

# Nanotoxicology: Health & Environmental Impacts

The BioPark Hertfordshire, Welwyn Garden City, AL7 3AX: 27<sup>th</sup> Feb 2009

*"The nanotechnology industry is rapidly growing with promises of substantial benefits that will have significant global, economic and scientific impacts, applicable to a whole host of areas from engineering and electronics to environmental remediation and medical healthcare. However, at present there is growing concern over the safety of nanomaterials with respect to occupational, consumer and environmental exposures. This timely symposium is aimed at bringing together eminent scientists at the forefront of the nanotoxicology field to present their current research findings and discuss the potential impact of nanomaterials on human health and the environment.*

*This event will therefore present an ideal opportunity for toxicologists, nanotechnologists, industrial members and governmental regulatory agencies to interact and discuss the latest developments in this controversial field"- Meeting Chair - Dr. Shareen H. Doak, University of Wales Swansea, UK*

***This meeting has CPD accreditation***

9:00 – 9:45      **Registration**

9:45 – 9:55      **Introduction by the Chair:** *Dr. Shareen H. Doak, University of Wales Swansea, UK*

9:55 – 10:30    **Health implications of Nanoparticles: Toxicokinetic Aspects**

*Professor Wolfgang G. Kreyling, Helmholtz Zentrum München Research Center for Health & Environment; Institute of Inhalation Biology, Focus Network Nanoparticles and Health, Germany*

Quantitative dosimetry (QD) of nanoparticles (NP) allows precise estimates of NP accumulation not only in the primary organ of intake but also in secondary target organs. Hence, QD is prerequisite for subsequent dose dependent toxicological investigations on responses of target cells in exposed organs and underlying mechanisms. Accumulation in target cells is likely to depend on complex formation of NP with proteins and biomolecules of body fluids in which they happen to be suspended.

10:30 – 11:05    **The carbon nanotube hazard**

*Professor Ken Donaldson, Edinburgh University, Edinburgh.*

The similarity of multiwalled carbon nanotubes (CNT) to asbestos in terms of their fibre shape, has been noted and failure to detect potential of CNT to cause mesothelioma, the hallmark tumour of asbestos exposure, early-on would be devastating for the nanotechnologies. The structure/activity paradigm that explains the pathogenicity of asbestos and other fibres is established, defining the long rigid needle-like shape of the fibres and their ability to persist in the lungs, as requirements for pathogenicity. The fibre pathogenicity paradigm has been shown to be applicable to asbestos, synthetic vitreous fibres and one organic fibre. The essential feature of the paradigm are that to be pathogenic:- 1) an appreciable number of thin fibres longer than about 20µm must enter the lungs; 2) the long fibres must be biopersistent in the lungs and not dissolve/break into shorter fibres. Such fibres over an extended time in the lungs cause inefficient or frustrated macrophage phagocytosis leading to chronic inflammation and diminished clearance of the long fibres leading to an accumulation of dose with ongoing exposure in an inhalation situation; epithelial and mesothelial injury and genotoxicity; secondary genotoxicity; fibrosis and cancer in the lungs, pleural and peritoneal cavity. We hypothesised here that the most important and unique response to asbestos is the mesothelial one and so we used a mouse model of mesothelial exposure. We exposed the mouse peritoneal mesothelium to two long rigid samples of CNT that looked like asbestos fibres and two CNT samples that were tangled and therefore essentially 'particles', rather than fibres. We also used a long fibre asbestos preparation (LFA) and a short fibre preparation made from it by milling (SFA) and a nanoparticle carbon black (NPCB) sample as controls. The only samples which caused any evidence of pathogenic effects, by causing inflammation and granuloma formation in the diaphragm and multinucleate foreign body giant cell reaction were those 3 samples that had long fibres – LFA, and the two long CNT samples. The study demonstrates that not all MW-CNT are created equal in terms of potential asbestos-like hazard to the mesothelium. Tangled and short forms of CNT presented very low or no fibre hazard whereas long rigid forms of CNT did. This study is hazard-based and there is a pressing need for exposure data for those who work with MW-CNT in order to assess and manage their risk and for further toxicology work using pulmonary exposure to determine whether CNT reach the mesothelium and cause mesothelioma there. Acknowledgement. This research was funded by the Colt Foundation

