



Report from the second SnIRC Day:

Nanotechnology Environment, Health & Safety Research at its best in Scotland

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The Safety of Nanomaterials Interdisciplinary Research Centre (SnIRC) held its second meeting on the 13th January at Napier University, Edinburgh. The day was organised by Professor Vicki Stone and Dr Lesley Young of Napier University together with SAFENANO, and brought together SnIRC members from the Institute of Occupational Medicine (IOM), the University of Edinburgh and Napier University. Building on the success of the first SnIRC day held in June 2008, the meeting provided an opportunity for members to reconvene, exchange new ideas and discuss recent developments in the field of nanotechnology health and safety. In addition, research students involved with SnIRC were again offered a platform to present their most recent findings to the group, allowing them the opportunity to receive feedback on their current work from the experts in attendance. The day was once again of great value to those who attended, and the research presented proved to confirm that Scottish research organisations remains at the forefront of the nanotechnology health and safety field.

About SnIRC...

SnIRC aims to become the leading UK and European centre for information, advice on health, safety and environmental impacts of nanoparticles. It is hoped that by generating a scientific evidence base for management of nanoparticle risks through integration with other UK and international researchers and regulators, through seminars such as SnIRC days and a variety of other activities, the growth of the UK nanotechnology industry may be facilitated in parallel to safeguarding workplace, public and environmental health. Furthermore, SnIRC actively assists Government Departments in developing sound strategies for managing public, workplace & environmental health and safety.

Multi-National Collaborations...

Following a brief welcome from Professor Vicki Stone, Dr Steve Hankin, Senior Consultant at IOM, opened the first scientific session of the day with an overview of current FP7 project EHRHES (Engineered Nanoparticles: Review of Health & Environmental Safety), involving IOM, Napier University, Technical University of Denmark (DTU), JRC European Commission and the Institute of Nanotechnology.

The overall aim of the ENRHES project is to perform a comprehensive and critical scientific review of the health and environmental safety of fullerenes, carbon nanotubes (CNT), metal and metal oxide nanomaterials. The review will consider sources, pathways of exposure and the health and environmental outcomes of concern, with a view to informing the regulation of the potential risks of engineered nanoparticles. Dr Hankin outlined the five thematic contexts which form the framework of the review (Production, Use & Exposure, Toxicity, Ecotoxicity, Epidemiology & Human Studies, Risk Assessment), describing the main topics to be discussed within each context.

Dr Hankin also highlighted the information management techniques that are being adopted within the ENRHES project to allow for coordinated information management across the consortium. The use of online reference management software with categorised literature



search results, and a Wiki platform to allow cross-partner editing and review compilation will facilitate a more streamlined and accessible review approach. In addition to informing regulators and contributing to the growing NanoEHS knowledge-base, it is anticipated that ENRHES will promote responsible development and use of nanomaterials, and allow for informed prioritisation of research needs.

Scientific Services for nano E,H&S and developing GLP

Dr Julia Varet, Nanotoxicologist for SAFENANO Scientific Services, followed on from this, providing an overview of both SAFENANO and the recently developed Scientific Services section, from her perspective in the Laboratory team. Complementing the SAFENANO Information Service and Community, SAFENANO Scientific Services has been developed to enable companies to understand and minimise the health and environmental risks of working with nanomaterials. Based on the extensive expertise of Napier University and other multi-disciplinary collaborators, the Laboratory Services team are able to develop methodology to assess toxicity and form study design specific to customer's needs.

Dr Varet highlighted the potential challenges facing such work, including the need to develop appropriate in vitro methods of testing nanoparticles, overcome particle interference with assays, choose relevant in vitro toxicity tests that correlate and predict in vivo outcomes, achieve standardisation of protocols and ensure the use of suitable reference materials. Dr Varet explained the methodology that the Laboratory Services team plans to use in order to meet these challenges and to achieve their long term goal: Good Laboratory Practice accreditation.

