and in the development of appropriate regulation.

2.3 Review methodology

2.3.1 Co-ordinated information management

Information was collected from the peer-reviewed literature in the public domain that was relevant to the health and environmental safety of each of the four material types.

At the outset of the review, the project team agreed a set of search terms for each work package theme (physico-chemical characterisation; production, use and exposure; toxicology; epidemiology; ecotoxicology; and environmental fate and behaviour). These were derived from recognised standard terminology and nomenclature (e.g. documents from the BSI nano 9 collection, CEN ISO/TS 27687 and ASTM E2456-06) and the US National Library of Medicine's Medical Subject Headings. These terms were converted into a specific Boolean search strategy (using the agreed search terms in combination with logic operators e.g. AND, OR, NOT) for each theme. The structured literature searches for all WPs were completed by the end of December 2008. No structured Boolean literature searching was undertaken after this date. However, authors maintained a general awareness of the literature relating to their technical area and tasks throughout the writing stages and any newly published literature of high relevance was considered for inclusion based on meeting the following criteria: i) the content appeared to provide novel information within a particular area that was not previously encountered within the literature; or ii) the content was able to provide confirmation of findings of other investigators.

The preliminary Boolean searches for each theme were undertaken in web-based sources, namely PubMed and Web of Knowledge. All searches and search results (in terms of numbers of references obtained) were recorded using Microsoft Excel spreadsheets. In the majority of cases, the initial searches resulted in the collection of extremely high numbers of papers, including a high proportion of irrelevant literature. This was due to the relatively broad initial search strategy employed. The Boolean search strategy for each theme was therefore further refined through assessment of the references and consultation with the review task leads.

The refined Boolean searches were then carried out and the obtained references imported into the online reference

management software 'RefWorks', categorised according to each theme. Through utilising the expertise of the report authors, the Refworks lists were further complemented with:

- additional literature that was known to be relevant to the area under investigation, but had not been obtained through the Boolean search strategy;
- other papers of interest, brought to the attention of the authors through reading of the available literature contained in the RefWorks lists;
- iii) results of more specific PubMed/Web of Knowledge searches, required to more fully develop the understanding of particular aspects of the literature, or provide further confirmation of findings of other investigators.

Following removal of reference duplicates within the individual Refworks folders, the review task leaders then selected a final set of references after initial assessment of the title and/or abstracts. These were converted into final electronic reference lists within RefWorks for each of the review tasks. Links to these lists were included in a Wiki platform, creating a shared reference resource for the project that allowed authors to access references from any of the sourced literature as required.

Provided in Table 1 are the number of papers obtained through the initial Boolean searches for each review theme, followed by the number of papers obtained after refinement of the search terms and final selection by the review task leads.

Table 1 Summary of the number of references obtained	Table 1 Summar	y of the number	r of references obt	ained
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Review Theme	No. of papers	No. of papers	No. of papers
	from initial	after search	identified of
	search	term	possible value
		refinement	for reviewa
Nanoparticle	~ 25, 000	1,815	240
Characterisation			
Exposure	~ 1,500	779	36
Fate and	~ 40, 000	3,776	294
Behaviour			
Toxicity	~19,000	558	111
Epidemiology	~ 14, 000	5,329	62
Ecotoxicity	~ 80, 000	6,105	89

a Additional references may have been identified independently by chapter authors

The implemented information management strategy served to assist the component activities of the review by means of establishing and maintaining a shared reference resource for the review that:

- constituted a common resource accessible remotely and in real time by all partners;
- employed a standardised and efficient searching process;
- added value by combining the reference-sourcing capacity of all partners;
- allowed cross-referencing of resources appropriate to any of the multiple review tasks;
- reduced duplication of reference sourcing.

2.3.2 Review activity

The objectives of the review were to appraise information on the production, use and exposure to the target engineered nanomaterials, persistence, bioaccumulation, toxicity (i.e. PBT) and interactions of the engineered nanoparticles in living and environmental systems.

Review of Materials Production, Use and Exposure

To establish the context of the hazard data for the review, literature on the production, use and exposure routes for the four material types was reviewed. Specifically, the following information was identified and reviewed:

- the types of nanomaterials in common production and their applications, by conducting a focussed survey with the nanotechnology industry. This was carried out by the Institute of Nanotechnology, to provide an objective and non-biased contribution to the review, that minimises the risk of conflicts of interest (e.g. commercial confidentiality issues from individual company representation) and maximises the benefit from integrating with relevant initiatives and recognised expertise. A more detailed methodology for this task is outlined in Appendix 2;
- methodological aspects of nanoparticle characterisation;
- the types of scenarios which may lead to human exposure and the nature of these exposures and their measurement;
- the transport of nanomaterials in indoor and outdoor air;
- the potential for materials to leach from soils to ground waters;
- the potential movement from soils to surface waters;
- the transport mechanisms in water bodies.

Review of Toxicity Data

- Available information pertaining to particle, ultrafine and nanoparticle toxicology across the four material types was reviewed, specifically literature relating to:
- methodological aspects;
- toxicokinetics of uptake (ingestion, inhalation, dermal adsorption and injection), distribution, metabolism and excretion of manufactured unfixed nanoparticles and nanotubes in and by the body. The relationship between route of exposure and toxicokinetics as well as subsequent toxicity was also investigated;
- persistence and bioaccumulation potential of nanoparticles and nanotubes in the body;
- evidence of genotoxicity and reproductive toxicity;
- differences in toxicokinetics and any subsequent toxicity posed by variations in nanoparticle size, physical structure, chemical composition;
- mechanisms of interaction of nanoparticles with cells and their components, and partitioning within and between tissues in organisms;
- mechanisms of nanoparticle induced toxicity in relation to nanoparticle physico-chemical characteristics (e.g.



size, surface area, surface charge, length etc.), in order to generate an improved understanding of the potential for nanoparticles to induce toxicity. Many of the studies published to date focus on acute endpoints related to inflammation, but chronic hazards have also been considered where there was available information.

Due to the paucity of data in relation to human exposure and toxicology, animal and in vitro studies have also been used and interpreted as appropriate. Information from older papers, in relation to model pollution particles (e.g. carbon black) and occupational dusts has been expanded to allow the understanding of the relatively small literature on engineered nanoparticles. As nanotubes are reported to have fibre-like morphology, a short summary of the major toxicological issue relating to fibres has also been included, in order to put into context the concerns relating to nanotube hazard.

Where possible, special attention was provided to papers conducting dose response relationships that might be relevant for risk assessment purposes.

Review of Ecotoxicity Data

The review of literature relating to the ecotoxicity of manufactured nanomaterials across the four material types, specifically pertaining to:

- the persistence, bioaccumulation and toxicity of manufactured nanoparticles in aquatic and terrestrial species, including invertebrates, vertebrates and plants. Special emphasis has been put on aquatic base set organisms used in the risk assessment of chemicals (i.e. fish crustacean, algae);
- differences in ecotoxicity posed by variations in nanoparticles' physico-chemical characteristics (e.g. chemical composition, size, shape), test conditions (e.g. static, renewal or continuous exposure) and organisms (e.g. species, ages, feeding).

Again, where possible, special attention was provided to papers conducting dose response relationships that might be relevant for risk assessment purposes.

Review of Epidemiology and Human Studies Data

In reviewing the epidemiology and human studies data, the project have examined:

- methodological aspects;
- published results on the epidemiology studies on carbon black and titanium dioxide industries;
- published data on environmental and occupational exposure to diesel exhaust particulates;
- epidemiological studies on particles with a known nano size range such as metals will be examined, including any available information on exposure to emerging engineered nanoparticles.

In all cases, case-reports of exposure-health effect relationships were identified for each study, where possible. The available human-studies tend to be short-term inhalation studies on



human volunteers. Most importantly, information on human dosimetry was also examined by considering existing studies of mathematical models of particle deposition, including those related to nanoparticles.

Risk assessment appraisal

The objective of this stage of the review was to perform a coherent evaluation of the feasibility of conducting a (regulatory) risk assessment for each material type and perform basic risk assessments to the extent possible based on the information presented within the review chapters. More specifically, the exercise has evaluated the extent to which a traditional risk assessment/chemical safety assessment can be made based on available information and the specific nature of the nanomaterials.

The risk assessment appraisal has been based upon the information presented within the review chapters and it was outside the scope of the exercise to study again the original literature. Consequently, also no further literature searching has been conducted and thus the cut-off date of December 2008 also applies to this section.

The exercise aimed to conduct a regulatory risk/safety assessment as would apply in relation to chemicals policy. This means that in principle the entire substance life cycle should be addressed, including manufacturing, downstream and consumer use of the substance on its own, in preparations and in articles, as well as the final disposal. However, the exercise has been further scoped based on the data available for the different life cycle stages. Specific applications (e.g. for pharmaceuticals, cosmetics, pesticides, biocides) are not normally considered in a regulatory chemical legislation risk/safety assessment and have therefore not been addressed. As data for physico-chemical hazards (flammability and explosivity) was not included in the review chapters, the risk assessment appraisal has not addressed these types of hazards/risks. It was also not the purpose to attempt suggesting classification and labelling of the studied nanomaterials. The risk assessment exercise has been conducted for Fullerenes, CNT, and the most data rich substances from the metal and metal oxide nanoparticle categories.

The basic risk assessments carried out in the ENRHES report were inspired by the REACH Guidance on Information Requirements and Chemicals Safety Assessment⁴, following its general methods and structure. However, given the availability of information (which is not complying with the REACH requirements both in terms of serious lack of knowledge of use and exposure, as well as data on inherent properties), the detailed assessments have been adapted to the available information and taking into account the overall structure of the ENRHES report. In a way the assessments therefore more resembles a risk assessment as carried out under the old chemicals legislation (for "existing substances") where the authorities did an assessment based on the available information. A qualitative and/or quantitative risk characterisation was conducted to the extent data allowed. Uncertainties and possible gaps in data and methodology have been identified and described. Based on the outcomes of the appraisal, recommendations for focus and further research have been elaborated and presented.

2.4 Executive Summary

Engineered Nanoparticles: Review of Health and Environmental Safety presents a comprehensive and critical scientific review of the health and environmental safety of four classes of nanomaterials: fullerenes, carbon nanotubes (CNT), metals and metal oxides. The review considers sources, pathways of exposure the health and environmental outcomes of concern, followed by a risk assessment based upon this information. The report includes an illustration of the state-of-the-art as well as on-going work, while identifying knowledge gaps in the field. Prioritised recommendations have been developed and set in the context of informing policy makers in the development of methods to address exposure as it relates to the potential hazards posed by engineered nanoparticles, and in the development of appropriate regulation.

The review first provides context for the materials chosen, in term of the **production techniques**, **applications and market value**. This is supplemented with the findings of an industry survey carried out in an attempt to gather up to date information on the quantities of various types of nanomaterials produced and used, the type of products in which they are used, any exposure data gathered and risk assessment practices employed. Whilst useful to an extent, the survey received a limited response and does not provide a complete overview of nanomaterial production and use worldwide.

The review subsequently highlights the essential role which nanoparticle characterisation plays in a variety of overlapping contexts ranging from fundamental and applied research, through process and product quality control and commercialisation, to health and environmental protection. The review highlights the effort being made towards improving the characterisation basis for toxicological studies, such as identifying the key physico-chemical characteristics of nanoparticles and how they can be measured. The body of literature published confirms that there is now a consensus that thorough and accurate particle characterisation is an essential part of assessing the potential toxicity of nanoparticles in biological systems. Appropriate and common characterisation of test materials is important to ensure that results are reproducible, and also to provide the basis for understanding the properties of nanoparticles that determine their biological effects. Some of the key physico-chemical parameters influencing the biological activity of nanoparticles remain unknown or to be fully understood at this point. Hence, the characterisation of test materials should be as thorough as possible and broad in scope. The review identifies the basis of a minimum set of characteristics that should be measured for test materials used in toxicity studies. In addition to composition, these include size and shape, state of dispersion, surface area, and surface chemistry.

⁴ http://guidance.echa.europa.eu/docs/guidance_document/information_ requirements_en.htm?time=1252482386, accessed 16th October 2009

In the context of **exposure assessment**, the review shows that there is, in general, a paucity of published data. For the materials of interest, eleven studies were identified which have reported measured exposure data. All of these are in the occupational setting, while no studies have reported consumer exposures or exposures in the environment. All but one of the studies have reported inhalation exposure only; one study reported dermal exposure and no studies reported ingestion exposure. Most of the studies were carried out in university settings, however, some industrial settings were also found. A wide range of instruments and approaches were used and exposures were reported in terms of number, mass and surface area concentrations, as totals and differentiated as a function of Most studies showed some evidence of elevated size. exposures although these were often associated with ineffective or deliberately disabled control systems. Studies which have assessed the effectiveness of respiratory filters have shown that, as theory predicts, they are efficient collectors of nanoparticles. Exposures are clearly plausible in occupational, consumer and environmental settings throughout the lifecycle of materials and products. The review also considers modelling studies and identified two which provide useful information relating to environmental and consumer exposure. Recommendations are made for further occupational, consumer and environmental exposure assessment to support effective risk assessment and characterisation.

Similarly, the review highlights the general paucity of data in the area of environmental fate and behaviour, which represents a major obstacle in developing a holistic view of the fate and transport of nanomaterials within the environment and therefore environmental exposure. Current knowledge of transport of nanomaterials within air, soil and water compartments is rooted in aerosol and colloid science. This background is used to provide preliminary information from which further understanding of nanoparticles' fate and transport can be developed. With respect to the aquatic environment, one consistent finding is that most nanomaterials interact with natural organic matter and other materials found in the environment, and that this influences the fate and transport of namomaterials in water and may also be of significance for their biological effects. The review identifies the need for systematic studies to be undertaken on different types of nanomaterials using a range of physico-chemical parameters (e.g. size, shape, form, surface area), in order to generate data which will support development of reliable and truly relevant Predictive modelling of emission scenarios and models. subsequent transport pathways will play an important role in furthering understanding of this area.

A substantial appraisal of the toxicity of nanoparticles is presented for each nanomaterial class. The review evaluates the toxic potential of the four classes of nanomaterials and identifies the underlying mechanisms driving each of their toxicities, and determines whether any generalisations can be made regarding nanomaterials as a whole. In addition, the review reveals material or particle specific attributes that are particularly relevant in driving nanomaterial toxicity, therefore allowing identification of key characteristics that can influence safety. In an attempt to achieve this, available information regarding the exposure conditions and characteristics of nanomaterials used within the described studies has been



outlined. The review highlights discrepancies regarding the dose metrics used when expressing the concentration of particles exposed to cells or animals, specifically whether dose is based upon the mass, surface area, or particle number administered. This is of relevance as it has been repeatedly demonstrated that the toxicity of particles is related to their size, so that as particle size decreases, toxicity generally increases, which is thought to be driven by their surface area. However, nanomaterials are a diverse group of materials, and it has become evident that other particle dimensions are also important in driving their toxicity, such as length. This has been clearly demonstrated in using both in vitro and in vivo studies of CNT. Surface functionalisation of nanomaterials also alters their potential toxicity (e.g. fullerenes), although at this time the mechanisms of such changes are not understood. Furthermore, the tendency of nanomaterials to agglomerate or aggregate is of concern, and has encouraged investigation into improving nanomaterial suspensions, including the use of dispersants, solvents, surface modification or mechanical processes.

As exposure to nanomaterials is expected to primarily occur through dermal, inhalation, ingestion or injection routes, the focus of the toxicological review employs studies using the lungs, skin, gastrointestinal tract (GIT), or blood as routes of entry, with the inclusion of both in vitro and in vivo models for each route. However, the realisation that nanomaterials can distribute from their exposure site, within the blood or even nerves, means that nanomaterial toxicity may be exerted at a number of targets including, for example, the liver, brain, spleen, and kidneys.

The review has considered the latest studies which have sought to assess the toxicity of nanomaterials including the utilisation of both in vivo (within mice and rats) and in vitro models (using cell lines and primary cells). The evidence-base from particle toxicology combined with the use of models provides a useful series of protocols to allow benchmarking of new nanomaterials to the relative potential toxicity of other substances of known hazard. Cell lines are frequently used to investigate the effects of potentially toxic substances. The types of cells considered in the review are diverse and represents a wide range of organ and cell types, including tumour derived and transformed cells. Their response is often representative of the in vivo response, but careful comparisons and controls are required to ensure relevance. The review highlights the limitations in using in vitro cytotoxicity (cell death) for risk assessment purposes, even if benchmarked against a material of 'known' risk, is questionable, as very few particle-induced diseases are associated with acute immediate cell death. Instead, sub-lethal effects measured in vitro are shown to have useful potential for risk assessment purposes. Measures of cytotoxicity are, more useful to ascertain sub-lethal concentrations for further investigation rather than explicitly for risk assessment purposes. Most pathological particles act via the induction of cellular and molecular changes such as oxidative stress and/or the induction of inflammation, both of which can lead to disease. These endpoints have therefore been assessed with greatest interest and highest priority, when assessing the toxicity of nanomaterials.



When appraising the available epidemiology and human studies, the paucity of data relating to CNT, fullerene, metal and metal oxide nanoparticles, has required a broader approach. This draws upon the depth and breadth of knowledge available for a small number of nanoparticles which have been manufactured at the industrial scale for decades. The results from the discussed epidemiology studies of the carbon black industry indicate some adverse effects of exposure to carbon black dust on respiratory health. However, the main findings are reassuring in that respiratory symptoms and lung function appear to be primarily associated with current exposure rather than being caused by cumulative exposures. A mortality study by Sorahan et al. 2001 clearly indicates no strong and little suggestive evidence of excess non-malignant respiratory disease associated with working in the carbon black industry. Despite the fact that two of the five factories investigated generated evidence that there was excess mortality from lung cancer, the study has failed to link this disease to carbon black exposure. Although the available TiO2 industry epidemiology studies provide little information to evaluate the health risks associated with ultrafine particle manufacture, as most work has focused on larger particles, it is unlikely that exposure to a true ultrafine or nanoparticle dust explains the variations in lung cancer mortality between studies and factories. The review considered the limited number of relevant epidemiological studies that assess particle number in ambient air which conclude that; (i) there are adverse health effects associated with the ultrafine fraction of respirable particles, with effects indicated on mortality in the general population and panels of susceptible individuals and (ii) death was related to particle numbers in the nano-size range. Overall, the findings of the review of human studies suggest that nanoparticles are capable of inducing physiological and inflammatory responses in humans.

The review of the literature on the ecotoxicity of nanoparticles for each of the four groups of nanomaterials has addressed aquatic toxicity, terrestrial toxicity, bioaccumulation, and degradability. Aquatic ecotoxicity is further sub-divided into studies dealing with fish, crustacean, algae and other taxa (covering studies on bacteria, non-crustacean invertebrates, and amphibians) with the view to providing data for risk assessment purposes. Due to the strong focus on regulatory use of the ecotoxicity data for the risk assessment aspects of the review, a special effort has been put into translating the effects, found in the reviewed papers, into the terminology traditionally used in risk assessment, e.g. ECx- and LCx-values and NOEC/LOECvalues. Large differences in behaviour, fate and effects, even in standardised test systems, were encountered for different metals and metal oxides within each class. Hence, as for the toxicology review, the ecotoxicity review of metals and metal oxide nanoparticles have considered studies pertaining to specific substances rather than considering them as one group of substances. The review identifies the range of studies that have been carried out with aquatic and terrestrial species and those carried out towards the base-set organisms used in the REACH risk assessment procedures for chemicals (fish, crustacean and algae). More studies are available using bacterial groups and, though they do not report the findings in traditional ecotoxicological endpoints, these studies may be of value for mechanistic interpretations of ecotoxicity in both the

aquatic and the terrestrial environment. The review highlights how initial studies used different solvents to suspend C60, but how more recent studies avoided the use of any solvents since, as for the mammalian toxicology studies, it has been demonstrated that not only C60/solvent interactions may affect toxicity, but also solvent degradation products may be responsible for some of the observed effects. Major knowledge gaps are identified within persistence and bioaccumulation of fullerenes since no structured studies, aimed to investigate this, have been reported in the reviewed literature. Only a few ecotoxicological studies of the effects of CNT on aquatic and terrestrial species have been carried out. The review identified studies on other taxa (ranging from bacteria and protozoans to amphibians), but the high variability in these studies means that it is not possible to draw any common conclusion on the effects of CNT on this basis. It was identified, however, that a number of studies do not find adverse effects after exposure to CNT at often very high concentrations. The review highlights that degradability of CNT still remains to be studied and testing difficulties in relation to obtaining, handling, purification and solubilisation are likely to have an influence in the very limited number of studies available for environmental risk assessment (i.e. ecotoxicity, persistency, and bioaccumulation). The review identifies that the major part of the published ecotoxicology literature deals with toxic effects of silver and copper nanoparticles and general conclusions on the toxicity of these are reported. Only a very few studies have dealt with bioaccumulation of metal nanoparticles even though this is a topic of high concern when considering past experiences with metals which, by definition, are not degradable. However, the review highlights that changes in the metal speciation can occur depending on redox conditions, salt content etc. The ecotoxicity studies of aluminium, gold, cobalt, and nickel nanoparticles have also been reviewed. However, the literature on these metals can best be described as extremely limited. Although general conclusions on metal oxide ecotoxicity are hampered by the large diversity of materials, the review presents summaries for three individual metal oxides types (TiO₂, ZnO and SiO₂) and identifies a number of trends. The review highlights the importance of considering the effect of functionalisation on bioavailability and hence toxicity and bioaccumulation of nanoparticles, which remains to be fully studied.

The penultimate chapter presents a basic **risk assessment**, inspired by the REACH Guidance, for the four types of nanomaterials under review based on the information provided by preceding chapters of the review. It includes an assessment for both the human health and the environment, limited to the extent that the available data allows. For each nanomaterial uncertainties and additional work needed to complete the assessment are also described.

Each of the four groups of nanomaterials under review fullerenes, carbon nanotubes, metals and metal oxides – different forms of the substances are included, e.g. fullerenes with different functionalisation, or single and multi walled carbon nanotubes. In particular, the metals and the metal oxide nanoparticles - like those in the conventional/'bulk' form cannot be considered as one group in terms of risk assessment due to the chemical, toxicological and ecotoxicological diversity between substances within one group. Therefore for metals and metal oxides the most data rich substance(s) were chosen as case studies in the development of a simple risk assessment approach. The case studies are presented as a purely scientific exercise which allows the exploration of key questions associated with the risk assessment of nanomaterials, and should not be used in any other way. These case studies do not reflect any opinion of the European Commission.

The limited availability of information (which does not comply comprehensively with the REACH requirements in terms of detailed information on the use, exposure, and data on inherent properties), means that the risk assessments are commensurate with an assessment as carried out under the old chemicals legislation (for 'existing substances') where the regulatory authorities conducted an assessment based on the available information. In order to follow this format, information has been extracted from previous chapters of the review and assimilated into a risk assessment. On the basis of the identified information, the risk assessments are carried out following both a quantitative and a qualitative approach. For human health, the quantitative approach requires establishing exposure values for the various routes of exposure (inhalation, dermal and oral) for consumers and workers and the establishment of a Derived-No-Effect Level (DNEL), typically based on extrapolation of animal data to the human situation by using appropriate assessment factors. For the environmental assessment, the quantitative approach requires the determination of the Predicted Exposure Concentration (PEC) and the Predicted No Effect Concentration (PNEC) for each environmental compartment. PEC and PNEC are then compared to identify any risk for environmental compartments. For both human health and the environment, the application of assessment factors is based on the REACH guidance. Qualitative risk characterisation was carried out in the event where no exposure value and/or no dose descriptors were available or estimated.

The risk assessments show a significant lack of measured and modelled exposure data of nanoparticles, for humans (occupational and consumer exposure) and for the environment. The limited amount of published measured data for human exposure may be due to the difficulties associated with the measurement of ultrafine or nanoparticles, the decision regarding which metric(s) to use, and their distinction from background particles. For the environment, this is further complicated by the challenges of "identifying" nanoparticles in environmental matrices. A few relatively simple exposure models have been used, however more sophisticated reliable models for predicting exposure to nanoparticles have not been identified. The risk assessment highlights that it is highly recommended to further establish good exposure data for all relevant exposure routes and targets, via measurements as well as to develop validated exposure models. Establishment of exposure data should address the issues related to a proper characterisation. It is also important to further study the interaction of nanoparticles with environmental matrices (e.g. natural organic matter, sediments, etc.), affecting the environmental fate and transport, and thus the exposure for aquatic and soil organisms.



For human health, it seems that the risk of metal and metal oxides is largely driven by the size and therefore surface area of the nanoparticles, and it seems that chemistry may (e.g. silver) or may not (e.g. TiO₂) influence the toxicity, possibly depending (at least partly) on the formation and toxicity of free metal ions. For the carbon-based nanoparticles it seems very relevant in addition to consider the three dimensional structure of the nanoparticle (e.g. fibre-like characteristics), the chemical composition (e.g. impurities from their production) and not least the various surface modifications, which are often added deliberately to promote a certain effect (e.g. increase water solubility). A particular challenge (both in terms of measuring exposure and assessing risks) is introduced by the fact that exposure data often refer to a distribution of particles of different characteristics and different sizes, whereas toxicity tests are often performed for nanoparticles of limited size ranges and of one type. In addition, nanoparticles will often aggregate to agglomerates (both relevant for exposure assessment and toxicity testing) and as evidenced in the risk assessments, it is not always clear what the agglomeration state was in the relevant studies. Even if known it is difficult to make general conclusions on how this will influence the toxicity and therefore the risk.

For the environment, it was not possible to determine an influence of the size or the shape on the ecotoxicity for any of the groups of investigated nanoparticles, although many studies lack particles of different characteristics allowing such comparisons to be made. The effects may however be affected by agglomeration and aggregation, especially at the very high concentrations used in the tests. Toxicity of metals and metal oxides seems to be driven by chemical composition, but the effect of coatings (e.g. in consumer products mostly coated nanomaterials are used) on their toxicity was not sufficiently studied. For example, coating can reduce or block the release of toxic ions from silver nanoparticles thus reducing their toxicity. Moreover, coating and surface functionalisation may improve the metal and metal oxide nanoparticle dispersion stability and hydrophilicity and consequently may increase the possibility of transport over long distances in the environment. The effects of carbon-based nanomaterials on organisms are influenced by functionalisation and the level of impurities (especially in CNTs).

The review considers one of the key issues in nanoparticle safety assessment, namely the prospect and validity of scaling risk information from bulk substances. It is often discussed to which degree the risks of nanoparticles can be assessed based on the toxicity of the bulk/normal substances; i.e. whether the risk of the bulk/normal substances can simply "be scaled" to the nanoform taking into account the smaller size of the particle or whether the small sizes triggers "nano-specific" behaviour/effects. To date no firm conclusion can be drawn which would be applicable to all nanoparticles. However, when considering whether scaling is possible, it seems to be a prerequisite that if the 'chemistry' (at least partly) drives the toxicity, it needs to be the same chemistry in the bulk/normal form as well as in the nanoform before such scaling can be considered. This already introduces some reservations for carbon-based nano-materials, which have surface modifications deliberately added to give them in order to generate specific properties. For more chemically 'inert' particles, it may be



possible to draw conclusions on their behaviour and scaled from larger inert particles simply based on the shape. However, this needs further investigation beyond the possibilities in this review.

For human health, there are indications that (some of) the toxic effects of nanosized TiO_2 can simply be scaled based on surface area considerations from the toxicity of the micro-sized TiO_2 . For silver, there is still too little known about the toxico-kinetics to give a fair judgement of this question. For the carbon-based nanomaterials, it does not seem obvious that the toxicity observed for the nanoforms could be found based on scaling from any normal/bulk state of carbon-materials. These observations should rather be seen as reflections than firm conclusions. For the environment, it is interesting to note that the toxicity seen for nanosized ZnO is indicated by some authors to be related to the release of zinc ions (Zn^{2+}) just as the toxicity of the bulk form of ZnO. Scaling may therefore be possible for this substance.

The influence of increased surface area of nano-forms with respect to the bulk form is to be verified on the amount and efficiency of ion leaching. However this does not include coated nanomaterials, which have peculiar properties. Concerning silver nanoparticles, no conclusion can be drawn yet, even if toxicity of silver seems to be related to Ag^+ ions. In conclusion, it seems possible to predict (part of) the toxicity of some nanomaterials based on the toxicity of the bulk/normal form, but this is not possible for all types of nanomaterials.

In **conclusion**, the review's findings strongly supports the further development of thorough characterisation (including proper considerations of agglomeration/aggregation) of the

nanoparticles in exposure media when conducting exposure assessment, as well as in the generation of data for determining exposure to both humans and the environment as well as assessing hazardous properties. This is a crucial prerequisite for carrying out a meaningful assessment of the risks. Further testing strategies are required to be established to cover all relevant endpoints needed for a risk assessment. At present, carrying out risk assessment of nanoparticles can only sensibly be done on a case-by-case basis. Only when more data becomes available may it be possible to group nanomaterials according to their physical, chemical and/or biological properties or mode of action, so that testing could be done for representatives of each group.

2.5 Dissemination

The dissemination activity of the review commenced from an early stage in the project, with the construction and launch of project the ENRHES website, hosted JRC by (http://nmi.jrc.ec.europa.eu/project/ENRHES.htm). The final report was published in the website in January 2010, accompanied by a press release for SAFENANO (http://www.safenano.org), serving to further raise awareness of the review amongst its international network of scientists, technologists and risk assessors, and a host of ancillary nanoscience newsgroups.

In additon, numerous peer-review publications have been, or are in the process of being published in the scientific literature, as outlined in Table 2.

Table 2 List of publications based on the ENRHES review

Title	Authors	Journal	Year of publication	Relevant pages	Status
Setting the limits for engineered nanoparticles in European surface waters – are current approaches appropriate?	Baun, A., Hartmann, N., Grieger, K.D., Hansen, S.F.	J. Environ. Monit.	2009	1774-1781	Published
Identification of the mechanisms that drive the toxicity of TiO2 particulates: the contribution of physicochemical characteristics	Johnston, H.J., Hutchison, G.R., Christensen, F.M., Peters, S., Hankin, S., Stone, V.	Particle and Fibre Toxicology, 6(33)	2009	1-27	Published
The biological mechanisms and physicochemical characteristics responsible for driving fullerene toxicity	Johnston, H.J., Hutchison, G.R., Christensen, F.M., Aschberger, K., Stone, V.	Toxicological Sciences	2009	-	Epub ahead of print
A critical review of the biological mechanisms underlying the in vivo and in vitro toxicity of carbon nanotubes: the contribution of physcio-chemical characteristics	Johnston, H.J., Hutchison, G.R., Christensen, F.M., Peters, S., Hankin, S. Aschberger, K., Stone, V.	Nanotoxicology	2010	-	Epub ahead of print
A review of the in vivo and in vitro toxicity of silver and gold particulates: particle attributes and biological mechanisms responsible for the observed toxicity	Johnston, H.J., Hutchison, G.R., Christensen, F.M., Peters, S., Hankin, S. Aschberger, K., Stone, V.	Critical Reviews in Toxicology	2010	-	Epub ahead of print

Title	Authors	Journal	Year of publication	Relevant pages	Status
Carbon fullerenes - assessment of the feasibility and challenges for human health risk assessment, based upon open literature	Ashberger, K., Johnston, H.J., Stone, V., Aitken, R.J., Hankin, S.M., Peters, S.A.K., Tran, L.C., Christenesen, F.M.	Regulatory Toxicology and Pharmacology	-	-	Manuscript submitted
Nano-silver - feasibility and challenges for human health risk assessment based on open literature	Christensen, F.M., Johnston, H.J. Stone, V.; Aitken, R.; Hankin, S.; Peters, S.; Aschberger, K.	Nanotoxicology	-	-	Manuscript submitted

2.6 Further information

Further information on the ENRHES project and its beneficiaries is available from the project website

(<u>http://nmi.jrc.ec.europa.eu/project/ENRHES.htm</u>), where the final report is available for download.



