

**1st International Conference on Nanotoxicology
(NANOTOX 2006)
Miami FL, 29 January – 1 February 2006**

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Executive Summary

The main topics covered in the meeting were generally organised around the following themes:

1. Characteristics and characterisation criteria for nanoparticles,
2. Experimental models for studying biological effects
3. Mechanisms of toxicity
4. Modes of translocation within tissues and to more distant tissues
5. Appropriate risk assessment paradigms for nanomaterials

The types of nanoparticles that were studied ranged from single and multi-walled carbon nanotubes (S/MWCNT), fullerenes, quantum dots, and nanoporous amorphous silicon spheres, as well as the conventional ultrafine metals and metal oxides (TiO₂, ZnO, nanogold) and some complex mixtures (e.g. diesel emissions, welding fumes). There were also references to various types of applications (e.g. biosensing and gas detection devices; DNA detecting nanotubes, drug delivery vehicles) and commercial suppliers of nanomaterials (e.g. Nanomix).

The studies presented at the conference highlighted the importance of physio-chemical characterisation of the nanoparticle being investigated. There were many reports of seemingly slight alterations in the surface chemistry and/or agglomeration state of nanoparticles that lead to large differences cytotoxicity and biodistribution. The conference also included discussions of regulatory efforts in the US and Europe and appropriate protocols for the safety testing of nanomaterials.

General Comments

The meeting was promoted by the University of Pittsburgh organisers as the first international conference focussing on nanotoxicology. While it attracted some 200 registrants from 13 countries, the program was somewhat insular and narrowly focussed. The invited speakers were primarily from the 5 or 6 US and German organizations which comprised the Organizing Committee or worked closely with them. It almost seemed that the program had been constructed primarily to showcase the work of the Pittsburgh group and its collaborators on the generation of reactive oxygen species and their role in inflammatory lung disease and fibrosis.

Some of the key speakers who had been advertised in the initial promotional brochures had withdrawn from the meeting. Notably absent were: Vicki Colvin (Rice CBEN), Vyvyan Howard (UK) and Eva Oberdorster (Texas).

However, some of the leading lights did attend (e.g. Gunter Oberdorster, Eileen Kuempl, Nancy Monteiro-Riviere, Peter Hoet and Vince Castranova) and there were some quality presentations from some of the US, German, Swedish and UK scientists. There were a few presentations designated as “young investigator”. However, it is not clear how the organizers selected “young investigators” for the program, since the quality of these presentations was quite patchy.

In general, the input from US agencies (NIOSH and EPA) was pertinent and well appreciated. There was a particularly useful talk by Vince Castranova outlining some of the NIOSH programs in nanotoxicology research and information collation, and on how these were being co-ordinating with the broader Nanotechnology programs of the US Government. The final session, on risk assessment, featured presentations from Philip Sayre (US EPA), Eileen Kuempl (NIOSH) and Lang Tran (UK IOM) and from my perspective, this was probably the most useful session of the whole meeting.

At the Opening ceremony, which was organised as a sit-down dinner around an opening keynote address by Martin Philbert (UK), it was announced that Germany would host the 2nd(!) NANOTOX in Karlsruhe in 2008.

There was a significant amount of overlap between information presented by different groups and at different sessions. This presumably reflects the nascent status of the discipline. Many people are working on much the same things. Also, it was notable that many abstracts opened with, or included, wording like “...nanomaterials are widely used but not much is known of their toxicity.....biological and ecological impacts are only beginning to be explored.....effects on biological systems are largely unknown, or little understood.” It is clear that many people are of the opinion that research in nanotoxicology is very much in its infancy!

The main topics covered in the meeting were generally organised around the following themes:

1. Characteristics and characterisation criteria for nanoparticles, including attention to sources, stability and dosimetry estimates
2. Experimental models for studying biological effects
 - in vitro (both cell culture and cell-free systems)
 - in vivo (mainly inhalational and intra-tracheal installation models)
 - newer technology (e.g use of gene microarrays to determine the patterns of gene expression activated or repressed by nanoparticles)
3. Mechanisms of toxicity – with a particular focus on the role of reactive oxygen species (ROS) and their downstream effects on cell signalling systems
4. Modes of translocation within tissues and from sites of exposure to more distant tissues
5. Whether traditional risk assessment paradigms can be applied to nanomaterials