Undiscovered territory - nanoparticles and reproductive health

Dr Gary Hutchison, Lecturer within Napier University's School of Life Sciences, was next to present, providing an overview of his current research in the area of in vitro male reproductive toxicology, using the testes as a new target organ for nanoparticle research. Dr Hutchison highlighted the current lack of data in this area, despite numerous publications indicating that nanoparticles can translocate to the testes via inhalation and ingestion, with the potential to cause detrimental effects on the testes and male fertility. He then went on to describe initial research undertaken during an undergraduate summer project in 2008, which focused on investigations using Sertoli cells. These cells play a key role in supporting the process of sperm generation (spermatogenesis) and formation of the blood testes barrier (BTB), which has an important immunological role similar to that of the blood brain barrier. During the first part of the project, Sertoli cells (TM4), were exposed to 35 nm silver (Ag) and 25 nm titanium dioxide

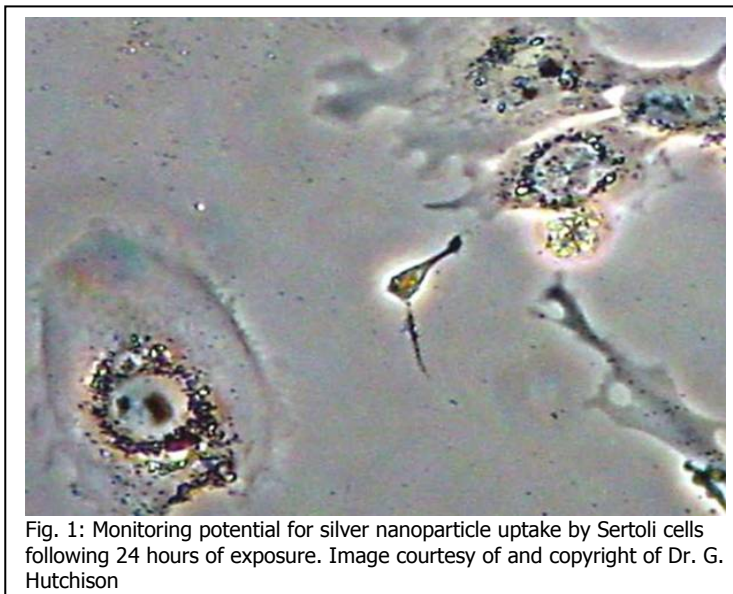


Fig. 1: Monitoring potential for silver nanoparticle uptake by Sertoli cells following 24 hours of exposure. Image courtesy of and copyright of Dr. G. Hutchison

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(TiO₂) nanoparticles over a 24 hour time course. Increased releases of lactate dehydrogenase (LDH), (an indicator of cytotoxicity, measuring release of the stable cytoplasmic enzyme LDH in the incidence of plasma membrane damage), were observed with increasing concentrations of both Ag and TiO₂ nanoparticles over time; and microscopy images indicated that the nanoparticles accumulated near the cell membrane, particularly for the silver samples (Figure 1). These results provide some evidence that Sertoli cells are affected by nanoparticle exposure, but Dr Hutchison emphasised that further research and imaging is needed. The second part of the project aimed to determine whether Ag and TiO₂ nanoparticles were capable of inducing iNOS (inducible nitric oxide synthase) production in Sertoli cells, an indicator of potential inflammation which, in the testes, can lead to infertility. Upon exposing Sertoli cells (TM4) to Ag and TiO₂ nanoparticles over a 24 hour time period, no increase in nitrite levels was observed in either case, indicating i-NOS production cannot be stimulated by nanoparticles alone.

Nanoparticles, platelets and thrombogenesis...

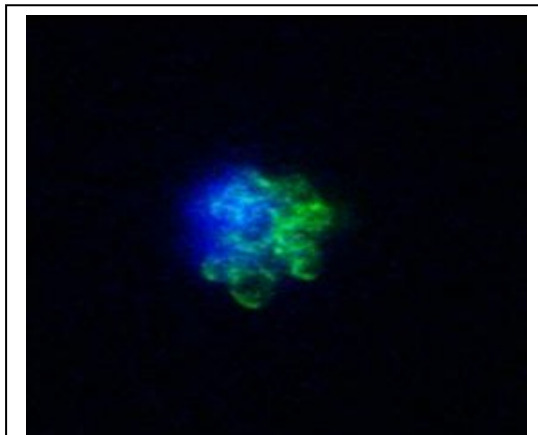


Fig. 2: Co-localised confocal image of platelet aggregates stained with CD42a FITC and fluoresbrite BB carboxylated polystyrene latex nanoparticles. Image courtesy and copyright of C McGuinness

