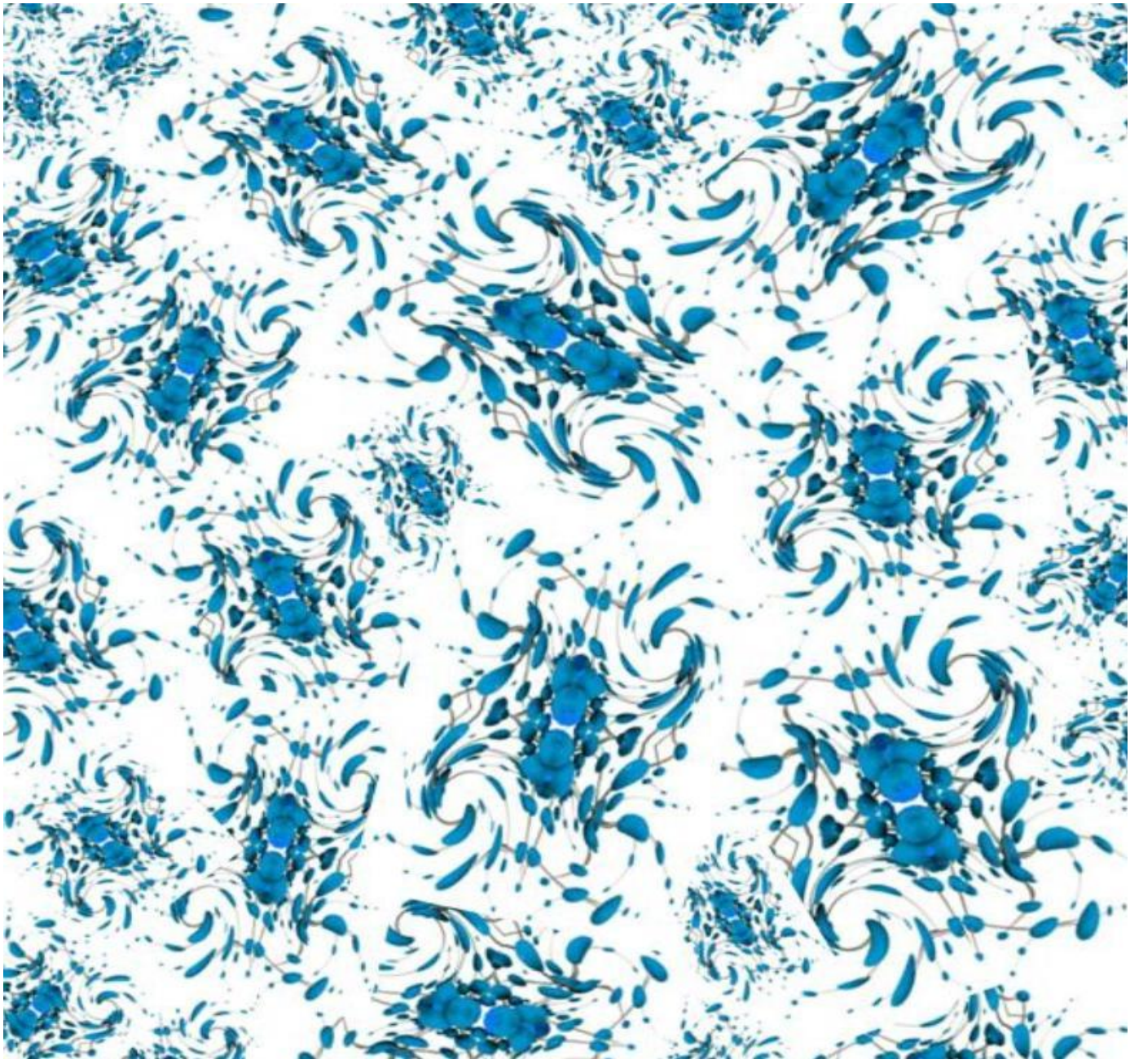
Scientific Review Report

Australian Government
Department of Health and Ageing
NICNAS

Review of 2007–09 literature

on toxicological and health-effects relating to six nanomaterials



Review of 2007–09 literature on toxicological and health-effects relating to six nanomaterials

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Preface

What this review is about
NICNAS commissioned this technical consultancy to review and analyse available literature from 2007-2009 on six industrial nanomaterials, chosen as they were considered to already be in, or close to, commercial use in Australia. The aim of this review was to identify any available scientific evidence of important toxicological/health effects that had not been covered by the scope of previous reviews and therefore supplement currently available scientific information on these substances.