11:05- 11:10    **Speakers photo**

11:10 – 11:30    **Mid-morning break**

- 11:30 – 12:05 **Consumer safety implications of the use of nanotechnologies in food**  
*Dr. Qasim Chaudhry*, Central Science Laboratory, United Kingdom  
 Like other sectors, advances in the field of nanotechnology are promising to revolutionise the food and related sectors. An increasing number of (health)food products is already available worldwide. Dr. Chaudhry will present an overview of the recent developments in this area, and will discuss whether the use of nanotechnology-derived materials in (health)food products will have any consumer safety and regulatory implications.
- 12:05 – 12:20 **Nanotest: Alternative testing strategies for the assessment of the toxicological profile of nanoparticles used in medical diagnostics**  
*Margaret Saunders*, Biophysics Research Unit, Bristol Haematology & Oncology Centre, University Hospitals, United Kingdom  
 The assessment of nanoparticle cellular interactions is fundamental for adequate risk assessment of engineered nanoparticles. The FP7 project NanoTEST addresses this in relation to the toxicological profile of nanoparticles used in medical diagnostics. NanoTEST will develop alternative testing strategies and high-throughput toxicity-testing protocols using *in vitro* and *in silico* methods to inform detailed risk assessment. NanoTEST will use carefully characterized nanoparticles in order to define their major physico-chemical properties, study interactions of nanoparticles with molecules, cells and organs, validate *in vitro* findings in short-term *in vivo* models, determine structure-activity relationships and adapt suitable assays for high-throughput automated systems and validation
- 12:20 -12:35 **Effects of single walled carbon nanotubes on mixed neuro-glial cultures from chicken spinal cord and dorsal root ganglia**  
*Dr Cordula Hirsch*, Materials Science & Technology, Laboratory for Materials-Biology Interactions, Switzerland  
 The increasing use of carbon nanotubes (CNTs) in consumer products and medical applications lays emphasis on the importance of understanding their potential toxic effects on human health and the environment. As there is only little knowledge about possible neurotoxic effects of CNTs, we studied the influence of dispersed single-walled CNTs (SWCNTs) with different degrees of agglomeration on primary mixed neuro-glial cultures from central and peripheral nervous system tissues of chicken embryos. We found that SWCNTs can have an acute adverse effect on glial cells as well as on certain neuronal subtypes in primary mixed neuro-glial cultures.
- 12:35– 12:40 **Introduction to the BioPark**  
 12:40 – 13:45 **Lunch and Poster Viewing**
- 13:45 – 14:00 **Capturing Nanotoxicity data – what is the optimum solution?**  
*DS Wilkinson*, Lhasa Limited, Leeds  
 With increasing use of nanotechnology researchers would benefit from being able to capture nanotoxicity data and compare this with the known toxicity effects of the corresponding bulk materials. We are seeking feedback from the wider scientific community to determine the optimum solution for incorporating nanotoxicity data into an existing structure-searchable toxicity database.
- 14:00 – 14:15 **Understanding the Genotoxic Potential of Ultrafine Superparamagnetic Iron oxide Nanoparticles (USPION)**  
*Neenu Singh*, University of Wales Swansea, UK
- 14:15 – 14:50 **Linking the Toxicology and Ecotoxicology of Nanoparticles**  
*Professor Vicki Stone*, Napier University, Scotland  
 Investigations of the effects of a range of nanoparticles on mammalian cells and rodent models clearly indicated that smaller particles have the potential to induce toxicity via reactive oxygen species, oxidative stress and activation of the immune system leading to inflammation. The potency of particle induced effects is related to size, surface area and a wide range of other physicochemical characteristics. How can this information be used to investigate the ecotoxicology of nanoparticles, and what evidence is there that nanoparticles behave similarly in different species?
- 14:50 – 15:25 **Possible Fate, Behaviour and Ecotoxicology of Nanoparticles in Natural Waters**  
*Professor Jamie Lead*, The University of Birmingham, UK  
 Manufactured nanoparticles (NPs) will interact with natural components in aquatic systems and this interaction will strongly influence the behaviour and impacts of these NPs. The interaction of primary importance and where there is fundamental uncertainty, is with natural colloidal and nanoparticulate material. These natural nanoparticles which are produced by weathering, microbial processes and other processes and are ubiquitous in aquatic and terrestrial systems. This talk will discuss these likely interactions and their effect on transport and on biological systems

- 15:25 – 15:50 **Afternoon Tea/Coffee and Last Poster Viewing**
- 15:50 - 16:25 **Toxic Effects of Nanomaterials to Trout: Implications for Fish Toxicity Testing, Characterisation of Test Media, & Regulation.**  
*Dr Richard Handy, Plymouth University, UK*
- 16:25 – 17:00 **Biological response of fish cell cultures and sticklebacks to cadmium and silver nanoparticles.**  
*Dr Matthew Sanders, CEFAS (Centre for Environment, Fisheries and Aquaculture Science), Weymouth Laboratory.*  
 Nanotechnology is a rapidly developing field, attracting investment from industry and governments. The range and variety of nanomaterials available is likely to require a specific risk assessment for each new compound with associated fit for purpose testing procedures. We are developing a tiered testing approach using cadmium (4nm ± 1nm) and silver (13nm ± 7nm) nanoparticles, which comprises an initial screen for cellular toxicity using cultured cells, followed by standardised (OECD) whole organism ecotoxicology tests using the freshwater Crustacean *Daphnia magna*. Finally the estuarine fish stickleback (*Gasterosteus aculeatus*) will be used to assess a range of toxicological, behavioural and reproductive endpoints.
- 17:00 **Chairman's summing up.**
- 18:00 **Soiree at \*The Best Western Homestead Court Hotel for all the participants**

### About the Chair

Dr Shareen Doak obtained her PhD in 2003 investigating the molecular mechanisms that drive neoplastic progression of oesophageal adenocarcinoma, and since has been researching the mechanistic basis of DNA damage induction and persistence, as well as their consequences upon human health. Shareen is currently appointed as a Research Councils UK (RCUK) Academic Fellow in Nanomedicine at Swansea University, where her specific research interests are focused upon the genotoxic responses of nanomaterials and developing the use of high resolution imaging and force measurement tools to study the biophysical properties of diseased cells at the nanoscale.