3 Directory

Table 1 Directory of people involved in this project.

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HINAMOX

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



Contract Agreement: NMP4-SL-2009-228825 – HINAMOX

Website: http://www.hinamox.eu

Coordinator: Sergio E. Moya, Centro de Investigación Cooperativa en Biomateriales - CIC biomaGUNE

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3	University of Leipzig	ULEI	Germany
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5	Centro de Investigación en Química Aplicada	CIQA	Mexico
6	Zehjiang 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

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1 Summary

The HINAMOX project is concerned with the impact in human health and biological fate of metal oxide nanoparticles (NPs) like TiO2, ZnO, Al2O3, CeO2, 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 will work 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, and Confocal Microscopy. Also the cytological and pathological response to NPs will be studied, considering aspects related to size, shape, and capping agents. We will study the inflammatory response of the alveoli as a possible vehicle for the introduction of NPs in the body. The interaction of NPs with alveoli type II cells will be 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

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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 TiO2 and ZnO as catalysts and UV protectors, CuO in anti-fouling paints, Al2O3 as a surface protector, CeO2 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:

- 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 metallochemical 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 TiO2 or ZnO in sun creams have led to concerted actions already within the 6th Framework Programme. For example, the Nanoderm aim of the project (http://www.unileipzig.de/~nanoderm/), which focused on the topical exposure of TiO2, 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

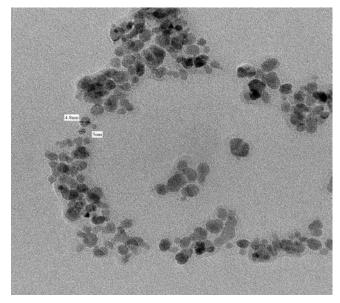


collaboration in a highly interdisciplinary consortium with expertise in synthetic chemistry, production technology, particle physics and characterization, biochemistry, toxicology and occupational hygiene.

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.



TEM image of Fe2O3 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 magnetitebased 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).

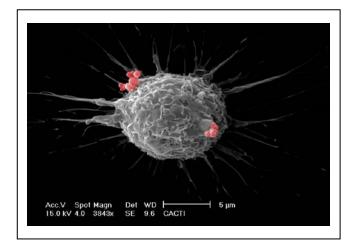
3) Quantification and distribution studies at cellular level, by Ion Beam Microscopy (IBM), Electron Microscopy (EM) and Confocal Laser Scanning (CLSM).

There is a profound lack of knowledge concerning the amount of NPs present in a cell for an applied NP dose. In other words, a quantitative relation between dose and uptake of NPs at both organ and cellular level is missing. The particle uptake depends on the activity of the cells, as well as the size, shape and physicochemical 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). 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). 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

3) Understanding the interaction of NPs with cellular and extracellular components

For assessment of the fate and interaction of NPs in the organism, investigations of NP-protein interaction and stability of NPs in different biological compartments will be carried out by biochemical methods focusing on measuring complementary agents and by means of binding studies with Fluorescence Correlation Spectroscopy (FCS). In FCS fluctuations in the fluorescence intensity from a confocal volume in a sample, which are caused by diffusional and rotational processes are measured and correlated temporally (Haustein et al., 2003).



SEM image of a macrophage surface in the presence of metal oxides

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.

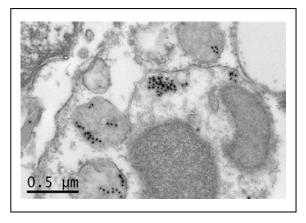
5) Risk of exposure and toxicological effects of metal and metal oxide NPs.

The projected work in HINAMOX encompasses in this way a complete approach to understanding the safety and human health implications of NPs and nanotechnology based materials. approach addresses aspects concerning hazard This characterization, human exposure, occupational exposure, and the inflammatory and toxicological response to NPs. The HINAMOX project is particularly strong with respect to the monitoring of NPs in cells and their biodistribution in animal bodies, employing techniques such as IBM and PET/SPECT, which have been little used up to now for studying the fate of NPs in biological systems. In order to achieve this task, a complex and innovative work of engineering and labelling of NPs is proposed. Cellular and body distribution studies together with biodurability tests will enable us to understand the biological fate, transport and transformation of NPs within biological entities.

Aspects such as the behaviour, fate, bio-persistence, biokinetics, 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.



TEM-Image of NPs within macrophages

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 the European to 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 Sate-of-the-art techniques engineering. 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.

Instituto Superior de Saude Alto Ave (Isave)

ISAVE is a private higher education establishment located in the north of Portugal. Its main mission is to promote "Health" in the population, through education and professional training, offering different degree and postgraduate courses and research specialities. The research group involved from ISAVE carries out projects related to the generation and evaluation of new therapeutic agents, possible applications and cytotoxic effects.

The role of ISAVE in HINAMOX is the study of citoxicity and genotoxicity of NPs

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InLiveTox

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



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

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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 cross the intestinal barrier? (iv) Which physico-chemical characteristics of NPs influence their uptake by intestinal

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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

<u>Context</u>

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

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

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)

¹ The term nanomaterials refers to engineered nanomaterials and particles.

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

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



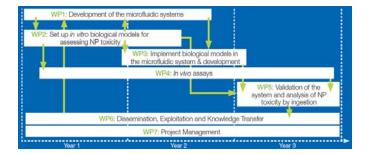
These objectives will be achieved by bringing together microfluidics technologies with cell culture models of human tissues to produce an *in vitro* test system that is more physiologically relevant. The microfluidics system will be flexible and modular so that the complexity of the system can be increased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

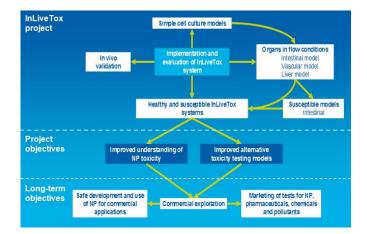
In a first phase of the project, in work package 1, a microfluidics device will be designed and fabricated for the simultaneous culture of different cell lines representing the intestinal epithelium, the vascular endothelium and the liver. In a second phase, feedback from the other work packages will 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.

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NanEAU

Toxicological effects of emerging nanoparticles in water on aquatic model organisms and uptake in humans from drinking water

Contract Agreement: FNR Core2008 (Co8/SR/07) Luxembourg

Coordinator: Arno C. Gutleb, Department of Environment and Agrobiotechnology, Centre de Recherche Public – Gabriel Lippmann, Belvaux, Luxembourg

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4	Norwegian Institute of Air Research	NILU	Norway

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1 Summary

NanEAU is a 2-year project funded by the Fonds National de la Recherche in Luxembourg (Co8/SR/o7). The aim of NanEAU is to adapt and develop test protocols for the evaluation of (environmental) toxicity of emerging NPs. In addition to classical assays using established biomarkers and standardized procedures, novel-predicting biomarkers applying proteomic and genomic approaches will be developed. The possibility of uptake from the water in higher vertebrates and intracellular localization will be studied.

NPs will be selected in close cooperation with the ongoing FP7 project NANOTEST and NorPol, a bilateral project of the Norwegian and the Polish Research Council to make best use of the expertise available in this project.

The experiments as proposed in NanEAU will allow a better description of hazards and risks associated with the uncontrolled release of NPs into the environment and will contribute to a sound scientific and holistic understanding of toxicological aspects as effects will be studied from a protein to the organism levels and from algae to complex vertebrates. The expected outcome of NanEAU is additional information on behaviour and interaction of NPs in the water, its potential risks for a range of organisms, and new biomarkers for the presence and effects of NPs supporting the sustainable use of water resources.



2 Directory

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NanEx

Development of Exposure Scenarios for Manufactured Nanomaterials



Contract Agreement: FP7-NMP-2009-CSA-3 Website: http://nanex-project.eu/ Coordinator: Martie van Tongeren, Institute of Occupational Medicine, Edinburgh, UK

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3	Eidgenössische Materialprüfungs- und Forschungsanstalt	EMPA	Switzerland
4	European Research Services GmbH	ERS	Germany
5	Institut universitaire romand de Santé au Travail, Lausanne [Institute for Work and Health]	IST	Switzerland
6	Fundación LEIA CDT	LEIA	Spain
7	Naneum Limited	NANEUM	United Kingdom
8	Nanocyl S.A	NANOCYL	Belgium
9	Nanotechnology Industries Association	NIA	Belgium
10	Netherlands Organisation for Applied Scientific Research	TNO	Netherlands
11	Joint Research Centre	JRC	Pan-European
12	National Centre for Scientific Research "Demokritos" -	DEMOKRITOS	Greece

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Key data::

Start date : 1 December 2009

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 the 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 aim of the NanEx project, a 12 month project which commenced in December 2009, is to develop a catalogue of generic and specific (occupational, consumer and environmental release) exposure scenarios for MNMs taking account of the entire lifecycle of these materials. NanEx will collect and review available exposure information, focussing on three very relevant MNMs: (1) high aspect ratio nanomaterials- HARNs) (e.g. carbon nanotubes); (2) mass-produced nanomaterials (e.g. ZnO, TiO₂,

carbon black); and (3) specialised nanomaterials that are currently only produced on a small scale (e.g. Ag). The exposure information will include both quantitative (measurement results) and qualitative contextual exposure information (risk management measures). We will also review the applicability of existing models for occupational and consumer exposure assessment and for environmental release from these scenarios. We will carry out a small number of specific case illustrations and carry out a gap analysis of the available knowledge and data. Finally, the project knowledge will be disseminated to relevant stakeholders, taking into account other relevant activities that are taking place in this field.

2 NanEx Overview

2.1 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 nanotechnology¹ there have been numerous reviews published considering the potential risk from exposure to nanoparticles. The reviews have been remarkable 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 is also likely to increase rapidly.

Human exposure to MNMs and environmental release of these materials 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.

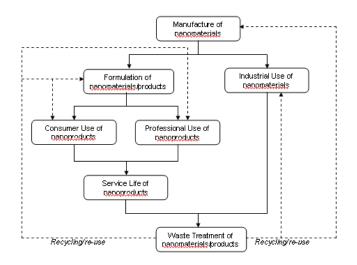


Figure 1: Simplified overview of life cycle stages of manufactured nanomaterials

2.2 Aims and objectives

The aim of the NANEX project is to develop a catalogue of generic and specific exposure scenarios for MNMs relevant for human exposure taking account of the entire lifecycle of these materials. NANEX will collect and review available exposure information and develop a set of generic exposure scenarios for three very relevant MNMs:

- high aspect ratio nanomaterials HARNs) (e.g. carbon nanotubes);
- 2. mass-produced nanomaterials (e.g. ZnO, TiO₂, carbon black); and
- 3. specialised nanomaterials that are currently only produced on a small scale (e.g nano-Ag).

Data collected will include both quantitative (measurement results if available) and qualitative, contextual exposure information (risk management measures). The project will review 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 will be carried out, covering occupational, consumer and environmental release/exposure scenarios and carry out a gap analyses of the available knowledge and data and define research priorities.

The NANEX project has the following specific objectives:

- 1. To describe generic requirements for the development of exposure scenarios for MNMs.
- To collect and review exposure data, exposure metrics, risk management measures and existing models for the development of occupational exposure scenarios for HARNs, mass-produced MNMs and specialised MNMs.

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



- To collect and review exposure data, exposure metrics, risk management measures, and existing models for the development of consumer exposure scenarios for HARNs, mass-produced MNMs and specialised MNMs.
- 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 HARNs, massproduced MNMs and specialised MNMs.
- 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 specific MNMs (HARNs, mass-produced MNMs and/or specialised MNMs).
- 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.

2.3 Key issues

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 from relatively small studies and usually not collected in a consistent manner. With respect to occupational exposure scenarios for MNMs, a very limited number of workplace air monitor studies have been published so far³. The exposure scenarios 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 been focussed on carbonaceous nanomaterials, e.g. carbon black ⁴, fullerenes⁵, carbon fibres⁶, and carbon nanotubes⁷, or

metal(oxide)s⁸. 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.

Specific and reliable measurement methods for nanoparticles still need to be developed while the performance of currently available models for estimating occupational and consumer exposure is unknown as they have not been specifically validated for these materials. Within the REACH guidance⁹, so called first tier models are recommended for assessment of both inhalation and dermal exposure, e.g. EASE, ECETOC-TRA, Stoffenmanager¹⁰ (inhalation) and RiskofDerm (dermal). For some use scenarios specific models have been developed, e.g. for dermal exposure during spraying¹¹, 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.

Within 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.

Due to the lack of information on the toxicology of NMMs 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. In addition, several organisations have provided guidance on the safe use of MNMs and have suggested control banding approaches.¹³ This information will be utilised to provide input into the development of generic exposure scenarios that can be used for a wide range of nanomaterial applications. Although such exposure scenarios may not be integrated into a quantitative risk assessment, they can be used to benchmark different exposure scenarios with respect to state-of-the-art process operations and control measures and provide guidance to reduce exposure.

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 FOPH, 2008; IRSST, 2009; HSE, 2009



For each stage of the life cycle of an MNM, the project will review existing exposure situations and develop generic exposure scenarios that effectively describe how exposure to humans and the environment occur and what measures are currently recommended and used to control the exposure. 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 released¹⁴, whether as (1) free nanoparticles, (2) aggregated and agglomerated nanoparticles, or (3) integrated in a nanomaterial or within a micrometer sized particle.

2.4 Overall strategy

The various activities within NANEX will be carried out in 9 complementary work packages. WP1 (Management) will implement a tried and tested management structure to ensure the timely and efficient implementation of the work plan. WP2 (Development of generic exposure scenario description) will identify and briefly describe the main generic exposure scenarios during relevant life-cycle stages for the three types of MNMs. A format for exposure scenario descriptions will also be developed for use in subsequent work packages. WP3 (Occupational exposure) will collect and review measurement and contextual information to describe and characterise occupational exposure and review available tools and models to predict occupation exposure to MNMs. WP4 (Consumer exposure) will collect and review similar information but focussing on consumer exposure and will characterise possible specific consumer groups being exposed. WP5 (Environmental release/exposure) will identify exposure scenarios that result in a release of MNMs to the environment and may subsequently result in human exposure. Generic descriptions of the relevant exposure scenarios where MNMs may be released to the environment will be identified and developed. Models and tools to predict environmental exposure based on release data during production, use and disposal will be reviewed. WP6 (Case illustrations) will collect information on specific exposure scenarios for a limited number of MNMs to test and illustrate the applicability of the generic exposure scenarios developed by WP2 and provide examples of specific exposure scenarios (occupational, consumer and environmental). WP7 (Scientific integration and gap) will integrate the information and knowledge obtained during work packages WP2 to WP6. In collaboration with WP2 a catalogue database for exposure scenarios will be developed and a gap analysis will be performed after all information has been included in this database. This database will be included in the NAPIRAHUB, hosted by JRC. The integration will include a classification of data type, data sources, data quality and completeness. With this information, a knowledge matrix will be created that will serve for the identification of gaps in knowledge, tools and research. WP8 (Dissemination) will develop and implement a comprehensive dissemination strategy to ensure a wide dissemination of project knowledge on occupational and consumer exposures and environmental release. To achieve this a NANEX website will be developed containing specific exposure modules which will be included in a global Safetypedia as European Technological Platform on Industrial Safety or GoodNanoGuidewebsite. A large audience final workshop with stakeholders including world-wide standardisation organizations will also be organised in collaboration with NanoImpactNet. **WP9** (Scientific Management) will coordinate the scientific activities of the project, monitor progress of the work packages, perform scientific review and prepare the scientific part of reports to be submitted to the European Commission.

2.5 Progress to date

The project is still in its early stages, however progress is well underway. Key achievements of note include:

- The project was officially launched with a kick-off meeting held in Hoofdorp, Netherlands during December 2009 in which detailed work plans were constructed;
- The project website (<u>http://nanex-project.eu</u>) was launched in January 2010, providing up to date project information, a platform for internal communication and a base for eventual report dissemination;
- The consortium has been agreed that within the three classes of manufactured nanomaterial classes, the project will focus on: i) carbon nanotubes; ii) mass-produced nanomaterials (TiO₂), and; iii) specialised nanomaterials (nano-silver);
- A training workshop on 'Exposure Scenarios' was held in January 2010 in Hoofdorp, Netherlands and attended by all project partners, providing a general overview of REACH and exposure scenarios. In addition, specific case illustrations of occupational, consumer and environmental exposure scenarios were presented and discussed by the team;
- Development of the format for the description of exposure scenarios is well underway, including the development of plans for its incorporation into the NAPIRA-hub;
- Key industry partners have been identified and engaged in the case study process;
- The first in a series of NanEx workshops has been planned and is due to be held on the 9th March at the Museé Olympique in Lausanne. The workshop has the following key aims:
- i.) engaging key stakeholders in NanEx;
- ii.) providing a definition of generic exposure scenarios and data requirements;
- iii.) defining details for the collection of data for the occupations, consumer and environmental exposure scenarios;
- iv.) gathering information from the key experts and stakeholders in attendance and identify further sources of relevant information; and
- v.) commencing the process of identifying gaps and research needs.

¹⁴ Koehler and Som 2008



vi.) Plans for eventual dissemination have been commenced, with the results of the NanEx project due to be presented at the NanoSafe conference in Grenoble, France in November 2010.

2.6 Further information

Further information on the NanEx project and its beneficiaries can be found on the project website, <u>http://nanex-project.eu</u>.

3 Directory

Table 1 Directory of people involved in this project.

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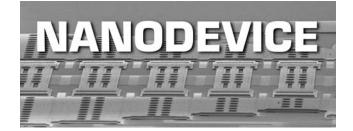
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NANODEVICE

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



Contract Agreement: Website: <u>http://www.ttl.fi/nanodevice</u> Coordinator: Professor Kai Savolainen, Finnish Institute of Occupational Health

No.	Beneficiary name	Short name	Country
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2	TEKNILLINEN KORKEAKOULU	ТКК	Finland
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1 Introduction

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

2 Project summary

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

- identification of relevant physico-chemical properties and metrics of airborne ENP, establishment of reference materials
- exploring the association between physico-chemical and toxicological properties of ENP
- analyzing industrial processes as a source of ENP in workplace air
- developing methods for calibration and testing of the novel devices in real and simulated exposure situations
- 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:

- 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.
- 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 Conclusions

NANODEVICE will provide new information on the physicochemical 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.

7 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: To be announced

Coordinator: Dr Claus Svendsen (csv@ceh.ac.uk), NERC - Centre for Ecology and Hydrology, Wallingford, UK

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2	VU University, Amsterdam	VUA	Netherlands
3	Oxford University	UOXF.DJ	United Kingdom
4	University of Aveiro	UAVR	Portugal
5	Faust & Backhaus	F+B	Germany
6	NanoTrade	NT	Czech Republic
7	Università degli Studi del Piemonte Orientale Amedeo Avogadro	UNIPMN	Italy
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

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1 Summary

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

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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-ofthe-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 elemental analysis. Further, by using cathodoluminescence spectroscopy it will be possible to detect the nanoparticles in small samples and investigate their degree Since the tagged ions will be inside the of aggregation. 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.

 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

(<u>http://www.nanotechproject.org/inventories/consumer/</u>) 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 CeO₂. Initial predictions will be generated based on worst case conditions.

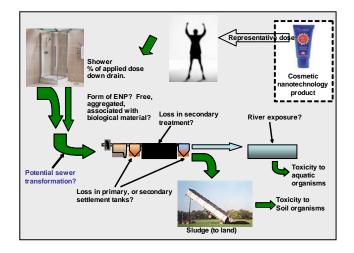


Fig. 1. Schematic illustrating key issues concerning the disposal, fate and environmental release pathways of an example "down the drain" nanotechnology product (e.g. ZnO ENP containing sunscreen).

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

- For CeO2 the assessment will focus on direct deposition of 1. 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).
- For both ZnO and Ag ENPs the major route of release to the 2. 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.

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

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);
- 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)

- 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.

<u>Obj.4: Study ENP fate and behaviour through wastewater</u> 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.

- Examination of the colloidal behaviour of ENPs in <u>real</u> 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).
- 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.

<u>Obj.5: Determine the chronic toxicity of ENPs of different</u> 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 noeffect 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.
- 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.

<u>Obj.7: Establish the mechanisms of uptake, internal trafficking</u> 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).

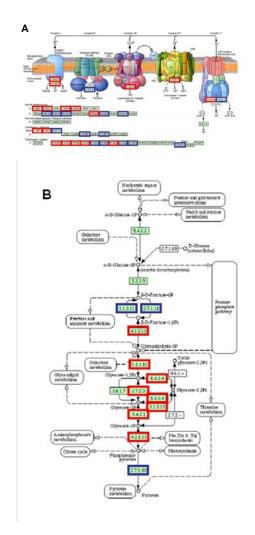


Fig. 2. Example analysis of the impact of ionic copper on oxidative phosphorylation (A) and on glycolysis/gluconeogenesis (B) of earthworms in a pathway based visualisation of the mechanism of toxicity. Transcripts outlined in bold are represented on the utilised microarray, those with < 2 fold change 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 CeO₂ in dissolved and nanoparticle form will be used to address issues relating to the relative contribution of ENP forms to toxicity. Information on bioavailability will be built using models developed in WP 4 that will build on the biotic ligand model and also information on particle properties including surface charge and dissolution. Such information will be of fundamental importance to the development of the concept of ecologically responsible design of nanotechnology products and is a key project outcome.
- 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.

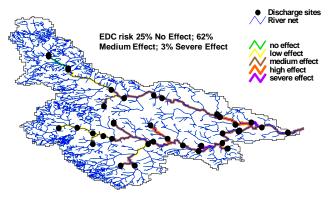


Fig 3. Catchment risk map of predicted endocrine disruption of fish from effects of oestrogenic chemicals for the Aire and Calder rivers, Yorkshire, UK.

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.

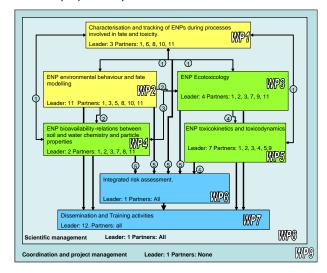


Fig 4. NanoFATE PERT diagram showing flow of data and products between the seven workpackages within particle chemistry and fate component (yellow), ecotoxicology and bioavailability



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 speciesspecific 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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NANOFILM

Characterisation and technological analyses of nanoparticles in liquid based nanofilm products (NANOFILM)

K.A. Jensen, A.W. Nørgaard, S.T. Larsen, P. Wolkoff

The National Research Centre for the Working Environment, DK-2100 Copenhagen, Denmark

1 Summary

NANOFILM is a four-year products ending ultimo 2010, which is focused around mainly a PhD-project, which is funded by the 2.500.000 DKK from the National Research Centre for the Working Environment, Denmark. NANOFILM products are popular nanotechnology-based chemical products for producing easy-to-clean or dirt-repellent surfaces. The active compounds may be titania (TiO₂) or silica (SiO_x) nanoparticles and/or chemicals such as siloxane (R_2 SiO, where R normally is an alkyle) and flour-silanes (FH₃Si, FH₃Si₂, F₃H₂Si₂...). These compounds are suspended or dissolved in water-based acidious solutions or alchols (methanole or ethanole). Challenging for risk assessment, the exact chemical composition of these products is often not readily available. It may not required by legislation, because their concentrations in the products often are below 1 wt%. However, for this product group, it may be very crucial to evaluate the exposure and toxicological risk based on user scenarios and not from the product composition itself. A 1 ml product may contain 10⁸ to 10¹¹ nanoparticles, which suggest a potential high exposure risk associated with the application of NANOFILM products. This was confirmed in Nørgaard et al. (2009) who also showed that free silane and siloxane particles are formed during spray application. The air-concentrations of specific chemicals may even exceed limit guidelines in poorly ventilated rooms. Moreover, it is clear that the identity and concentration of the chemicals in the products are seldom reported in sufficient detail and knowledge of the toxicity may be insufficient for risk assessment. An inhalation study showed that a specific chemical one the four products tested have a high potential to cause dramatic acute air-way effects (Nørgaard et al., submitted). More publications are underway.

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3 Current project publications

1. Nørgaard, AW, Jensen KA, Janfelt C, Lauritsen FR, Clausen PA, and Wolkoff P. Release of VOCs and particles during use of nanofilm spray products. Environmental Science & Technology 2009;43(20):7824-30.

2. Nørgaard AW, Larsen ST, Hammer M, Poulsen SS, Jensen KA, Nielsen GD, and Wolkoff P. Lung damage in mice after inhalation of nanofilm spray products: The role of perfluorination and free hydroxyl groups (submitted).

4 Links to information

http://www.arbejdsmiljoforskning.dk/Aktuel%20forskning/NANO FILM%20Karakterisering%200g%20toksikologisk%20analyse%20af %20nanopartikler%20i%20v%C3%A6skebaserede%20nanofilmprod ukter.aspx?lang=en





NanoHouse



Cycle of Nanoparticle-based products used in house coating

Contract Agreement: NMP-2009-2-247810 Website: http://www-nanohouse.cea.fr Coordinator: Dr Francois Tardif, CEA Commissariat à l'Energie Atomique et aux Energies Alternatives, France

No.	Beneficiary name	Short name	Country
1	Commissariat à l'Energie Atomique et aux Energies Alternatives	CEA	FR
2	Eidgenössische Materialprüfungs- und Forschungsanstalt	EMPA	СН
3	Consorzio Venezia Ricerche	CVR	IT
4	Katholieke Universiteit Leuven	KULeuven	BE
5	Université Joseph Fourier - Laboratoire de Géophysique Interne et Tectonophysique	JOSEPH FOURIER U.	FR
6	MATERIS PAINTS ITALIA	MATERIS PAINT	IT
7	GFC CHIMICA	GFC	IT
8	AKZO NOBEL COATINGS S.A.	AKZO	NL
9	PPG Europe BV	PPG	NL

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1 Summary

NanoHouse project in some words:

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

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

This project aims at promoting a responsible and sustainable development of nanomaterials in building industry through a Life Cycle Thinking approach.

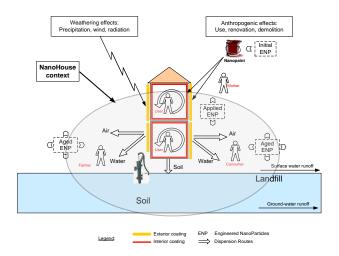
Nanomaterial can bring great advantages to building industry for sustainable development. Indeed, nanoparticles can increase the resistance to ageing (UV, mechanical stress, etc.) of construction materials particularly paints and coatings, they can replace toxic organic biocides and they can be advantageously used for air purification, thermal insulation, self cleaning, etc. Nevertheless, the development of nanomaterials in this economic area can develop dynamically only if the safety of

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humans and the environment is satisfactorily resolved. 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 nine partners involved in NanoHouse project are generating missing data on the potential exposure levels and the hazard due to this chronic exposure for 2 nanoparticle types: nano silver and nano titanium dioxide contained in indoor and outdoor coatings and paints. Both direct and indirect exposures (through the environment to human: vegetables, drinking water) are considered.

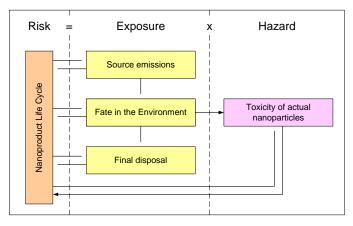




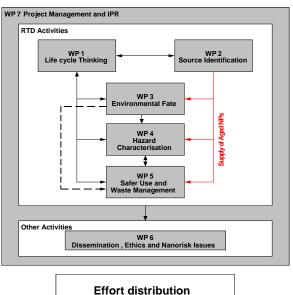
2 Project description

2.1 Project structure

NanoHouse project covers the whole risk assessment by evaluating the exposure and the hazard. Through a combination of knowledge from Life Cycle Thinking and risk assessment, NanoHouse outlines a holistic and prospective overview on the potential EHS impacts of paints containing ENPs throughout all life stages of the paints. This approach applied to an illustration case will allow identifying missing knowledge in view of risk assessment and control of nanoproducts, and deriving recommendations to fill in gaps in relevant regulations and standards.



The NanoHOUSE project is structured around five scientific work packages (WP1-WP5):





2.2 Research activities

The main innovation of NanoHOUSE work is to study the environmental behaviour and the potential effects on health of **actually released Engineered NanoParticles ("aged ENPs")**. The work thus extends beyond the current research which focuses almost exclusively on pristine ENPs received directly from producers or synthesised in the laboratory. NanoHOUSE is comparing the actually released ENPs from coatings to pristine ENPs.

The project adopts a **holistic approach** covering the whole life cycle of ENPs containing paints and coatings to study their potential Environmental Health and Safety (EHS) impact in home building. NanoHOUSE develop a new **concept of Life Cycle Thinking** that combines two complementary aspects: on the one hand investigating the risks and opportunities during the nanoproduct life cycle, and on the other hand investigating and assessing the environmental impacts of the nanoproduct.

The project study the kinetics of the release of ENPs, understanding the release mechanisms from nanoproducts, characterisation of the whole mixture of particles released.

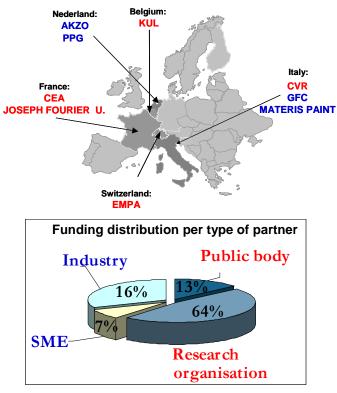
Furthermore, NanoHouse develop quantification methods and characterisation of ENPs in complex matrix and study new compartments: plants, and estimation of ENPs flows between soil, plant and water.

Finally NanoHouse quantify ENPs airborne release during incineration process and evaluate the tightness of landfill geomembranes against ENPs.



3 Consortium

NanoHouse comprise nine partners: 4 industrials and 5 academics



Coordinator:

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4 4. links

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NanoImpactNet

European Network on the Health and Environmental Impact of Nanomaterials



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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
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1 Summary

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, assessment, Communication, Integration Impact 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

Recent technological advances allow the targeted production of objects and materials at the nanoscale. 'Nanotechnology' refers to structures smaller than 100 nanometers (about 1/80th the diameter of a human hair) and the term 'nanomaterials' under its most commonly used definition refers to materials that have at least one structural dimension at the nanoscale. Nanomaterials often have different chemical, physical and bioactive characteristics from those of larger entities of material with the same chemical composition, and from molecular forms where these exist. The nanotechnology industry is expanding rapidly and nanotechnology is considered to be a key enabling technology for the 21st century. A wide range of applications are emerging. This new technology is 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 the database



of the Woodrow Wilson Institute, 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 the 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 parameters 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 under 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 continues to adapt to the challenge and to develop initiatives to promote crosstalk between projects.