Scope of the review
The consultant was asked to draw out knowledge of toxicological/health information of: fullerenes, carbon nanotubes and nanoforms of zinc oxide, titanium dioxide, cerium oxide and silver. To build technical knowledge and avoid unnecessary duplication specific 'data gaps', not covered in previous reviews, were identified and addressed in the consultancy (depending on the availability of published papers).

In addition, any other toxicity/health related information on the six nanomaterials that were not captured in the previous reviews was also expected to be included in the report.

Conduct of the review
This consultancy was carried out in three phases, a comprehensive literature search to collect articles available on each data gap identified by NICNAS[1], analysis of the literature on findings in relation to data gaps and compilation of the report based on these findings.

Use of the review
This review will be one source of information used by NICNAS in determining the risks posed by the use of these substances in Australia. NICNAS continues to monitor and keep up to date on scientific research papers on these nanomaterials as they are published, work that will provide the basis for future risk assessments on these materials, as new information arises, contributing to the key NICNAS strategic direction in relation to industrial nanomaterials - to protect human health and the environment through appropriate regulation.

[1] See *Section 12.2* of this report for more information.

Contents

Glossary of abbreviations used

AAS	Atomic absorption spectrometry
ACGIH	American Conference of Governmental Industrial Hygienists
BET	Brunauer-Emmett-Teller (algorithm describing surface area of NPs)
CB	carbon black
CHO	Chinese Hamster Ovary cells
CeO	cerium oxide
CNS	central nervous system (incorporating the brain and spinal column)
ENPs	engineered nanoparticles
Fe_2O_3	iron oxide (a common contaminant in manufactured CNTs)
HRA	health risk assessment
ICON	International Council on Nanotechnology
IP	the intraperitoneal injection route of administration
IV	the intravenous route of administration
LOAEC/L	Lowest Observable Adverse Effect Concentration/Level
MDA	malondialdehyde (a product used to measure lipid peroxidation)
MWCNTs	multi-walled carbon nanotubes
NOAEL	No Observable Adverse Effect Level
NICNAS	National Industrial Chemicals Notification & Assessment Scheme
NMs	nanomaterials
NPs	nanoparticles or nanoparticulates
OHS	occupational health and safety
PCR	polymerase chain reaction
PM10	dust and other airborne particulates with a size $\leq$ 10 micrometers
PVA	polyvinyl alcohol
SA	Surface area
SC	the subcutaneous route of administration
SEM	scanning electron microscopy
SWCNTs	single-walled carbon nanotubes
ROS	reactive oxygen species
STIM	Scanning Transmission Ion Microscopy
TEM	transmission electron microscopy
TiO_2	titanium dioxide
TLV	threshold limit value
UF	ultrafine (also UFPs ultrafine particles)
UV	ultraviolet (subclassified UVA and UVB according to wavelength)

Executive summary

The literature on the potential applications of the nanotechnologies is expanding at a tremendous rate. This report focuses of literature which specifically relates to health and safety assessments of six nanomaterials (NMs – mainly as nanoparticulates, or NPs) specified to be of interest to NICNAS (zinc oxide, titanium dioxide, cerium oxide, carbon nanotubes, fullerenes and nanosilver). It complements other literature reviews which have been done in recent years by focussing only on papers published from late 2007 to September 2009 which have not been covered in these previous reviews.

Several reviews have been published during the 20 month period covered by this report, as well as a large number of experimental studies. The literature search identified some 264 relevant papers, most of which have been discussed in this report. In addition to summarising individual reports and information relating to the toxicity of the six NMs, attempts were made to assess the extent to which the literature informs health risk assessment (HRA) and broader issues of occupational health and safety (OHS) assessment. In this context, the report does address the plethora of *in vitro* studies with the six NMs, but these probably do not contribute substantially to informing HRA or OHS assessment. The limitations of *in vitro* testing were well discussed in the Toxikos review, and this report supports those views, and suggests that their utility may be limited to characterising cellular mechanisms associated with inducing toxicity, and possibly showing how modification of nanoparticle (NP) size and surface structure may influence relative cytotoxicity. The innate, medium-dependent tendency of NPs to aggregate in air and in biological media can result in changes in size and surface area which may further confound the interpretation of both *in vivo* and *in vitro* toxicity studies.

Some special features of NP-induced toxicity have been singled out for more detailed analysis in this report. These are:

- The extent to which repeated-dose vs. single dose and/or *in vitro* studies can contribute useful information to undertake a HRA; including the role of tests for genotoxicity to reveal carcinogenic potential, as opposed to being simple markers of the induced formation of reactive oxygen species (ROS)
- How likely is it that fibrous NMs (e.g. single- and multi-walled carbon nanotubes) can reproduce the adverse health effects of asbestos fibres; specifically, the mesothelioma risk associated with exposure to CNTs
- Whether there is new information about the extent to which NPs can be absorbed across unbroken skin (there is already substantial evidence that NPs penetrate broken skin more readily)
- Whether some NPs represent a specific risk of neurotoxicity under conditions where they can actually reach the brain, or whether NP exposure can result in cardiovascular toxicity, given the known potential for ultrafine air pollution to contribute to human cardiovascular morbidity and mortality, and
- Knowledge gaps which might help to inform HRA, and in particular, whether advances in knowledge of the effects of surface modification, or other structure-activity relationships, or application of genomic techniques, might fill some of these gaps.