### About the Speakers

*Professor Vicki Stone*, is a Professor of Toxicology at Napier University, Edinburgh. She leads research investigating the effects of nanoparticles on a wide range of cell types from the lung, liver, immune system and gastrointestinal tract. Collaborations also investigate effects of reproductive toxicity and ecotoxicology of nanoparticles. Projects are funded by EC FP6 and FP7, NERC, EPSRC, Defra, The Colt Foundation and Unilever.

*Dr. Matthew B. Sanders* is engaged in full time research and development projects in the fields of ecotoxicology, fish physiology, endocrinology and nanotoxicity at the Centre for Environmental, Fisheries and Aquaculture Science (Cefas) in the United Kingdom. The majority of his research involves the development of biomarkers for environmental pollutants. For the past 2 years he has been working with material scientists to assess the risks presented by nanoparticles to the aquatic environment. Working primarily with quantum dots, fullerenes and fullerols, Dr Sanders and colleagues have recently been using microarray technologies in an attempt to correlate behavioural and molecular responses in fish following nanoparticle exposure.

*Professor Jamie Lead*, The University of Birmingham, UK

Jamie Lead is Professor in Environmental Nanoscience at the University of Birmingham, UK and is interested in the behaviour and impact of both natural and manufactured nanoparticles (NPs) in aquatic and terrestrial systems. His research group is performing fundamental research into the chemistry and transport of a range of NP types. He is also investigating methods to accurately quantify concentration and physico-chemical form in the environment and to quantify dose appropriately. He is also investigating the relationship between these physico-chemical aspects and biological behaviour (uptake, accumulation and effects).

*Dr Richard Handy*, Plymouth University, UK

Dr Handy is an expert in environmental toxicology (20 years experience), with special expertise on the effects of nanoparticles on fish, and also has interests in wildlife pathology and organ-level effects of pollutants. He is also an expert on organ perfusion methods for fish and other invitro methods. Dr Handy has been working with nanoparticles since 2006, and has published a number of primary works on fish, as well as dietary/food chain issues for nanoparticle exposure. The latter includes environment and human health issues.

*Dr Margaret Saunders* is a Clinical Scientist at University Hospitals Bristol NHS Trust heading up the Biophysics Research Unit. The group's research interests largely address environmental effects upon fetal development as part of an NHS research programme led by Dr Saunders. Studies funded mainly through the EU Framework programme are currently under way to investigate transport of chemicals and nanoparticles across the placental barrier, potential cellular toxicity and effects on cellular immune function. Dr Saunders is a workpackage leader within the current FP7 funded NanoTest project to investigate nanoparticle toxicology in a broad range of in vitro systems

*Dr Cordula Hirsch* studied Biology at the University of Konstanz (Germany) receiving her diploma in 2002 working on a myelin-associated protein (Nogo-A) in the central nervous system of the frog. In 2007 she finished her PhD thesis analysing influences of the canonical Wnt-signalling pathway on murine neural progenitor cells at the Institute of Molecular Medicine and Cell Research at the University of Freiburg (Germany). Since 2008 she's working as a postdoc for the Institute of Materials-Biology Interactions at the Empa in St. Gallen (Switzerland) investigating effects of single-walled carbon nanotubes on neuro-glial cultures from the embryonic chicken nervous system

*David Wilkinson* studied Chemistry with Pharmaceutical and Forensic Science at University of Bradford and then went on to do an MSc in Toxicology at University of Birmingham. He has worked at Lhasa Limited as a database scientist for 2 years.

*Professor Wolfgang G. Kreyling*, is a biophysicist coordinating all aerosol-related research within the GSF Focus Network Aerosols and Health spanning R&D work over five GSF-institutes ranging from material sciences to toxicology and epidemiology. He also is deputy director of the GSF-Institute for Inhalation Biology. He chairs the R&D program on dosimetry of ultrafine aerosol particles and engineered nanoparticles in the respiratory tract and secondary target organs like the cardiovascular and the central nervous system. His research interests range from aerosol sciences and nanoparticle technology to biophysics of the lungs reaching from the characterization of ambient aerosols to particle dosimetry and nanoparticle lung interactions on the level of the entire organism, cells like alveolar macrophages, and molecular compounds.