3 What is NanoImpactNet

NanoImpactNet is first and foremost a network and a platform for information and idea exchange. Its unique position is already generating a lot of interest, and since the start 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 their and institutes. representatives of major research projects (European, but also national and non-European) and experts from stakeholders such as industry, NGO 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.

The NanoImpactNet work plan consists of six work packages (WPs: Human hazards and exposures, Hazards and fate of nanomaterials in the environment, Impact assessment, Communication, Integration and nomenclature, and Coordination and management). The work plan will be implemented over four years.

NanoImpactNet's primary objective is to create a scientific basis for ensuring the safe and responsible development of manufactured nanoparticles and nanotechnology-based materials and products, and to support the determination of regulatory measures and implementation of legislation in Europe. 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.

NanoImpactNet has a strong commitment to openness, and explicitly invites all researchers and diverse stakeholders to participate in our planned activities. Indeed this framework includes strong two-way communication which ensures efficient dissemination of information to the various stakeholder groups while at the same time consulting with these groups about their needs and questions.

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 will be accessible to all.
- NanoImpactNet will communicate stakeholders' needs directly to researchers.
- NanoImpactNet will ensure stakeholders receive the information they need and in a format that is of direct use to them.
- NanoImpactNet will become the focal point for exchange of information between the scientific community and the stakeholders in the EU and beyond.

4 Organisation of NanoImpactNet

The overall goal of NanoImpactNet 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 school, 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 interrelated and interconnected 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. In all work packages, existing data is taken into consideration and every attempt is made to include results and contributions from other ongoing projects.

Table 1 Workpackages	(WP)) of NanoImpactN	let
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WP	Title	Торіс
1	Human hazards and exposures	This workpackage 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 the material's (product) lifetime.



2	Hazards and fate of nanomaterials in the environment	This workpackage 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 these different environments 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 workpackage 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 these different environments 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. Discussions on computer simulations relating the physicochemical characteristics of nanomaterials to their fate, behaviour and hazard in the in the environment are also included.
4	Communication	This workpackage 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 (human hazards and exposure) and WP2 (environmental hazard and exposure) in order to integrate their knowledge, methodologies, and achievements into an assessment of the overall impact of nanomaterials.
5	Integration and nomenclature	This workpackage initiates, stimulates and facilitates cross-talk and collaboration between the workpackages of the NanoImpactNet CSA. 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 workpackage 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 first two years of the project, NanoImpactNet organized a series of events and training schools and produced several reports, which are described here to illustrate the wide range of questions being addressed within NanoImpactNet.

5.1 Events

5.1.1 The Dublin Workshops, Ireland

On the 19th and 20th of June 2008 NanoImpactNet's first two workshops were held:

1. Standardization of materials and protocols

2. Most relevant material metrics for different needs (for both hazard and exposure assessment).

Despite the very short announcement period with the workshops being held only 2 months after the kick-off of the project, a very large number of researchers from all over Europe and also from the USA attended these first two workshops (over 50 participants each). On the first day 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 the second day the need for standard and reference materials and protocols was emphasized. It became clear that robust and established protocols are needed for a field of science that is under heavy societal scrutiny, which has emerged so recently and is undergoing a phase of very rapid development. On both days, a good balance was struck between excellent introductions from two respected speakers, followed by 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 applications of the technology. This is also 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 of the existing toxicity tests, and as such, a significant validation procedure is required to ensure that the 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 required urgently.

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 nonprofit 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.

Some of the major conclusions drawn from the workshop include:

- 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

Strategies to standardize nanomaterials for environmental and ecotoxicological research

A first workshop entitled, 'Strategies to standardize nanomaterials for environmental and ecotoxicological research', took place on 3-4 September 2008 in ETH Zurich, Switzerland. Over 40 delegates attended the meeting. Prior to the 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. The workshop focused on three key questions:

- 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?

These questions were specifically and solely focused on environmental studies. The workshop participants, through a series of discussion and reflection sessions, generated the following conclusions.

- 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 nanomaterials 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 topic of the second part of the Zurich workshops was the development of approaches and methodologies for assessing the whole life-cycle of nanomaterials and nanoproducts. In particular the workshop considered:

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

The goals of the workshop were to:

- Support the development of approaches and methodologies for assessing the impacts of nanomaterials during their life cycle in the environment, by assessing the fate and behaviour of nanomaterials in the environment.
- Elaborate upon current data regarding health and environmental exposure to nanomaterials throughout the life cycle of nanoproducts
- Provide advice regarding the identity, quantity and properties of relevant nanoparticles released into different compartments

An important aspect of the workshop was the group discussions where the participants were split up into three groups which discussed several pre-defined questions. The groups discussed 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 to the participants was how risk assessment methods and methods based on a life



cycle perspective can complement each other. One group also focused the discussion on life cycle assessment methods and on the main elements of life-cycle assessment that are missing in relation to nanotechnology products.

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

The third part of the Zurich 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 nanomaterials throughout their lifecycles;
- the fate and persistence of nanoparticles in humans, animals and the environment;
- the associated risks of nanoparticle 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 the workshop provided an overview of the main stakeholder perspectives, including presentations from representatives of industry, regulatory authorities, NGOs, insurers and the European Commission, followed by break-out discussions. During this part of the workshop, stakeholders contributed actively to the discussions regarding information needs, communication, the safe use of nanomaterials, whether more or other regulation was needed and whether enough information was available to make informed decisions regarding the safety of nanomaterials and products containing nanomaterials.

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 nanomaterials 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 nanomaterials was raised, as in the public mind safety and labelling are connected. This may need to be addressed soon since the issue of nanomaterials 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 the uncertainties, adaptations of frameworks such as REACH may be necessary for nanomaterials. Regulation is often needed even if voluntary measures are welcome 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 Integrated Conference with Training School and Workshops in Lausanne, Switzerland

Scientists, industry, policy makers and civil society from around the world converged in March 2009 at the University Hospitals of Lausanne, Switzerland, to discuss the challenges and limitations of exploring and characterizing nanomaterials. The conference was structured into 5 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 which provided 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

The 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 nanomaterials. The focus of this first training school was on protocols for handling nanomaterials and protocols for toxicology testing. The issues such as controlled dose (understanding of aggregation of nanoparticles in the presence of biological fluids), controlled presentation of nanoparticles to the test system, and development of appropriate testing strategies taking into account the novel aspects of nanomaterials which can influence the testing were tackled. 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 sub-groups in order to ensure that the group size was optimal for encouraging discussion and engagement of the students. Each sub-group attended each of the three training sessions in sequence, meaning that the sessions were run in parallel and repeated three times.

Workshop - Protocols for assessment of biological hazards and biological responses

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

A limitation for determining the health and safety of nanomaterials 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 nanomaterial 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). Following these presentations, participants divided into break-out groups to consider how to develop and apply different approaches within the nanotechnology field. The break-out 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 industry 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 Workshop, The Netherlands

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

6 Directory

Table 2 Directory of people involved in this project.

Nanoparticle metrics in the air, exposure scenarios and exposure routes

Particle number and particle size distribution are *de facto* standards to describe nanoparticle 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 nanomaterials, 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 nanomaterials. 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 nanomaterials. It brought support to the integration of existing knowledge and newly gained insights to aid in the development of risk assessment methodologies adequate for nanomaterials.

Reports on each of these workshops are in progress.

5.1.5 The Bratislava Training School, Slovakia

This training workshop for young researchers was conducted in Bratislava 9-11 November 2009 on "Life cycle-based methods for assessing nanomaterials".

The increasing production and use of engineered nanoparticles raises concerns over their safety to human and environmental health. The training workshop 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 nanoparticles. A basis for the development of adequate methodologies, tools and indicators for assessing the life cycle of nanomaterials was presented. Students got an overview on life cycle methods together with practical training in using life cyclebased tools. The targeted audience was young researchers, PhD students and junior scientists from different fields with an interest in the fate and life cycle of nanomaterials.

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NanoInteract

Development of a platform and toolkit for understanding interactions between nanoparticles and the living world



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1 Summary

NanoInteract was an EU FP6 STReP funded under the NMP theme, running from 01/01/2007 to 31/12/2009. The overarching objective of NanoInteract was to create a firm scientific and technical basis for understanding and potentially predicting the likely biological impacts of engineered nanoscale particulates interacting with living systems. NanoInteract was built around the belief that a fundamental understanding of the space- and time-resolved interaction of nanoparticles in their *in situ* biological context (i.e. with their corona of proteins and other biomolecules) with living systems is essential to ensure the safe implementation of nanotechnologies.

The NanoInteract program acknowledged from the outset that there were significant limitations in the direct application of the traditional toxicological approaches (as applied to pesticides and air pollutants for example) to the assessment of (potential) nanoparticle hazards. Besides the potential for new toxicological end-points which are not addressed within existing testing strategies (such as nanoparticle-induced protein fibrillation), there are many problems of a more technical nature that cast uncertainty on the reliability and applicability of current methods for assessment of nanoparticle impacts. Important objectives of the NanoInteract program were therefore to produce controlled and reproducible dispersions of nanoparticles in biological fluids and to correlate the nature of the protein corona with the sub-cellular localisation and functional impacts of nanoparticles in a time-resolved manner.

To date the program has lead to no newly identified hazard (solely due to nanoscale elements) for nanoparticles, but it has highlighted several issues requiring further investigation, in particular related to the need for consideration of appropriateness of several of the established OECD (and other) tests for chemical toxicity to assessment of nanoparticle toxicity. NanoInteract has resulted in over 40 original peerreviewed publications on various aspects of nanoparticle interactions with living systems, including round-robin studies to validate test methods, and 2 books are in preparation.

2 Project Description and Organisation

NanoInteract seeks to understand how engineered nanoparticles interact with living systems, from the moment of first contact, through uptake and localisation, and finally to the onset of functional and signalling impacts induced by the presence of the nanoparticles. Thus, a first step was to identify some of the routes via which nanoparticles enter and accumulate in cells, aquatic flora and fauna and some terrestrial species. Then, using advanced methods of chemical, physical, biological and toxicological sciences Nanointeract attempted to connect nanoparticle properties to the mechanisms via which they interact with, and disrupt, cellular processes. This knowledge was then connected to the outputs from classical toxicology testing (such as those relevant to REACH and OECD considerations), in order to evaluate whether these existing toxicity tests are sufficient to predict nanoparticle toxicity, or whether they need to re-designed, or supplemented with newer approaches more tailored to the issues implicit in the nanoscale.



The interactions and knowledge-flows within NanoInteract are summarized in Figure 1. Fundamental to the project was the effort to establish protocols and standards via which every step of the project could be controlled, including across partner laboratories, as we sought to eliminate the factors that currently cause irreproducibility in terms of the outcomes from tests performed on identical systems in different groups. Some of these protocols are already being made available to the woder reserach community via the NanoImpactNet protocols database, and those that are unpublished as yet are being compiled into a book for publication as an output from the NanoInteract project, in order to enhance progress in the field and to share our experiences in this arena with others.

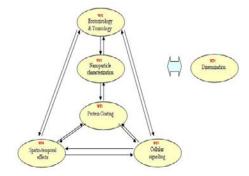


Figure 1 Overview of the interactions and interconnections between the different scientific disciplines, within NanoInteract, and the division of the project into workpackages.

The NanoInteract objectives are summarized as follows:

- Establishment of experimental protocols for every aspect of the study of nanoparticle interaction with cells, and several types of aquatic plants and organisms, ensuring complete reproducibility.
- Understanding the effect of adsorbed protein on nanoparticle stability and nanoparticles on protein conformation and function, ultimately connecting this to biological impacts.
- Connecting final cellular location of nanoparticles with the intra- and inter-cellular processes disrupted.
- Combining these results, along with the expertise from diverse disciplines, to point towards a 'standard approach to nanosafety assessment'.



2.1 Summary results and outputs

The NanoInteract programme acknowledges that even after the 3 years of this research project, the field of nanosafety assessment is an emerging field, with few established paradigms or standard practices or protocols. A key driver for the NanoInteract consortium was the recognition of its responsibility to ensure that any contributions to the literature that emerged from the NanoInteract project were of the highest scientific quality, including internal checking and confirmation, so as not to add further confusion to an already complex arena. The first interlaboratory study of a test for nanoparticle-induced geneotoxicity, using the Comet assay emerged as an early output from the project (Barnes 2008), as well as an interlaboratory study showing that nanoparticle can modulate the rate of protein fibrillation in solution (Linse 2007).

The depth and breadth of the issues are unique, and the foundation of the field on a rational and quantitative basis is of paramount importance for all concerned, from governments to NGOs to industry and beyond. As there are still so many unknowns, a key aspect of the NanoInteract programme is the use of internal round robin type approaches, whereby important results are reproduced and replicated in at least one completely separate laboratory using the identical protocols and starting materials, in order to ensure that the results are scientifically valid and reproducible. In addition to addressing known risks from nanomaterials, such those endpoints for which there are established mechanisms of action (e.g. DNA damage), NanoInteract is also addressing the potential for new toxicological end-points, such as nanoparticle-induced changes in protein aggregation and fibrillation, as well as changes in subtle gene and protein regulation pathways.

A key aspect in the success of the NanoInteract project has been the development of secure processes for controlling the systems used across all partners. Within the NanoInteract project, the investigations have focussed on several key classes of nanoparticles – silicon dioxide, cerium oxide, aluminium oxide (both particulate and monolithic surfaces), polystyrene, quantum dots and gold nanoparticles, being the principle materials used across the studies. Not all studies have been performed on all particle types, but certain particles have been carried through the majority of the studies in order to have a complete story. In this case, the test particle is the silica dioxide, which also has significant commercial interest, as it is already in use in industry. All particles were held centrally by partner 1, and cleaned and dispersed for periodic shipment to the partnerss in the appropriate solutions (concentration, pH, etc.).

2.1.1 Toxicology and Ecotoxicology

An early highlight from the toxicology work was the first crossinstitutional example of a round-robin type toxicology experiment, where identical results were obtained in two separate institutions using an identical protocol, and common cells, serum and nanoparticles. The test used was the COMET assay for genotoxicity, and the particles used were silica nanoparticles from a commercial source (Sigma) and from within the NanoInteract consortium (Glantreo), in a range of nominal sizes (10-400nm) and with different surface properties (surface charge and coating). Using the 3T3 cell line, no

genotoxicity was observed and the same answers were obtained in all laboratories. One year of intra-laboratory visits was required before the problems with the round robin were identified. Importantly, no genotoxicity was observed using these nanoparticles, in this cell line, and over the concentration range tested, as shown in Figure 2. More recently, the same range of nanoparticles have also be tested using other tests for genotixicity, such as micronucleus assay and the lacZ assay, which look at chromosomal damage and gene mutations respectively. In these studies measuring different end points compared to the Comet assay some genotoxicity was observed at non toxic concentrations. Localisation studies showed no evidence of direct contact between DNA and the nanoparticle, which were never detected in the nuclei, and were always localised to vesicular structures, likely lysosomes. Results are currently being prepared for publication.

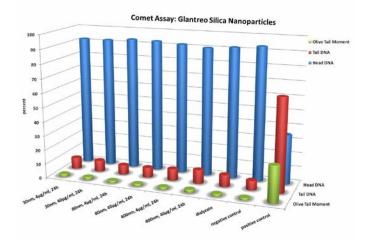


Figure 2: Summary of Comet assay results on the different sizes of Glantreo silica nanoparticles tested following 24 hours of exposure. No genotoxicity was observed in 3T3-NIH cells at the range of concentrations tested.

The ecotoxicological research of NanoInteract involved mainly aquatic studies with SiO_2 and CeO_2 nanoparticles of various sizes. The unicellular green alga *Pseudokirchneriella subcapitata* was used as the primary test organism in algal growth inhibition assays in accordance with the OECD protocol No. 201. Algae form the basis of the aquatic food chain and were found to be the most sensitive test organisms.

The main aims of the effects assessment were (1) to investigate the difference in toxicity between nanoparticles and their corresponding bulk material, (2) to assess the importance of nanoparticle surface area for their ecotoxicity, (3) to determine if nanoparticles were taken up by algal cells, (4) to evaluate if any important interactions between nanoparticles and the ecotoxicity test medium occur that indirectly can cause toxicity, and (5) to investigate the influence of abiotic factors, like the addition of natural organic matter (NOM), pH and ionic strength, on the toxicity of engineered nanoparticles.

No toxicity was found for either SiO_2 or CeO_2 bulk powder at the maximum test concentrations of 1000 mg/l. On the other hand, 12 and 27 nm SiO_2 NPs resulted in $E_rC_{10}s$ of 10.9 and 15.0 mg/l, respectively. For 14, 20 and 29 nm NPs, $E_rC_{10}s$ of 2.6, 3.4 and 5.4 mg/l were obtained, respectively.



It was clearly demonstrated that reduction in size to the nanoscale enhanced toxicity of the materials, in comparison to their inert bulk material.

Secondly, it was demonstrated that for both NP types ecotoxicity towards *P. subcapitata* was related to the NP surface area. In case the concentration was expressed as mass, the smaller NPs were more toxic than the larger NPs. However, when concentration was expressed as surface area, no difference in toxicity was found for various particle sizes. It was furthermore demonstrated that despite the aggregation of CeO_2 NPs, the nominally smaller particles were still more toxic on a mass basis.

No evidence was found for NP uptake in algal cells. SiO_2 NPs adhered strongly to the cell wall and covered the entire surface. CeO_2 NPs clustered around algal cells, but no strong physical interaction between particles and cells was observed.

NP dissolution behaviour and adsorption of test media constituents was also assessed. The SiO₂ NPs dissolved up to a maximum reactive silica concentration of 4.1 mg/l, while dissolution of the CeO₂ NPs was below the ICP-MS detection level. Hence, any toxic response could not be due to the presence of solutes released from the NP surface. The nutrient phosphate was found to strongly adsorb to the CeO₂ NP surface. The amount of phosphate adsorbed was also governed by the CeO₂ NP surface area. However, an additional experiment elucidated that the maximum of 50% phosphate depletion of the medium due to adsorption to the NP surface could not be responsible for the observed CeO₂ NP toxicity. Bare SiO₂ NPs did not adsorb nutrients, but total depletion of phosphate was observed with Al₂O₃ coated SiO₂ NPs at low pH (6.0-6.8). In the latter case, the phosphate depletion significantly contributed to the decrease in algal growth rate.

Finally, one of the major achievements in the ecotoxicology section of NanoInteract is the experimental assessment of the influence of NOM on NP toxicity. At 2 mg C/I NOM, the E_r C10 values of SiO₂ NPs increased by a factor of 20. A similar detoxifying effect of NOM was obtained with CeO₂ NPs. For example, at 2.2 mg C/I and 7.4 mg C/I, the E_r C10 of 14 nm particles increased 7.7 and 31.5 fold, respectively. In addition, the NOM was able to prevent CeO₂ NPs from aggregation to micrometer size particles. In a multivariate study using 14 nm CeO₂ NPs, pH was also identified as an important parameter. Toxicity was strongly reduced at the lower pH values of 6.0-6.6.

The full studies are reported in (Van Hoecke 2008 and Van Hoecke 2009). Significant other work on the assessment of toxicity and ecotoxicity (both facte and effecst) of nanoparticles has been performed, some of which is already published, and some of which is still in preparation for publication. However, it should be noted that no significant nanoparticle-associated hazards have been identified within the project, except in some very specific cases at high concentrations (e.g. Park, 2009)

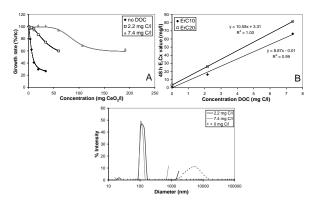


Figure 3: A: concentration-response curves of 14 nm CeO_2 NPs in absence and in presence of 2.2 and 7.4 mg C/I NOM. B: Illustration of the linear relationship between concentration of NOM in the test medium and the effect concentrations on algal growth rate. C: Illustration of the stabilizing effect of NOM on 14 nm CeO_2 NP suspensions. In presence of NOM, small aggregates are stabilized at about 100 nm.

2.1.2 Nanoparticle dispersion & Protein Corona

Establishment of a solid common ground and understanding for comparing the results from the very different groups and experimental approaches used in the NanoInteract project was a key goal. This was quite a significant challenge in many cases, as each cell line or test often required a different growth medium with different salts and pH, all of which affect the stability of the nanoparticle dispersion, and as such complete characterisation of the nanoparticle dispersions in each medium is necessary. This is a significant draw-back of the current ecotoxicology testing framework, as well as restricting direct comparison between different cell lines. Within the NanoInteract programme, we have overcome this limitation by having (where possible) a single protocol, and where this is not possible to compare the recommended protocol to our protocol. While this significantly increased the effort required from all partners, it also significantly increased the reliability and reproducibility of the outcomes and results.

The huge variability between batches of nominally identical particles from the same source was not fully appreciated when writing the NanoInteract project description, but during the project we learned that this is not an insignificant issue in determining nanosafety. For example, we found that different batches of positively charged polystyrene from the same supplier and with identical specifications induce different degrees of cellular apoptosis in identical cells at identical nanoparticle doses (50 μ g/mL). The implications are profound for the field in general.



Perhaps one of the most striking aspects of the nanoparticlescell interaction story, that clearly distinguishes nanomaterials from chemicals, is the issue of the 'protein corona'. This concept was included in the NanoInteract proposal, but we did not fully foresee the implications of the corona. This arena has been clarified by several partners within NanoInteract (Cedervall 2007a, 2007b, Lundqvist, 2008), and lead to the award of the 2007 Cozzarelli prize of the US National Academy of Sciences. In essence, chemicals (generally) 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. This has also fed into the exciting results emerging from AFM studies of cell-nanoparticle interactions in the presence and absence of proteins, where very different forces are observed, and in cellular uptake studies in the presence and absence of serum, where again very significant differences are observed in the uptake rate, amount and overall behaviour

Figure 4 makes the issue clear by showing the competitive processes that occur between nanoparticles, proteins and cell membranes, and which ultimately determine uptake of nanoparticles by receptor-mediated processes. 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 considering the particle and its adhering proteins, and the interaction of this object with biological membranes and barriers.

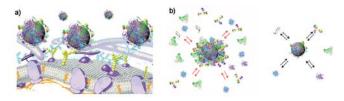


Figure 4: Kinetic processes between the effective bionanoscience particle in a biological fluid and the cell. (a) Cartoon representation of the relevant kinetic processes between the nanoparticle-corona biomolecules and cell surface. (b) Schematic drawing of the structure of NP-protein complexes in plasma: the "core" nanoparticle is surrounded by the protein corona composed of an outer weakly interacting layer of protein (left, full red arrows) rapidly exchanging with a collection of free proteins and a 'hard' slowly exchanging corona of proteins (right, black dotted arrows).

Very considerable progress has been made within the NanoInteract project towards elucidating the nature, identify, conformation and aggregation of proteins incorporated into nanoparticles-coronas, and to understanding the consequences of these interactions for both particle and protein stability. Significant effects of particle composition and size on the nature of the adsorbed proteins has been observed, using a series of polystyrene nanoparticles of different surface charge and different sizes. Important proteins from the lipoprotein class have been observed to show differential binding to the differently charged nanoparticles, and differential biological responses to the same sets of particles have also been observed, suggesting a link between the corona and the biological or functional impacts from nanoparticles, However, it is still too early in the research to draw any definite conclusions regarding specific proteins or signals, and much more work towards this long term goal is required, far beyond the scope of NanoInteract.

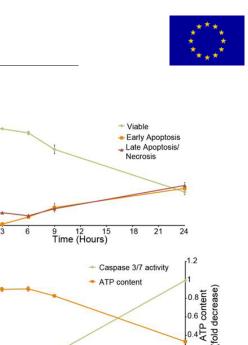
2.1.3 Spatio-temporal uptake & distribution of nanoparticles

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 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 5A where uptake and export of a molecular dye are tracked by fluorescence flow cytometry (Salvati and others 2010).

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 5B where we show effects of cell energy depletion on nanoparticles uptake), driven by active biological processes that are currently being uncovered by follow-on projects, such the FP7 NeuroNano project (page 181). Sufficient preliminary information now exists (Salvati and others 2010; Walczyk 2009) (although publication will in some cases take some time more for a complete story) 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.

High content analysis (HCA) has been used to screen several nanoparticles in a multiplexed approach, and the effects observed correlate well with the detailed uptake studies, and indicate that for the nanoparticles tested, the nanoparticles selectively localise to the lysosomes, which are known to be the waste-bin of the cell. The uptake studies have shown that nanoparticles uptake is energy-dependent, highly regulated and likely occurs by several routes. For several exemplar nanoparticles (including polystyrene and silica) we have not observed any evidence of exocytosis in the cells studies performed in the presence of serum, suggesting that nanoparticles may bioaccumulate in cells. Correlation of the spatio-temporal uptake studies, the HCA studies, and the functional impact studies (below) are underway, and will be published shortly.

C



18 21

STS

15

0.2

_____0 24

Time (Hours) PARP-Cleaved PARP-1

GAPDH

Α

в

С

Caspase 3/7 activity

(fold increase

100

80

60

40

20 0

12

10

8

6

4

2

0

Percentage of cells

Figure5: 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) (Salvati and others 2010).

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D

Nanoparticles Functional of 2.1.4 impacts nanoparticles

As stated above, no significant toxicological effects have been observed with any of the nanoparticles studied, except in some specific cases, such as in embryonic stem cells. However, NanoInteract has never intended to study only simple cytotoxicity, but has always been more interested in subtle functional or signalling changes induced by the presence of nanoparticles, and understanding these and the consequences of such functional impacts.

Much of the functional impacts work has been performed during the second half of the project, and as such several manuscripts are in the final stages of preparation, and so the data cannot be presented here. One very interesting observation has been the induction of an apoptosis-like pathway in cells by amine-modified polystyrene nanoparticles, which we observed in A549 cells, and which has also been reported by Nel in macrophages (Xia 2009). The initial data suggested that the programmed cell death was occurring via apoptosis, and that the process was occurring in a time and concentration-dependent manner. 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 is shown for illustrative purposes (Bexiga 2010). However, as we have probed deeper into the mechanisms, it seems to be not as straightforward as simple apoptosis, and is instead a mixture of apoptosis and autophagy. By probing the processes in a timeresolved manner, we have been able to map out the onset of each of the effects observed, and several markers of autophagy are also observed (manuscript in preparation).

Figure 6: NH₂-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 NH₂-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 NH2-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 50 [a]/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.

9 12 1 Time (Hours)

Global characterization of the effect of a range of nanoparticles on mRNA (gene) expression using DNA microarrays was performed on a series of reference (polystyrene and silica nanoparticles). The first experiments were exploratory, and did not involve any attempt to remove the particles prior to isolating and extracting the m-RNA. This was as planned in the project, and in accordance with known practices where the perturbant is not removed prior to m-RNA extraction. However, a key finding of the project is that the nanoparticles interfere with many read-out approaches, and as such a more complete study of each of the extraction and isolation steps was performed, to determine best practice for performance of gene expression in the presence of nanoparticles.



For example, physical interactions between mRNA and nanoparticles may affect the transcription process, and effects of ozone have been shown to be significant for the fluorescence efficiency of the Cy3 and Cy5 dyes during the hybridization steps. A review on these issues is currently in preparation.

Very significant progress has been made to address the question of functional impacts of nanoparticles, and there are a series of publications in preparation from this aspect of the project.

2.1.5 Summary of NanoInteract outcomes

To date the NanoInteract program has lead to no newly identified hazard (solely due to nanoscale elements) for nanoparticles, but it has highlighted several issues requiring further investigation, in particular related to the need for consideration of appropriateness of several of the established OECD (and other) tests for chemical toxicity to assessment of nanoparticle toxicity, as well as highlighting important issues such as the lack of comparability across data generated using different standard (e.g. OECD) test media which have different ionic strengths and pH, different sources of serum and treatments of the serum. This is in addition to the particlerelated challenges such as the different surface properties for the same material made by different synthesis routes, not to mention issues such as batch-to-batch reproducibility for a single nanoparticle synthesis route.

NanoInteract has resulted in over 40 original peer-reviewed publications on various aspects of nanoparticle interactions with living systems, including round-robin studies to validate test methods, and several more are in preparation, including 2 books or protocols and best practice are in preparation. Some of the detailed protocols that resulted from NanoInteract are already being shared with the wider scientific community via the NanoImpactNet protocols database (see also page 99).

An enduring output from NanoInteract is the issue of the nanoparticle-protein complex *in situ* is the relevant "biological entity" that interacts with living systems, and is what the cell (or organism) sees. The fact that a similar argument holds for natural organic matter in ecotoxicity testing is a further confirmation, and suggests that for both human and ecological safety testing of nanoparticles, detailed characterisation of the nanoparticles as they exist *in situ* is vital.

The need for careful controls, assessment of all of the potential interactions of nanoparticles with the test system and all potential artefacts from the presence of nanoparticles in the test system need to be addressed in all studies. The use of interlaboratory or round-robin type studies to ensure robustness of protocols and reproducibility of test outcomes has also been demonstrated for nanosafety testing using the Comet assay for genotoxocity and for protein fibrillation assays in the presence of nanoparticles, among others. This approach gives increased confidence in the protocol, in the data and in the interpretation of the data, as it involves an iteration of the experimental approaches until convergence of outcomes across different users and different laboratories is achieved.

Another key output is the demonstration that truly quantitative studies are possible using nanoparticles interacting with living systems, including quantitative uptake and sub-cellular distribution studies. Such quantitative data can then be further utilised in modelling the interactions of nanoparticles with cells, another aspect that is underway, and will be further developed in follow-on projects, funded nationally and via the EU FP7 programme.

Many of the concepts developed in the NanoInteract project have been taken up into the FP7 project NeuroNano, as well as informing the CSA NanoImpactNet and other projects. We expect the impact to extend for several years to come.

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NANOKEM

Nanoparticles in the paint- and lacquer industry. Exposure and toxic properties

Contract Agreement: Danish Working Environment Research Fund (grant #20060068816)

Website:http://www.arbejdsmiljoforskning.dk/Aktuel%20forskning/Nanopartikler_i_farve_og_lakindustrien_-_NANOKEM.aspx?lang=en

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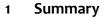
No.	Beneficiary name	Short name	Country
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3	DHI-group	DHI	DK
4	The Danish Paint- and Lacquer Organization	DPLO*	DK
5	Flügger	Flügger	DK
6	Boesens Fabrikker	Boesens	DK
7	Akzo Nobel Decorative Coatings	Akzo Nobel	DK
8	Dyrup A/S	Dyrup	DK
9	Beck & Jørgensen A/S	Beck & Jørgensen	DK
10	Teknos A/S	Teknos	DK

Unofficial short names

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7 Dissemination
-



NANOKEM is a 3-year Danish project with 5 mio Kr external funding from the the Danish Working Environment Research Fund, which focuses on determination of several hazard endpoints (inflammatory, carcinogenic, cardiovascular, reprotoxic and allergenic effects) and the risks of exposure to engineered nanoparticles used in the paint and lacquer industry and to dust generated during sanding nanoparticle-doped paint and lacquer products. The results are used to conduct an overall risk assessment for production and use of nanoparticle-doped paint and lacquer products. Seven engineered "nanomaterial" additives are tested (three TiO₂ materials, kaolinite, carbon black, two silica materials), two paint groups (polyvinyl acetate and Acryl), one lacquer type (UV Hardcoat) and two fillers (normal and fine). The paints are added 2-28 wt% engineered nanoparticles, normally replacing similar amounts of pigments and/or fillers. Six peer-reviewed articles have been published or submitted at this point and approximately 8 additional papers are expected at the end of the project.