In relation to each of the above dot points, the literature over the past 20 months has offered some advances in knowledge, although there remain substantial gaps. The main areas of advancement have been in relation to *in vitro* mechanistic studies and sub-chronic inhalational toxicity studies in rodents, which have demonstrated:

- Biopersistance (long-term retention of NPs in target tissues) is a factor quite critical in determining whether NPs penetrate cells and are retained long enough to provide the continuing inflammatory signals and other biological stimuli which lead to pathological changes.
- Most biopersistent NPs induce adverse cellular oxidative stress events dependent on reactive oxygen species, but that initiation of apoptosis can be a further factor inducing cell necrosis. Furthermore, biological signals indicative of apoptosis can occur at dose levels lower than those producing frank cytotoxicity. This in turn suggests that cellular antioxidant mechanisms are partially protective against cytotoxicity.
- Modification of surface activity (particularly that leading to solubilization of otherwise hydrophobic NPs) modifies the toxicity potential of NPs. However, as yet, there is no clear pattern of structure activity relationships (SAR) which would permit predictability of NP toxicity
- Formal HRA is still hampered by a lack of useful chronic exposure studies, especially those which clearly demonstrate dose-response relationships, or provide for clear NOAELs. An innovative approach to HRA was demonstrated in a case study relating exposure to CeO NPs used as a diesel fuel additive and comparing estimated human lung internal doses with *in vitro* toxicity data, and
- Characterization of NPs administered in either *in vitro* or *in vivo* experimental studies remains critical to the interpretation of the outcomes. Most studies now report the characteristics of the NPs they use, including the critical surface area in terms of Brunauer-Emmett-Teller values (an algorithm describing surface area of NPs).

1. Review methodology and scope

The literature on the potential applications of the nanotechnologies is expanding at a tremendous rate. Indeed, Ostrowski *et al* (2009)[1] , in reviewing just the nanotoxicology literature for the period 2000-07, estimated that the peer reviewed literature had expanded by around 600%, with publications spanning at least 58 different journals. The distribution of articles by route of administration in Figs 3 and 4 from that review indicate that the inhalational route in mammalian species predominates over other routes of exposure, but the number of papers where the exposure route is not specified is also large. Some of these may be *in vitro* studies.

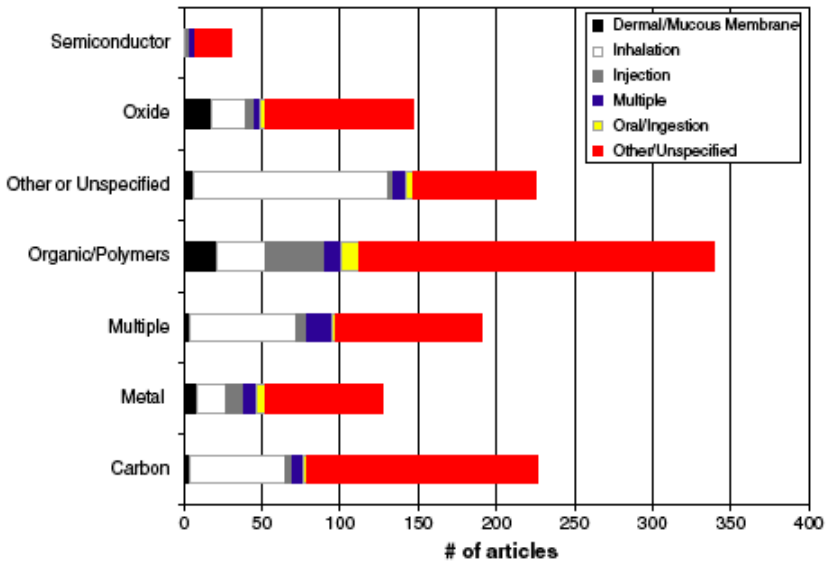

Fig. 3 Number of articles by material type and exposure pathway (Collected from ICON)