The types of nanoparticles which were studied ranged from single and double-walled carbon nanotubes (S/DWCNT), fullerenes, quantum dots, and nanoporous amorphous silicon spheres, as well as the conventional ultrafine metals and metal oxides (TiO₂, ZnO, nanogold) and some complex mixtures (e.g. diesel emissions, welding fumes). There were references to various types of applications (e.g. biosensing and gas detection devices; DNA detecting nanotubes, drug delivery vehicles) and commercial suppliers of nanomaterials (e.g. Nanomix,)

Some of the key issues which emerged as a general theme from the meeting were:

1. Many speakers noted the potential for nanomaterials to aggregate and change their surface chemistry during use and emphasised the need for a comprehensive characterization of the nanomaterials being studied. Reference was made to minimum characterization criteria published in a landmark paper by Oberdorster et al (2005) (<http://www.particleandfibretoxicology.com/content/2/1/8>) arising from an ILSI-sponsored consultation. In a survey of published papers, it was noted that, while 90% characterised the nanomaterials used in terms of particle size, only 29% reported on surface characteristics. It was noted that NIOSH are establishing a database of nanomaterials, which should contain quite a lot of relevant characterisation information. Among the factors considered important in determining the toxicological profile and performance characteristics of nanoparticles were: surface roughness; fibre aspect ratio; crystallinity.

2. Several speakers referred to the potential impact of trace metals (particularly Fe) which may remain as impurities in manufactured nanoparticles like SWCNTs. These retained metals can significantly enhance the toxicity of nanoparticles by catalysing Fenton-chemistry production of ROS.
3. The significance of surface chemistry in moderating toxicity was shown by studies with C60 fullerenes. Substitution with up to 20 hydroxyl groups on the surface was shown to significantly decrease cytotoxicity (now published by Sayres et al in *Nanoletters* **4**(10) 1881-1887, 2004). Similar results were obtained with hydroxylation derivatization of nanotubes, and another paper (Kreuter) reported that coating of drug-delivery nanoparticles with surfactants (particularly polysorbate 80) modified the biological distribution characteristics after IV injection, and protected against cardiotoxicity when doxorubicin was the entrained drug.
4. There were a couple of papers which addressed the surface characteristics of nanoparticles which influenced their recognition by macrophages, the main mechanism whereby particles may be cleared from tissues. Non-functionalized SWCNTs are not readily recognised or phagocytosed by macrophages, but coating the nanotubes with phosphatidylserine (PS) provided the universal “eat me” signal which is expressed on the outer cell membranes of apoptotic cells, triggering phagocytosis. Coating SWCNTs with phosphatidylcholine did not provide the same signal. The PS phagocytic signal on SWCNTs could be blocked by Annexin V, which binds this site.
5. Several speakers noted the difficulty of preparing nanoparticle solutions, particularly with the hydrophobic SWCNTs, and preventing their aggregations prior to administration. Some used sonication to achieve an acceptable dispersion, while others used nonpolar solvents, with and without added surfactants. In a dramatic illustration of this problem, Oberdorster's ecotoxicology group showed that the LC₅₀ to various aquatic species could be lowered from >35 ppm when fullerenes were prepared by simple stirring in water, to as low as 0.8 ppm, when the same materials were first solubilised in tetrahydrofuran (the unresolved issue was whether this increased toxicity was attributable to entrained THF).
6. There was discord between some groups over the significance of ROS-initiated cell apoptosis as the dominant cytotoxic mechanism associated with NPs. Pathogenic fibres (asbestos, silicates) appear to activate the same cell signalling pathways as nanoparticles, leading to local inflammation and cell proliferation. Microarray and Bioplex assays were used to differentiate the responses which were associated with cell death (primarily via apoptosis) and cell growth and proliferation. There was an apparently unresolved paradox with some types of nanoparticles and

inflammatory responses. Some types could produce lung toxicity following intrapleural administration without much inflammatory response, while others produced strong cascades of ROS, inflammatory cytokines and significant inflammation.

7. An important technique used to study inflammatory responses after intratracheal instillation of nanoparticles (e.g. 14-260 nm carbon black, 64-535 nm polystyrene beads) included neutrophil counts in bronchoalveolar lavage (BAL) solution, and fluorescent dyes or cell GSH and intracellular Ca as measures of ROS formation. The measurement of selected cytokines on either side of an artificial lung epithelial monolayer was used to demonstrate toxic responses at an air/tissue interface.
8. Studies with ultrafine radiolabelled iridium (^{192}Ir), TiO_2 and radiolabelled gold in rats after 1 hr inhalation showed distribution to distant cardiovascular sites, and raised the possibility that ROS and platelet activation in the vasculature might be associated with cardiovascular toxicity similar to that observed with $\text{PM}_{2.5}$ - PM_{10} air pollutants. More significantly, a single 1 h inhalational exposure gave peak exposures in the liver, kidney, spleen and brain within 1-2 h, but clearance was very slow, particles being detectable in tissues for up to 160 days.