Irene McGuinness, Research Assistant with Professor Ken Donaldson's Research Group at the University of Edinburgh, continued the session by presenting results of her investigations into the interactions between nanoparticles and platelets. Her work forms part of the FP6 project 'Particle_Risk' and follows the central hypothesis that inhaled nanoparticles may be able to cross the epithelium, gain access to the blood and lead to enhanced thrombogenesis via contact with endothelial cells and platelets. To study potential interactions of nanoparticles with human blood and platelets, Irene used 50 nm polystyrene latex beads (PLNP) with varying charge as model particles (unmodified, aminated [positively charged] and carboxylated [negatively charged]). In vitro testing demonstrated that nanoparticles in whole blood could associate with platelets,

with both aminated and carboxylated forms causing platelet aggregation (Figure 2). It was also demonstrated that different mechanisms are involved in platelet aggregation, as only the carboxylated-PLNP caused classical upregulation of adhesion receptors. The aminated-PLNP appeared to act by perturbing the platelet membrane and directly revealing anionic phospholipids. These findings suggest that surface charge is an important factor in platelet activation and aggregation, which may be a mechanism for the cardiovascular effects of nanoparticles.

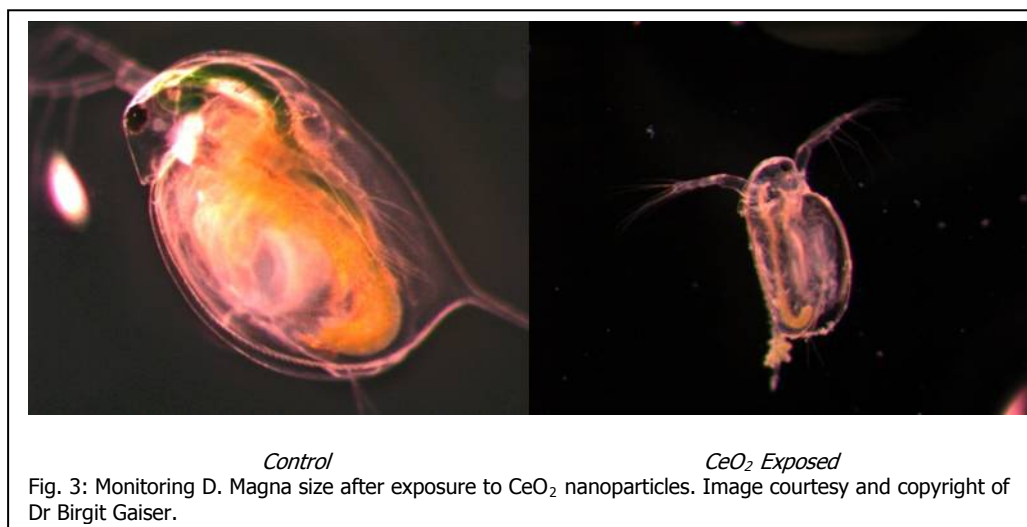
Linking Ecotoxicology & Toxicology using waterborne nanoparticle exposure

Birgit Gaiser, Post Doctoral Research Fellow at Napier University, closed the first session with results from her research that aims to link the ecotoxicology and toxicology of water-borne nanoparticle exposure. In the first part of her project, Birgit investigated the effects of acute nanoparticle exposure by aquatic invertebrates, using *Daphnia magna* as a model species. As test particles, silver nanoparticles (~35 nm), which are increasingly utilised in bactericidal and fungicidal consumer products, and cerium dioxide (CeO₂) nanoparticles (< 25 nm) which are currently in use as fuel additives for diesel engines were selected, and exposure to their bulk

counterparts (μm sized) was also tested for comparison. Mortality studies indicated that silver nanoparticles possess much greater toxicity than both its bulk Ag equivalent or cerium dioxide nanoparticles. Exposure to both nano and bulk forms of the silver and cerium dioxide particles caused a reduction in size and moulting of the *D. magna*, suggesting nanoparticles have an ability to affect growth and health, further indicators of toxic effects (Figure 3).