2 Project overview

2.1 Description

The NANOKEM project focuses specifically on issues related to the application of engineered nanoparticles in the paint- and lacquer industry. The project focuses on two main scenarios; 1) the exposure risk during use of ENP powders and slurries as well as during sanding ENP-doped paints 2) comprehensive studies on the potential health risks associated with these exposures, and 3) a integrated risk assessment based on the obtained research results and a risk assessment model developed in the project. By focusing on a single industrial sector, the consortium aims at a rapid use of the used in praxis. The project contains four sub-projects outlined below.

1. Assessment and exposure risks of nanoparticle handling and sanding nanoparticle-containing paints.

2. Characterization of physical and chemical properties of nanoparticles and dust generated from sanding nanoparticle-containing paints.

3. Characterization of toxic properties of nanoparticles and dust generated from nanoparticle containing paints.

3.1. Translocation of nanoparticles and dust generated from nanoparticle-containing paints across tissue barriers.

3.2. Genotoxic effects of nanoparticles and dust generated from nanoparticle-containing paints.



3.3. The effects of nanoparticles and dust generated from nanoparticle-containing paints on the cardiac system.

3.4. Embryogenic effects of nanoparticles and dust generated from nanoparticle-containing paints.

3.5. Allergenic effects of nanoparticles and dust generated from nanoparticle-containing paints.

4. Development of model for risk assessment of nanoparticles, for use or expected use in the paint- and lacquer industry.

2.1.1 Time Schedule

Project's time frame is 2007-2010.

2.1.2 Organizational structure of the NANOKEM project

The structure and management of the NANOKEM project and inter-relationships by which the research and communication activities are coordinated across is shown in Fig. 1. Contacts, members of the consortium and the international advisory board are presented below.

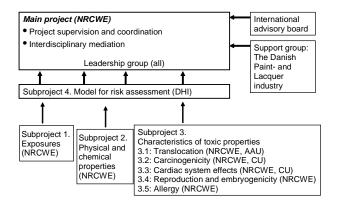


Figure 1. Organization diagram and communication flow in the NANOKEM project

2.1.3 International advisory board

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Lang Tran: Institute of Occupational Medicine (IOM), Scotland, $\mathsf{U}\mathsf{K}$



Steffen Loft: Institute of Public Health, Copenhagen University, Denmark

Håkan Wallin: National Research Centre for the Working Environment, Denmark

3 Background

Nanotechnology is a rapidly growing field and the existing knowledge about the exposures and toxic effects of nanoparticles is sparse. There is a growing need for more practical and theoretical knowledge about nanoparticles and their health effects. It is evident that particulate chemicals gain new properties and extra reactivity when they are reduced to the nanoscale. However, it is currently not possible to anticipate the toxic characteristics of new nanoparticles, and more so, draw analogies from one nanoparticle to the next.

The existing epidemiological knowledge about the possible health effects of engineered nanoparticles comes primarily from knowledge from exposures to particles of similar sizes, e.g. diesel exhaust particles, air pollution particles and technical carbon particles such as carbon black. Airborne particles are recognized to be potentially: carcinogenic, increasing the risk for pulmonary or cardiac diseases, or cause allergies. There is only a sparse knowledge of the potential embryogenic effects of particles. New research indicate adverse health effects on fine particles, especially on particles under 100 nm (nanoparticles). It is the particulate surface reactivity, and ability to cross cellular membranes or other body barriers, that induce inflammation and oxidative stress, that in many cases seem to correlate to these diseases.

The main focus of this project is on nanoparticles in the paintand lacquer industry. The industry already uses a large amount of nanoparticles and there is a substantial exposure risk while handling nanoparticles as powders or aerosols while spray painting, or as they are released from their matrices during sanding or welding painted materials. The Paint- and Lacquer Industry is expecting a boom in the use of technical nanoparticles. One advanced possibility is the use of quantum dots (semiconducting nanocrystals) that upon excitation emit light in well a defined wave-length, which is perceived by the eye as a specific color. They are often composed of a metalic complex core, covered by a layer of protective material, such as zinc sulphide. The surface is composed of active groups, giving quantum dots special properties (<u>http://www.evidenttech.com</u>). There are developed other nanoparticles with attractive properties as well, for example nanoparticles creating hard surfaces (in lacquers), nanoparticles that may remove organic dirt and odors, thereby improving the indoor climate, or nanoparticles with UV-filter function for prolonged life-time of the paint.

4 Objectives

The goals of the project are to identify and characterize the potential risks caused by exposure to nanoparticles in paint- and lacquer industry. The main project will combine knowledge of four research fields and carry out close cooperation with the Paint- and Lacquer Industry. Discussions of results will be conducted with relevant stakeholders from the industry, occupational branch organizations and the Danish Working Environment Authority with the anticipation of formulating principles of ensuring a safe working environment and development of safe products.

The goals of the four subprojects are:

- Evaluation of exposure risks while handling nanoparticles and while sanding paint and lacquers containing engineered nanoparticles.
- Characterization of the selected physico-chemical properties of the engineered nanoparticles used in paints and lacquer as well as dusts created by sanding these products.
- Identification and evaluation of the toxic effects of engineered nanoparticles used by the paint- and lacquer industry and comparison to the effects created by sanding dust from paint products. Translocation of particles across barriers, carcinogenicity, effects on cardiac system, embryogenic and allergenic effects will be part of the study.
- Development of model for risk assessment of nanoparticles, for use or expected use in the paint- and lacquer industry. Overall risk assessment of work with nanoparticle powders and sanding paint and lacquers with nanoparticles.

5 Work description

5.1 Assessment and exposure risks of nanoparticles and dust generated from nanoparticle-containing paints.

The goal of this subproject is to evaluate exposure risks involved with handling of technical nanoparticles released during sanding and polishing surfaces treated with nanoparticle-containing paints, lacquers and polishes. In cooperation with the paint- and lacquer industry, seven engineered "nanomaterial" additives were selected (three TiO_2 materials, kaolinite, carbon black, two silica materials) as well as two paint groups (polyvinyl acetate and Acryl), one lacquer type (UV Hardcoat) and two fillers («heavy « and « fine »). The paints are added 2-28 wt% engineered nanoparticles, normally replacing similar amounts of pigments and/or fillers. Exposure potentials from handling nanoparticle powders are simulated by a dustiness test for identification of diameter dependent dust index, based on European standard (EN15051) and modified by NRCWE for testing nanoparticles. Differences in the dust index will be used



for evaluation of differences in exposure risks and compared with results from two field measurements. Sanding operations will be evaluated in full scale simulation exposure chamber at NRCWE. Dust fractions smaller than app. 5 μ m (most relevant for human evaluation and mouse models) will be collected in a sample bank and will be used in projects three and four. Finally exposure risks from sanding will be done by determination source strength analysis under different user scenarios.

5.2 Physical and chemical characterization of nanoparticles and dust generated from sanding nanoparticle-containing paints.

The goal of the project is to carry out toxicologically relevant physical and chemical characterization of the technical nanoparticles and dusts produced by sanding the final products. Further, particulate chemical reactivity in synthetic biological fluids will be studied. This will enable the evaluation of the solubility and biodegradability of individual particles, as well as their capability to create oxidative stress and thus damage cells and living organisms. Results will be compared with the toxicological studies, to eventually identify subtle causal mechanisms; and will be used in risk assessment analysis. Scanning and Transmissions Electron Microscopy will be used to characterize the particle physical size, form, crystalinity and physical and chemical complexity on micro- and nanoscale. Crystalline compounds will be identified and quantified by analysis powder X-ray diffraction analysis. The total chemical and elemental composition will give information about the potential chemical inhalation exposure caused by the technical nanoparticles or dust created from painted and lacquered products. The particles' redox activity and ability to form free radicals and their biodurability in synthetic physiological fluids will be studied.

5.3 Translocation of nanoparticles and dust generated from nanoparticle-containing paints across tissue barriers.

The goal of this project is to investigate if nanoparticles mixed in test paints can cross the tissue barrier and thus can translocate into the body. Particles can travel in nerves at speeds up to 2.5 mm/hr. The project investigates to what extent three different nanoparticles can pass over the lung barrier in mice. Nanoparticles will be chosen from particle sample bank, after the initial screening described in project 3.2. Mice will be exposed to particles via intratracheal installation and the distribution of particles in different tissues will be studied by light- and transmission electron microscopy. Exposure times and choice of tissues will be determined based on experience gained during work with other nanoparticles in an on-going study.

5.4 Genotoxic effects of nanoparticles and dust generated from nanoparticle-containing paints.

The goal of the project is to investigate if nanopaticles and dust containing nanoparticles can cause inflammation or DNA damage, associated with carcinogenicity. Tested nanoparticles and dust containing nanoparticles will be screened in *in vitro* assay for cytotoxicity in the murine epitelial cell line MML. All the materials will be tested by intratracheal installation in C57 mice. DNA damage and inflammation will be identified. Inflammation will be measured by quantitative real-time PCR mRNA expression of cytokines in vivo (murine lung tissue). The DNA damage will be detected by the Comet assay. Assay quantifies the total amount of DNA damage and oxidative stress.

5.5 The effects of nanoparticles and dust generated from nanoparticle-containing paints on cardiac system.

The goal of this project is to investigate if nanoparticles and paint dust that contains nanoparticles can cause cardiac diseases. Tested particles will be screened for their abilities to induce systemic inflammatory effects. The most potent particles (2-3 particles) will be examined to understand if they can:

1) induce production of acute phase proteins

2) influence endothelial function in the form of vasomotoric response in aorta segments in vitro.

In vivo imaging of transgene HO-1/NF-κB mouse: The production of acute phase proteins and transcription factors, and thus response to particles in the vascular system, will be exhibited in mice with luminescent protein (luciferase). Response can be followed by in vivo imaging in living animal over time. Acute phase proteins will be measured as mRNA expression of acute phase proteins in liver, lungs and aorta of exposed mice. Biomarkers for vascular response: Aorta of mice with minimal calcification will be examined after the exposure to several nanoparticles. Studied will be effects on endothelial cells and induction of inflammatory response.

5.6 Embryogenic effects of nanoparticles and dust generated from nanoparticle-containing paints.

The goal of this project is to examine if nanoparticles can have adverse effect on the fetal development and cause birth defects in the offspring.

Pregnant mice will be exposed via inhalation to nanoparticles (chosen based on results of project 3.2) and microparticles. Animal wellbeing during the exposure will be monitored, and after birth the effects on the offspring survival and development will be registered. Registered parameters will be obvious birth defects, growth during lactation and effects on



the nervous system. The nervous system will be evaluated by a series of specific behavioral tests. Effect of nanoparticles on pregnancy and birth will be registered as weight gain during pregnancy, gestation length, litter size, offspring weight, number of aborts. Effects of nanoparticles on offspring's nervous system will be evaluated by methods of behavioral teratology developed at NRCWE. Exposure to particles may affect basic brain functions such as learning speed and memory. Further, effects can be expressed as level of activity and motoric- and cognitive function of offspring.

5.7 Allergenic effects of nanoparticles and dust generated from nanoparticle-containing paints.

The goal of the project is to investigate to what extent exposure to nanoparticles via respiratory system can affect the immune system and cause inflammatory reactions and/or cause development of allergies. The project will be divided into four sections. The first two sections will study the inflammatory response to reference- and industrial nanoparticles and the other two will study the adjuvant effects. Acute study with quartz as the reference/standard particle will be used to determine particular inflammatory properties. Dose response to intratracheal installation of this standard particle in mice will be reported. Inflammatory effect will be reported as number of inflammatory cells in the Bronchoalveolar lavage (BALF), as well as development of cytokines at mRNA and protein level. Inflammatory effects of the two most potent industrial nanoparticles (project 3.2. criteria) will be compared to the effects of the standard particle. Adjuvant effect of nanoparticles will be evaluated as a production of antibodies in blood (IgE, IgG1, IgG2). Adjuvant effect of standard nanoparticles and industrial nanoparticles will be compared to the effect of standard adjuvant $Al(OH)_3$. Standard particles will be studied at four concentrations and at four sizes to examine the importance of particle size and adjuvant effect. Industrial particles will be studied based on results with standard particles. Particles will be compared for their potency.

5.8 Development of model for risk assessment of nanoparticles, for use or expected use of paintand lacquer industry.

The goal of this project is to develop a model for risk assessment of nanoparticles. The model will contain a collection of internationally published data and knowledge of nanoparticles that will be generated in other subprojects. Project will characterize toxic properties and dose response relationships of nanoparticles, to allow comparison to the actual/estimated exposure scenarios.

Part of this project is to obtain supplementing literature concerning risk assessment of particle exposure, especially exposure to small particles via airways, digestive system, skin and other exposure routes. Parameters for uptake of particles will be evaluated. Existing models for nanoparticle toxicokinetic will be tried and evaluated. Based on the collected knowledge, a model will be developed for evaluation of toxicological risks to nanoparticles.

6 Conclusion

Conclusions of this project will be presented in scientific journals (see below) and on the Nanokem homepage (see *links to information*) when the project publications have been completed. Additonally, knowledge from the project will be disseminated at a final international meeting or conference as explained below.

7 Dissemination

We have published 4 peer reviewed articles and two articles are submitted (see below). We are planning further 8 peer-reviewed articles within Nanokem. Additionally, we are planning to arrange an international scientific meeting in 2011, where the data and conclusions obtained in Nanokem will be presented. In addition, we have held regular meetings with representatives from the paint and lacquer industry. At the end of the project we have planned to present the obtained result at a meeting with the paint and lacquer industry.

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2. I. K. Koponen, K.A. Jensen and T. Schneider: Sanding dust from nanoparticle-containing paints: physical characterisation. *Journal of Physics: Conference Series*, 2009, 151.

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NANOMMUNE



Comprehensive Assessment of Hazardous Effects of Engineered Nanomaterials on the Immune System

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1 Summary

Engineered nanomaterials (ENs) present tremendous opportunities for industrial growth and development, and hold great promise for the enrichment of the lives of citizens, in medicine, electronics, and numerous other areas. However, there are considerable gaps in our knowledge concerning the potential hazardous effects of ENs on human health and the environment. The NANOMMUNE consortium is committed to filling these knowledge gaps through a comprehensive assessment of ENs, with particular focus on effects on the immune system. The immune system is designed to respond to pathogens and foreign particles, and a core concept underpinning the current project is that the recognition versus non-recognition of ENs by immune-competent cells will determine the distribution as well as the toxicological potential of these materials. Our international, multidisciplinary consortium will focus on the procurement, synthesis and

detailed physico-chemical characterization of representative categories of ENs, and the monitoring of potential hazardous effects using an array of in vitro and in vivo systems, as well as transcriptomic and oxidative lipidomic profiling strategies to determine specific nanotoxic profiles (signatures) of these materials. The final and integrative component of our research project is modeling and risk assessment of potential adverse effects of ENs on human health, and the dissemination of our findings. Through our comprehensive approach, which combines analytical procedures from many different disciplines and leading experts from several national institutes devoted to occupational and environmental safety, we aim to establish a panel of read-out systems for the prediction of the toxic potential of existing and emerging ENs, thus enabling a continuous and sustainable growth of the nanotechnologies. Overall, the results generated through this international program will contribute to the understanding and mitigation of possible adverse effects of nanomaterials.

2 Overview of project

2.1 Introduction, scientific/industry needs, problem addressed

Nanotechnologies are viewed as being the driving force behind a new industrial revolution which is expected to have profound socio-economic effects (Royal Society and Royal Academy of Engineering, 2004; United States Congress Joint Economic Committee, 2007). Nanotechnologies comprise a disparate array of technologies that cut across many traditional scientific disciplines, including chemistry, material science, engineering, physics, biosciences, medicine, and environmental sciences. The only unifying feature is the nanoscale dimensions at which the material concerned is being manipulated. Nanoparticles have all three dimensions in the nanoscale, whereas nanotubes have two dimensions in this regime, and nanosurfaces have one dimension in this regime. It is important to note that nanomaterials can be on the same scale as elements of living cells, including proteins, lipids, nucleic acids, and organelles (Shvedova et al., Annu. Rev. Pharmacol. Toxicol. 2010). Therefore, one must focus particular attention on how ENs can interact with or influence biological systems, which may be desirable for certain medical applications, but may cause unanticipated hazardous effects upon occupational or environmental exposure to nanomaterials.

The properties of materials are different on a nanoscale for two reasons. First, ENs have, relatively, a larger surface area than the same mass of material produced in a larger form. This can make materials more chemically reactive, and affect their functional properties such as mechanical strength or electrical properties. Second, below 50 nm, the laws of classical physics give way to quantum effects, provoking optical, electrical, and magnetic behaviors different from those of the same material at a larger scale. However, the very same properties that make ENs so uniquely useful, such as a high degree of chemical reactivity and the ability to cross biological barriers may also be associated



with unforeseen adverse effects on health and the environment. Moreover, small size *per se* may contribute to the failure of immune recognition and hence to adverse or unexpected effects of nanoparticles. Indeed, numerous physico-chemical attributes, including size, shape, surface area, surface chemistry, solubility, charge, porosity, etc have been suggested to be associated with the potential adverse effects of ENs. However, much more research is required to ascertain the relevance of a given physico-chemical parameter for EN-associated toxicity following human exposure.

One of the members of our consortium co-authored the original proposal for a new subcategory of toxicology, namely nanotoxicology, to be defined to address gaps in our knowledge and to focus on the specific problems that are related to ENs (Donaldson et al., Occup. Environ. Med., 2004). Maynard, Tran and other leading scientists have also proposed that the pursuit of responsible and sustainable nanotechnologies can be tackled through a series of grand challenges to stimulate the global research community, including the development and validation of methods to evaluate the toxicity of ENs, and the development of risk assessment models for predicting the potential impact of ENs on human health and the environment (Maynard et al., Nature, 2006). Indeed, despite the tremendous growth potential of the nanotechnologies, there is still a considerable lack of information on bioaccumulation, biotoxicity, and biodegradation of ENs in humans as well as in other species. However, previous epidemiological studies have documented a strong association between so-called ultrafine air pollution particles and respiratory and cardiovascular morbidity and mortality in humans. Some, but not all of these effects, may be related to indirect actions of particles on components of the immune system, for instance through modulation of inflammatory cytokine secretion. Indeed, 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.

2.2 Scope, objectives

Engineered nanomaterials present tremendous opportunities for industrial growth and development, and hold great promise for the enrichment of the lives of citizens, in medicine, electronics, and numerous other areas. However, there are considerable gaps in our knowledge concerning the potential hazardous effects of ENs on human health and the environment, as pointed out in a recent report from the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) of the European Commission (SCENIHR, 2007). Our research consortium, which we have designated NANOMMUNE, is committed to filling these knowledge gaps through a comprehensive assessment of ENs, with particular focus on effects on the immune system, our primary defense system against foreign invasion.



One challenge in evaluating risk associated with the production and application of nanomaterials is the diversity and complexity of the types of materials available, and the many different routes of entry and possible sites of interaction with biological systems. Our interdisciplinary project will focus on the manufacturing and detailed physico-chemical characterization of several representative classes of nanomaterials, and the monitoring of deleterious effects of these nanomaterials on the immune system, using an array of in vitro and in vivo methodologies, as well as state-of-the-art in silico approaches for the assessment of genomic and oxidative lipidomic "nanotoxicity-signatures". Our studies will also include several examples of commercial ENs that are currently on the market. Moreover, we also aim to modify specific features of various classes of ENs, in order to mitigate toxic responses to these materials.

The immune system, present throughout the body, and on constant surveillance, has the capacity to respond to invasion by pathogens and foreign particles. The core concept underpinning the current project is that the recognition versus nonrecognition 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 antigenpresentation 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.

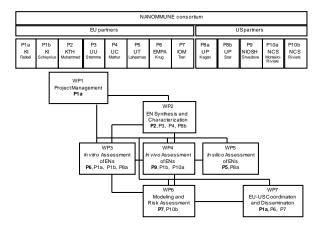


Figure 1. Schematic representation of the consortium and work packages.

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.

2.3 Technical approach, work description

2.3.1 Synthesis and characterization of nanomaterials

Our studies are focused on three categories of nanomaterials of technological and/or biomedical importance: a) metallic nanoparticles; gold and silver; 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. TiO₂ is common in numerous consumer-based and other products, and FeO-ENs have been applied for various biomedical applications, including their use as magnetic resonance imaging (MRI) contrast agents for almost 20 years. Mesoporous silica nanoparticles are of fundamental and applied interest for their potential in diverse applications in areas ranging from catalysis to photonic crystals, biomimetic engineering to sensor technology and drug delivery. They offer several attractive features for hosting molecules of various shapes, sizes and functionalities. However, it remains to be determined whether these materials also may exert adverse effects on immune-competent cells.

Carbon nanotubes, particularly SWCNT, have found numerous applications in different fields of industry due to their excellent strength and high electrical conductivity; moreover, functionalized (surface-modified) CNTs are emerging as novel components in nanoformulations for delivery of therapeutic molecules. The spread and distribution of CNTs in the body is dependent, to a large extent, on their specific interactions with cells of immune system. Indeed, we hypothesize that the recognition or non-recognition of ENs by the immune system will determine the toxicological potential of these nanomaterials, as well as their distribution in various tissues and organs. Systematic study of the therapeutic efficacy of CNTs is anticipated in the near future; however, detailed investigations of potentially hazardous effects on cells of the immune system remain to be performed, and are of paramount importance for the successful application of CNTs in nanomedicine. Moreover, there is a pressing need for careful consideration of the hazardous effects of CNTs for industrial workers.

Our consortium will standardize the synthetic methods as well as the characterization techniques of various classes of nanomaterials. Commercially available nanomaterials of various



sources will also be procured and characterized, for benchmarking purposes. Of note, members of our consortium have highlighted the need to avoid contamination with lipopolysaccharides (LPS) in the production of ENs, since LPS can interfere with the in vitro assessment of biological responses of immune-competent cells (Vallhov et al., Nano Lett., 2006). The data obtained by the different partners within WP2 and the in vitro (WP3) and in vivo (WP4) work packages (see Figure 1 for a schematic representation of the different work packages) will be summarized as standard operation procedures (SOPs), and compiled into the NANOMMUNE Quality Handbook (QHB), which will be made available to academic researchers and industries. The consortium will also develop methods for tuning (controlled modification) of the various physico-chemical properties (crystallinity, size, morphology, surface area, charge, hydrophilicity/hydrophobicity, coating molecules, etc) of custom-designed and commercial nanomaterials, to determine which properties are driving the immunotoxic responses. Finally, we aim to generate nanomaterials that are modified in terms of texture properties, composition and structure, in order to improve their biocompatibility. For instance, the presence of nitrogen in carbon nanotubes can promote the biodegradation of such materials, and our studies may thus aid in the safe development of ENs for medical and other purposes.

2.3.2 In vitro effects of nanomaterials: focus on immune-competent cells

There are several routes for nanomaterials to come into contact with living organisms. Inhalation, ingestion, and dermal routes are the most relevant for most occupational exposure scenarios. In addition, nanomaterials are produced for medical applications or may be released from medical implants upon mechanical stress. Such internal exposure (intentional or non-intentional) will lead to an increased number of nanoparticles within the bloodstream.

The immune system protects us from foreign materials that enter our body and we will therefore focus on possible adverse effects of nanoparticles on immune-competent cells. This is of particular importance, since immunotoxic effects of ENs have not been addressed in much detail to date. An excellent, recent review on this topic summarizes aspects of immunotoxicity of nanomaterials, but deals mostly with those materials that are produced for medical use, such as dendrimers or polymeric nanoparticles (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 nonbiodegradable 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, antigenpresentation, 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 immunecompetent cells. 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 cellular communication pathways. These studies will not only inform us on the toxic potential of ENs, but may also provide novel insight into this mode of communication between cells of the immune system. Taken together, these in vitro studies will form an important basis for *in vivo* studies and subsequent risk assessment, and may also generate fundamental insights into the handling of foreign materials/particles by the immune system.

2.3.3 In vivo effects of nanomaterials: focus on immune responses

Inhalation and deposition on the skin are the most likely routes of exposure to ENs in the environment. Indeed, one of the most important target organs for airborne particles, for obvious reasons, is the respiratory system. Members of our consortium have reported a remarkable degree of pulmonary granuloma formation and fibrosis in mice upon pharyngeal aspiration of engineered carbon nanotubes (CNTs) (Shvedova et al., Am. J. Physiol. Lung Cell Mol. Physiol., 2005). These studies suggest that if airways of workers are exposed to CNTs at the current permissible level (for graphite particles), they may be at risk of developing some lung lesions. Our project aims to further understand the potential for hazardous effects of ENs on the



lung, focusing on specific interactions of ENs with cells of the immune system, and to use these data as the basis for risk assessment of hazardous effects of ENs for humans. 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 interactions of ENs with different organ systems, using real-time *in vivo* monitoring in live animals, and will provide insights into the role of lymphatic uptake after exposure to nanomaterials.

2.3.4 Novel biomarkers of nanomaterial exposure: genomics and lipidomics

Microarray technologies for gene expression profiling (transcriptomics) can be used for identification and characterization of toxic responses, and could provide for more sensitive and earlier detection of adverse effects in, for instance, animal toxicity studies. Moreover, such studies could yield novel hypotheses concerning the mechanisms underlying nanotoxic responses. Recent developments in genome-wide technology platforms have been extremely rapid. Hence, it is now possible to carry out genome-wide gene expression profiling on a very small sample size starting only with 100 ng of sample material. Moreover, the data analysis and mining tools have made it possible not only to discover individual genes or gene lists that are influenced by a particular treatment but to obtain insight regarding the molecular pathways that are activated or shut down. Members of our consortium have exploited a number of techniques and functional genomics tools that enable holistic approaches to identify known and novel genes involved in the regulation of T cell, DC and macrophage responses. Some studies are available on differential gene expression in ENexposed 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 nonoxidized phospholipids from a complex mixture (Kagan et al., Nat. Chem. Biol., 2005). Developments in the field of gene expression profiling and oxidative lipidomics have thus provided increasingly valuable and feasible approaches to search for potential mechanisms underlying physiological or cellular processes, to generate new hypotheses concerning the mechanisms involved, as well as to identify novel biomarkers characteristic for toxic responses. We will apply these protocols to the assessment of cells (WP3) and tissues (WP4) exposed to various classes of nanomaterials.

2.3.5 Risk assessment of nanomaterials: steps towards safe management

Epidemiological studies on ambient particles incidentally produced in industrial processes and from traffic have demonstrated a correlation between ambient air concentration of particles and respiratory and cardiovascular morbidity and mortality rates. These adverse health effects of particles highlight the urgent need for research also on nanoparticles that are intentionally produced. This is also the final, integrative component of our current NANOMMUNE project i.e. guidelines for safe handling of nanomaterials. Risk assessment will be performed in close collaboration between all consortium members. Members of our consortium possess considerable expertise in Physiologically-Based-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 socalled "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.

2.4 Achievements: a progress report

At the time of writing, the first 18 months of the NANOMMUNE project have been completed. 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 by our consortium).

2.4.1 Interaction of carbon nanotubes with immunecompetent 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 "eatme" 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.

2.4.2 Internalization of mesoporous silica particles by macrophages

Macrophage recognition and ingestion of apoptotic cell corpses, a process referred to as programmed cell clearance, is of considerable importance for the maintenance of tissue homeostasis and in the resolution of inflammation (Witasp et al., Curr. Immunol. Rev., 2008). Moreover, macrophages are the first line of defense against microorganisms and other foreign materials including particles. However, there is sparse information on the mode of uptake of engineered nanomaterials by primary human macrophages. Members of our consortium have recently reported on the interactions between mesoporous silica particles with human monocyte-derived macrophages. To this end, mesoporous silica particles with cubic pore geometries and covalently fluorescein-grafted particles were synthesized through a novel route; these particles are endowed with very high surface areas due to their porous structure (Witasp et al., Toxicol. Appl. Pharmacol., 2009). Efficient and active internalization of mesoporous silica particles of different sizes was observed by transmission electron microscopy and flow cytometry, and studies using pharmacological inhibitors suggested that uptake of particles occurred through a process of endocytosis. It is noteworthy that macrophage uptake of the mesoporous silica particles was independent of serum factors. The particles did not impair cell viability or function of macrophages, including the ingestion of different classes of apoptotic or antibody-opsonized target cells. These findings are relevant to the development of mesoporous materials for drug delivery and other biomedical applications.

2.5 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-theart, high-throughput global transcriptomics and oxidative lipidomics approaches, to obtain "nanotoxicogenomic" signatures, and to define novel biomarkers of nanoimmunotoxic 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 is expected to conclude in 2011, at which time a summary report will be made available.

2.6 Dissemination of results

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

- A public-access website has been developed as a portal of dissemination of our results.
- Members of the consortium will present scientific findings at international scientific conferences, and will also publish scientific findings and reviews in international peer-reviewed journals.
- Members of our consortium are actively involved in the organization of international conferences on nanotoxicology, including, in the past, the 1st International Meeting on Nanotoxicology, Miami, FL, 2006 (Dr. Valerian Kagan and Dr. Anna Shvedova); and 1st Nobel Forum Mini-Symposium on Nanotoxicology, Stockholm, Sweden, 2006 (Dr. Bengt Fadeel), and during the current project: 2nd International Meeting on Nanotoxicology, Zurich, Switzerland, 2008 (Dr. Krug); 3rd International Meeting on Harald Nanotoxicology, Edinburgh, United Kingdom, 2010 (Dr. Lang Tran); and 2nd Nobel Forum Mini-Symposium on Nanotoxicology, Stockholm, Sweden, 2010 (Dr. Bengt Fadeel). The latter event is an official part of the Karolinska Institutet Bicentennial celebration.
 - Members of the consortium are also involved in the organization of the Nanosafety Autumn School in Venice, Italy (Dr. Lang Tran, Dr. Bengt Fadeel); the first event took place in November 2009, and will be followed by 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, Saltsjöbaden/Stockholm, Sweden, 2009 (co-organized with the Royal Swedish Academy of Sciences; a collection of review articles by prominent speakers was published in the January 2010 issue of *The Journal* of Internal Medicine, and several NANOMMUNE participants contributed as speakers and as authors of invited reviews).
- 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).