Other studies using Tecnegas (Te-labelled carbon) and SWCN (1-4 nm diam; $>5\ \mu\text{m}$ length) produced by high pressure CO conversion (HiPCO) showed that thrombotic events related to platelet activation occurred much earlier than inflammatory responses; that these events were not histamine-mediated (although later inflammatory events could be partially blocked by antihistamine) and that ROS-mediated atherosclerotic events had a much longer time course (7-28 days, with some persistent mitochondrial damage persisting for up to 6 months). In a mouse model of atherosclerosis (ApoE $^{-/-}$), the combination of multiple SWCNT and a 1 month atherogenic diet produced a markedly increased incidence of atherosclerotic plaques in the vasculature.

9. The significance of size distribution and surface chemistry was well illustrated by the studies of Christie Sayres, using TiO_2 (anatase and rutile, with different size and surface chemistry) and with nanotubes derivatized with sulphonated benzene or dicarboxylic acids (both of which markedly reduce cytotoxicity). She also showed that uV light strongly activates the anatase form of TiO_2 .
10. An interesting immunotoxicity study was presented by a group from NIOSH. They exposed F_{344} and SD rats to welding fumes (10-100 nm; which can agglomerate to 100-400 nm) in inhalational exposure chambers, 3 h/day for 10 days at 15 or 40 mg/m^3 . F_{344} rats and older rats were much more susceptible to IT bacterial challenge with *Listeria*

monocytogenes, so young SD rats were used in subsequent studies. These showed that Cr was the most active component of welding fumes responsible for suppressed lung defence to bacterial infection, significant lung damage (LDH and albumin in BAL), but without much pulmonary inflammation.

11. There were a number of key projects sponsored by NIOSH as part of their contribution to the National Nanotechnology Initiative (NNI), including the launching in September 2005 of its strategic plan (http://www.cdc.gov/niosh/topics/nanotech/strat_plan.html), co-operation with the International Council on Nanotechnology (ICON) and Rice University CBEN to launch a database on nanotechnology articles, formation of work groups addressing dosimetry and risk assessment, workplace exposure and risk communication strategies, as well as a forthcoming web-based information library, featuring information on the production, composition, size range and commercial sources of nanomaterials.
12. There was an interesting presentation from W. Tyler Beaty, a lawyer with a firm specialising in environmental torts. He noted that the US Toxic Substance Control Act (TSCA), like NICNAS legislation, discriminates between new and existing chemicals. If a nanomaterial is sufficiently consistent with the description of an existing chemical, it would not be assessed under TSCA. He noted that the EPA is investigating a voluntary notification scheme to get around this loophole. Rather ominously, he warned that US juries had a nasty habit of awarding large \$ judgements against phantom risks (i.e perception of risks in the absence of hard scientific evidence). He noted that uncertainties around the risks of nanomaterials could give rise to such lawsuits.

The focus on lung toxicity and mechanisms of toxicity after inhalational exposure or intratracheal instillation (IT) at the expense of other routes was disappointing. There was really only one session devoted to dermal exposure (led by Nancy Romeiro-Riviere) and this session provided useful information on the extent to which different types of NPs penetrate the dermis, where they deposit, and how they may initiate toxicity in different types of skin cells. Nanomaterials studied included MWCNTs, quantum dots, substituted fullerenes, all of which penetrated into skin, but had different sites of deposition. TER was used to demonstrate distribution within the dermal layers. Bioplex and other assays (IL-8 release) were used to assess skin inflammatory responses, while keratinocyte viability and proteomics were used to characterise cell toxicity. In another paper, Murray et al demonstrated that cytotoxic, ROS-mediated inflammation and skin thickening could be produced by 5 days dermal exposure to SWCNTs. However, the doses used (2,4,8 mg/kg) were quite high, and only the 8 mg/kg dose produced any marked skin thickening.