*Dr. Qasim Chaudhry* is a Principal Research Scientist at the Central Science Laboratory of Defra in York. Dr. Chaudhry, a Chemist and Biochemical Toxicologist by training, is leading a team of scientists undertaking research into the safety of nanotechnology applications to human health and the environment in a variety of products and applications, including food and food packaging

*Professor Ken Donaldson* BSc, PhD, DSc, CBIol, FIBiol, FRCPath, FFOM is the Scientific Director of the ELEGI Colt Laboratory in the Medical School of the University of Edinburgh, where he is Professor of Respiratory Toxicology. Prior to this he was Professor of Pathobiology, Napier University and before that Head of the Toxicology Unit, Institute of Occupational Medicine, Edinburgh. KD is recognized as an expert in the mechanisms of lung disease caused by inhaled agents especially particles and fibres and in this capacity has provided expert opinion and consultancy to the US Environmental Protection Agency (North Carolina), US Health Effects Institute (Massachusetts), World Health Organisation, International Agency for Research on Cancer (Lyon France), WHO Air Quality and Health (Bonn, Germany), UK Medical Research Council, UK Health and Safety Executive, etc. KD sits on three government committees pertaining to toxicology of air pollutants – Committee on the Medical Effects of Air Pollution (COMEAP) and Expert Panel on Air Quality Standards (EPAQS) and Advisory committee on Hazardous Substances. KD has given advice on the toxicology of fibres to the US EPA and UK HSE. In relation to inhaled nanoparticles (NP) and nanotubes, KD was one of the initial proponents of the NP theory of the toxicity of particulate air pollution and has acted as a consultant to various bodies on the risk from NPs such as EU (SCEHNIR, COST), European Science Foundation, Health and Safety Executive, ECETOC and the WHO. He has published over 280 scientific papers, a large number on mechanism of lung injury caused by inhaled agents and currently has a research programme into the adverse effects of nanoparticles on the lungs and cardiovascular system. He is Founding Editor of the journal 'Particle and Fibre Toxicology' and Co Editor of 'Particle Toxicology', 2007, CRC Press.

*This meeting was **organised by Euroscicon** ([www.euroscicon.com](http://www.euroscicon.com)), a team of dedicated professionals working for the continuous improvement of technical knowledge transfer to all scientists. Euroscicon believe that they can make a positive difference to the quality of science by providing cutting edge information on new technological advancements to the scientific community. This is provided via our exceptional services to individual scientists, research institutions and industry. The event was hosted by **'BioPark** ([www.biopark.co.uk](http://www.biopark.co.uk)), a research and development centre in Welwyn Garden City providing specialist facilities and support for bioscience and health technology businesses to grow, and to develop new products and technologies*

## POSTER PRESENTATIONS AND EXTENDED ABSTRACTS

### **Effects of single walled carbon nanotubes on mixed neuro-glial cultures from chicken spinal cord and dorsal root ganglia**

C. Hirsch, L. Belyanskaya, S. Weigel, U. Tobler, H. Krug, P. Wick

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The unique chemical and physical, as for example optical and magnetic, properties of carbon nanotubes (CNTs) suggest an enormous potential in many areas of research and applications, including biosensors, field emission, energy storage, molecular transporters for drug delivery and neural prosthetics. The increasing use of CNTs in consumer products and medical applications lays emphasis on the importance of understanding their potential toxic effects on human health and the environment. Most of the recent research studies addressing the potential health impairment due to CNTs and other nanoparticles focused on cells and tissues that are likely to get immediately into contact with airborne particles. As there is only little knowledge about possible neurotoxic effects of CNTs, we studied the influence of dispersed single-walled CNTs (SWCNTs) with different degrees of agglomeration on primary mixed neuro-glial cultures. These cells were isolated from the spinal cord (SPC) or the dorsal root ganglia (DRG) of chicken embryos thus allowing us to differentiate between effects on cells derived from a central nervous system (CNS), or a peripheral nervous system (PNS) tissue, respectively. We found that SWCNTs significantly reduced the total DNA content of mixed cultures from both nervous system tissue types. However, agglomerated SWCNTs (SWCNT-a) were more toxic in this context than the better dispersed SWCNT bundles (SWCNT-b) which indicates that the aggregation state of SWCNTs was an essential factor in determining their toxic potential. To assess the contribution of different cell types to the observed DNA reduction we used a neuron- and glia-specific ELISA, as well as purified glial cultures and found that glial cells were generally affected in cultures of both CNS and PNS tissue. While we could not detect any effects of SWCNTs on spinal cord derived neurons in terms of cell number, neurite outgrowth velocity and electrophysiological properties, we found that the number of dorsal root ganglia derived sensory neurons was reduced and their inward conductivity was diminished. These data suggest that SWCNTs can have an acute adverse effect on glial cells as well as on certain neuronal subtypes in primary mixed neuro-glial cultures.

### **Capturing Nanotoxicity data – what is the optimum solution?**

DS Wilkinson & KA Briggs

*Lhasa Limited, 22 - 23 Blenheim Terrace, Woodhouse Lane, Leeds, LS2 9HD*

Nanotoxicology has the potential to become a very large and important area of toxicity. With rapid advances in nanotechnology increasing numbers of nanoparticles are being used in both research and industry. Indeed there are currently over 150 different “nano” products available from Sigma Aldrich. Clearly researchers would benefit from being able to capture nanotoxicity data and compare this with the known toxicity effects of the corresponding bulk materials. Therefore we have investigated the schema modifications necessary to incorporate nanotoxicity data into an existing structure-searchable toxicity database.