As a means to study the effect of nanoparticle exposure to bony fish, the bioavailability of both bulk- and nano- silver particles in the common carp *Cyprinus carpio* was investigated by sampling the liver, gill, kidney, brain, blood, gall bladder and intestine after a sub chronic 21 day exposure. The effects of CeO_2 on this species are already documented (Scown, T., manuscript in preparation), thus to avoid duplication of in vivo studies exposures using CeO_2 were not carried out. Significant uptake of both sizes of Ag particle was observed in liver, gills, gall bladder and intestine, with ingestion found to be the main route of uptake.

In vitro cytotoxicity studies were also undertaken to investigate the effects of nanoparticles on a key body system, the liver, using both human and primary trout hepatocytes. Both nano- and micro- silver particles were found to be cytotoxic in both cell lines, but significantly more so in the human hepatocytes with an LD50 (dose lethal to 50% of cells in the sample) of $50 \mu\text{g}/\text{mL}$. Cerium dioxide, however, displayed no such cytotoxicity even at high doses, despite observed uptake into cells.



In the final part of her research to date, Birgit investigated the potential for nanoparticles to be transported through gastrointestinal tract (GIT) barriers using an in vitro M cell model. M Cells are key to the immune system of the GIT, having the unique ability to sample antigens from the intestinal lumen and transfer them to lymphocytes and other associated immune cells on their basolateral side. Interfocal imaging revealed both silver and cerium dioxide nanoparticles to be taken up into and transported through M cells, compared to little or no transport from their bulk counterparts. This suggests potential for nanoparticle uptake into the intestinal epithelium and transport through the GIT.

In conclusion, Dr Gaiser summarised that particle toxicity is dependant on material type ($\text{Ag} > \text{CeO}_2$), particle size (nano > micro) and aggregation state (disperse > aggregated). In addition, uptake of particles in to many species (within the laboratory setting) occurs by ingestion, and subsequent uptake and transportation of these particles by intestinal epithelium may be possible.

Metal Oxide Nanoparticles - Environmentally Realistic Scenarios

The second session was opened by Jon Mullinger, who is currently undertaking a Unilever-sponsored PhD at Napier University. His research aims to investigate environmentally realistic exposure scenarios for nanoparticles and test the ecotoxicology of nanoparticles using those models. Jon described his recent investigation into the toxicity of TiO₂ nanoparticles to freshwater algae *Pseudokirchneriella subcapitata* (a standard OECD test species) with and without the presence of humic acid, a natural dispersant present in all freshwater systems. The toxic effect of TiO₂ nanoparticles was found to increase significantly in the presence of humic acid by comparison of EC50 values (concentration provoking a response halfway between the baseline and maximum responses), with Transmission Electron Microscopy (TEM) images indicating adherence of the nanoparticles to the algal cells. Jon emphasised that these findings are based on range-finding concentrations (EC50) & single tests which need to be repeated in order to ensure reliability of results. The mechanism of the increase in toxicity also needs to be investigated in future work.

Aquatic Invertebrate toxicology: uptake, particle toxicity & bioindicators for oxidative stress

Philipp Rosenkranz, PhD student at Napier University continued the ecotoxicology discussion with an overview of results from his investigations into the ecotoxicity of nanoparticles to *D. magna*. Philipp's work was split into three sections. The first examined the uptake of nanoparticles by *D. magna* using fluorescent polystyrene beads (PS beads) both qualitatively and quantitatively. Daphnids were exposed to 20 nm and 1000 nm PS beads. 4 hour observations revealed that uptake of 1000 nm PS beads was approximately 25 times higher than for 20 nm PS beads when expressed as mass dose. However, clearance following exposure was found to be over 90% after 4 hours for 1000 nm particles, compared to only 50% for 20 nm PS beads.