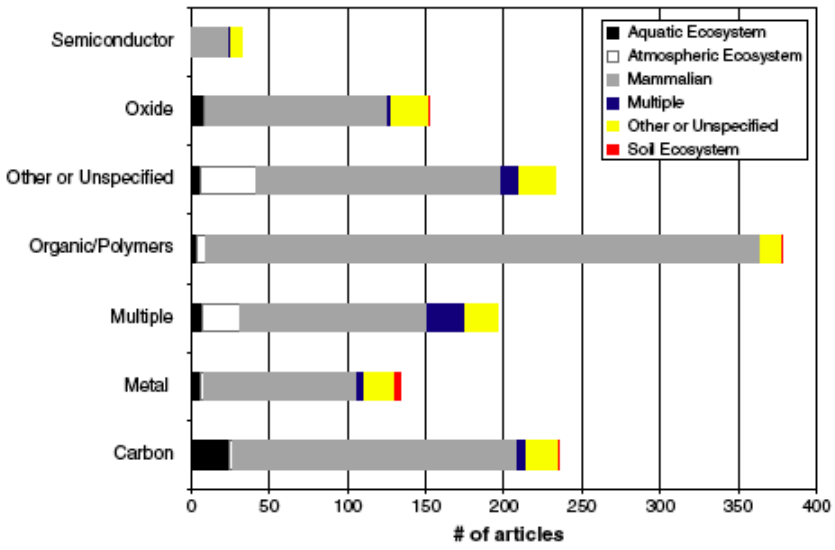

Fig. 4 Number of articles dealing with ecosystem and material type (Collected from ICON)

The range of materials being developed for use at the nanoscale is also increasing. These nanomaterials may, or may not, have distinctly different properties compared to the same chemicals presented at other than nanoscale, and these different properties may influence the health and environmental risks they pose during different lifecycle stages – from manufacture through to disposal.

This review focuses only the potential health effects and risk management associated with six nanomaterials, specified by NICNAS to be of particular interest in an Australian context. These six nanomaterials are:

zinc oxide (ZnO)	titanium dioxide (TiO_2)
cerium oxide (CeO)	carbon nanotubes (CNTs)
fullerenes (C60)	nanosilver

It is intended that this review will complement other recent (2006-09) reviews of relevant nanomaterials and several other reviews which preceded them. The reviews specifically identified by NICNAS as being complementary and therefore out of scope for the current review are:

- *A Review of the Toxicology and Health Hazards Associated With Engineered Nanomaterials* – a 2008 report prepared by Toxikos Pty Ltd for Safework Australia (draft dated 10 December 2008 provided to me)
- *Evidence on the effectiveness of workplace controls to prevent exposure to engineered nanomaterials* – a 2008 report prepared by RMIT University for the Department of Education, Employment & Workplace Relations (DEEWR)
- *A review of the scientific literature on the safety of nanoparticulate titanium dioxide or zinc oxide in sunscreens* – a 2006 report prepared by the Therapeutic Goods Administration
- *EMERGNANO: A review of completed and near completed environmental health and safety research on nanomaterials and nanotechnology* – a 2009 report prepared by the Institute of Occupational Medicine (IOM) for the UK Department of the Environment Food and Rural Affairs (DEFRA), and
- *Examining the British Safety Institute Guide on Safe Handling & Disposal of Manufactured Nanomaterials* – a report under preparation by the Monash Centre for Occupational & Environmental Health (MonCOEH) for the Department of Education, Employment & Workplace Relations (DEEWR).

This means that the present review represents only a snapshot of relevant literature over a roughly 20 month period, from late 2007 to September 2009. It does not purport to be a comprehensive summary of all known facts about the health and safety of nanomaterials, since it deliberately avoids considering papers which were cited in the above reviews, and which generally surveyed the toxicological literature up to the middle of 2008 or more recent OHS-related literature.

An important objective of this current review is to summarise new information which has come to light since the publication of the previous reviews, and to identify where significant gaps in knowledge may remain.

The literature search was undertaken primarily using standard databases (e.g. PubMed) using the following search terms:

nanotoxicology	nanoparticle + toxicity
nanoparticle + health effects	nanoparticle + risk assessment
nano zinc oxide	nano cerium oxide
nano titanium dioxide	fullerenes
carbon nanotubes	nanosilver

A significant source for many citations was the International Council on Nanotechnology (ICON) database on recent research (http://icon.rice.edu/virtualjournal.cfm) and the EMERGNANO database on research. In addition, recent issues of key journals were surveyed to find papers which may not have been picked up yet in the above databases. These key journals were:

Nanotoxicology	J. Nanoparticle Res
Nature – nano	Particle & Fibre Toxicol
Nanomedicine	Env. Sci. Technol.
Toxicol. Sci.	J. Toxicol Sci
Inhalational. Tox.	J Nanosci. Nanotech.