As a first step we reviewed the types of data generated as part of a nanotoxicity study. Consideration was then given to the ways in which researchers might want to query the data in order to determine how it could best be presented. We then identified an example paper from the published literature containing in-vivo carcinogenicity data for a group of nanoparticles and their bulk equivalents [Reference 1] and did a trial extraction of this data into an existing structure-searchable toxicity database to illustrate how it could be adapted. The existing carcinogenicity schema contains fields for key experimental conditions as well as more detailed tissue/tumour incidence data. Any changes needed to the database schema to capture nanotoxicity data were noted.

#### Four possible solutions for capturing nanotoxicity data were identified:

- 1) Use the existing database schema. It is possible to extract nanotoxicity data into the existing database schema but this solution is not ideal. Data on nanoparticles is grouped with the data on the equivalent bulk materials and although information on particle size can be added to the free text this is not searchable.
- 2) Enter each nanoparticle as a separate substance. For example the database would have two or more entries for titanium dioxide. One for the bulk material and then one or more entries for titanium dioxide nanoparticles depending on whether it was considered acceptable to combine toxicity data for nanoparticles of different particle size.
- 3) Enter information on particle size in each study record. Although data on nanoparticles is grouped with the data on the equivalent bulk materials the user would then have the option of searching for data on a particular particle size or range of particle sizes.
- 4) Capture the data in a separate folder or table e.g. nanotoxicity or nanocarcinogenicity. Using separate tables means the schema could be customised for nanotoxicity studies but would make comparisons with data on bulk particles more difficult.

In conclusion, to meet the needs of researchers investigating nanotoxicity we would need to implement one of the suggested schema changes. The impact of these schema changes would need to be discussed with the wider scientific community to determine the optimum solution for incorporating nanotoxicity data.

#### Reference

1. Hansen T et al. Journal of the Royal Society Interface (2006), 3(11), 767-775 [doi:10.1098/rsif.2006.0145]

### **Nanotest: development of *in vitro* tests to measure potential toxicity of nanoparticles to placental function**

L. Cartwright, S. Correia Carreira, S. Thawley & M. Saunders

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The objective of this ongoing investigation is to examine the transport mechanisms of a range of well-defined and representative medically relevant nanoparticles in models of the human placental barrier, in order to estimate the extent of potential nanoparticle transfer to the fetus following human exposure. We aim to identify the potential impact of nanoparticles on this barrier by measuring a range of toxicity parameters and to determine whether exposure to nanoparticles affects the existing transport mechanisms functioning at this barrier.

The human *in vitro* placental choriocarcinoma BeWo cell line is maintained in cell culture and seeded on permeable membranes in Transwells® to generate monolayers. Model development and optimisation have been completed to produce a robust monolayer model as determined by confocal and electron microscopy, visualisation of tight junctions by antibody staining, and transport marker permeability. Confluence and barrier integrity are determined by trans-epithelial electrical resistance (TEER) measurements which must fall within a defined range. Preliminary experiments have demonstrated that fluorescently-labelled latex Fluoresbrite® nanoparticles (40-60 nm diameter) can cross the placental barrier in the Transwell model and the other nanoparticles (which include titanium dioxide, paramagnetic metal oxides, metal fullerenes, polymeric materials such as polylactic glycolic acid, chitosan, and hyaluronic acid, and quantum dots) will be evaluated in this model with permeability measurements enabling direct comparison of nanoparticles. Appropriate inhibitors and controls will be used to determine the relevant mechanisms involved in the transport of nanoparticles across the placental barrier.

A range of assays will be utilized to evaluate the cytotoxic and genotoxic effects of NP upon placental function in this model. These include DNA damage as determined by apoptosis and necrosis, Comet and micronucleus assays; viability assays including Alamar blue, WST-1 and MTT/MTS or LDH assays; differentiation/proliferation assays such as BrdU and hCG production; oxidative stress and ROS production and pro-inflammatory assays including cytokine mRNA production and cytokine secretion. Assay optimisation for the evaluation of the effects of nanoparticle exposure upon the placental cell line has been completed for LDH and WST-1, and validated using cobalt-chrome nanoparticles (29 nm diameter). The WST-1 assay has shown a BeWo dose response when exposed to CoCr nanoparticles (0.005-0.04 mg/mL) for 24hr

Developmental work is underway with the BeWo model to establish a more *in vivo*-like cell culture structure in order to obtain a model of improved relevance. This involves addition of co-cultured cell populations such as endothelial cells (HUVEC) and an assessment of the effects of co-culture on specific parameters evaluated in the monolayer model.