The second part concentrated on determining the toxicity of engineered nanoparticles carbon black, silver and cerium dioxide both acutely (96h) and chronically (21 day) as a function of both mass dose and surface area. To ascertain this, endpoints of mortality, moulting, growth and reproductive ability were examined. Mortality results allowed a clear gradient of toxicity to be drawn, with silver being the most toxic to cerium dioxide the least. The toxicity of carbon black appeared to be mainly surface area dose related. In comparison, results for silver suggest that surface area dose is not the only factor responsible for the mode of toxicity. Although the endpoints of moulting (Figure 4), growth and offspring were more sensitive than mortality, no clear gradient of sensitivity could be established from these studies.



Fig. 4: Effect on moulting of a 21 day exposure to 0.001 mg/l Ag nanoparticles. Image courtesy and copyright of P. Rosenkranz



Finally, Philipp's work had examined potential bioindicators for measurement of oxidative stress within daphnia exposed to nanoparticles. The first technique examined was the TEAC assay (Trolox-Equivalent Antioxidant Capacity assay). This assay measures the antioxidant capacity of a sample substance, by measuring its ability to quench (and thus decolourise) the blue-green ABTS⁺ radical, a blue-green chromophore. The measure of absorbance obtained for the test sample is compared to the quenching activity of trolox (a water soluble analogue of vitamin E, a known powerful antioxidant) at a given concentration, in order to indicate the degree of oxidative stress. Philipp's investigations revealed a clear effect of background absorbance in all treatments, with the carbon black treatments exceeding control by up to four fold, and rendering the TEAC assay unsuitable for use as a bioindicator.

The second assay examined was the Glutathione Assay, which measures oxidative stress via quantifying the ratio of reduced state glutathione – GSH (which buffers endogenously induced oxidative stress) to total glutathione. Exposure of daphnia to carbon black, Ag and CeO₂ for 3 hours revealed a significant increase in reduced glutathione for nano Ag and carbon black (early stage apoptotic cells extrude reduced glutathione), however a significant reduction in the GSH:total glutathione ratio following addition of the nanoparticles to daphnia homogenate means that the usefulness of the glutathione assay for this use may not be ideal.

Nanoparticles & the Immune System

Morag Prach of Napier University then provided an outline of her proposal to research the potential immunotoxic effects of manufactured nanoparticles. The work is based on evidence which suggests that nanoparticles may interact with natural allergens such as dust mites and potentiate their effects. Morag's aims are twofold: i) to investigate whether nanoparticles present in commercial products (e.g. food additives & sunscreens) may interact with antigens to generate an exaggerated immunogenic response; and ii) whether nanoparticles may form a safer and more efficient adjuvants for e.g. vaccines than the currently employed yet far from ideal aluminium adjuvant. Using human monocytic cell lines and peripheral blood mononuclear cells, Morag plans to monitor various immuno-responses upon nanoparticle stimulation, including modulation of cell surface markers, cytokine production, cell proliferation and alterations in gene expression.

And finally...Carbon Nanotubes & the fibre paradigm: taking the next steps

The presentation session was closed by Fiona Murphy, first year PhD student with Ken Donaldson at the University of Edinburgh, with an overview of her project aims in the area of Carbon Nanotube (CNT) toxicity. Using an intrapleural injection model to compare the effects of long CNTs and tangled CNTs to amosite and carbon black, Fiona's work builds on previous studies in this field with relation to the structure/activity paradigm for fibre pathogenicity and the intraperitoneal model (e.g. Poland et. al, 2008). Her central aims are to:

- i) investigate the effects of intrapleural injection of CNT,
- ii) determine the kinetics of CNT translocation from the lung space to the pleura and
- iii) determine whether mesothelial cells in culture can be used as an in vitro model to show similar differentiation of toxic effects between long and short CNT.

As the pleura is such a small compartment, Fiona explained that her first challenge is to develop a suitable method for intrapleural injection which avoids injection into the lung itself.



In summary, the second SnIRC meeting provided another excellent forum for discussion and served to highlight the wide range of novel and innovative research that is taking place within the field of nanotechnology EHS. We look forward to the next one!

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