The bibliography for the review had a publication cut-off date of 13 September 2009. While the searches and databases originally yielded several hundred references, these were narrowed down to the 264 papers which this reviewer considered to be relevant for the project and which were referred to NICNAS. Of these, NICNAS prioritised 48 papers as requiring special attention. This review report ultimately cites only 204 of the more relevant papers from the bibliography. The cited papers are arranged in numerical order of their citation.

2. General reviews and commentaries

Many reviews and commentaries outlining the current status of health and safety assessment of NMs have been published in the past 20 months[2-21]. Some focus more on occupational exposure as the most significant source of NM exposure[22-28], while others comment on potential health effects of deliberately administered nanomedicines[29-33], the safety of specific types of NMs (e.g. CNT toxicity[34-38], and nanosilver toxicity[39-41]), nanomaterials used in food production[42], the development of regulatory regimes[43-47], membrane transport and skin absorption[48-50], and community perceptions of risks[51-52].

Genaidy *et al* (2009)[53] used a meta-analysis approach to review the toxicity literature on CNTs, and graded the studies (from very poor to excellent) in terms of their methodology, result reporting and utility in defining risk characteristics. They concluded that, while studies generally defined a potential for cytotoxicity, few studies reach a level of quality which could be useful for putting research findings into practical risk management.

Tervonen *et al* (2009)[54] proposed a risk-based classification system for NPs to assist their regulatory assessment. The proposed classification system is based on a multi-criteria decision making approach which uses performance metrics that measure both the toxicity and physico-chemical characteristics of the original materials, as well as the expected environmental impacts through the product life cycle. The performance metrics were: particle size, bioavailability, bioaccumulation, toxicity potential, agglomeration, reactivity/surface charge and the presence of critical functional groups. Some of these could be expressed quantitatively, while others required subjective assignment of ordinal scores using expert judgement.

Mailander & Landfester (2009)[55] reviewed the mechanisms by which NPs interact with cell membranes. A significant finding in their review was that cellular uptake is not only influenced by NP size, but that surface modification (positively or negatively charged side groups, or adsorption of amino acids, peptides and proteins) is an important factor in enhancing cellular uptake. Elder *et al*, 2009[48] also reviewed the surface and size characteristics of NPs which influence their ability to penetrate cell membranes (including layers of the skin) and commented that skin is a fairly solid barrier to the passage of most types of NPs. However, prolonged exposures over time may result in small amounts reaching immune-competent cells or the microcirculation.

Nel *et al* (2009)[56] reviewed the biophysical interactions which can occur at the interface between NPs and biological systems. They detailed the nature of forces which influence binding of proteins and other ligands and the potential impacts of these interactions on NP solubility, membrane transport and imaging possibilities. The review includes table and diagrams which illustrate how such surface modifications have modified NP toxicity. The authors speculate that better understanding of such interactions may enable better prediction of NP behaviour and toxicity, and to fulfil the aim of “safer-by-design”.

Simeonova & Erdely (2009)[57] picked up on the fact that UF air pollutants are known to be epidemiologically linked to cardiovascular diseases and that similar toxicity may be associated with ENPs. They reviewed the range of inflammatory cell markers released after inhalational exposure to a variety of NPs, and their possible utility as biomarkers of cardiovascular toxicity, along with genomic techniques to identify changes in gene expression patterns. While iron NPs were not one of the specified NMs to be covered in this review, a study by Apopa *et al* (2009)[58] showed that one of the effects of such NPs

was the ROS-dependent increase in the permeability of human microvascular cells. This suggests a potential mechanism for other NPs to influence cardiovascular toxicity. Other studies on cardiovascular toxicity of NMs specified for this report are summarised in Section 9.2.

2.1 Definitional and mechanistic aspects

The working definition of nanomaterials emphasises that the dimensions of at least one plane of the NP should be between 1 and 100 nanometres. CNTs fall within such a definition on the basis of diameter, even though their length may exceed this figure manyfold.

It is also axiomatic that the very small NP size, along with their surface characteristics and different surface activity, confer unique properties on NMs, as compared to materials of the same chemical composition, but of larger size. This paradigm drives the call for a separate HRA process for NMs, since it presumes that these unique characteristics of NPs may translate into enhanced or unusual toxicological properties.

While there is an ongoing international debate on whether the 100 nm size boundary should be extended to 200 or even 300 nm, a contrary view has been put forward. Auffan *et al* (2009)[59] propose that novel size-dependent properties should be a more important determinant than simply NP dimensions alone, and that, at least in regard to metallic oxide NPs, only those with a size <20-30 nm are likely to have a sufficiently different toxicity profile to warrant a separate HRA. This is based on an assumption that only NPs <20-30 nm have a substantial proportion of their molecules on the surface and give rise to different crystallinity and thermodynamic properties. Auffan *et al* (2009)[60] further postulated that chemically stable metal oxide NPs would have little or no cytotoxic potential, while those which are able to be oxidized, reduced or solubilised are more likely to be cytotoxic or genotoxic. This is partly due to the propensity for surface catalytic and redox effects to favour the generation of ROS.