*Outcome:* A placental cell barrier model has been developed and parameters established for assessment of nanoparticle transport and toxicity using the BeWo cell line. Preliminary results suggest that nanoparticles are likely to be able to cross the placental barrier, and ongoing research into toxicity will reveal some of the implications of this on placental function.

## **Nanotest: Alternative testing strategies for the assessment of the toxicological profile of nanoparticles used in medical diagnostics**

M. Saunders, L. Cartwright, S. Correia Carreira, S. Thawley, M. Dusinska, L.M. Fjellsbo, E. Heimstad, M. Harju, A. Bartonova, L. Tran, L. Juillerat-Jeanneret, B Halamoda-Kensaoui, F. Marano, S. Boland, M. Whelan, C. Housiadas, K. Volkovova, J. Tulinska, M. Beno, K. Sebekova, L. E. Knudsen, T. Mose, J. V. Castell, M. R. Vilà, L. Gombau, M. Jepson, P. Verkade, G. Pojana, A. Marcomini

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**Introduction:** The unique properties of nanoparticles (NP) whilst likely to benefit many aspects of our lives, are also the cause of concern over inadequate toxicological assessment of their possible impact on human health. With some NP-based products already in general use, and many more soon to follow, it is critically important that the potential risks from this new technology are properly assessed. Clearly, there is a pressing need to understand how engineered NP can interact with the human body following exposure as patients or consumers, in the workplace or from the environment. Fundamental to developing an increased understanding of NP risks is the assessment of NP cellular interactions, particularly at biological barriers. The FP7 project NanoTEST<sup>1</sup> ([www.nanotest-fp7.eu](http://www.nanotest-fp7.eu)) addresses these requirements in relation to the toxicological profile of NPs used in medical diagnostics.

**Aims:** The overall project aim is to develop alternative testing strategies and high-throughput toxicity-testing protocols using *in vitro* and *in silico* methods which are essential for detailed risk assessment. NanoTEST will specifically aim to: a) carry out a detailed characterization of selected NPs in order to define the major physico-chemical properties b) study specific and nonspecific interactions of NP with molecules, cells and organs and to develop *in vitro* methods which can identify the toxicological potential of NPs; c) validate *in vitro* findings in short-term *in vivo* models, to study manifestation of particle effects in animals and humans, and to assess individual susceptibility in the response to NPs; d) perform both Structure-Activity modeling and physiologically-based pharmacokinetic (PBPK) modeling of NP; e) adapt the most advanced and promising assays for high-throughput automated systems and to prepare them for validation by the European Centre for the Validation of Alternative Methods (ECVAM).

**NP and characteristics:** Critical detailed NP characterization is underway and is focused on high purity clinically relevant and benchmark/control NP: a) TiO<sub>2</sub> (benchmark); b) paramagnetic metal oxides (contrast enhancement, cancer treatment); c) Metal fullerenes (MRI); d) polylactic glycolic acid (PLGA), chitosan, hyaluronic acid (polymeric materials for macromolecule delivery); e) Quantum dots (QDs) (cellular labeling, imaging and tracking); f) Fluorescent latex particles (benchmark for transport studies). All pertinent physico-chemical properties of concern, e.g. size distribution, shape, specific surface area, porosity, chemical composition, purity, impurities of concern, surface chemistry, surface charge, crystal structure, are being determined in order to ensure high quality of results. Dispersion and stability of selected NP in aqueous and biological media, as well as their interactions with culture media components, are also being investigated.

**Experimental models:** Development of *in vitro* models using cell lines and cells from several different organs (blood, vascular system, liver, kidney, lung, placenta, digestive, renal and central nervous system) is in progress together with optimizing protocols for biomarkers of oxidative stress, inflammation, immunotoxicity, cellular toxicity and genotoxicity. Preliminary results with Fluoresbrite® NP in a placental model indicate dose-dependent transfer and uptake which may have implications for fetal development. The toxicological NP profile will be validated *in vivo* using rats and exposure effects determined in a range of organs correlating with *in vitro* studies and using similar biomarkers and endpoints.

**Outcome:** NP characteristics will be related to possible adverse health effects, results validated *in vivo*, and recommendations for evaluating potential risks associated with medical NP will be communicated to the scientific and industrial communities.

<sup>1</sup>Supported by EC FP7 [Health-2007-1.3-4], Contract no: 201335.

## Understanding the Genotoxic Potential of Ultrafine Superparamagnetic Iron oxide Nanoparticles (USPION)

N.Singh, PM Williams, GJS Jenkins, SM Griffiths, CJ Wright, SH Doak

**Background:** Ultrafine superparamagnetic iron oxide nanoparticles (USPION) are being widely used for various biomedical applications e.g. magnetic resonance imaging (MRI). Although, the potential benefits of USPION are considerable, there is a distinct need to ensure the safety of these agents and to identify any potential adverse health effects. However, at present there is little information on the toxicity of nanoparticles, which is of growing concern. This toxicity in turn, is determined by the size, surface area, concentration, chemical structure and surface coating of these nanoparticles. The aim of this study was to characterize a range of USPION particles, to investigate cellular uptake and their potential in vitro cytotoxic and genotoxic effects.