2.7 Selected publications

- Shvedova AA, Kisin ER, Murray AR, Kommineni C, Castranova V, Fadeel B, Kagan VE. Increased accumulation of neutrophils and decreased fibrosis in the lung of NADPH oxidase-deficient C57BL/6 mice exposed to carbon nanotubes. *Toxicol Appl Pharmacol.* 2008;231:235-40.
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NANOPLAST

Nano-technological materials and products in the plastics industry: Exposure assessment and toxicological properties

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1 Project Summary

NANOPLAST is a three-year project running from ultimo 2007 to primo 2011. The aim is to investigate physico-chemical, and toxicological properties of engineered nanomaterials (NMs) that may obtain massive use in production of plastic products. The project focuses on risks associated with the production of polymer nano-composites (PNCs) of thermoplastics that can be injection moulded. Those PNCs consist of a polymer matrix with uniformly dispersed NM's, which in this project are different organically modified clay minerals nanoclay (or organoclay) or carbon nanotubes (CNTs). The different polymers include (polyethylene (PE), polystyrene (PS), and poly(methyl methacrylate) (PMMA)). The main project will organize the obtained knowledge across three work packages and carry through a comprehensive dissemination in collaboration with The Danish Plastics Federation, The Central Organization of Industrial Employees in Denmark, The Danish Working Environment Authority, and other relevant parties. The three subprojects are:

Work package 1. Characterisation of toxicologically relevant physical and chemical properties of nanoclays and CNTs

Work package 2. Characterization of toxicological properties of ENMs for use in plastic products – Special focus is set on potential mutagenic effects of nanoclays and CNTs.

Work package 3. Assessment of the risk of exposure to ENMs in the plastics industry – Dustiness and Workplace measurements carried out during test productions in the laboratory.

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3 Funding

Project is financed by 2.500.000 DKK from the Danish Working Environment Research Fund and co-financed by a similar amount by internal funding.

4 Current project publications

Jensen K.A., Koponen I.K., Clausen P.A. and Schneider T. (2009) Dustiness behavior of loose and compacted bentonite and organoclay powders: What is the difference in exposure risk? Journal of Nanoparticle Research **11**, 133-146.

5 Links to information

http://www.arbejdsmiljoforskning.dk/Aktuel%20forskning/Nanot eknologiske_materialer_og_produkter_i_plastindustrien_-__NANOPLAST.aspx?lang=en





NanoPolyTox



Toxicological impact of nanomaterials derived from processing, weathering and recycling of polymer nanocomposites used in various industrial applications

Contract Agreement (Under negotiation): FP7-NMP-ENV-2009-247899 Website: not available Coordinator: Socorro Vázquez-Campos, LEITAT Technological Centre, Barcelona, Spain

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3	Institut Català de Nanotecnología	ICN	Spain
4	Polyrise S.A.S.	POLYRISE	France
5	L'Urederra Technological Centre	LUREDERRA	Spain
6	DHI Water & Environment	DHI	Denmark
7	Laviosa Chimica Mineraria, S.p.A.	LAVIOSA	Italy
8	Lati Thermoplastic Industries S.p.A.	LATI	Italy

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1 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, processing, aging and recycling to their end of life (disposal) covering their migration and/or release to the environment during their life cycle. The biological and environmental fate of these nanomaterials will be established

taking into account the physical-chemical properties and the toxicological data obtained. 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. Moreover, the overall human health and environmental impact will be assessed by LCA analysis specifically designed for nanomaterials. Additionally, three recycling strategies will be considered in order to give solutions for the disposal of both toxic and innocuous nanomaterials. For this purpose, exhaustive evaluations including the selection of adequate digestion and extraction methods to separate the



nanomaterials from the polymeric matrix will be developed. 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.

2 Concept, objectives and work plan

2.1 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).

2.1.1 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. Therefore, NanoPolyTox is proposed to study the evolution of nanomaterials (physical and chemical properties) during their life cycle and their toxicological impact on human health and environment related to this evolution. Moreover, NanoPolyTox will allow the development of innovative strategies for the disposal of nanocomposites at the end of their life cycles.

2.1.2 Objectives

The main goal of NanoPolyTox is to improve the understanding the potential environmental/health impacts of 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, final and/or treatment and disposal of recycling 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 (toxicology and ecotoxicology), exposure, 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.

2.1.2.1 Specific Objectives

- The preparation of highly pure and monodisperse nanomaterials from three different families (carbon nanotubes, nanoclays and metal oxide nanoparticles) including adequate tailoring 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 properties and toxicity 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 metal oxide nanofiber filters
- Development of new solutions for the disposal of toxic nanomaterials as complement for recycling processes based on the inclusion of specific metal oxide nanofibers filters (containing the toxic nanomaterials) in xerogel matrices by sol-gel processes and sintering
- Evaluation of the human health and environmental impact of nanomaterials that are highly used in many industrial sectors during their life cycle by LCIA analysis specifically amplified by the data obtained during this 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 manufactured nanomaterials.

2.2 Methodology and associated work plan

For achieving the study on the human health and environmental impact of nanomaterials during their life cycle as fillers in polymeric matrices, the following work plan has been proposed.

Carbon nanotubes (one selected type), metal oxide nanoparticles (three types of nanoparticles) and nanoclays (two types) will be prepared and tailored to match their surface properties (polarities, chemical functionalities) with three different types of polymeric hosts, selected during the project.

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. Therefore, it could be expected to be able to reproduce the degradation occurring in nanomaterials because the environmental conditions and the chemical composition are the same for each nanocomposite. This control over the nanomaterials is of high importance to determine the environmental impact of released nanomaterials knowing their characteristics (structure, chemical composition and toxicity) at different stages of their life cycle.

The synthesized nanomaterials (samples 1, Figure 1) will be included in polymeric matrices by double screws extrusion process (samples 2, Figure 1). The final step of the processing will be the injection of these nanocomposites to obtain polymeric demonstrators (samples 3, Figure 1). The concentration of nanofillers after the injection processing will be measured in order to detect possible migration and release during the transformation process. The nanomaterials included in the composites obtained after compounding and injection will be extracted from the polymeric matrices for evaluation of the physical and chemical and toxicological properties.

The nanocomposites will be weathered in normalized conditions (DIN 75220) in order to observe the degradation occurring into the nanomaterials during their use. Therefore, all the nanomaterials and nanocomposites generated in this project will be submitted to:

- 12 cycles of weathering (samples 4, Figure 1)
- 60 cycles of weathering (samples 5, Figure 1)
- 120 cycles of weathering (samples 6, Figure 1)

The nanomaterials will be extracted from the polymeric matrices by methods developed from existing technologies (centrifugation, membrane nanofiltration). The extracted nanomaterials will be characterized (physical and chemical properties) in order to:

- Evaluate the degradation of the nanomaterials occurring over their life cycle
- Quantify the migration of the nanomaterials from the polymeric matrices

Additionally, new organic/inorganic filters based on nanofibers will be generated by electrospinning as new filtration methodology for the extraction of nanomaterials from the polymeric matrices.

The samples 1, 3, 6, 7 (Figure 1) will be submitted to toxicological (human toxicity and ecotoxicity) analysis to determine their impact in human health and the environment. These samples will be tested in preliminary *in vitro* assays to evaluate their toxicity (cytotoxicity and ecotoxicity). The more toxic nanomaterials obtained from this screening (number determined during the course of the project) will be tested by *in vivo* assays to determine their biological and environmental fate.

Physical, chemical and toxicological data of the different nanomaterials will be described in technical cards. The technical cards will consist of the ID cards of the nanomaterial during the project containing all the relevant data about their physical and chemical properties. These cards will be useful tools for sharing information between the several partners.

All the data obtained on the physical, chemical and toxicological properties of the nanomaterials over their life cycle will be used for the development of theoretical models to predict the human health and environmental impact of nanomaterials.

Moreover, methods for recycling and disposal of the nanomaterial will be proposed and developed. Three strategies for recycling and disposal will be carried out during NanoPolyTox:

- Direct mechanical recycling of the nanocomposites (samples 7) for new applications. These samples obtained after recycling will be fully analyzed in order assess the risk of migration during this process
- Filtration and extraction of the innocuous nanomaterials from the polymeric host using nanofiber-based filters specially designed for nanomaterials filtration
- Filtration, extraction 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 calcinations



Finally, the data collected in the whole project will be used to generate a new methodology for the life cycle impact assessment of nanomaterials. At the end of this study a comprehensive framework describing the impact and risks associated nanomaterials will be obtained.

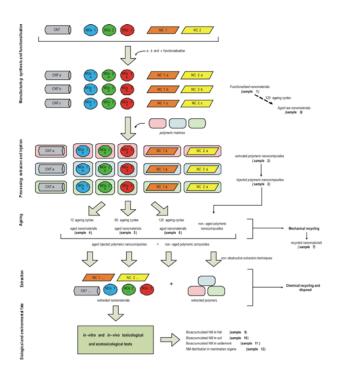


Figure 1: Scheme of the work plan of the NanoPolyTox project

2.3 Dissemination and exploitation

2.3.1 Dissemination

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. The first project brochure will be printed within the 6th month of the project and uploaded versions will be delivered at months 12 and 24 or when required from the Management Committee. Videos & demos will be regularly produced for demonstration and dissemination purposes. All this information will be uploaded in the electronic format on the project's website.

The website will be created at the beginning of the project. The website will produce an extensive record of publications and reports originated on the course of the project. It will consist of

a public area, a registration area and a private area. Public area will contain:

- General information on the project
- Useful links to the EC services, nanotechnology portals...
- An area for downloading project brochures, videos, demos, and other useful material such as public deliverables
- News for communication of events, workshops, conferences related to the project or to the area of nanosafety

The registration area will allow every public user to upload personal data (name, affiliation, area of interest and email address) in order to participate in the project's mailing list.

The private area will be exclusively used by the Consortium for exchanging and sharing electronic data. The partners will be able to access the private area by using their own username and password.

Finally, the interactions with relevant technology (e.g. nanotechnological and advanced materials) and environmental management platforms will allow the consortium to widen the potential applications and the dissemination of the results.

On a scientific level, the dissemination activities will be carried out through publications in specialized journals in the areas of nanotechnology, toxicology, polymer 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. This strategy includes the following activities:

The dissemination of knowledge in cooperation with European organisations, such as European Polymer Federation, European Chemical Industry Council (CEFIC), Federation of European Toxicologists and European Societies of Toxicology (EUROTOX), and the Society of environmental Toxicology and Chemistry (SETAC). Through the websites and annual meetings of these organizations, NanoPolyTox can have a direct link with their related scientific community, industry, and affiliations. In general, any useful contacts and coordination/networking with national programs, industrial associations and related consortia within and outside Europe will be pursued. Limited results will be made available for public dissemination after the conclusion of the project, where possible links will be established with existing EC projects and relevant Coordination Actions.

The results of the project will be presented at different events (workshops, technical conferences, fairs and exhibitions) organised by the members of the consortium and in other potentially interesting events that could be planned by other organizations, such as, NanoImpactNet (European Network on the Health and Environmental Impact of Nanomaterials), OECD (Organization for Economic Co-operation and Development),



ICON (International Council on Nanotechnology), NIOSH (Nanoparticle Information Library), REACH, ...

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. Common dissemination activities are expected and contact will be established with the coordinators of the different projects, so efforts are joined and the four projects can provide improved information to the Community on the aspects of toxicity tests and screening of NP toxicity, environmental fate, biological accumulation and degradation, predictive models for NP toxicity and standardisation and validation conditions limiting risk assessment of NP and nanomaterials.

2.3.2 Exploitation

There are several major areas of research activity, which are expected to generate new technology and may therefore generate new intellectual property opportunities and ultimate products. Some of them can be outlined:

- New recycling and/or material recovery methodologies for polymers, nanocomposites and nanomaterials may find practical industrial applications in different sectors, such as, automotive industry, plastic/polymer industries, packaging industry and waste management industry
- Filtering/protection technologies are foreseen to be developed and might have applications on innovative

solutions dedicated to the protection of workers and researchers handling nanomaterials

The novel methods of recycling developed during the NanoPolyTox project will provide solutions for the re-use of nanomaterials included in a variety of consumer products. These methods will be exploited as part of the design criteria for nanomaterials production and applications to promote the sustainability in the development of nanomaterial-based products. The disposal methods proposed by NanoPolyTox for the identified toxic nanomaterials will offer solutions to decrease the environmental impact of nanomaterials.

The new test methodologies developed for the assessment of the human health and environmental impact of nanomaterials will contribute to the generation of valuable information for both industry and academia, as well as to bodies with environmental, health and safety responsibilities. The results obtained following these test methodologies will allow the generation of predictive models of toxicity and contribute to risk management.

NanoPolyTox will contribute to the development of novel procedures for life cycle assessment that are suitable for engineered nanomaterials, including a focus on determining the stages in a product life cycle that introduce the greatest potential for risk. The results from these studies will give information on the material choices to industries so that risks may be reduced. Consequently, industries will offer manufacturing approaches that minimize environmental impact through "green design" principles or to determine if there should be any limitations or restrictions when using certain modes of transportation or waste disposal.

The partners will analyse and validate the primary and secondary market potential, and structure a market penetration & development plan accordingly.



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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: <u>http://www.nanoretox.eu</u> Coordinator: Dr Eugenia Valsami-Jones, Natural History Museum

No.	Beneficiary name	Short name	Country
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2	Imperial College of Science, Technology and Medicine	IMPERIAL	UK
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	UK
11	United States Geological Survey	USGS	USA
12	Intrinsiq Materials Ltd	IML	UK

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1 Summary

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 European Commission's Action Plan for Nanotechnology (2006), the EPA's Nanotechnology White Paper (2005), 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.

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 by addressing five key questions:

[1] How does the environment into which engineered metal nanoparticles are released affect their physicochemical properties and their bioreactivity?

[2] How does this impact on their ability to interact with and/or penetrate mammalian and aquatic cells, and organisms (bioavailability) 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 discovered is most likely to pose a risk to human health and the environment?

[5] How can this information be incorporated in a risk assessment model?

2 NanoReTox: Risks to the Environment and Human Health

2.1 Overview

NanoReTox is an international, integrated, interdisciplinary, strategic program, addressing the environmental and human implications of exposure to engineered metal nanoparticles. The consortium is building upon the existing knowledge and experience of the partners in particle synthesis and reactivity, as



well as existing knowledge of risks from metal and natural (nano)particles. We propose that methodologies and in vitro and in vivo models that the partners have developed for studying natural nanomaterials and metal toxicity can provide a valuable scientific and technical platform from which to assess the effect of environmental exposures, and toxicity of engineered metal nanomaterials. We suggest that concepts used in the models that determine environmental bioavailability of metals are a valuable starting place from which to evaluate risks from engineered nanoparticles. Finally, we introduce a range of novel approaches to study engineered nanomaterial reactivity/toxicity, including a novel method for detecting metal particle bioavailability (creation of particles with unique stable isotope ratios), which could be of significant value in applications for following certain different types of engineered nanomaterials through their life cycle.

2.1.1 Relevance

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 affected¹. Clues from these human studies suggested the possibility that particles may enter the circulation and translocate to other organs and/or that there are "knock-on" systemic effects due to locally produced proinflammatory 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 spleen². 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.

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

¹ Peters A. 2006 Commentary: inflamed about ultrafine particles? Int J Epidemiol.: 35(5):1355-6.

² Kreyling et al, 2nd International Conference on Nanotoxicology, Venice, April 2007.



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.

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 environmental harm. However, until the mechanistic associations between nanomaterial characteristics and putative toxicity are understood, determination of nanorisks will not move forward. Many recent toxicological studies have fallen short of this; furthermore many studies have led to contrasting results and interpretations about risks, possibly reflecting the diverse sources and nature of the test materials. This illustrates the importance of studying commercial and designer particles that have been fully characterised before, during and after toxicity studies. Of the many different types of engineered nanoparticles currently produced, industrially or in the laboratory, environmental risks from non-carbon-based nanoparticles are the least studied. This is despite the rapidly growing use of particles such as TiO₂, ZnO, SiO₂, Ag, CuO, and CdS. The chemical composition of metal nanoparticles may contribute to their having significant additional toxicity, but few studies address this.

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).

Ecotoxicological studies with engineered nanomaterials are almost non existent^{3,4}. Most of those 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 body⁵.

2.1.2 Strategy

Our consortium's overall strategy is to apply a highly multidisciplinary approach (combining three main research strands: "Synthesis", "Ecotoxicology" and "Toxiolcogy") to a focused

³ Owen R, 2005. *Marine Pollution Bulletin* 50: 609-612.

set of scientific questions. This will result in a detailed and comprehensive assessment of engineered nanoparticle reactivity/toxicity, established on a wide range of nanoparticle properties and biological indicators, so that the new dataset can be extrapolated beyond the available data and applied to other engineered metal nanoparticles not considered in our study.

Our team is uniquely qualified across the necessary disciplines and committed to working and communicating in close collaboration to achieve an optimum outcome. We have established an international **"Joint Advisory Group"**, which has extensive experience with risk communication and regulation. The industrial members in NanoReTox provide a unique connection to the realities of that sector, both in terms of materials with which to work and appreciation of the industry's needs. To further enhance international collaboration NanoReTox includes an active partner from outside the EU (US Geological Survey, Menlo Park, CA USA).

2.1.3 Scientific and Technical Objectives

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. The specific scientific and technical objectives are:

- 1] To synthesise and fully characterise a set of engineered metal nanoparticles with a range of physicochemical properties using industrial and laboratory methods (size, shape, phase, concentration, composition, surface modification, method of synthesis). Why? To study how carefully controlled differences in physicochemical properties influence particle reactivity, bioavailability and toxicity and to see whether physical (e.g. size) characteristics override chemistry and vice versa. To generate engineered metal nanoparticles with properties that vary systematically, so that robust links to toxicity can be made.
- [2] To study the abiotic reactivity^{*} (transformations) of the synthesised nanoparticles in simulated environmental and biological media. Why? To monitor the behaviour of engineered metal nanoparticles in a range of simulated environmental (hard/soft freshwater, seawater) and biological (simulated body fluid, lung fluid, gastric fluid) media. To investigate if increased reactivity is an indication of potential for toxicity and whether this can then be used as a proxy for rapid hazard assessments. To

⁴ Colvin BL 2003, *Nature Nanotechnology*, 21(10): 1166- 1169.

⁵ Maynard A et al, 2006. Safe handling of nanotechnology, *Nature* 4444: 267-269

^{*} In this document we define "**abiotic reactivity**" as the tendency to undergo dissolution, aggregation, phase transformation or to change surface properties in the absence of any organisms or cells.



couple this work with monitoring nanoparticles in the biological models below.

- [3] To investigate *in vivo* uptake of nanoparticles by aquatic species and study mechanisms and paths of internalisation. *Why?* To determine if engineered metal 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. To assess if selected aquatic species show stress responses when nanoparticles are accumulated, and to characterize the nature of those responses.
- [4] To investigate *in vitro* uptake and reactivity of nanoparticles and to discover putative mechanisms of toxicity.

by aquatic species primary cells. *Why*? To determine cellular reactivity to, and possible uptake of engineered metal nanoparticles by aquatic species primary cell cultures. To assess if cell traits influence reactivity and bioavailability *in vitro* in aquatic invertebrate cells.

by primary mammalian and human cells and existing human cell lines. Why? To assess engineered metal nanoparticle reactivity and uptake by mammalian/human cells and investigate if toxic responses can occur.

- [5] To consider the genotoxicity and carcinogenicity of metal nanoparticles. *Why*? To use emerging molecular tools to determine whether engineered metal nanoparticles can induce changes in DNA that might impact on health.
- [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. Why? To assimilate the data from the previous objectives to discover whether there is a hierarchy of nanoparticle reactivity and uptake. To determine which physicochemical characteristics of the nanoparticles confer toxicity and/or predict internalisation and in which models.
- [7] To establish universal approaches to risk assessment model and risk communication. Why? To develop a universal set of criteria to define hazard and risk from nanomaterials and a similar set of standards for communicating risks. To maximise the practical application of study results and assist regulation/legislation.

2.2 Work package list/overview

Below are described the individual work packages (WP). The partner in bold is the lead partner for that component of the project.

2.2.1 Synthesis and characterisation (WP1)

Key partners: 1(NHM), 2(IMPERIAL), 7(JRC), 9(DSL), 12 (IML)

Many nanoparticles are already used in various consumer products and this was a key criterion in the selection of study material for NanoReTox. Of 356 currently available products containing nanoparticles, the most common nanomaterial is silver. This is followed by carbon-based materials (nanotubes and fullerenes – which, however are excluded from NanoReTox mainly because their physical properties are very different from metal nanoparticles and because they have been studied more). silica, zinc oxide, titania and gold (Woodrow Wilson International Center for Scholars, 2007, http://www.nanotechproject.org/consumerproducts). The nanoparticles chosen for this study are either already present in consumer products, or likely to be included in the near future. A list, including their applications are shown below.

Table 1. Nanoparticles for testing and their applications

NP	Applications
TiO ₂	cosmetics and personal care products, paints and coatings, photocatalysts, food packaging
SiO2	electronics, sensors, paints and coatings, biolabeling, oral care
ZnO	cosmetics and personal care products, photocatalysts
CuO	biocides, catalysts, cosmetics
CeO ₂	fuel catalysts
CdS	photocatalysts, electronics
Ag	biocides, cosmetics and personal care products, food packaging, biolabeling, electronics
Au	cosmetics and personal care products, supplements
Fe	water treatment and environmental clean up





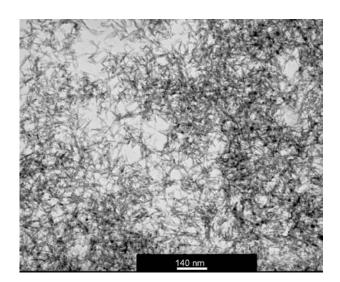


Figure 1. TEM image of in-house synthesised TiO2 nanorods.

This workpackage will generate sets of well characterized nanoparticles using different methods of synthesis and displaying 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, we intend to apply both top down (i.e. nanoparticles produced from bulk materials by milling, Partner DSL) as well as bottom up (wet chemical synthesis, Partners NHM & JRC; microfluidics, Partner IMPERIAL; plasma synthesis, Partner IML), 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 nanomaterials produced will be 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 (BET). Multicollector ICP-MS will be used for analyses of stable isotope labelled particles. Focused Ion Beam Scanning Electron Microscopy for visualising the nanomaterials produced and their inner structure. Xray Photoelectron Spectroscopy (XPS) and Time-of-Flight Secondary Ion Mass Spectrometry (ToF SIMS) for nanoparticle surface composition.

2.2.2 Abiotic reactivity (WP2)

Key partners: 1(NHM), 7(JRC)

Using the nanomaterial 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, 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 and hydrophobicity.

Behaviour of nanoparticles released in biological or environmental media is currently unknown. Predictions are currently based on studies of natural colloids (but note that significant differences exist between natural and engineered particles, as discussed previously) and on incidental observations in toxicity studies. 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 will be 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 will be investigated.

2.2.3 In vivo exposure, aquatic organisms (WP3)

Key partners: 1(NHM), 3(RUC), 4(UNS), **5(UCO)**, 6(UPV-EHU), 11(USGS)

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.

Nanoparticles must be bioavailable to exert toxicity. *In vivo* experiments are an essential part of any assessment of risks from bioavailable toxins. Bioavailability to whole organisms is influenced by many traits that are not simulated in *in vitro* exposure of cell cultures⁷. For example, some whole organisms filter massive quantities of water across their gills (e.g. bivalves) expanding their exposure to even very low concentrations of



nanoparticles; while others (e.g. fish) are exposed to much smaller flows of water across their gills. Different organisms eat different kinds of foods, some of which may contain nanoparticles and some may not. Organisms have behaviours and live in ways that may either expand or reduce their contact with nanoparticles. External barriers to nanoparticles in epithelium of the gills and gut of animals may also influence whether nanoparticles can reach sites where they may be toxic.

If nanoparticles are bioaccumulated internally then adverse effects are possible, but not inevitable⁶. For example, animals and plants have mechanisms to sequester metals into innocuous non-toxic forms that prevent toxicity if these mechanisms are not overwhelmed⁷. It is not known if nanoparticles will be similarly detoxified (or are simply innocuous on their own). If the nanoparticles are recognized by the cell as a toxin, and/or are attracted to molecular sites that disrupt important functions, the reaction of the organism can be determined.

Bioavailabilty will be addressed using particles made with artificially enriched stable isotopes⁸, radioisotopes and fluorescent labels to quantify biodynamic uptake and loss Bioaccumulation will be modelled from characteristics. 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 (partner 6) in mussels by means of autometallography (AMG) at both light and electronmicroscope level, and X-ray microanalysis9. 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. AMG allows the visualization in tissue sections of very small traces (i.e 6-8 atoms) of metals associated to AMG precipitates (black silver deposits, BSD). Image analysis allows quantification in mussel digestive gland²⁷ to determine the relationship between exposure, type of nanoparticle and levels of metal accumulation in cells and tissues. AMG will be also applied in liver of zebrafish.

These experiments will accompany studies of adverse responses. Partners will experiment with different animals in order to compare implications of different biological traits. Bivalve molluscs will be compared that filter at different rates

⁷ Rainbow, P. S. (2002). Environmental Pollution, 120, 497-507.

⁸<u>Croteau, M.N., Luoma, S.N</u>. Environmental Toxicology and Chemistry, V. 24, pp. 2871-2878. 2005.

⁹ Soto M., Marigomez I. 1997. Ecotox Environ Safety 37:141-151; Soto M, Zaldibar B, Cancio I, Taylor MG, Turner M, Morgan AJ, Marigómez I. 2003. Histochem J. 34:273-280.

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 organisms¹⁰ 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 vitro⁶. If organisms show such responses to bioavailable nanomaterials, in vivo, it is unequivocal evidence that nanomaterial uptake causes the Visual evidence of internal organisms to respond. nanomaterials, evaluation of internal dissolution and manipulation of experimental design will be used to determine if responses are due to internal dissolution of the metal oxide particle or due to disruption by the particle itself.



Figure 2. Platynereis dumerilii (ragworm), a marine polychaetous annelid is used for in vivo testing.

2.2.4 In vitro exposure, aquatic species primary cells (WP4)

Key partner: 6(UPV-EHU)

This workpackage will address 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

⁶ Durou C., Smith B.D., <u>Roméo M., Rainbow P. S., Mouneyrac C.,</u> Mouloud M., Gnassia-Barelli M., Gillet P., Deutsch B., <u>Amiard-</u> <u>Triquet C.</u> 2007. Ecotoxicology and Environmental Safety, 66 (3): 402-411.

 ¹⁰ Selck, H., Forbes, V. E. 2003. Marine Environmental Research.
 57: 261-279.



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 defense system in mussels. Effects on this cell type could reflect damage on the inmune system, which could have consequences at higher levels of biological organisation, ie, individuals and communities¹¹. In previous studies, isolated cultures of mussel haemocytes showed changes in cell viability and cell cytoskeleton associated to increased oxidative stress upon exposure to metals¹². Cells comprising the gill epithelium of mussels were also recently characterized *in vivo* and *in vitro*¹³. Primary cultures of gill cells are a useful model because gill cells are in direct contact with water pollutants, accumulate those pollutants and show measurable changes after pollutant exposure.

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.

2.2.5 Cellular/molecular mechanisms of action; human (WP5)

Key partners: 2 (IMPERIAL), 7(JRC), 8(UNIPI), 9(DSL)

In vivo experiments using animal models can be used to determine whether (and in what quantities) inhaled, ingested, topical or intravenously delivered nanoparticles might be translocated to other body compartments¹⁴. Furthermore, such studies will also indicate pathological effects on target organs. Using these 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. We therefore intend to use in vitro models to examine cellular responses to nanoparticles; this approach is also in line with the 7th amendment to the EU Cosmetics Directive (European Commission, 2003; Council Directive 76/768/EEC) to avoid excessive animal testing.

We hypothesise that the cellular reactivity of the particles will critically depend both on the target tissue and the function of the cell type within that tissue. Thus, whilst some nanoparticles may be overtly cytotoxic, even at low levels, others may not, but they may adversely affect cell function, for example stimulating inflammatory mediator release or compromising epithelial barrier integrity. Conversely, the magnitude and profile of the cellular response will depend on the physicochemical properties of each type and format of particle and its exposure dose. We will initially concentrate on Ag, TiO₂, SiO₂, ZnO, CdS (and test all different sets of nanoparticles synthesised), which we expect will have a broad range of activity for comparative purposes. If time permits, or if data from WP2 indicate, we will study other particles. In the following studies we want to know:

1) Which cell types are most vulnerable to nanoparticle exposure?

2) Which cellular functions are affected?

¹¹ <u>Cajaraville MP</u>., Olabarrieta I., <u>Marigómez I.</u>, 1996. Ecotox. Environ. Saf. 35, 253- 260

¹² Olabarrieta I., L'Azou B., Yuric S., Cambar J., <u>Cajaraville MP.</u>, 2001. Toxicol. In vitro 15, 511- 517; Gómez-Mendikute A., Etxeberria A., Olabarrieta I., <u>Cajaraville MP</u>., 2002. *Mar. Environ. Res.* 54, 431- 436; Gómez-Mendikute A., <u>Cajaraville MP</u>., 2003. Toxicol. In Vitro 17, 539- 546.

 ¹³ Gómez-Mendikute A., Elizondo M., Venier P., Cajaraville MP., 2005. Cell Tissue Res. 321, 131- 140

¹⁴ Oberdorster et al 2005.. Environmental Health Perspectives 113:823-839.



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 by Prof Terry Tetley (partner 2, IMPERIAL) and Dr Oron (partner 9, DSL), who have access to and utilise primary cell systems and where Prof Tetley¹⁵ is currently studying nanoparticle-cell interactions and other inhaled toxicants while Dr Oron¹⁶ is studying a human skin model. The cell line studies will be performed under the guidance of Dr Rossi¹⁷ (partner 7, JRC) and Professor Migliore¹⁸ (partner 8, UPI), whose groups use these models to screen other toxicants.

Radiolabelled nanomaterials will be used to study the interaction of labelled nanomaterials with cells following a mechanistic approach (behaviour in culture media, uptake, intracellular distribution and interaction with bio-molecules) using advanced radio-analytical techniques (partner 7, JRC). State of the art fixed and live cell microscopy (partner 2, IMPERIAL) will also be used to study particle-cell interactions; air-liquid interface models will be 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; iv) confocal for 3D imaging and v) scanning ion conductance microscopy combined with confocal microscopy for topographical, surface imaging.

2.2.6 Genotoxic and carcinogenic potential (WP6)

Key partners: 6(UPV-EHU), 7(JRC), 8(UNIPI)

¹⁶Ginsburg, Sadovnic M, <u>Oron M</u>, Kohen R. Inflammopharmacology. 2004;12(4):305-20.

¹⁷ J. Ponti, L. Ceriotti, B. Munaro, M. Farina, P. Colpo, E. Sabbioni, <u>F. Rossi,</u> A. Munari, M. Whelan, 2006, *ATLA-Alternative to laboratory animals*, Vol. 34.

¹⁸ Bergamaschi E, Bussolati O, Magrini A, Bottini M, Migliore L, Bellucci S, Iavicoli I, Bergamaschi A. Int J Immunopathol Pharmacol. 2006 Oct-Dec;19(4 Suppl):3-10.