Several reviews[15,61-65] have addressed the mechanisms of cytotoxicity associated with NPs, including the ability of NPs to generate ROS, induce inflammatory responses, initiate apoptosis and have direct effects on cell membranes. Most of these findings are extrapolated from *in vitro* studies using cell cultures of different types, but including many where human cell types have been used. Other reviews[30,66] address the potential for enhanced NP cytotoxicity to combine with enhanced delivery to target sites. The potential genotoxicity of NPs has also been extensively canvassed in review articles[67,68], as well as studies on individual NPs (see section 5).

Cytotoxicity mechanisms independent of ROS-mediated effects have been explored for several NPs. An important finding by Frohlich *et al* (2009)[69] was that some types of NPs (they used polystyrene NPs) could generate cytotoxicity by mechanisms involving apoptosis and/or effects on cell membranes and cell proliferation, at exposure levels lower than those required for ROS generation. Some of the NMs of concern to NICNAS also seem to be able to induce apoptosis as a critical pathway leading to cell necrosis (see Section 4).

Given the rapid advancements being made in the genomic techniques in toxicity assessment (toxicogenomics, proteonomics), it is perhaps surprising that a genomic approach has not been advocated to a greater extent in addressing problems in nanotoxicology. Haniu *et al* (2009)[70] have used such an approach, and while there remain

many difficulties in interpreting the outcomes of such studies, they found that proteonomic techniques were uniquely sensitive to carbon black NPs, with some proteins associated with metabolism, response to stress, signal transduction and cell differentiation being turned in human monoblastic leukaemia cells at quite low exposure levels. See also Section 3.2.1 for a mouse inhalational study with nanosilver, and Section 3.2 for a mouse intratracheal instillation study with TiO_2, where the toxic outcomes were assessed using a microarray genomic technique. Erdely *et al* (2009)[71] have referred to the utility of genomic assays demonstrating activation or alteration of key genes and proteins as possible biomarkers.

Another approach has been the use of a genetically modified mouse model to enhance sensitivity to toxicity. Jacobsen *et al* (2009)[72] describe the differential toxicity of five different NPs (CB, fullerenes, SWCNTs, gold and quantum dots) after intratracheal instillation in apolipoprotein knockout mice (ApoE-/-) and normal C57 mice.

Vesterdal *et al* (2009)[73] also used apolipoprotein knockout mice (ApoE-/-) to show that the extent of atherosclerosis in aging ApoE-/-mice influenced vascular responsiveness to fullerenes. Acetylcholine-induced endothelium-dependent vasorelaxation of isolated aortic strips was inhibited to a greater extent in the younger mice, while the phenylephrine-induced vasoconstriction response was increased, 1 hr after IP injection of C60 (0.05 or 0.5 mg/kg).

2.2 Dose-response metrics

The most appropriate metric for constructing dose-response relationship has also been the subject of extensive discussion, both during the past 20 months and from the earliest papers on nanotoxicology. Sager *et al* (2008, 2009)[74, 75] have elaborated on the importance of using surface area as a dose metric, rather than mass, in some comparative studies of the intratracheal toxicity of fine and ultrafine CB in rats, compared to TiO_2 NPs. The transient inflammatory and cytotoxic responses seen after ultrafine CB installation were approximately 65 x greater than that seen after fine CB, when expressed on a mass basis. However, the differences were largely abolished when expressed on a surface area basis. The same group found similar findings when fine and ultrafine TiO_2 particles were administered by intratracheal instillation and the results expressed on a mass or SA basis. Comparison between UF CB and TiO_2 NPs showed that, when administered on a comparable SA basis, the inflammatory responses were similar on day 1 after dosing, but only the TiO_2 doses produced a prolonged inflammatory response.

2.3 Tracking and characterising NPs

One challenge which is common to many NPs is the difficulty, or impracticality, of developing analytical techniques which permit the tracing of NP distribution through cells and tissues after *in vivo* exposure. Gul *et al* (2009)[76] propose the use of a fluorescently labelled functionalised and physically stable (i.e. non-aggregating) NP of polyvinyl alcohol (PVA) to facilitate NP tracking in distribution and pharmacokinetic studies. They contend that the PVA NP cores are devoid of some of the troublesome aggregating and biomembrane interactive effects of commonly used latex NPs.