**Methods:** Human MCL5 lymphoblastoid B cells and HFF-1 fibroblast cells were exposed for 1, 2 and 3-cell cycles to different concentrations (ranging from 0 µg/ml to 250 µg/ml) of USPION. Genotoxic effects were studied by performing the micronucleus assay. Cytotoxicity and hence, the cell viability was inferred from the ratio of binucleated to mono- and multi-nucleated cells. The colorimetric Ferrozine assay was used to quantitate the iron accumulation, while for histologic analysis, treated cells were stained with Prussian blue and counterstained with nuclear fast red. Characterization studies on USPION were also performed to determine physico-chemical properties such as particle size, distribution, agglomeration and zeta potential under test conditions.

**Results:** It was demonstrated that the iron concentration in both cell lines increased significantly in a time-dependent and dose-dependent manner. The particle size of the USPION, its chemical composition including the oxidation state and the serum concentration of the culture media seemed to influence the USPION uptake. The uptake of USPION showed a correlation to the induction of genotoxicity; kinetochore labelling indicated a clastogenic mode of action. The most suitable micronucleus assay methodology to assess the genotoxicity following exposure to USPION was determined and was an important factor to consider as the nanoparticles interacted with a number of assay components. It was also observed that the USPIONs did not induce any apparent cytotoxic effects as measured by the cytokinesis-block proliferation index (CBPI).

**Conclusions:** An understanding of the physico-chemical characteristics of USPION in the current study was critical for providing insight into their effects and mechanism of action under different experimental conditions. Interestingly, despite a clear lack of cytotoxicity in the current acute exposure study, USPION clearly showed a genotoxic response. However, further investigation is required to determine the more long-term impact and the fate of these nanoparticles on cellular processes that may govern the observed genotoxicity. The present study indicates that the USPION may have the potential to cause DNA damage that could lead to the initiation and progression of cancer.

### Iron oxide nanoparticles interact with fluorometric and colourmetric dyes

S.M.Griffiths, N. Singh, G.J.S. Jenkins and S.H. Doak

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The reactivity of nanoparticles is often an unknown entity, consequently the possibility of direct interaction between nanoparticles and experimental assay components cannot be ignored, particularly when these tests are reporting on the nanoparticles' ability to induce cellular damage. Such interactions have the potential to result in false or misleading information. Some such instances have been documented in the literature, for example it has been shown that single walled carbon nanotubes interact with both fluorometric and colorimetric dyes, to give unexpected results when these probes are used for cell viability assays (Davoren et al. 2007; Herzog et al. 2007; Casey et al. 2008).

In the present study the fluorometric dyes dichlorofluorescein (DCF) and 3'-(*p*-Aminophenyl) fluorescein (APF) were used to quantify the oxidative stress response induced by dextran coated ultrafine super paramagnetic iron oxide (Fe<sub>2</sub>O<sub>3</sub> and Fe<sub>3</sub>O<sub>4</sub>) nanoparticles. In a cell free system increasing concentrations of dextran coated Fe<sub>3</sub>O<sub>4</sub> nanoparticles (100ng/ml 1µg/ml, 10µg/ml, and 100µg/ml) induced a dose dependent decrease in DCF (2µM and 4µM) signal as compared to control levels. In contrast, the same concentrations of dextran coated Fe<sub>2</sub>O<sub>3</sub> nanoparticles induced a dose dependent increase in DCF signalling. Similar results were seen when the fluorogenic probe APF was used as an alternative to DCF. The present study also suggests that dextran coated iron oxide nanoparticles may directly interact with other fluorescent and colorimetric probes. For example, interactions between MTS (a colorimetric dye used for cell proliferation assays) and dextran coated Fe<sub>2</sub>O<sub>3</sub> have also been observed in a cell free system, typified by an increase the colorimetric signal 2.5 fold as compared to control.

This study emphasises that the possibility of nanoparticles interacting with other fluorometric and colorimetric assays must be considered and controlled for in any experiment. Interestingly, the present study also draws attention to the importance of the oxidative state of iron oxide nanoparticles in relation to their interactions with the fluorometric dyes as distinct differences were observed. Thus, where colourimetric or fluorometric dyes are to be relied on for experimental test systems, potential interactions with nanoparticles need to be considered if the investigators wish to reliably use these assays in a quantitative manner.

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### **Potential genotoxic risks of single walled carbon nanotubes**

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**Objectives:** Nanotechnology is a fast growing industry surrounded by an atmosphere of uncertainty regarding the toxic and genotoxic health effects of nanomaterials. The fibre pathogenicity paradigm which holds true for asbestos fibres is based on aspect ratio (>3:1) and biopersistence. Hence, the geometry and surface chemistry of nanomaterials play an important role in the potential of these nanomaterials in inducing toxic effects. Therefore, the validity of the asbestos paradigm with respect to single walled carbon nanotubes (SWCNT) was evaluated after thorough material characterisation in parallel with the genotoxicity assays.