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^{19,20}. Previous studies at UPI and JRC (partners 8 and 7) of the potential genotoxic effects of cobalt nanoparticles (CoCl₂, Co_{nano} e Co_{micron}) and other metals of environmental interest, on human peripheral lymphocytes, show DNA damage and lead us to hypothesise that some metal nanoparticles might be genotoxic and therefore have carcinogenic potential²¹; 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

This work package aims to examine the mechanism of genotoxicity and carcinogenic potential, and particularly the role of oxidative stress, induced by selected metal nanoparticles using different *in vitro* and *in vivo* models. The chosen cell models have been fully characterised and are based on human leukocyte cultures (obtained from healthy volunteers), and on

¹⁵ Witherden IR, Vanden Bon EJ, Goldstraw P, Ratcliffe C, Pastorino U, <u>Tetley TD</u>. 2004. Am J Respir Mol Cell Biol 30 (4), 500-509

¹⁹ Valko M, Morris H, Cronin MTD. 2005. Curr Med Chem 18: 1161-1208; Leonaed SS, Harris GK, Shi XL, 2004. Free Rad Biol Med 37: 1921-42,

²⁰ **Cajaraville, M.P**., et al 2003b. Chapter 2: In: Lawrence, A.J., Hemingway, K.L., (Eds.), *Effects of Pollution on Fish*. Blackwell Science Ltd, Oxford, pp 14-82

²¹ Bonassi ... <u>Migliore L</u>. et al 2001 ... Environ Mol Mutagen 37: 31-45, <u>Migliore L</u>, Frenzilli G, Nesti, C, Fortaner S, Sabbioni E. 2002. Mutagenesis 17:411-17



cell lines relevant to occupational and environmental exposure²²²³. 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 hepatocarcinogenesis²⁴. In addition, possible carcinogenic effects will be also studied in mussels, where haemic or haemocytic neoplasia and gonadal neoplasia have been reported²⁵.

2.2.7 Interpretative comparison and interlinkage of reactivity, bioavailability and health effects (WP7)

Key partners: **1(NHM)**, 2(IMPERIAL), 3(RUC), 4(UNS), 5(UCO), 6(UPV-EHU), 7(JRC), 8(UNIPI), 11(USGS)

The experiments above will compare 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?

²³ L Ceriotti , **J. Ponti** , F. Broggi , A. Kob, S. Drechsler , E. Thedinga , P.Colpo , E. Sabbioni , R. Ehret <u>, **F. Rossi**</u> Sensors and Actuators B 123 (2007) 769–778.

²⁴ Law, J. 2003. Toxicologic Pathology 31:1, 49 - 52

2.2.8 Risk model and communication (WP8)

Key partners: 1(NHM), 3(RUC), 7(JRC), 10(KCL)

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 our joint studies will be development of a conceptual model to guide evaluation of hazards and risks from nanoparticles. Because our studies build a basis for evaluating risks; assessing what our results mean in a systematic way is necessary. The model will be developed to be applicable to the body of evidence that will surely grow quickly as knowledge of nano-materials grows.

Risk communication: The profile of nanotechnology and any associated risk is high in the media; so inadvertent miscommunication is possible. Another goal of NanoReTox is to develop a risk communication strategy that will guide how we release our results, but more important help recipients of our results (government, industry) communicate risks in a balanced, robust manner. It is essential to "get the risks from nanoparticles right" because the technology offers many potential benefits. The costs of over-stating or under-stating risks could be high. Although general risk assessment procedures are well known, there are many unique attributes of nanoparticles that may require new or adjusted methodologies. Communicating new results in an unbiased, balanced and value free way is critical to public credibility. Communicating risks appropriately also requires a holistic view of the issues, as well as a careful, rational and transparent approach.

2.2.9 Project management (WP9)

Key partners: all; Lead: 1(NHM)

NHM will be ultimately responsible for both scientific and financial/administrative coordination. Scientific coordination will cover technical issues, including scientific reports to the Commission, management of workpackages, quality control, progress meetings and publications whereas financial/administrative coordination will cover financial issues, meeting organisation, financial reports, cost control, deadlines, contacts with the Community and dissemination.

2.3 Current status

NanoReTox is a 4-year project, which started in December 2008. Highlights of the project at the end of the first year include:

 ²² <u>Migliore L</u>, Petrozzi L, Lucetti C, Gambaccini G, et al 2002 Neurology 58: 1809-1815. <u>Migliore L</u>., Fontana I., Trippi F., Colognato R., Coppede' F., Tognoni G., Siciliano G. (2005 Neurobiol of Aging; 26(5):580-86

²⁵ Barber, B. J. 2004. Aquatic Living Resources. 17: 449-466.



2.3.1 Synthesis and characterization of nanoparticles ("Synthesis Group")

The main objective of the Synthesis Group is to produce sets of well-characterised nanoparticles with properties that vary systematically, so that robust links to toxicity can be made. In order to establish that the nanoparticles tested by NanoReTox are representative of what is currently and in the future released in the environment, we apply both top down (milling) and bottom up techniques (e.g. aqueous synthesis). Furthermore, different quality of samples will be tested; from industrial large-scale techniques (plasma synthesis) generating polydispersed nanoparticles to the fine microfluidic approach producing highly monodispersed nanoparticles. Also, samples will be tested in both powder and suspension forms. One has to bear in mind that powder exposure is one of the most realistic exposures at this stage and therefore has to be considered. Suspensions mainly produced using surfactant molecules modifying the surface of the particles will also be investigated.

In order to transfer existing knowledge and skills from previous projects to this multi-disciplinary team, CuO nanoparticles produced via plasma synthesis was supplied by Intrinsiq, characterized by NHM and used to set-up protocols by the toxicology groups. This sample was important to get the wide number of partners geographically split to work together and communicate in order to form a cohesive group and tackle challenges together. The availability of this sample in large quantities allowed us to set-up protocols without constrain on batch variability and subsequent change of physicochemical properties.

Following that first step, the Synthesis Group produced a variety of "model" particles, tailor-made for toxicity studies, of various well-controlled size, as well as other properties. Similar particles were produced using different methodologies to assess any potential effects of the synthesis method on toxicity. Along with these tailor-made particles, "standard" particles where available (specifically: TiO₂ P25 Degussa) and bulk particles were purchased to be included in the next set of toxicity experiments. The particles included in this second step were: CuO, TiO₂ and Au. These have now been distributed to (eco)toxicologists for experimentation. Next batches to be produced are: SiO₂, Ag and ZnO.

2.3.2 Ecotoxicological effects of engineered nanoparticles ("Ecotoxicology Group")

The main objective of the Ecotoxicology Group is to determine 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 will be investigated. Ecotoxicological studies with engineered nanomaterials are currently limited. Most of those 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. A key aim of NanoReTox is to generate such systematic knowledge that will enable to develop reliable standardised tests using new simpler more relevant screening techniques.

Whole aquatic organism studies of CuO nanoparticle toxicity have been carried out. In the first year of the project, the important challenge for the Ecotoxicology Group 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 (see above) was used by partners for preliminary experiments to fine-tune their methodologies.

The next step of the ecotoxicological studies will be to try and establish the bioavailability of nanoparticles, followed by the biodynamic studies and finally, the understanding of stress generated by nanoparticles to aquatic organisms will be investigated.

2.3.3 Toxicological effects of engineered nanoparticles ("Toxicology Group")

Using animal 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 present many disadvantages. We therefore intend to use in vitro models to examine cellular responses to nanoparticles.

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. This research strand 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.

In the first year of the project, the important challenge for the Toxicology Group 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 (see above) was used by partners for preliminary experiments to fine-tune their methodologies. Further screening tests using titania nanoparticles have now started.



3 Directory

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NANOSH

Inflammatory and genotoxic effects of engineered nanomaterials



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No.	Beneficiary name	Short name	Country
1	Finnish Institute of Occupational Health	FIOH	Finland
2	Institute for Surgical Research, University of Munich	LMU-MUENCHEN	Germany
3	Central Institute for Labour Protection - National Research Institute	CIOP-PIB	Poland
4	Netherlands Organisation for Applied Scientific Reseach	TNO	Netherlands
5	Health and Safety Laboratory	HSE.HSL	UK
6	Deutsche Gesetzliche Unfallversicherung eV	DGUV	Germany
7	Cancer Biomarkers and Prevention Group, University of Leicester	ULEIC	UK

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1 Project summary

Nanotechnology, *i.e.* production based on different nano-sized particles, is a rapidly increasing area of industry providing new and innovative solutions that are being introduced into many industrial sectors. In the near future, it will have a major impact on the everyday life of people in industrialized countries and, therefore, there are increasing demands by society for reliable and understandable information on the possible health effects of engineered nanoparticles. The present research project focused on occupational exposure to nanoparticles and their health effects.

The overall goal of the research was to characterize the levels of exposure to specific engineered nanoparticles and to delineate the health effects of selected nano-sized particles relevant to the occupational environment. Exposure levels were evaluated both under laboratory conditions and during the manufacture of the particles. The particles were characterized with respect to their morphology and particle-size distribution, surface activity, and potential for agglomerate formation. The health effects studied included genotoxicity, pulmonary inflammatory responses, and effects on the vasculature.

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The information gathered together with the state-of-the-art technology utilized in these studies increase our knowledge on nanoparticles and help to create a reliable basis for the evaluation of possible health risks associated with these new materials.

This project brought together expertise from different research areas highly relevant for assessing the safety of nanoparticles and will thereby significantly promote the formation of new centers of excellence and a competitive European Research Area in this rapidly evolving area.



Scientific and technological objectives of the NANOSH project

Particle and exposure characterization:

- to define exposure levels of selected engineered nanoparticles under laboratory conditions and in workplaces
- to delineate particle size distribution, dissolution, agglomeration properties, surface area and surface activity of various engineered nanoparticles

Genotoxicity of engineered nanoparticles:

- to delineate nanoparticle-induced oxidative DNA damage in pulmonary cells
- to explore nanoparticle-induced DNA strand breakage in pulmonary cells
- to study nanoparticle induced chromosomal damage in pulmonary cells

Pulmonary inflammation induced by engineered nanoparticles:

- to investigate direct effects of nanomaterial exposure on pulmonary inflammation
- to investigate modulatory effects of nanomaterial exposure on the development of allergic asthma

Effects of engineered nanoparticles on microcirculation:

- to investigate the effects of nanoparticles on microvascular thrombus formation
- to investigate potential prothrombotic and proinflammatory effects of nanoparticles in the microvasculature of healthy mice
- to investigate the role of nanoparticles in consequences of post-ischemic injury

2 Introduction

The project consists of two main parts; **Particle and exposure** characterization and Biological effects of engineered nanoparticles.

Particle and exposure characterization

Particle characterization gathered information on the physical and chemical characteristics of the nanomaterials in bulk, air and biological / tissue samples.

Exposure characterization assessed exposure to nanoparticles and associated control issues. The main thrust of the work was the measurement of levels of airborne engineered nanoparticles in a wide range of research and industrial settings; each partner carried out measurements in several settings giving 18 different datasets. The monitoring programme was planned carefully, involving the company and the workers, so that both parties benefit from the monitoring exercise. The exposure data will become part of the database. Particle and exposure characterization were in a key-strategic position for the biological studies, as they are instrumental in providing information on particle characteristics and realistic levels of exposure to nanoparticles in different settings.

Biological effects of engineered nanoparticles

Information of the potential health effects of manmade nanomaterials on airways is still extremely limited. In this project the potential of nanoparticles to induce genotoxic effects and inflammatory responses in addition to the prothrombotic effects in the microcirculation of experimental animals was examined.

As to the genotoxicity of nanomaterials, it is presently poorly understood which nanomaterials are genotoxic and how their genotoxicity should be assessed. Nanomaterials may have primary or secondary genotoxic effects. Primary genotoxicity may be direct, if the nanomaterials themselves interact with DNA or the mitotic apparatus, or indirect if they act through reactive oxygen species (ROS). Secondary genotoxic effects may occur via inflammation, oxidative stress, or lipid peroxidation and involve the generation of ROS, malondialdehyde, or other reactive species. The project approached these issues by investigating the induction of oxidative DNA damage, DNA strand breaks, and chromosomal damage in pulmonary cells *in vitro* and *in vivo*.

The parameters to evaluate pulmonary inflammation included alterations in the panorama of pulmonary inflammatory cells *in vivo* as well as expression of biochemical markers of inflammation, i.e. cytokines and chemokines, also *in vivo*. Parameters of pulmonary inflammation measured *in vitro* included increased expression of chemokines and cytokines, and markers of cell death. The *in vitro* studies were designed to answer specific questions raised during the *in vivo* studies and questions that could be solved by using experimental animals only.

Nanomaterials, once they have entered the body, end up in the microcirculation, i.e. the small vessels (< 100 μ m) present in all organs and tissues. The microcirculation is essential to many functions of the organism. In addition to delivering nutrients and removing waste products, it plays an essential role in fluid exchange between blood and tissue, regulation of flow and blood pressure, inflammation, hemostasis, and angiogenesis. To better understand potential biologic and toxic effects of engineered nanomaterials, increased knowledge of their distribution, fate, and effects in the microcirculation is needed.

3 Technical approach

Particle characterization

Nanomaterial characterization for commercial nanoparticles in general, nanoparticles collected from the workplaces and the nanoparticles used in toxicity studies is divided into four categories: 1) primary particle size and morphology was studied by electron microscopy coupled with image analysis, 2) state of



agglomeration was studied both in air (dustiness tests) and in specified standard liquid (state of dispersion) by imaging the representative samples, 3) crystallinity and phase structures were studied with electron diffraction and x-ray diffraction, while the composition of nanoparticles was analyzed by energy dispersive spectroscopy (EDS) and inductively coupled plasma mass spectrometry (ICP-MS), 4) specific surface area was measured by adsorption using the BET isotherm.

Assessment of potential worker exposure to engineered nanoparticles

A harmonized measurement strategy was developed for the project, based on knowledge of the state-of-the-art and practical feasibility for the four partners. Toxicological evidence suggests that current exposure methods based on mass concentrations may not correlate with the potential heath effects from inhalation of emerging nanoparticles. A suite of instruments was therefore deployed at workplace to measure airborne nanoparticle levels. Instruments for measuring near real-time particle size distributions, such as the SMPS (scanning mobility particle sizer) and the ELPI (electrical low pressure impactor), formed the basis for the air measurements during the nanoparticle-related activities. In addition, near real-time active surface area concentrations were measured with diffusion charging devices, and particle number concentrations were measured using a variety of condensation particle counters. Electrostatic precipitators and specially adapted filters were used to collect airborne particles during the activities, for physical and chemical characterisation by electron microscopy.

In order to discriminate between levels of nanoparticles generated from the processes being studied and those from external sources (e.g. traffic exhaust, etc) and from other indoor processes, the times of all process activities were carefully logged and observations of incidental activities such as opening doors and the passage of vehicles both inside and outside the workplace were noted as far as possible. This enabled the nanoparticle concentrations produced by the processes to be determined by a statistically enhanced subtraction of the background levels before and after each process. Estimates of (the potential for) exposure were then derived by processing the collected data in a structured way, and a preliminary "decision logic" was developed to assist the evaluation of the data. This had four stages and used calculations from the results of the monitoring instruments, together with detailed particle characterisation by electron microscopy and consideration of other workplace issues, to derive a "likelihood of exposure" to nanoparticles from the process being studied.

Measurement of filter efficiency for engineered nanoparticles

A range of different filter materials, both mechanical and electrostatic, that are currently used in RPE and ventilation devices for filtering nanoparticles were tested in the laboratories of three of the partners using tests rigs based on the principles of the current European Standard for measurement of filter penetration. Tests were carried out by each partner with one of the test aerosols of nano-sized aluminium oxide, carbon black and titanium dioxide, generated by atomization of suspensions. Sodium chloride aerosol was used as the reference for comparison of the results among the three partners. Tests were carried out at two different face velocities and in triplicate for each combination. Results of filter penetration as a function of particle size were obtained by using SMPS instruments to measure the number size distributions of the challenge and penetrating aerosols.

Genotoxicity of nanoparticles

The genotoxicity of various nanomaterials was assessed in human bronchial epithelial BEAS 2B cells, mesothelial MeT 5A cells, and lymphocytes in vitro. The doses tested were chosen on the basis of cytotoxicity assessment by the analysis of cell counts, mitotic index, or proliferation index. Oxidative DNA damage was determined using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for 8-oxo-7,8-dihydro-2'deoxynucleoside adducts of guanine (8-oxodG) and adenine (8oxodA) and an immunoslot blot assay for N-1,N² malondialdehyde-2'-deoxyguanosine (M₁dG) adducts. DNA strand breaks were studied by the single cell gel electrophoresis (comet) assay and chromosome damage by the micronucleus assay and the chromosome aberration assay. Centromeric and telomeric fluorescence in situ hybridization (FISH) was used to study the contents of micronuclei induced by nanosized TiO₂ anatase. Cytotoxicity was assayed by viable cell counts. The genotoxicity of nanosized TiO₂ aerosol (anatase) to lung cells of C57BI/6J mice in vivo was examined by the analysis of DNA strand breaks and micronuclei in alveolar type II cells and Clara cells after a 5-day inhalation exposure using techniques developed in the project.

Pulmonary inflammation

In vitro:

Mouse macrophages and bone-marrow derived dendritic cells in addition to human macrophages and fibroblasts were exposed to different nanomaterials. After the exposures, cell death was calculated by the Trypan blue -assay, and cells were collected for protein secretion by ELISA (enzyme linked immunosorbent assay), RNA expression (TaqMan) and co-stimulatory molecule expression by FACS (fluorescent activated cell sorter) analysis.

In vivo:

Mice were exposed by inhalation for 2 h, 2 h on 4 consecutive days, or 2 h on 4 consecutive days for 4 weeks to several commercial nanoparticles and to nanosized titanium dioxide generated in a gas-to-particle conversion process at 10 mg/m³. After the exposure, the following samples were collected and analyzed:

- airway reactivity to methacholine was measured by a plethysmograph

- blood \rightarrow changes on protein level by ELISA from serum

- bronchoalveolar lavage (BAL) \rightarrow inflammatory cell infiltration analysis



- lung tissue \rightarrow expression of cytokines and chemokines relevant to inflammation

- \rightarrow immunohistochemistry
- \rightarrow morfology

Microvascular effects

Various nanomaterials, such as nano-sized titanium dioxide particles, diesel exhaust particles, carbon black particles, pristine carbon nanotubes, and surface-modified quantum dots, were used in these studies. First, a practical method to disperse nanoparticles in physiologic solutions for biological in vitro and in vivo studies was established and validated. Second, the fate of nanomaterials and their potential to exert prothrombotic or proinflammatory effects was analysed in various murine models using state-of-the-art in vitro methods, ex vivo (confocal microscopy) and in vivo microscopy (transillumination and fluorescence microscopy, 2-photon laser-scanning microscopy), immunohistochemistry, and electron microscopy. Third, the question was addressed of whether a 24-h inhalation exposure to nano-sized carbon particles evokes thrombogenic effects in hepatic and cardiac microvessels and whether these effects are associated with pulmonary or systemic inflammation.

4 Achievements

Particle characterization

Characterization of nanomaterials in general and for toxicity tests indicated the relevance and importance of material analysis in order to verify the specifications provided by manufacturers. As a conclusion for nanoparticle dispersion studies, it can be stated that for preparation of suitable nanomaterial dispersions for toxicity tests, the use of dispersion additives is beneficial. Within the workplace sample analyses, it TEM/EDX (transmission was noted that electron microscopy/energy dispersive x-ray spectroscopy) analysis is a very valuable tool to confirm the presence of engineered nanoparticles in workplace air. In addition, within the experimental work on the assessment of the efficiency of different sampling methods, a similar size distribution was found on the TEM grids using the two collecting devices: ESP (electrostatic precipitator) and the filter / TEM grids assembly. The filter / TEM grids assembly, developed through the NANOSH project, is a cheap alternative to the ESP.

Assessment of potential worker exposure to engineered nanoparticles

The raw results were collated in a spreadsheet-based draft database that included contextual information about the process and activity being monitored and the ventilation and exposure controls in use, as well as other important factors. The entries were anonymised, thereby allowing further use and analysis by external bodies. Eighteen different companies or institutes were involved, with 124 measurement sets and 426 individual measurements. Of these, 40% were carried out in commercial premises and 60% in research laboratories; 30% involved production (pilot and full scale) of new ENPs and 70% the downstream use of already manufactured ENPs. A wide range of different types of ENPs was encountered in the studies, including many of the most common nanomaterials (e.g. carbon nanotubes, TiO_2 , ZnO, carbon black, fumed silica, nanoclays) and some less common ones.

In general, levels of ENPs in the workplaces monitored were low and close to background. As expected, the highest emissions were found during the various industrial powder handling activities; and small-scale research carried out in laboratories and clean rooms were the lowest. Following the decision logic process, in 28% of the exposure situations monitored, exposure to ENPs was found to be "likely"; in 20% of the situations it was found to be "possible/not excluded", and in 52% of the situations it was found to be "not likely".

Measurement of filter efficiency for engineered nanoparticles

Key parameters (e.g. test aerosol neutralisation) have been identified that affect the testing of the penetration efficiency of filter materials for nanoparticles. More rigorous and detailed specification of the generation, testing and measurement systems will need to be addressed in future work of this nature, to allow inter-laboratory comparability of data. This is of particular importance to European standards for filter testing.

No evidence was found to suggest that nanoparticle penetration through the filters tested would increase at sizes lower than those considered in this work (i.e. <10 nm). It was found that penetration curves for mechanical filters tend to suggest that the most penetrating particle size is outside the tested range of particle sizes (~10 to 150 nm), and probably at larger particle sizes, whilst those for electrostatic filters tend to show a maximum penetration in the region of 20 to 50 nm for the particle size range tested. It was also found that filter penetration increases as face velocity increases for the majority of cases studied. No conclusive evidence was found to suggest that nanoparticle composition significantly affects filter penetration.

Genotoxicity of engineered nanoparticles

Various TiO₂ nanoparticles increased the level of 8-oxodA adducts in MeT 5A or BEAS 2B cells in a dose-dependent manner, probably as a consequence of Fenton chemistry leading to the generation of •OH radicals in the vicinity of DNA. None of the nanoparticles studied increased M₁dG adducts in BEAS 2B or MeT 5A cells, suggesting no formation of secondary oxidation products such as malondialdehyde as a consequence of lipid peroxidation. Most metal oxide nanoparticles (including nanosized and fine forms of TiO₂) and carbon nanomaterials examined, with the exception of nanosized amorphous silicon dioxide (SiO₂), were able to induce a dose-dependent increase in DNA strand breakage in the comet assay with BEAS 2B or MeT 5A cells. A stronger effect was usually seen with the mesothelial MeT 5A cells than the epithelial BEAS 2B cells. Nanosized TiO₂



anatase, zinc oxide (ZnO), long carbon nanotubes (mixture of singlewall and other carbon nanotubes), and graphite nanofibers produced micronuclei as well, but a dose-response was only seen with nanosized ZnO which was also clearly more cytotoxic than the other particles. The chromosome-damaging effect of nanosized TiO₂ anatase and short single- and multiwall carbon nanotubes was also studied in human lymphocytes in vitro and all of them increased chromosomal aberrations in a dose-dependent fashion after a continuous treatment for 48 h. FISH analysis indicated that micronuclei induced by nanosized TiO₂ anatase in human lymphocytes contained chromosomal fragments and whole chromosomes, indicating both clastogenic and aneugenic mode of action. No induction of DNA strand breaks or micronuclei could be seen in type II alveolar cells or Clara cells of mice after a 5-day inhalation exposure to nanosized TiO₂ anatase.

Pulmonary inflammation

In vitro

The analysis of cell death showed that all particles studied were dose-dependently cytotoxic in both mouse macrophes and dendritic cells. Macrophages were the most sensitive to ZnO, followed by TiO_2 and carbon nanotubes. Dendritic cells were very sensitive to all studied materials. The most significant induction of inflammatory mediators was seen in macrophages, dendritic cells showing much less inflammatory effects in response to nanomaterials.

In vivo

Experiments on the direct effects of repeated exposure with different nanomaterials on pulmonary inflammation in mice showed that the particles accumulate in the alveolar macrophages. Silica coated titanium dioxide nanoparticles were the only sample tested that elicited a clear-cut pulmonary neutrophilia in healthy mice. Pulmonary neutrophilia was accompanied by an increased expression of other essential inflammatory markers in the lung tissue. Asthmatic mice showed a remarkable suppression of most mediators and signs of allergic asthma when exposed to either nanosized or coarse TiO_2 . The levels of leucocytes, cytokines, chemokines and antibodies relevant in allergic asthma, as well as airway hyperresponsiveness were all decreased or even returned to a normal values in healthy mice.

Microvascular effects

The findings demonstrated that nano-sized diesel exhaust and titanium dioxide particles injected into healthy mice had no effect on platelet activation or thrombus formation, whilst pristine single-walled carbon nanotubes induce activation/aggregation of platelets and exerted prothrombotic effects in both small arteries and microcirculatory arterioles. Moreover, the results showed that even minor basic surface modifications of quantum dots dramatically influenced their in vivo deposition and clearance after systemic application. Furthermore, quantum dots were shown to modulate leukocyte adhesion and transmigration, depending on their surface modification. Carboxyl-modified quantum dots were rapidly

taken up by perivascular macrophages involving the activation of mast cells, thus potentiating leukocyte adhesion and transmigration in an ICAM-1-dependent manner. In addition, based on the present findings and previously published work, it could be suggested that exposure to moderate doses of nanosized carbon black particles exerted thrombogenic effects in the microcirculation of healthy mice, independent of the route of administration, i.e., inhalation or systemic intra-arterial administration. Such nanomaterial-induced thrombogenic effects were associated with neither a local nor a systemic inflammatory response and seemed to be independent of pulmonary inflammation.

5 Conclusions

Particle characterization

The consortium developed models for a thorough characterization of nanoparticles and their dispersions, which were used in the toxicity tests performed during the project. The models have been published and can be utilized by others, as well. A number of nanomaterials were selected for the NANOATLAS database (available both in printed and electronic form), providing examples of the most relevant nanomaterials used in commercial applications such as carbon nanotubes, fullerenes, metal particles, metal oxide particles, quantum dots and some experimental nanoparticles.

Exposure assessment

A strategy for assessing exposure to ENPs in a range of workplaces was developed. Solutions for background discrimination were explored, A decision logic for determining whether workers were likely to be exposed to ENPs was developed. Measurements were carried out in many types of workplaces from university research laboratories to large-scale production plants, where a wide range of ENPs are produced or handled. The nucleus for a database was developed where results of workplace measurements are stored with the aim to be accessible for anyone to use and add data to.

Genotoxicity of nanoparticles

Methods were developed for genotoxicity assessment of nanomaterials *in vitro* and *in vivo*. Most nanoparticles were able to damage DNA *in vitro*, and for TiO_2 this seemed to be due to primary oxidative DNA damage. No induction of malondialdehyde DNA adducts was seen, suggesting that secondary genotoxic effects due to lipid peroxidation were not involved. Mesothelial cells were more sensitive than bronchial epithelial cells to the DNA-damaging effect of nanoparticles. Some nanomaterials were also capable of increasing chromosome damage *in vitro*, zinc oxide showing the clearest effect. In human lymphocytes, structural chromosomal aberrations were only obtained by a prolonged *in vitro* treatment. Inhalation of TiO_2 did not affect the level of DNA or chromosome damage in mice. These findings were presented in a number of scientific publications.

Pulmonary inflammation

We assessed the toxicity and immune activation ability of five nanomaterials (TiO₂, silica coated TiO₂, SWCNT, MWCNT, ZnO) on antigen presenting cells that are the first responders of the immune defence and showed an induction of macrophage activation after exposure to all the materials studied. In vivo tests showed that healthy mice elicited pulmonary neutrophilia accompanied by chemokine CXCL5 expression when exposed to nanosized TiO₂. Asthmatic mice showed remarkable suppression of most mediators and signs of allergic asthma when exposed to either nanosized or coarse TiO₂. We could see that levels of leucocytes, cytokines, chemokines and antibodies allergic asthma relevant in as well as airwav hyperresponsiveness were all decreased or even returned to a level normal in healthy mice. Our results suggest that repeated airway exposure to TiO₂ particles modulates the airway inflammation depending on the allergic status of the exposed mice. Interestingly, the level of lung inflammation could not be explained by the surface area of the particles, their primary or agglomerate particle size, or radical formation capacity, but was rather associated with the surface coating. Our findings emphasize that it is vitally important for risk assessment to take into account that modifications, e.g., by surface coating, may drastically change the toxicological potential of nanoparticles. The findings were published in the scientific literature.

Microvascular effects

These *in vitro* and *in vivo* findings strongly corroborate the view that, in addition to size and shape, the surface chemistry of nanomaterials strongly affects their fate as well as their biological effects *in vitro* and *in vivo* and thus is a crucial parameter to be considered with regard to their toxicity but also concerning potential biomedical applications. The results obtained were reported in several original articles.

6 Intentions for use and impact

The findings of the project have a significant socio-economic impact on European capability of conducting research and innovation in the area of nanotechnology. Assuring the safety of



new nanomaterials is a crucial prerequisite for successful promotion of nanotechnological innovations and their applications in the future. This research creates a reliable and sound foundation for the assessment of safety of the chosen nano-sized particles and products containing nanoparticles and in this way encourages nanotechnological advances to support European national economies and the prosperity and wellbeing of citizens in the EU Member States.

The project provides essential information which can be used on a wider basis for assessing occupational and other safety risks associated with the production and use of nanoparticles. Essential products that serve these scientific and technological goals are means and methods to characterize particle properties, ways to carry out reliable exposure assessments, and models for assessing key-health effects - all important components of the safety evaluation of engineered nanoparticles.

7 Continuation plans

The presentation of the results of the project in scientific publications and conferences will continue. There is a need for effective dissemination of the obtained new knowledge, subsequent to appropriate digestion of the latest results. This activity is deemed highly important and should concern a wide variety of audiences.

Studies on exposure to various nanomaterials and on their toxicity will be carried on in new research projects, such as NANODEVICE, SUNPAP, and NANOGENOTOX. These projects aim at further development of methodologies and strategies in exposure assessment and nanotoxicology and are expected to result, e.g., in new approaches to nanoparticle measurements and in recommendations for guidelines on nanomaterial toxicity testing.

The consortium is actively participating in relevant European networks such as NanoImpactNet and Nanosafety Forum which provide excellent opportunities for further integration and future collaboration in health and safety research of engineered nanomaterials.



8 Directory

Table 1 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

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6	Veneto Nanotech	VN	IT
7	European Commission Joint Research Centre, Institute for Environment and Sustainability	JRC	BE
8	Kaunas University of Technolgy	KTU	LT
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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 nanoproducts 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

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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 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, labscale experiments will explore new technical solutions for the innovative design of nanomaterials and associated products and their sustainable use, re-use, recycling and/or final treatment and 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 behaviour 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 nano-technology 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 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 will be 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 reuseability, 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' will be 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 will consider 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 nanotechnologybased products.

Specific objectives are:

- to assess the hazard of representative groups of nanomaterials based on a comprehensive data survey on their properties (physico-chemical 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, ecotoxicology, 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 will be investigated in detail within the planned project:

- nanocellulose based products;
- CNT based products;
- nano ZnO based composites, and
- nano TiO2 based products

4 Technical approach and work description

A variety of available advanced analytical, characterization and biological test methods will be 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 will be 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 will be 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 treament.