Cheng *et al* (2009)[77] describe an approach to visualisation of MWCNT penetration through cell membranes of human macrophages, using 3D-dark field scanning TEM, in conjunction with confocal microscopy and SEM. The important thing about this study, apart from the

novel visualisation technique, was that it was able to differentiate cytotoxicity associated with purified MWCNT and the Fe_2O_3 impurities which commonly persist in unpurified materials. Fe_2O_3 was not cytotoxic in these studies, while purified MWCNTs were cytotoxic. They also demonstrated that the oxidative stress which led to the cytotoxicity was due to partial piercing of the cell membrane by MWCNT fibres, with incomplete phagocytosis of the cells. On the other hand, Elgrabli *et a*l (2008)[78] showed that intratracheally-instilled MWCNTs are biopersistent in lung tissue for at least 6 months, but do not cross the pulmonary barrier.

The importance of characterising NPs used in experimental studies and occupational exposure scenarios has been emphasised time and time again. The importance of controlling, or at least considering, the effects of NP aggregation or agglomeration has been well reviewed by Pauluhn (2009)[79] who has also pointed out the importance of understanding the biopersistence of the administered NPs. NPs generally have an innate medium-dependent tendency to aggregate in air and in biological media, so that their size and surface characteristics can undergo significant change. Baveye & Laba (2008)[80] criticised a published inhalational toxicity study of TiO_2, on the basis that the inhaled material appeared to be extensively clustered, while it was claimed to be dispersed. This was despite attempts made by the authors to ensure a dispersed suspension, using sonication in suitable solvents.

Elgrabli *et al* (2008)[81] noted disparate findings in some published toxicity studies where MWCNTs were administered by inhalation. Some unexpectedly failed to demonstrate pulmonary toxicity seen commonly after intratracheal instillation. They postulated that this may be due to lack of control over aggregation. When albumin solutions were used to minimise aggregation, they were able to demonstrate the expected profile of pulmonary toxicity.

The importance of controlling, or at least specifying, the dispersion medium in *in vitro* experiments has been well highlighted by Tabet *et al* (2009)[82] and is also discussed elsewhere in this report (section 11.1). Tabet *et al* found that MWCNTs formed agglomerates on the surface of human epithelial cell cultures, but that the agglomerates were much larger and more numerous in phosphate-buffered saline dispersion fluid, compared to fluids containing dipalmitoyl lecithin (a component of pulmonary surfactant) or ethanol. MWCNTs caused decreased cellular metabolic activity, similar for all dispersants, but there was no evidence of cellular internalisation or oxidative stress. In contrast, both CB and asbestos were internalised, but only asbestos produced expected toxic effects on cell metabolism and apoptosis.

2.4 Towards a more refined toxicity testing approach

A common theme in many of the review articles and commentaries which have been published over the past 20 months is that there remains much work to be done to develop appropriate *in vitro* and *in vivo* tests which could assist with human health risk assessment of NMs. Given the known limitations of *in vivo* tests which use exposure routes inappropriate for HRA (e.g. intratracheal installation; IP injection) and inhalational studies where the generation of respirable NP dispersions is influenced to a great extent by the choice of dispersion media or method of aerosol generation, there is a need for the development of a well-standardised test battery, preferably one formalised by international agreement (Warheit *et al* 2009[83]). The development of such a battery of useful *in vitro*

tests has been reviewed in depth[15, 84-87] including consideration of the vast methodological difficulties.

Meng *et al* (2009)[88] have set this out quite explicitly, noting that the predictive efficacy of such a test battery will depend on choosing tests which consider how the physico-chemical characteristics of NPs could influence the cellular and molecular pathways which lead to pathogenic diseases.

Lu *et al* (2009)[89] evaluated the ability of a battery of *in vitro* tests to predict the inflammatory toxicity of a range of 13 metal oxide NPs. The exposure doses were equalized for surface area. They found that the relative potency to produce ROS *in vitro* did not correlate well with inflammogenic responses *in vivo,* with 4/13 inducing ROS but only one (NiO) producing a significant inflammatory response when instilled intratracheally in rats. In contrast, alumina NPs were inflammogenic, but produced little ROS.

Donaldson *et al* (2009)[90] pointed out that *in vivo* pathogenic responses to three different types of particulates (PM_{10}, asbestos and quartz) result in a broad range of adverse health outcomes in the lungs and cardiovascular system, but their *in vitro* cytotoxicity, mediated by oxidative stress, is not so readily differentiated.