**Methods:** A range of techniques were utilised to characterise the physico-chemical features of the test materials (SWCNT with a manufactured length of 400-800nm; 1-3µm; 0.5-20µm; and 5-30µm), including size range, surface area, morphology, zeta potential, homogeneity, agglomeration, and impurity evaluations. Human bronchial epithelial (BEAS-2B) cells were then treated with SWCNT (1-3µm length) for 24, 48 and 72 hrs and the genotoxic potential of the SWCNT was determined using the cytokinesis blocked micronucleus assay.

**Results:** Physico-chemical analysis revealed that after purification the SWCNTs evaluated were at least 97% pure. Under test conditions, they were mainly found to exist in small spherical agglomerates and as bundles of parallel aligned tubes. When applied to BEAS-2B cell cultures, no significant cytotoxic effects were observed up to 100µg/ml at any of the time ranges assessed. However, significant increases in micronucleus frequency (and therefore chromosomal damage) were encountered in a concentration and time-dependent manner. Interestingly, the data set generated by the micronucleus assay varied according to the specific methodology used as a result of interaction with assay components. It was therefore evident that careful attention must be given to potential confounding factors in classical test systems to avoid confounding results.

**Conclusion:** This study demonstrates that SWCNTs induce significant levels of chromosomal damage at sub-cytotoxic concentrations. Furthermore, altering test conditions have revealed the dramatic impact of the methodology used upon the resultant information provided by a test system. Thus it is imperative that potential interactions between assay components and nanomaterials are fully investigated.

### **Measuring the Impact of Nanoparticles Used in Sunscreens on Human Health**

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Metal oxide nanoparticles contained in many commercially available sunscreens have been shown to be photoactive and produce free radicals when exposed to UV light (Barker & Branch, 2008). Whilst current evidence suggests that these nanoparticles are not absorbed through healthy human skin (Nohynek *et al.* 2007), previous studies have not adequately addressed the potential for absorption or biological impact over lifetime exposure across a range of different skin conditions, nor have they adequately addressed the potential influence of UV radiation. The Commonwealth Scientific and Industrial Research Organisation (CSIRO) in Australia has a

research program to study the health, safety and environmental impacts of manufactured nanomaterials. In the area of sunscreens we are asking two questions: 1. Are metal oxide nanoparticles in sunscreens absorbed through human skin? 2. What is the biological impact of long-term use of commercially available sunscreens in immuno-competent hairless mice? These experiments will be described.

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## Health Implications of Nanoparticles: Toxicokinetics Aspects

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### Introduction

Nanoparticles are increasingly used in a wide range of applications in science, technology and medicine. Since they are produced for specific purposes which cannot be met by larger particles and bulk material they are likely to be highly reactive, in particular, with biological systems. On the other hand a large body of know-how in environmental sciences is available from adverse effects of ultrafine particles after inhalative exposure. Since nanoparticles feature similar reactivity as ultrafine particles, health effects cannot be excluded and a safe and sustainable development of new emerging nanoparticles is required.

Cardio-vascular effects observed in epidemiological studies triggered the discussion on enhanced translocation of ultrafine ambient particles from the respiratory epithelium towards circulation and subsequent target organs, like heart, liver, spleen and brain, eventually causing adverse effects on cardiac function and blood coagulation, as well as on functions of the central nervous system. There is clear evidence that nanoparticles can cross body membranes and reach and accumulate in the above mentioned secondary target organs.

### Methodologies

To determine accumulated fractions in such organs the ultimate aim is to quantitatively balance the fractions of nanoparticles in all interesting organs and tissues of the body and include the remainder body and total excretion collected between application and autopsy. Otherwise substantial uncertainty remains if only selected organs are analyzed. Since these gross determinations of nanoparticle contents in organs and tissues do not provide microscopic information on the anatomical and cellular location of nanoparticles such studies are to be complemented by electron microscopy analysis as demonstrated for inhaled titanium dioxide nanoparticles.

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### Results and Discussion

Based on quantitative biokinetics analysis in a rat model we found small fractions of nanoparticles (iridium, carbon, gold) in all secondary organs studied including the brain, heart and even in the foetus. Organ fractions were usually below 0.1 % of the administered dose to the lungs but depended strongly on particle size in an inverse fashion. Also negatively ionic surface charged nanoparticles translocated more rapidly than positively charged nanoparticles of the same size. Furthermore, nanoparticle accumulation in the rat brain results from both pathways: via the olfactory bulb versus circulation. (Kreyling et al., 2002, 2009; Semmler-Behnke et al., 2004, 2007, 2008)

These data suggest nanoparticle parameters such as size, hydro- / lipophilicity, surface charge, surface ligands and their possible exchange in various body fluids needs to be considered. The current knowledge on systemic translocation of nanoparticles in man and animal models and an estimate of accumulating particle number, surface area and mass in secondary target organs during short-term and chronic exposure will be presented in order to demonstrate the relevance of translocated fractions of nanoparticles.

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