To identify potential impacts arising from production, application/use up to final recycling, treatment and disposal, existing LCA-methods and exposure models for LCIA will be 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

Short description of the technical work:

WP2 will collect and evaluate all available and relevant data for the selected types and groups of nanomaterials and products and provide the processed data to all other WPs in an appropriate form. It will 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).

WP3 will generate representative test samples for consequent measuring and testing the source strengths from handling and reworking dust emissions, the toxicological and ecotoxicological relevant physico-chemical 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 doseresponse 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 ecotoxicology 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, (8) develop and test accurate and repeatable analytical methods to detect, characterize and quantify nanoparticles in environmental matrices, and (9) apply these methods to real samples.

WP4: Based on results from WP2 and WP3, WP4 will develop (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) carry out 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 will be developed, tested and improved as well as design guidelines for improved recyclability and precautionary design applied to the newly developed technical solutions in WP5.

WP5: Specific nanocellulose based materials and associated endproducts will be 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 will 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 will be tested by waste incineration and characterization by using various detection and characterizsation techniques, including also gaseous and particulate emissions from incineration of selected products by standard techniques such as CPC, SMPS, ELPI, BLPI, FTIR and EMA.

Established glass recycling processes (e. g. melting) will be 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 will be 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.

5 Expected impact

NanoSustain will help improve our current insufficient knowledge on the hazard, impact and sustainability of nanomaterials and products, in particular in relation to end-oflife stages, like reuse/recycling, final treatment and disposal. It will substantially contribute 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 will generate solid scientific data on potentiat risks and their probability of occuring during reuse/recycling and final treatment or disposal. For the first time new and innovative technical solutions will be explored and developed on a labscale 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. А dedicated project website (www.nanosustain.eu) will enable 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 will be linked with other information sources such as NanoArchive (www.nanoarchive.org), NanoImpactNet (www.nanoimpactnet.eu), and observatorvNANO (www.observatory-nano.eu) and will provide presentations and proceedings from events planned during the project. Users will be required to register online, providing basic information on their organisation 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 regular newsletter will be 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 organised 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 organisations and associations demonstrating the functionality and applicability of the knowledge generated within the project to the processes employed within these organisations.



7 Directory

Table 1 Directory of people involved in this project.

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NANOTRANSPORT

Behavior of Aerosols Released to Ambient Air from Nanoparticle Manufacturing – A pre-normative study

Contract Agreement: NMP4 NMP4-CT-2006-033371 Website: http://research.dnv.com/NANOTRANSPORT/ Coordinator: Qinglan Wu, Det Norske Veritas AS, Veritasveien 1, N-1322 Hoevik, Norway

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1 Summary

The NANOTRANSPORT project addressed the behaviour of aerosols released to ambient air from nanoparticle (NP) manufacturing. We intended to elucidate and document the need for standardised test aerosols adapted to the scope of nanotoxicology and occupational health studies. The objective of NANOTRANSPORT was to investigate physical changes which NP aerosols undergo after release into the workplace environment under realistic scenarios. This information is essential to understand the characteristics of NPs when they reach a human receptor after transport over a distance from a NP source.

Within the project, we have proposed model exposure scenarios based on analysis of real workplace situations. Experimental work has been carried out to simulate the scenarios in an exposure chamber. The agglomeration dynamic of primary Pt nanoparticles released in a simulated workplace environment was thoroughly characterised, using both experimental data and aerosol dynamic modelling.

Knowledge obtained from the study was used to develop recommendations to the European Commission regarding:

- Test aerosols for nanotoxicology studies;

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- Testing of filters and protective equipment in the workplace;
- Metrology of nano-aerosols;
- Research priorities.

2 Major project results

2.1 Development of model exposure scenarios

Exposure scenarios were proposed on the basis of a literature study, visits to relevant companies, experiences of the project partners and discussions with invited experts at a scenarios development workshop. By compiling all the information we defined model exposure scenarios, and developed the experimental program accordingly.



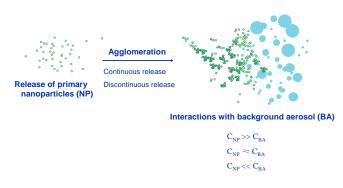


Figure 1: Model exposure scenarios used for the study (C_{NP} : nanoparticle concentration, C_{BA} : background concentration).

Model exposure scenarios used for this study are illustrated in Figure 1, including the release of primary NPs into ambient air with background aerosols (BA) in different concentration ratios to NPs (C_{NP} >> C_{BA} , $C_{NP} \sim C_{BA}$ and C_{NP} <
< C_{BA}). Both a continuous release and a pulse-wise discontinuous release of NPs are considered relevant. In case of a clean room environment the concentration of background aerosols can be much lower than NPs. In workplaces where background particles from ambient air are presented in a concentration range which is similar to or even higher than engineered NPs, interactions may occur among NPs and between NPs and background aerosols. In a typical industrial environment the realistic concentration range of background aerosols lies between 10³ to 10⁵ particles/ cm³.

2.2 Experimental results –Aerosol dynamics of nanoparticles in workplace exposure

An experimental program was designed to investigate aerosol dynamic behaviour of freshly generated Pt nanoparticles after release in an exposure chamber under different conditions defined in the model exposure scenarios in Figure 1. Main results of the study are published in the journal: *Annals of Occupational Hygiene*¹. Some high lights are given in this report.

2.2.1 Experimental set-up

The experimental set-up in Fig. 2 was built at University of Karlsruhe with technical and instrumentational support from Grimm Aerosol Technik GmbH. The exposure chamber had a dimension of $1\times1\times2$ m and a volume of 2 m³. A hot wire generator was used to generate Pt nanoparticle aerosol. Freshly generated Pt nanoparticles and reference background aerosols (oil droplets or silica monospheres) were released into the chamber through the inlets on the wall and ceiling, respectively. On the bottom of the chamber a speed-adjustable fan was

placed, allowing a variation of the flow pattern from unstirred air to a fully turbulent flow.

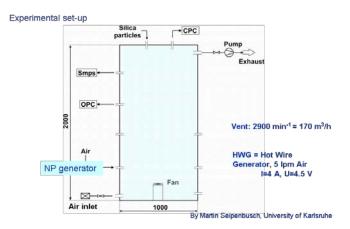


Figure 2: Experimental set-up used for studying behavior of nanoparticles after release in simulated workplace environments.

Physicochemical properties of Pt nanoaerosol were characterized both at the source (see Fig. 3) and after release in the exposure chamber. Particle-size distribution of NP aerosol was monitored on-line at different places along the wall and on the ceiling of the chamber by using SMPS (Scanning Mobility Particle Sizer) and OPC (Optical Particle Counter). Parameters, such as, convective flow rate, concentrations of NPs and background aerosols were varied to simulate different exposure scenarios. Using the designed experimental setup, agglomeration dynamics of freshly generated NPs were quantitatively documented in time dependences of particle number size distributions.

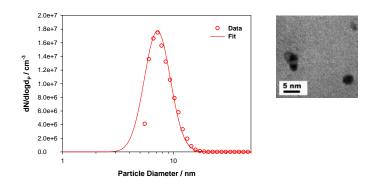


Figure 3: Particle size distribution (left) and TEM image (right) of freshly generated Pt particles used in the study.

2.2.2 Major experimental results

Nano-aerosols emitted from an NP source evolve considerably with time, both due to auto-agglomeration among NPs and/or due to attachment to pre-existing background aerosol particles. Which one of these two mechanisms will prevail depends on the given workplace scenario (i.e. the strength of a primary NP source relative to background concentration, and the elapsed

¹ SEIPENBUSCH, M., A. BINDER, and G. KASPER, Temporal Evolution of Nanoparticle Aerosols in Workplace Exposure. Ann. Occup. Hyg., 2008. **52**(8): p. 707-716.



time during transport from source to receptor). For simplicity and without limiting the conclusions, we disregard the attachment of NP to macroscopic surfaces such as "walls" or workbenches. According to our experimental results, the influence of NP deposition in the chamber is under 14%.

Nanoparticles release in a clean room environment

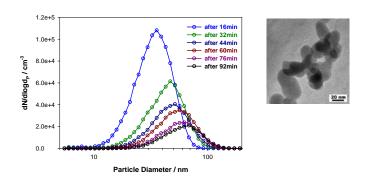


Figure 4: Particle size distributions of Pt nanoparticles measured after release of a pulse of NPs in a clean chamber (left); transmission electron microscope of Pt nanoparticles collected in the chamber (right).

When a pulse of Pt NPs are released into a clean chamber ($C_{NP} >> C_{BA}$), the average particle size increases with the time while the number concentration decreases, implying agglomeration among NPs (see Fig. 4). The auto-agglomeration process stabilizes typically in the size range between 100 and 200 nm, where particle collision processes slow down.

The time scale of size evolution is mainly dependent on the concentration of NP. At a lower concentration of $< 10^4$ particles/cm³ the agglomeration process of Pt is very slow, in the order of an hour, and therefore, workers may be exposed to primary NPs. When NP concentration is higher then $> 10^7$ particles/cm³, agglomeration occurs within seconds; even workers standing close to the source are very likely exposed to agglomerates rather then primary NPs.

Continuous release of NPs into a chamber with background aerosols

When nanoparticles are continuously released into an exposure chamber with background particles, the aerosol dynamic processes are largely driven by NP concentration at the source and concentrations of background particles and the ratio between the two concentrations. If the NP concentration is high compared to the background concentration, then autoagglomeration will initially dominate the evolution of size and concentration, forming a significant new population of agglomerates NP with two characteristic peaks in the range of 20 -100 nm. If NP concentration is relatively low, background particles act as scavengers for NPs, resulting in formation of heterogeneous agglomerates. The attachment of small NP to relative larger background aerosol particles leaves the size of the latter practically unchanged. The scavenged NPs therefore merge into the ambient aerosol between about 0.1 and 1µm. If NPs and background particles are comparable in concentration,

then both mechanisms are likely to run concurrently, producing both agglomerates of NPs and mixed particles.

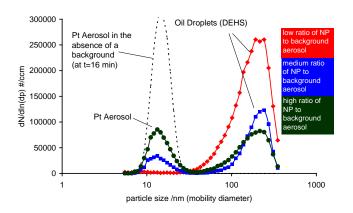


Figure 5: Size distributions of Pt particles continuously released in a simulated workplace environment with background particles (oil droplets), the background concentration is quasi-stable during the entire experiment.

An example of mixed agglomerates is given in Fig. 6. The particles on the electron microscopy were collected from the chamber after Pt nanoparticles were released to and interacted with the background aerosol, silica particles. The image shows the formation of both auto-agglomerates among Pt particles and heterogeneous agglomerates between Pt particles and silica particles.

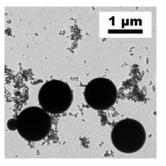


Figure 6: Transmission electron microscopy of Pt nanoparticles (d= 10 nm) in presence of silica monospheres (d=1 μ m) as background particles, particles were sampled from the exposure chamber in an impactor.

An aerosol dynamic model was derived based on coagulations through Brownian collisions among NPs as well as between NP and background aerosols by using Smoluchowski equation. The model was applied to fit the experimental data. A good agreement between experimental data and model calculation was achieved. The result demonstrates the possibility to predict the concentration of NP in a given workplace environment. Details of the aerosol dynamic model can be found in the paper by Seipenbusch et al 2008, in Ann. Occup. Hyg., 2008. 52(8): p. 707-716. Results of the modelling also suggest that aerosol agglomeration and/or attachment process is driven predominantly by thermal (Brownian) collisions. Hence, the adhension within the agglomerates will be mostly of the van der



Waals type and thus fairly weak. It will be important to know how the agglomerates behave once they become immersed into cellular fluid. Information on agglomerate stability and toxicity of agglomerates are needed.

2.3 Recommendations

Recommendations are developed based on experimental results of the study, taken into account the accepted practices in production and use of NPs and relevant research needs described in literature.

2.3.1 Suitable test aerosols for nano-toxicology studies

Based on the results of the study, two scenarios must be considered in emulating exposure to NP in the workplace. In one case, the receptor is located immediately at the source of a NP aerosol (the nose near the "leak", so to speak); in the other, the receptor is located further away (e.g. a person working elsewhere in the lab). Scenario 1 requires testing with primary, un-agglomerated NP, while Scenario 2 requires aged aerosols, either auto-agglomerated or adhering to neutral particles, or both. Scenario 2 may well be the more prevalent case for many exposure situations. Studying the deposition and effects of "aged" nano-aerosols is thus at least as important as those of un-agglomerated particles from a primary NP source.

A point of clarification with regard to the above mentioned scenarios: the range within which a receptor will likely be exposed to predominantly un-agglomerated NP (the "near-field exposure" radius) can be expressed in terms of the average distance traveled by the aerosol during the time required for the primary aerosol to becomes significantly agglomerated. The "near-field" exposure radius is thus a function of the primary emitted concentration (hence the agglomeration rate) as well as air velocity. Analogous considerations apply to the far-field exposure scenario.

For either scenario it becomes critical to control the state of agglomeration of the particles at the receptor: either as little agglomeration as possible, or a significant but defined state of agglomeration with corresponding, well defined structural characteristics of the aged particles.

The state of agglomeration matters of course for exposure assessment studies, because deposition is governed by aerosol particle size. The agglomeration stability and sizes are also important for toxicological studies. Currently our knowledge about the toxicological effects of the NP agglomeration is rather limited. Further research need to be done to investigate the potential for deagglomeration in cellular environments, or how the structure of the airborne agglomerate carries over into the liquid state.

Liquid NP suspensions or pre-prepared powders are generally difficult to aerosolize with controlled particle sizes. Particle structures and agglomerate strengths obtained via redispersion

are generally not representative of those formed in the aerosol state, except in cases where NP redispersion is the release mechanism to be investigated.

For exposure and toxicology studies it is therefore recommended to produce and age NP directly via on-line aerosol processes, including aging the presence of a relevant background aerosol. Aerosol processes best ensure NP structures and size distributions representative of workplace releases into the air. Only after the effects of agglomerate structure in cellular tissue environments, the transport processes of agglomerates in such environments, possible deagglomeration mechanisms etc. have been understood may it become possible to relax these requirements.

2.3.2 Testing of filters & protective equipment

The mechanisms of particle filtration in air are well understood. In particular we know that the efficiency of filters increases steadily with decreasing particle size in the submicron range. Recent studies confirm this down to sizes of about 1 or 2 nm. We can thus conclude that a filter deemed sufficient for larger particles will be even more effective in capturing "true" NP (<10 nm).

On the other hand, the growth of aged NP by autoagglomeration or their attachment to ambient background particles can shift their size into the range of the Most Penetrating Particle Size (MPPS) of typical filter media, which is typically in the range of 80 - 200 nm. If adequate protection against nano-aerosols is required, it becomes critical to assure adequate filter efficiencies also in the MPPS region.

The methods for filter testing, and in particular for testing at the MPPS, are well developed and have been incorporated into various standards; aerosol generation and testing equipment for the submicron size range such as mobility spectrometers and CPCs are also available on the market. Mobility spectrometers classify irregularly shaped particles by their mobility size, which is also the relevant parameter for diffusion deposition in filters.

The chemical or surface composition of the particles is of no importance for the deposition efficiency in filters and other protective equipment and therefore, does not have to be considered in the choice of test aerosols.

A notable exception to this statement are nano-fibers and carbon nano-tubes (CNTs), which by virtue of their highly nonspherical shape do not behave in the same way as isometric particles during deposition in a filter. Filtration behavior of fibrous particles merits a more detailed investigation. The agglomeration state of CNTs is again critical for such tests.

2.3.3 Metrology of nano-aerosols

The fact that aging nano-aerosols can move up the size scale rapidly by various agglomeration processes has important implications in choosing suitable metrological strategies for purposes of workplace assessments.



Aged NP aerosols are not recognizable by a specific particle size range, such as "<10 nm". Moreover, it is not sufficient to look for NP in the "nano size range" where they may have originally been generated/emitted, unless one measures near a primary source. Agglomerated or scavenged NP will populate a size range where they become nearly indistinguishable from ubiquitous background aerosol by straightforward size distribution measurements. It is well known that additional ENP concentrations are often marginal, unless there are gross emission sources.

One alternative are highly selective real-time (!) analytical techniques with some kind of species sensitivity. This would allow detecting the presence of a NP even when it is attached to other particles. Today such methods are not broadly available and those which exist, such as single particle mass spectrometry or aerosol catalysis, are untested in the context of workplace assessments. A significant effort is necessary in this critical area, akin to the development of real-time detection for bio-aerosols, which seemed nearly impossible a decade ago but has made great strides since then for well known reasons.

An alternative strategy to hazard assessment of NP emissions is based on the use of aerosol dynamic models to predict relevant information for specific workplace scenarios such as concentrations and size range of the NP aerosol at the receptor. Such models have been developed in past years to predict the evolution of an aerosol during catastrophic releases of radioactive material. Although not yet validated for such new applications, aerosol dynamic codes require as input

- the background aerosol size distribution,
- the characteristics of an emission source (strength, primary size range), hence the identification of release mechanisms for each specific NP hazard;
- and assumptions about an age distribution and possible dilution of the aerosol during transport to the receptor. This can be done via model scenarios.

Finally, the prevalence of aged and therefore agglomerated NP brings up the question of their ability to fragment, which presumably has a strong influence on biological effects. Thus there is a need for effective techniques to quantify agglomerate strength in aerosol particles.

2.3.4 Open questions & priorities for future research

As a result of the preceding analyses, a number of important open questions can be formulated.

The release mechanisms and sources of NP into ambient workplace air need to be characterized and typified regarding source strength, size range and agglomerate structure. This is relevant for the development of realistic aerosol generators, realistic doses, as well as for the effective use of predictive aerosol dynamic models.

The development of *model aerosol sources* for exposure and toxicological studies needs more focus and effort. Simultaneously, a systematic effort should be undertaken to use aerosol sources for such studies, rather than powders or liquid suspensions and to advance the appropriate techniques for their application. Model aerosol sources are required for relevant classes of species and release mechanisms, including aging and scavenging methods where appropriate.

Existing *predictive dynamic models* for nano-aerosol evolution between source and receptor need to be adapted, validated and further developed as needed. While the basic mechanisms are known, the specific input parameters for the workplace environment are not.

Real-time methods for highly selective, species-specific NP detection in the aerosol state are not broadly available and require a major effort, including the sustained funding of basic research to develop and validate new concepts. This includes methods capable of detecting NP attached to background particles as well as new methods to characterize the surface chemical state of airborne particles.

The fate of structure and strength of nano-agglomerates during their transition from the aerosol to the cellular liquid phase, and the influence of these structural parameters on their toxicological effects needs a concerted investigation. Techniques for quantifying the strength of homogeneous and heterogeneous agglomerates, especially on-line methods would be very useful.



3 Directory

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NEPHH

Nanomaterials Related Environmental Pollution and Health Hazards Throughout their Life Cycle



Contract Agreement: CP-FP 228536-2

Website: http://www.nephh-fp7.eu

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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	TECNALIA-INAS	INASMET	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

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1 Summary

NEPHH - NANOMATERIALS RELATED ENVIRONMENTAL POLLUTION AND HEALTH HAZARDS THROUGHOUT THEIR LIFE CYCLE is a Collaborative Project funded under 7th FWP (Seventh Framework Programme) in the research area of NMP-2008-1.3-2: Impact of engineered nanoparticles on health and the environment.

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. Conversely, there exist apprehensive domains with a planetary impact like environment and new products, and functions for health and safety of people. 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.

Coordinator: EKOTEK S.L.

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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.

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 the NEPHH project are:

- 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.

- Characterization of nanomaterials has proven to be more 3. 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 welllocated. 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.
- Health, safety and environmental risks that may be 5. with products and associated 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 socioeconomic 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.

The aim of NEPHH therefore is to identify and rate important forms of nanotechnology-related environmental pollution and health hazards that could result from activities involved in nanostructures throughout their life cycle, and to suggest means that might reduce or eliminate these impacts. The NEPHH project will consider the safety, environmental and human health implications of nanotechnology-based materials and products.

The nanomaterials selected are Silicon based laboratory materials which will be supplemented with nanomaterials from



industry. On the one hand, Silicon based nanoparticles including (nano)silica (SiO2), layered silicates (MMT), glass (nano)fibres and foam-glass-crystal materials have been selected. On the other hand, a total number of three engineering polymeric matrixes have been selected, including polyamides and polypropylenes as bulk materials and polyurethanes as foamed polymeric materials, which will be used to produce nano-induce 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.

The specific objectives of the project are the following:

- Development of a systematic, continuous practice for selecting and prioritizing engineered nanomaterials in order to assess their safety, environmental and human health.
- 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. Achievement of a better understanding of the health impacts of the selected nanomaterials.
- 4. Assessment of the human and environmental exposure throughout the life cycle
- 5. Assessment of the potential of nanomaterials to damage the environment (or human health through the environment).
- 6. Selection and dissemination of the best practices (in the fields of manufacture and disposal mainly), and actuation guidelines for exposed workers.
- 7. Contribution to the 'Code of Conduct for Responsible Nanosciences and Nanotechnologies Research' action to ensure that nanotechnologies are developed in a safe manner.

8. Contribution to the regulatory frameworks which are applicable to nanomaterials (chemicals, worker protection, environmental legislation, product specific legislation, etc).

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:

- During the 1st stage, the set up of a Technological Surveillance System will be carried out (WP1). The target is to develop a systematic, continuous practice for the selection of engineered nanomaterials in order to assess their safety, environmental, and human health implications. These nanomaterials will be selected in relation to their applications and relevance. Moreover, a survey will be carried out to assess the occupational health and safety procedures in place.
- During the WP2, the selected engineered nanomaterials will be synthesised and macro-scale structural specimens will be manufactured. This will enable the consortium to analyse the implications of such nanomaterials from synthesis to disposal.
- The WP3 involves the generation of nanoscale dust particles from the macro-scale fibre reinforced nanostructures fabricated in WP2, to consider the exposure throughout the whole life cycle of nanomaterials in as near 'real life' exposure as possible.
- The 4th and 5th stages will assess the health implications (WP4) and environmental implications (WP5) of the selected engineered nanomaterials in parallel. The health effects of nanoparticles (1) on lungs; (2) the structural study of cells and protein expression, and (3) the genotoxicological effects will be studied. WP4 will be led by IBC and CRAN, INASMET and CNRS will participate. During WP5 the potential of nanomaterials to damage the environment (or human health through the environment) will be assessed, based on persistence, bioaccumulation and eco-toxicity studies. Moreover, the environmental performance of nanocompounds form cradle to grave will be assessed.
- The 6th stage will carry out an integrated assessment of the results from WP4 and WP5. WP6 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.

Finally, the 7th stage of the project will envisage the dissemination of the research results (WP7). In parallel to all above, the optimisation of the allocation of resources within the project and among the partners will be controlled, ensuring all aspects of the EC requirements for communication and reporting are met (WP8).

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.

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.

Morever, 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.

Dissemination and exploitation of the results is considered by the consortium to be a vital component of the proposed work programme. Any Dissemination Activity will be reported in the **Plan for the Use and Dissemination of Foreground.** A wide dissemination of project results and their social and environmental benefits in the Nanotechnology sector will contribute to the success of the project.

The dissemination activities can be summed up as follows:

• Selection of target audience (NEPHH partners, other project partners, Industry, Standarisation bodies, Regulatory Administrations, etc.)

• Development of dissemination materials and media (Newsleters, Flyers/brochures, Press releases, Best Practices Manuals, etc.)

Project Website

• Organizing and participating in dissemination events (workshops and seminars).

• Preparation of scientific publications, journal and conference articles, posters...

• Networking activities



2 Directory

Table 1 Directory of people involved in this project.

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Nephh is a Small Collaborative Project under the NMP Theme of the European Commission's 7th Framework Programme.

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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 Websit

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4	University of Ulster	UU	United Kingdom
5	HelmholtzZentrum Munchen	HELMUC	Germany
6	Institute for Health and Consumer Protection	JRC-IHCP	EU JRC
7	University of Rochester	UR	USA
8	The Regents of University of California / University of California, Los Angeles	UCLA	USA
9	Rice University	Rice	USA
10	National Institute of Materials Science	NIMS	Japan
11	Universidade Federal do Ceará	UFC	Brazil

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1 Summary

NeuroNano is a Small Collaborative Project funded by the European Commission 7th Framework Programme. The project started on February 1st 2009 and will 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.

The background issues that led to the formulation of the NeuroNano project include the following issues:

 Nanoparticles may reach the brain –evidence that nanoparticles less than 40 nm particles can potentially pass through the blood-brain barrier.



- Nanoparticles *may* induce oxidative stress in living systems. Oxidative stress from ambient or combustion particles contribute to cell damage, including DNA damage.
- The large surface area of nanoparticles means that they can modulate the fate of protein fibrillation in solution. Whether this has significance *in vivo* is a key question that will be determined within the NeuroNano project.
- Oxidative stress and protein fibrillation are both associated with neurodegeneration.

Based on these issues, the key questions that 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 Project background and aims

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 a toxicity due to the nanoscale will be implemented.

There is incontrovertible evidence that some engineered nanoparticles (for example 6nm and 18nm gold nanoparticles), entering intravenously or via the lungs can reach the brains of small animals.(Kreyling 2007) Furthermore there are suggestions that nanoscale particles arising from urban pollution reach the brains of animals raising the possibility that nanosized particles encountered in ambient air or the workplace will gain access to the CNS and be retained for long periods of time. The relevant particle fractions arise from pollution but their structure and size are similar to those of intentionally engineered carbon nanostructures. Secondly, any nanoparticles in contact with tissue induce oxidative stress, (Brown 2001; Xia 2006; Duffin 2007) as well as various inflammatory mechanisms that could themselves lead to further oxidative stress (Block 2004; Nel 2006). Finally, it has recently been discovered that nanoparticles (many of significant industrial interest) can have highly significant impacts on the rate of fibrillation of key proteins associated with neurodegenerative diseases,(Linse 2007) and we will report here significant new findings for the proteins associated with Alzheimer's (amyloid β) and Parkinson's disease (α-synuclein).

Whilst the precise mechanisms leading to neurodegenerative diseases are not fully clarified, it is broadly agreed that the key effects involve the presence of early pre-fibrillar protein structures, neuroinflammation and Reactive Oxygen Species (ROS)-related processes. Thus, all of the links in the causal chain are now present for a credible expectation that nanoparticles could have impacts on the onset, progression or severity of neurodegenerative diseases. The main ideas and interconnections between them are laid out in Figure 1.

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 the endpoints of two diseases, 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.

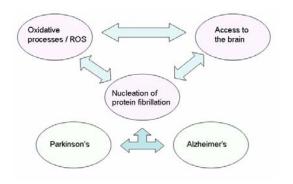


Figure 1 Overview of the interplay between various factors regarding nanoparticle interactions with living systems that could pose a risk for the development of neurodegenerative diseases.

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).

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 nanoparticleinduced 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.

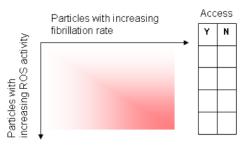


Figure 2: The FIBROS map as a potential screening assessment for nanoparticle risk in terms of neurotoxicity. Engineered nanoparticles will be tested for their oxidative effects, fibrillation effects and access to the brain.

2.1 Workplan and project interlinkages

The NeuroNano project is organised into 5 scientific and 1 management Work Packages, as illustrated graphically in Figure 3. The inter-dependencies of the Work Packages are also illustrated here. The flow of work in the 3 central experimental Work-Packages 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.

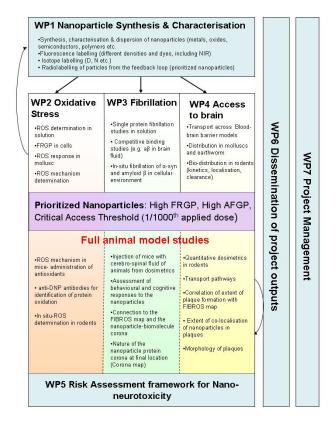


Figure 3: Workflow and ideological layout of the work packages and the information flow for the NeuroNano project

2.1.1 Progress to date

Very significant progress has been made already, and the project has just completed its first year of activity. All workpackages are progressing well, and the preliminary data is very promising. The first set of candidate nanoparticles chosen for the programme are 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 fluorescentlylabeled particles in a range of sizes, and their dispersion, cellular interaction and uptake are already well characterized via the NanoInteract (FP6 STReP project, NanoInteract;, page 109). Titania and ceria were chosen as candidates for radio-labelling to enable quantitative in vivo dosimetrics studies to be performed, as suitable strategies for labeling are available, obviously with some significant research element to optimize the procedure such that the resultant particles can be redispersed back into solution as nanoscale entities.

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.

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, intertracheal, 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.

2.1.2 Preliminary Results

Radiolabelling and dispersion of nanoparticles

Industrial TiO₂ nanoparticles have been successfully radiolabelled in dry form, then dispersed, size-selected, and delivered to various partners for assessment of the corona following labelling, and for *in vivo* studies of dosimetry. Gold nanoparticles have been successfully radiolabelled, resulting in an activated suspension with up to some hundreds of kBq of ¹⁹⁸Au per mg of gold nanoparticles, enough for some *in vivo* studies. Higher ¹⁹⁸Au activities may be produced by reactor



irradiation (not foreseen in the workprogramme, but possible to arrange). DLS studies indicated no significant changes to the NP powder size distribution, and leaching studies in water indicated no significant radiotracer release from the activated NPs. Work towards radiolabelling of ceria and carbon nanotubes is well underway, and these particles will also be available to the consortium in due course.

Dispersion of the industrial samples from dried powders and irradiated dried powders has also progressed well, and novel strategies using biomolecules are being optimized, such that they can be applied by all groups within the consortium, without the need for processes such as sonication, which are extremely variable and hard to standardize. Publications on this are in preparation.

Modulatory effect of nanoparticles of protein fibrillation in solution – interplay between different mechanisms

At the outset of the project, it was known that nanoparticles in solution can modulate the rate of protein fibrillation, dramatically accelerating the nucleation step by adsorbing the proteins and increasing their local concentration, thereby promoting the interaction and onset of critical nuclei, as shown via the TEM images in Figure 4.

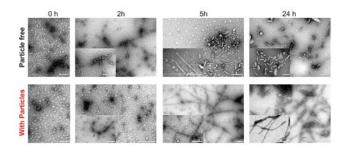


Figure 4: TEM images for a fibrillation time course in absence and presence of 40 nm diameter polymeric nanoparticles at 30 µg/ml and peptide concentration 25 µM. Top row shows images in the absence of particles and bottom row includes images of samples with nanoparticles prepared at 0, 2, 5 and 24 hours of fibrillation reaction. Scale bar indicates 200 nm. Due to the low concentration and presumably low binding to the grid, nanoparticles were not observed in the TEM specimens prepared (Cabaleiro-Lago 2008)

Within the NeuroNano project, a much more complex modulatory behavior has been observed using cationic polystyrene nanoparticles, where a dual effect has been observed, as shown in Figure 5. The fibrillation kinetics of the amyloid beta peptide analyzed in the presence of cationic polystyrene nanoparticles of different size. The results highlight the importance of the ratio between the peptide and particle concentration. Depending on the specific ratio the kinetic effects vary from acceleration of the fibrillation process by reducing the lag phase at low particle surface area in solution to inhibition of the fibrillation process at high particle surface area. The kinetic behavior can be explained if we assume a balance between two different pathways. First, fibrillation of free monomer in solution and second, nucleation and fibrillation promoted at the particle surface. The overall rate of fibrillation will depend on the interplay between these two pathways and the predominance of one mechanism over the other will be determined by the relative equilibrium and rate constants.