There were only two papers which addressed oral absorption. Alexander Florence reviewed the nature of the GI barrier, the role of Peyer's patches and enterocytes in facilitating membrane transport, and indicated that nanoparticles are absorbed across the GI epithelium by much the same mechanism that bacteria, viruses and prions are absorbed. A paper by De Jong & Gatti studied the effects of nanoparticles of Co (120 nm) and ZrO₂, administered to rats by gavage once weekly for 13 weeks. ZrO₂ particles could be found in liver, spleen and kidney for up to 12 months, but Co particles appeared not to be absorbed. Notably, there was no toxicity recorded in any organs associated with this chronic exposure, but the group sizes were very small (n=6).

The presentation by Gunter Oberdorster focussed on his studies of the mechanisms of systemic absorption from the respiratory passages. He emphasised the potential significance of transport via olfactory neurones as a pathway to the brain. Coating nanoparticles (e.g. 240 nm latex coated with LPS, 8 nm albumin-coated gold) facilitates cellular uptake through alveolar and tight junctions, and also intracellular translocation into mitochondria and cell nuclei).

Session on risk assessment

There were three very good presentations on risk assessment.

- Philip Sayre (Assoc. Dir, RA Sect. OPPT, EPA) outlined the networking arrangements under the NNI. He described the TSCA as the main legislative tool available to US government regulators, and its shortcomings with respect to assessing nanoscale materials if they did not qualify as new chemical entities. For example, only one submission relating to carbon nanotubes had been reviewed (under a low release/low exposure exemption program), and 12 submissions were currently under consideration. Sayre described work towards a voluntary notification under TSCA, or a stewardship program, which would invite manufacturers to notify basic nanomaterial characteristics and risk management practices to a NPPTC database. He indicated that conventional HRA methods could be applied to nanomaterials, but that there needed to be better information on exposures, consideration of which analytical techniques would be best to characterise materials (surface area and surface chemistry) and to develop appropriate dosimetry for dose-response assessment. He indicated co-operation with the ISO program (ISO TC229) which is setting out to characterise nanoparticles and develop international standards for terminology and nomenclature; metrology and characterization; and health, safety and the environment
http://www.ansi.org/news_publications/news_story.aspx?menuid=7&articleid=1084.

He also made reference to the possible development of a tiered screening system for nanoparticles, such as that outlined in Morgan et al (*Risk*

Analysis **25**(6) 1621-1635, 2005). The Tier I could include a variety of *in vitro* tests addressing potential cytotoxicity and mode of action, with an *in vivo* test component including a 2 wk inhalational exposure, or single intratracheal installation, followed by a 28 d observation period. However, work remains to be done to establish criteria for outcomes which would lead to a Tier II study, and for what endpoints. He suggested that the need for more extensive testing will probably be driven by the exposure potential.

Finally, he indicated that an EPA White Paper calls for the development of case studies to facilitate the HRA development (<http://www.epa.gov/osa/nanotech.htm>)

- Lang Tran (IOM Edinburgh) provided a European perspective on nanotoxicology research. Stimulated by the UK Royal Society Report, UK DEFRA established a series of taskforces to address measurement, exposure, toxicology, ecotoxicology and societal impacts of nanotechnology, and Tran had a key role in developing the HRA components of the EU's FP6 Framework Program, which promotes integration of research and funds specific thematic, such as nanotoxicology. For example, €2.5m was allocated to nanotoxicology research in FP5, €8m in FP6, and he was hoping for more in FP7.

He has developed two distinct research programs. Particle_risk (<http://www.snirc.org/index.html>) addresses HRA of nanoparticles through air, food and water using *in vitro* and *in vivo* toxicological models. It includes several UK and European collaborators, and appears to be a useful model on which to base Australian collaborative efforts.

Nanosafe 2 (<http://www.nanosafe.org/node/75>) is a broader program, to develop HRA and risk management options and includes a component which addresses safe production, social aspects and regulation.

- Eileen Kuempl (NIOSH) presented an overview of the how to translate existing HRA knowledge from human disease relating to ultrafine particles and deposition characteristics in the human and rodent respiratory passages. She emphasised the importance of appropriate dose metrics, and outlined some quantitative dose-response modelling based on the Benchmark Dose approach, extrapolations based on lung mass/surface area deposition, and how to translate this back to an equivalent airborne exposure for humans. Much of what she presented is summarised in her recent publication (with Andrew Maynard; *J Nanoparticle Res.* **7** 587-614, 2005). She also cited a useful paper (Stayner et al; *Am. J. Indust. Med* **34** 207-219 2005) which provides a basis for comparing the cancer risk estimates for diesel fume exposure in humans and rodents.