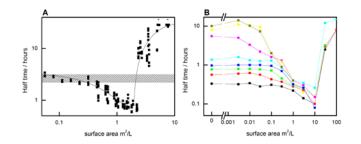


Figure 5: A) Half time of fibrillation versus particle surface area in solution for different experiments performed in presence of 120 and 180 nm polystyrene nanoparticles at a constant concentration of A6(1-40) (8 μ M). Horizontal bar indicates the half time value range in the absence of particles. Lines are guides to the eye. Asterisks indicate estimated values that fall outside the experimental time range. B) Half times of fibrillation versus particle surface area in solution in the presence of 57 nm amine modified polystyrene nanoparticles at different A6(M1-42) concentrations; (\blacksquare) 6 (\blacksquare) 4 (\blacksquare) 2.7 (\blacksquare) 1.8 (\blacksquare) 1.2 (\blacksquare) 0.78 (\blacksquare) 0.54 (\blacksquare) 0.36 μ M. (From Cabaleiro-Lago et al., 2010)

The effect of amine modified nanoparticles on the fibrillation of amyloid β peptide is dependent on the surface chemistry of the nanoparticle, which determines the strength of the protein-particle association, and the conformation that the peptide adopts at the nanoparticle surface, as well as being dependent on the relative concentrations of particles and peptide. Variation of the ratio between peptide and surface area may cause the formation of different particle-peptide entities, which can be reactive or nonreactive, that contribute in an opposite fashion to the overall kinetics of fibril formation. The results of the present study implies that to reach mechanistic insights into the effect of nanoparticles on protein fibrillation, the process needs to be studied as a function of both protein and particle concentration.

Further mechanistic studies in solution are in progress, using advanced approaches such as Fluorescence Correlation Spectroscopy to track the space and time resolved interaction between labeled particles and proteins. This approach is producing some very promising data, which will be reported in future publications.

To connect the effects observed in solution with effects in vitro and in vivo, cellular fibrillation studies are also underway, as a bridge towards the in vivo results on plaque formation in animals in the presence of nanoparticles. This work is exciting, yielding promising early results, but is at too early a stage to report in detail here.

Thus, significant progress is being made in the FIB part of the FIBROS story.

Reactive oxygen species generation by the test nanoparticles in solution and in cells



The ROS aspects of the FIBROS story are also progressing well, but as less has been published, we are only providing a snapshot summary of the efforts here.

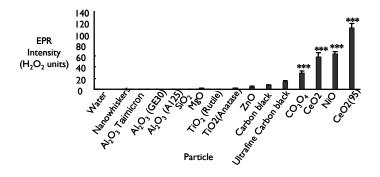


Figure 6 : Free radical generating potential (FRGP) of nanoparticles at equal surface dose.

Much of the work on this aspect has been focused on the copper oxide particles, as these have been shown to have a high oxidative stress capacity (Free radical generating potential, FRGP) and as such, they offer the certainty of observation of cellular and organism level reactive oxygen species effects. Redox proteomics is showing some very promising outputs in the model organism, the mollusc, and the data are being correlated with cellular impacts and protein coronas before and after the onset of the oxidative stress repsonse.

Nanoparticle passage to the brain – model Blood-brain barrier studies and *in vivo* studies in rats

Nanoparticles have been shown both *in vivo* and *in vitro* to cross barriers such as the blood brain barrier, although the amounts are extremely small compared to the amounts (dose) of nanoparticles presented. Broadly speaking, a variety of unfunctionalized nanoparticles penetrate the protective barriers in the body (the blood-brain barrier-BBB, the gut barrier, the lung airways barrier and others) by biological processes. The mechanisms driving this are as yet not fully understood *in vivo*, but experimental work using simple models, such as the human Blood-brain Barrier model established within NeuroNano is being used for this purpose.

Data from our transwell filter model using primary or co-culture cells, is revealing, and suggests that transcytosis is an active, energy dependent, endogenous process, Transcytosis is switched on as a significant process in these barrier models when the cell polarization is restored. Our preliminary data already suggest that the characteristics of successful translocation are connected more to the nanoparticle surface, and any specific protein adhesions, and nanoparticles size, and can in no way be simply matched to the chemical identity of the substance under consideration. Size dependence of transcytosis has also been observed, in parallel with the uptake of the particles in the non-polarised cell culture system where the cells are grown on a standard tissue culture dish.

In vivo work using radiolabelled gold and titania particles confirms a size dependence of the uptake, and the critical role

played by the biomolecule corona, as depending on the route of exposure the fate and biodistribution are different.

Publications on all these findings are currently in preparation, and as such, only the summary outcomes are presented here. However, it is clear that the "access" part of the FIBROS map is being addressed well, and the activity of the second year will also involve the integration of the various datasets into the risk assessment model.

Nanoparticle corona – stability confirms likely role in nanoparticles trafficking and biodistribution

Perhaps one of the most striking (and unforeseen) aspects of the nanoparticle-cell interaction story, that clearly distinguishes nanomaterials from chemicals, is the issue of the 'protein corona'. This arena has been clarified by several authors from FP6 NanoInteract, (Cedervall 2007a, Cedervall 2007b, Lundqvist 2008) and lead to the award of the 2007 Cozzarelli prize of the US National Academy of Sciences to NanoInteract for applications in this arena, and is a fundamental underpinning concept other NeuroNano project. 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. Recent findings include the fact that uptake of nanoparticles by cells is very dramatically different in the absence of proteins, where non-specific interactions appear to dominate over the more specific proteinreceptor mediated processes. It is important to note also that nanoparticles 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.

Within the medical device community, it is now well accepted that 'material surfaces' are modified by the adsorption of biomolecules such as proteins in a biological environment, and there is some consensus that cellular responses to materials in a biological medium reflect greatly the adsorbed biomolecule layer, rather than the material itself.(Norde 1991; 2000) An early paper in the field of protein interactions with planar surfaces drew attention to the fact that distortion of the protein may occur upon adsorption.(Klein 1986) However, the importance of the adsorbed protein layer in mediating nanoparticles interactions with living systems has been slower to emerge.(Lynch 2006) We introduced the concept of the nanoparticle-protein corona as the evolving collection of proteins that associate with nanoparticles in biological fluids in the EU FP6 NanoInteract project, and suggested that it is in fact the "biologically relevant entity" that interacts with cells (Cedervall 2007a, 2007b). However, whilst we focus mainly on particle-plasma and serum interactions here, remarkable advances have recently taken place to allow the corona from inside the cell (in specific organelle locations) to be isolated and identified.



The practical implications are that it is now possible to recover and study these nanoparticle-protein complexes in isolation, as shown in Figure 7, and the corona is remarkably stable. We can thus study their layer composition, size and zeta potential, and studies of protein corona identity, already somewhat advanced, will now become of much higher quality as we have learned to work with them in isolation, as well as in situ. The fact that particle-hard corona complexes of several materials have low zeta potentials suggests a different dispersion stabilization mechanism from that (such as charge and steric) typical for bare nanoparticles. Likely their stability is conferred by the specific protein layer characteristics, reminiscent of the design feat by which thousands of different proteins in plasma are colloidally stable despite their crowded environment. All of these quantities can now be studied in such detail as to allow theory to be developed, which would be a clear next step for this research field.

There are implications of these studies for the deeper question of 'what living organisms see' in mixtures of nanoparticles and biological fluids. In some specific cases the cell receptor may have a higher preference for the bare particle surface, but the timescale for corona unbinding illustrated here would still typically be expected to exceed that over which other processes (such as non-specific uptake) have occurred. Thus, for most cases it is more likely that the biologically relevant unit is not the particle, but a nano-object of specified size, shape, and protein corona structure. Naked particle surfaces will have a much greater (non-specific) affinity for the cell surface than a particle hiding behind a corona of 'bystander' proteins - that is proteins for which no suitable cellular recognition machinery exists. The evidence suggests that, in comparison to typical cell-membranebiology event timescales (most occurring over timescales much less than 30 minutes, many only several minutes) the particle corona is likely to be a defining property of the particle in its interactions with the cell surface, whether it activates cellular machinery or not. Similar observations and outcomes exist for particles inside the cell, in key locations.



Figure 7: (a) DCS results for particle-corona complexes free from excess plasma measured 1 hour and 6 hours after the redispersion in PBS. Bare 100 nm PSCOOH NPs in PBS (open circles) are reported for reference. The left most peak is for nanoparticles without serum, and the groups of peaks corresponds to the nanoparticle monomer and corona, as well as the dimmers, trimers etc (b) TEM picture of the protein-particle complex (free from excess plasma) for 100 nm PSCOOH NPs fixed in 2.5% glutaraldehyde solution. The white circle is a guide to the eye. Bar = 100nm-inside is the particle, outside is the corona. (c) DLS intensity-weighted size distribution obtained by CONTIN for 100 nm PSCOOH NPs (bare) and 100 nm PSCOOH protein-particle complexes free from excess plasma in PBS. Data from P1.(Walczyk 2009)

2.1.3 Next Steps

The promising results from the first year of effort will be built on during the second and third years of the project, and success in all arenas is expected. The design of the project is such that even in the absence of connections between nanoparticles and disease, we plan to emphasize the modules of research, the constituent endpoints of nanoparticle access to the brain, neurological oxidative stress, and neurologically based fibrillation processes. Each of these research modules has been laid out in some detail, and in the absence of any disease we will still seek to identify classes of materials, sizes and other factors leading to high scores on the FIBROS scheme, thereby highlighting those nano-particulates that have all, some, or none of the relevant risk factors. There is no technical risk in achieving these outcomes, and the information produced will be extremely informative to the relevant stakeholders.

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4 Directory

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PROGETTO

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Ttalian Ministry of Education, University and Research Programs of Relevant National Interest

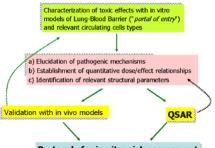
Interaction of newly synthesized nanomaterials with biological systems: experimental models for human health risk assessment

Years 2006/2007 - Grant PRIN 2006069554 Amount: 204.000,00 Euros

Main goals of the Research Project

- > To clarify the mechanisms underlying the toxicity of different NM, such as carbon nanotubes or metal oxide NP, so as to yield a solid toxicologic rationale based on the relationships between structural features and biological responses in relevant systems;
- > To develop experimentally validated, reliable in vitro methods to assess NM toxicity, so as to characterize a battery of tests suitable for the assessment of human health risks associated to newly synthesized NM.

Project flow chart



Protocols for in vitro risk assessment

Main characteristics of carbon naotubes used in the Project. All CNTs were sintesized by the CVD method. L and S refer to the nominal lenght of carbon nanotubes

Туре	Dimension (diam. x length)	Density (g/cm ⁱⁱ)	Surface area (m²/g)	Purity and metal contaminants
L-MWCNT	110-170 nm x 5-9 μm	1.35	2.2.10	C > 98% Fe < 0.1%; 1.5% Ni
S-MWCNT	40-70 nm x 0.5-2 μm	2.10	ND	C > 97% Fe < 0.1%
L-SWCNT	1.1 nm x 2-100 μm	1.70	1.7·10 ³	Co ~ 1.5% Mo ~ 1%
S-SWCNT	1.2-1.6 nm x 0.5-2.0 μm	1.90	4.8·10 ²	Co ~ 2.9 % Mo < 1%

a. L-MWCNT lower TEER of Calu-3 cell monolayers

Research Units and Institutions	People	Expertise
Dipartimento di Clinica Medica, Nefrologia e Scienze della Prevenzione e Dipartimento di Medicina Sperimentale Università degli Studi di Parma	Enrico Bergamaschi (<i>Project leader</i>) enrico bergamaschi@unipr.it O. Bussolati, B.M. Rotoli, M. Bianchi	Identification of the structural determinants affecting the trans-epithelial permeability of NM; characterization of dose-effects and -response relationships of distinct types of NP on biologically relevant cell types
Istituto di Medicina del Lavoro, Università Cattolica del "Sacro Cuore" e INFN Frascati, Roma	Antonio Bergamaschi antonio.bergamaschi@rm.unicatt.it 1. Iavicoli, S. Bellucci	Exposure assessment and characterization of micro- and nanoparticles; synthesis and functionalization of new NP; advanced microscopy techniques
Dipartimento di Bio-patologia e Medicina del Lavoro, Università di Tor Vergata, Roma e Burnham Institute for medical research, La Jolla, CA, USA	Andrea Magrini andrea.magrini@uniroma2.it A. Pietroiusti, M. Bottini, J.M. Legramante, L. Ghibelli, M. DeNicola	Cell internalization and intracelular distribution on NM; CNT with different chemical functional groups and of supramolecular nanoassemblies; animal models to assess cardiovascular effects
Dipartimento di Scienze dell'Uomo e dell'Ambiente, Università di Pisa e NMI Unit, European Commission, DG-Joint Research Centre, Institute of Health and Consumer Protection, Espra, (VA)	Lucia Migliore I.migliore@geog.unipi.it R. Colognato, A. Bonelli	Molecular and cellular effects on DNA: primary oxidative and chromosome damage by single cell assays (Comet and micronucleus); subcellular and molecular targets of NP and CNT genotoxicity
Dipartimento di Scienze Biomediche, Università degli Studi "G. d'Annunzio" Chieti	Paolo Boscolo boscolo@unich.it	Characterization of the effects of distinct types o NP on immune cell types

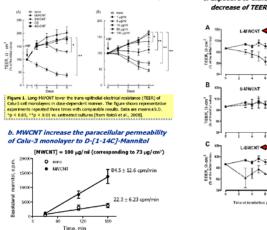
Understanding the biological basis of the interactions between NM and living cells at increasing level of complexity

- Bioavailability of NM and the interaction at the lung-blood barrier (LBB) by investigating the consequences of NM exposure on its barrier properties and on the main cell types that concur to its structure and function (airway epithelial cells, endothelial cells, inflammatory cells)
- ✓ Identification of the structural determinants that influence the trans-epithelial permeability of NP across the airway epithelium
- ✓ Characterization of the effects on biologically relevant cell types: airway epithelial cells, inflammatory cells of monocyte-macrophage lineage, stem cells, human endothelial cells and lymphocytes
- Mechanisms of cell survival/apoptosis, cell cycle/proliferation, expression of genes involved in the inflammatory response and oxidative stress
- \checkmark Molecular and cellular effects on DNA: primary oxidative and chromosome damage
- Identification of toxicological parameters, obtained from in vitro models, consistent with toxic effects observed with in vivo or ex vivo cell models
- ✓ In vivo studies to understand whether toxic effects or adaptive responses of cardiovascular and autonomic nervous systems may occur after exposure to NM

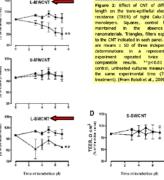
Main findings from the study of interactions of carbon nanotubes with biological systems:

- Bergamaschi et al., Int J Immunopathol Pharmacol. 19(4 SUPPL): 3-10, 2006.
- Rotoli et al., Toxicology Letters 178(2):95-102, 2008.
- Rotoli et al., Human & Experimental Toxicology, Vol 28: 361–368, 2009.
- Legramante et al., Human & Experimental Toxicology, Vol 28: 369–375, 2009.
- Migliore et al., Env Mol Mutagenesis, 2010 (in press)

1. Interactions of carbon nanotubes with airway epithelial cells: how physical features affect the biological behaviour. [Ref. Rotoli et al., 2009] Rotoli et al., 2009]

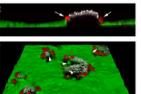


c. Exposure to either L-MWCNT or L-SWCNT causes a decrease of TEER



d. Airway epithelial cells adhere to MWCNT agglomerates and undergo adhesion-mediated cytotoxicity

Confocal microscop



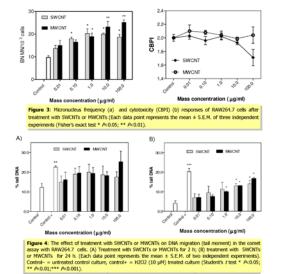
After 24h of exposure to 100 mg/ml MWCNT, the cell monolayer is mostly spared but some cells close to the aggiomerate are PT-positive (red, arrows). The dump top is free (reflected light, grey). Some viable (green) cells are climbing on the aggiomerates (arrowheads





Interaction of newly synthesized nanomaterials with biological systems: experimental models for human health risk assessment

2. Carbon nanotubes induce oxidative DNA damage in RAW 264.7 cells. [Ref. Migliore et al., 2010]



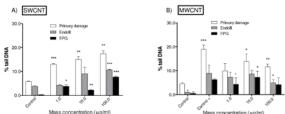


Figure 5: Oxidative DNA damage of SWCNTs (5 A) or MWCNTs (5 B) in the cell cultures (treatment for 24 h). DNA strand breaks, Endo-III and 'Fpg sensitive sites in treated, untreated and positive control (H202, 10 µ/h) releade cultures of RWX547 Cells. (Each data point represents the mean ± S.E.H. of two independent experiments). (Student's r test * A-0.05; * A-0.01;** A-0.01).

- Both the MN and comet assays can reliably detect small amount of damaged DNA at both chromosome and nuclear levels in RAW 264.7 cells.
- The modified version of the comet assay allows the specific detection of the induction of oxidative damage to DNA, which may be the underlying mechanism involved in the CNT-associated genotoxicity.

3. Carbon nanotubes affect the cardiac autonomic regulation [Ref. Legramante et al., 2009]

1. Cardiovascular Autonomic Control: models

- Repeated intra-tracheal instillations (3-month) of S-SWNT in healthy animals and in animals made susceptible to cardiac autonomic dysfunction
- Analysis of the baro-reflex system before and after each instillation
- Analysis of carotid baro-receptors and of cerebral integrating system for histologic damage and SWNT presence
- > Size of experimentally induced myocardial infarction in instilled and non-instilled rats

2. Cardiovascular Autonomic Control: methods

- Continuous (beat-by-beat) arterial pressure and heart rate monitoring
 Computerized analysis of the following parameters:
- a) number of baroreflex sequences,
- b) baroreflex sensitivity (which is given by the mean individual slope of the baroreflex sequences),
- c) heart rate standard deviation (which is a measure of the heart rate variability).

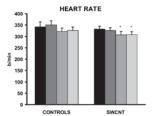
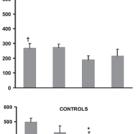
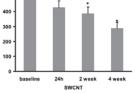


Figure 6: Heart rate values during the PBS (Controls) and single-walled carbon nanotubes (SWCNT) instillations at different experimental time. Data are shown as mean ± SEM. *P < 0.05 vs baseline.

Figure 7: Number of baroreflex sequences during the PBS (Controls, upper panel) and single-walled carbon nanotubes (SWCNT, bottom panel) instillations at different experimental time. Data are mean ± SEM. *P < 0.05 vs baseline. 1P < 0.05 control vs SWCNT-instilled group baseline values.



BAROREFLEX SEQUENCES



CONCLUSIONS AND FURTHER WORK HYPOTHESIS :

- a) Cells of the Lung-Blood Barrier exhibit differential acute sensitivity to CNT. (Epithelial Cells << Macrophagic cells <<< Endothelial Cells)</p>
- b) Exposure to MWCNT increases the permeability of the airway epithelium. (Synergy with inflammatory changes?)
- c) Epithelial cells adhere to MWCNT agglomerates, include them in the monolayer, exhibit contact-mediated toxicity. (A novel mechanism to penetrate airway wall and to explain access to subepithelial structures, e.g. inflammatory cells?)
- d) In macrophagic cells MWCNT and SWCNT cause genotoxicity at nominal doses which are not associated with significant decrease in cell viability. (An effect referable to oxidative damage?)
- e) SWCNT may affect the autonomic control of cardiac activity and in particular the arterial baroreflex control of sinus node. (Implications in terms of public health? Histological analysis of carotid baro-receptors and of cerebral integrating system and biomarkers of systemic inflammation?)

These effects are dose-dependent and may yield the basis for further research work



SIDANO

The parallelogram approach to assess the risk of **engineered** nanotechnology materials

Contract Agreement: -- Website: <u>http://www.inano.dk</u> Coordinator: Herman Autrup, Profesor, PhD

No.	Beneficiary name	Short name	Country
	School of Public Health and INANO center	AU	DK
	University of Aarhus		
	Chinese Academy of Sciences	NCNST	China
	Laboratory for Biomedical Effects of Nanomaterials and Nanosafety		
	Beijing		

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1 Abstract:

It is expected that engineered nanomaterials will increasingly be used as drug delivery systems, in consumer products as well as mechanical devices. Whereas there is a clear benefit using nano formulations as drug delivery systems, there is a health concern especially in relationship to exposure from nanoparticles present in consumer products, e.g. cosmetics, and when nanoparticles are released from the consumer products into the environment. As nano particles have different physio-chemical properties than the bulk chemical, it has been suggested that a new paradigm for risk assessment should be developed, e.g., different exposure metrics. Another concern if the current testing methods validated by OECD appropriate to test safety of nanoparticles released from engineered nanotechnology materials. The objective of the project is to use an in vivo/in vitro approach to identify the target organs of nanoparticle toxicity, and to investigate the molecular mechanism of the toxicity with focus on genotoxicity and inflammatory responses. The focus of these studies will be on routes relevant for human exposure, i.e., inhalation, oral intake. An in vitro/in vitro approach will be used to compare the effects in animal and human cells, to explore the similarities of the toxicological mechanism in human and animal cells as well as in different cell types. It is expected that these studies will provide some data on the replacement of animal studies with cell culture models, and in addition the molecular studies may result in identification of biomarkers that can be used to assess human exposure. An important aspect of nanoparticles is their ability to aggregate, agglomerate and to

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bind cellular macromolecules, thus the particles will be characterized both prior to investigations, in biological media and in target tissues.

2 Objectives:

- 1. to identify the potential target tissues of nanoparticle toxicity following inhalation and oral exposure
- 2. to identify the molecular pathways of toxicity and identify potential biomarkers of exposure and effects using different exposure metrics
- to compare the toxic response in *in vivo* and *in vitro* systems in order to validate *in vitro* systems for toxicity screening
- 4. to explore the relationship between oxidative stress using molecular markers for oxidative damage i.e., lipids, protein and DNA, induction of antioxidant response genes.

3 Work description:

In hazard assessment, data is normally obtained from epidemiological or animal studies. In case of a new technology, like NP, no human data is available, and thus data from experimental animals and in vitro systems will have to be used. Thus, the major part of the studies will be conducted in cell culture models, but a link between the two levels of biological organisation will be made. Linking in vivo and in vitro data will provide information on the potential application of cell culture models to assess the risk of NP. Furthermore, a comparison of the effects in tissues of the same origin from mouse and human, could indicate whether the sensitivity and mechanism of toxicity is similar In the in vitro models the questions on the appropriate dose metrics will be addressed, information that is important for the risk characterisation of NP. In order to validate the parallelogram testing approach, 4 different NP have been selected – CeO2, TiO2, Ag and HAR nanotubes.

Nanoparticles in biological fluids: One of the major issues in the area of nanotoxicology is the characterization of the NP in biological fluids, as the intrinsic physicochemical properties can change by the incorporation of biological materials (e.g. macromolecules), a process that is likely to modify the uptake and the toxicity of the NPs. It is anticipated that this modification may influence bioavailability and potentially toxicity. The interaction of biological molecules with the NPs will be studied in model experiments and by identification of macromolecules (through proteomic approaches) recovered from NPs exposed to biological fluids. The influence of modification on uptake and toxicity will be addressed by the use of model NPs with well defined and systemically modified properties. An example study will involve the use of NPs premodified with lung surfactant components studied in comparison to control NPs. NP modified with lipids and The detailed characterisation and surfactant proteins evaluation of the effects of modifications occurring to the NPs en route to cells and during entry into tissue will provide new evaluation criteria for specific NPs hazard and exposure in terms of risk assessment, for example the relative potential for a specific NP to be modified in particular ways .

Characterization of NP: The particles will be characterized by a range of analytical techniques to investigate their physicochemical properties (size, surface area, chemical and molecular composition and surface interactions) before and after exposure to biological fluids. The analysis will make use of information rich spectroscopic tools such as Time of Flight Secondary Ion Mass Spectroscopy (TOF-SIMS), X-ray Photoelectron Spectroscopy (XPS), X-ray Diffraction (XRD), Small Area Electron Diffraction (SAED). A combination of in situ (in fluids) and ex situ nanoimaging and spectroscopic tools will be used to evaluate the size and surface chemistry of the NPs e.g. Dynamic Light Scattering (DLS), Small Angle X-ray Scattering (SAXS), Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM) and optical spectroscopies (for metal NPs such as Ag). The ICP-MS will provide useful information for chemical and molecular composition.

Toxicological investigations in vivo: The relevant routes of exposure for NP are the lung by inhalation, the GI-tract or skin,



thus the focus will be on inhalation and oral uptake. The distribution of the NP following inhalation and oral uptake will be assessed in mouse as previously described (using both physical/chemical methods and biomarkers). The major focus will be on lung, kidney, spleen and liver that have previously been identified as target organs, and colonic tissues. In order to verify the systemic distribution, tissues will be analysed for the presence of NP as described above. For the metal or metal oxide NPs, ICP-MS technique is used to quantify their distribution and accumulation in vivo. Tissues from similar/same animals will be removed and analysed by different assays. The primary cells like macrophage and T cells will be isolated and collected for the inflammatory response exposed to NPs. Gene expression assays for toxicity pathways and DNA damage (SABiosciences) and induction of DNA damage will be analysed in the animal tissues following exposure, and relevant genes will be identified. See toxicological investigations in vitro.

Toxicological investigations in vitro:

Cells: Most of the studies on the biological effects of NPs have been conducted in cultured human cells of different origin, mostly carcinoma cells. In this project immortalised human epithelial cells from tissues initially in contact with the epithelial cells, e.g. bronchus, colon and target cells e.g. endothelium will be used, and the effects will be compared to similar cells from the mouse species used in the in vivo study. The in vitro toxicological studies will be investigated in pairs of human and murine cells. Lung cells A549 (human lung carcinoma), BEAS-2B (SV40-human bronchial epithelial cells), mouse LA-4 (epithelial; lung adenoma) Human colon Caco-2 (adenocarcinoma), mouse monocyte/macrophage (RAW 264.7) and human monocytes THP-1, and human brain HCN-1(neuronal) and mouse SW40 (neuronal) cells. The cells will be cultured according to the published procedure. All toxicological studies will be conducted in 1% serum.

Cytotoxicity and viability assays: Standard assays for LDH release and CCK-8 kits will be applied as previously described by us. The dose response relationship of biomarkers will be investigated as a function of different exposure metrics, surface area, number and weight. The IC10 and IC50 will be determined. These concentrations will be used in all other toxicological studies in order to cover response at low and intermediate/high exposure.

Induction of ROS: Oxidative stress is considered central to the toxicological mechanism of action for nanoparticles. Damage to DNA is primarily induced by reactive oxygen and nitrogen species. Thus, the intracellular production of reactive oxygen species will be assessed by the fluorogenic dye H₂DCF-DA, oxidative stress will be estimated by the GSH/GSSG ratio and malondialdehyde (MDA) levels and damage to the cells and apoptotic cells will be measured using the annexin V/propidium iodide flow cytometric assay.

Gene expression assay: Key genes in the toxicological pathways will be identified and PCR-based assays will be developed for quantification. One of the proposed toxicological mechanisms by which NPs exert their proinflammatory effects are through



the generation of oxidative stress. Cellular responses to oxidative stress include activation of antioxidant defence.

Inflammation is a complex biological process involving different cell types, and thus cannot be measured in simple *in vitro* assays. In this project we will focus on markers of proinflammatory signalling, e.g. cytokines and chemokines that play specific roles in promoting or controlling inflammation. A PCR based assay will be used to quantify TNF α , IL1 α , IL1 β , IL6, IL8. These markers have been selected as sporadic studies indicate that the levels are altered by UFP, including diesel exhaust particles (DEP) and some NPs.

Carcinogenicity: The concern for potential carcinogenicity of NPs is based upon the increased cancer risk following exposure for asbestos and DEP and recent studies showing the induction of mesothelium by carbon nanotubes in p53 deficient mice at high doses. In addition, some NP have demonstrated genotoxic activity in different in vivo models using transgenic animals and in cell cultures Development of cancer can operationally be divided into genotoxic and non-genotoxic events. Genotoxicity of NP will be investigated in the potential target tissues using three different assays, induction of chromosomal mutations, induction of aneuploidy, formation of DNA strand breaks as measured by the comet assay, and unspecific bulky DNA adducts. International standard protocols for chromosomal mutations (chromosomal aberrations), the comet assay and aneuploidy have been established (OECD protocols). The former two effects are assumed to be due to direct DNA damage, whereas aneuploidy could be induced by interference of the NP with the spindle-apparatus. Bulky DNA adducts will be analysed by the P32 postlabelling technique as previously described. Preliminary studies have shown that NPs induce bulky DNA adducts in cultured human cells in a dose-dependent manner and the level depends on the type and size of the NP. These adducts may be formed as a secondary effect of oxidative stress induced by nano-particles, e.g. lipidperoxidation products .The relevance of this marker to assess risk has been demonstrated in epidemiological studies where bulky DNA adduct level is a risk indicator for lung cancer . Different mechanisms for nongenotoxic carcinogens have been proposed, one of these is change in methylation pattern that will result in altered gene expression. Hypermethylation of the CpG island in region of certain tumor repressor genes have been shown to be associated with many human tumors. Both global methylation level and gene specific methylation will be investigated. We have developed several methods to study epigenetic mechanisms on regulation specific gene expression. E.g., transferrin receptor methylation specific PCR (MSP) technique, which is a bisulfite conversion based PCR technique . In addition bisulfite sequencing which can quantify the methylation status of cytosines located in specific DNA regions has also been established. A chemiluminescent electrophoretic mobility shift assays (EMSA) to study protein-DNA interactions involved in DNA epigenetic regulation is established.

Inhibition of intracellular communication has been proposed as an important step in carcinogenesis by an epigenetic mechanism that can be modulated by specific chemicals most likely through the MAP kinase pathways. In a preliminary study we have shown that Ag NPs can induce intracellular communication by increasing the expression of the connexin 43 gene. A quantitative assay will be developed based upon these preliminary findings.

4 Exploitation:

It is anticipated that the information obtained from this project mostly will be of interest to the regulatory and academic communities as a contribution to the ongoing debate on the evaluation of the safety of materials/products that contain nanoparticles. The focus of this approach is on the 3R-principles

5 List of publications:

Foldberg R, Olesen P, Hougaard M, Dang DA, Hoffman HJ, Autrup H. PVP coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. Toxicol Letters , Tox Letters

Deng F, , Olesen P, Foldberg R Dang DA, Guo X, Autrup H. Silver nanoparticles up-regulate Connexin 43 expression and increase gap junctional intracellular communication in A549 cells J.Nanotox In press

Foldberg R, Dang DA, Autrup H Uptake and cytotoxic effects of silver nanoparticles in the human epithelial cancer cell line, A549. Arch Toxicol In press



6 Directory

Table 1 Directory of people involved in this project.

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