Walker & Bucher (2009)[91] reviewed the strategies for evaluating the health hazards of NMs in the context of recent developments in toxicity testing, under the US NRC "Toxicity Testing in the 21st Century" program. They pointed out that, while the new NRC approach emphasises the gradual replacement of *in vivo* toxicity tests with *in vitro* tests, genomic approaches and better understanding of mode of action and the use of information technology to describe patterns of toxic behaviour, these approaches may not be so fruitful to help unravel the complexities involved in predicting the toxicity of nanomaterials

It has also been proposed (Erderly *et al* 2009)[71] that information on the array of biological responses after NP administration may have utility in developing suitable *in vivo* biomarkers of toxic response. Those responses involving activation of cytokine pathways of immune system may be particularly useful.

3. Chronic inhalational toxicity studies

It is generally accepted that chronic exposure studies underpin the development of health-based exposure standards. Where these are based on animal studies, they should be conducted using a route of exposure relevant to HRA. The inhalational route is the one considered most relevant to undertaking HRA for NPs in the occupational setting, although there is always some potential that dermal exposure may be relevant in some settings.

There have been relatively few inhalational toxicity studies done during the past 20 months using even multiple exposures, let alone chronic exposure, for any of the NMs of interest. Those that have come to light are discussed under the individual NMs of interest.

A more common study design for most NP inhalational toxicity studies is where the exposure is short term (sometime just a single dose), with observations of the time-dependence of the development of the toxic responses followed over periods up to 90 days.

Because of the significant interest generated by the studies by Poland *et al* (2008)[92] (reviewed by Toxikos) which demonstrated asbestos-like pathological effects of CNTs after IP injection, the potential for CNTs to mimic the adverse effects of chronic asbestos inhalational exposure is discussed in more detail in Section 8.1.

The issue of potential NP carcinogenicity has also been addressed in a review article by Roller (2009)[93] who noted that although IARC found inadequate epidemiological evidence to classify CB and TiO_2 as carcinogenic, the potential for such biopersistent NPs to increase the risk of lung cancer in exposed workers should not be underestimated.

3.1 Studies with ZnO

No new repeat inhalational exposure studies with ZnO were identified during the 20 month period of interest for this review.

3.2 Studies with TiO_2

Some significant inhalational toxicity studies done with TiO_2 have been published in the past 20 months.

Van Ravenzwaay *et al* (2009)[94] compared the uptake and systemic distribution of three types of particulates in mice after 5 days of 6/h day nose-only inhalation. The doses used were 100 mg/m^3 nanoscale TiO_2 (20-30 nm mixed anatase/rutile), 250 mg/m^3 pigmentary TiO_2 (200nm rutile) and 100 mg/m3 quartz dust (315 nm). The BET comparisons showed higher values for the TiO_2 NPs (48.6 m^2/g) compared to the other two particulates (6 and 5.9 m^2/g). Most of the inhaled TiO_2 was deposited in the lung, with some transference to mediastinal lymph nodes (pigmentary>nanoscale>quartz). In contrast, nano-TiO_2 injected intravenously accumulated mainly in the liver and spleen. The inhalational studies produced relatively mild neutrophilic inflammation and macrophage activation, which comparable for all three particulates. The lung changes were reversible for pigmentary and nanoscale TiO_2, but progressive for quartz.

Ma-Hock *et a*l (2009)[95] proposed that shorter-term inhalational studies could predict toxicity which might occur with more chronic (e.g. 90-day) exposures. They exposed male rats (head-nose only) to respirable dusts containing TiO_2 NPs (2, 10, 50 mg/m^3) 6 h/day for 5 days, using a special dust generator designed to deliver disaggregated NPs (<3000nm). They demonstrated that adverse pulmonary effects (increased lung weight, inflammation, increased bronchial cell proliferation) were comparable to those reported in a similar 90-day exposure study using female F344 rats (Bermudez *et al* 2004 – reviewed by Toxikos), although lung TiO_2 deposition was somewhat higher in the 90d study. More importantly, they demonstrated that the LOAEC (2 mg/m^3), based on increased terminal bronchial cell proliferation, was the same in both the 90d and 5d studies.

Although it was not a chronic study, a paper by Park *et al* (2009)[96] is worth noting because of the use of genomic techniques to assess toxicity. They administered a single dose of TiO_2 NPs by intratracheal instillation in mice (5, 20, 50 mg/kg). They demonstrated inflammatory pulmonary responses at day one, sustained for up to 14 days, with the dose-related induction of T-helper type cytokines. Using microarray genomic techniques, they also demonstrated the induction of multiple genes associated with antigen presentation and the induction of chemotaxis of immune cells.

3.3 Studies with CeO

No new repeat inhalational exposure studies with CeO were identified during the 20 month period of interest for this review. It seems unlikely that such studies have been done previously with this NP either.

3.4 Studies with CNTs

The only studies identified over the relevant 20 month period of this review were done to explore the repeated dose toxicity of MWCNTs. There were none identified which used SWCNTs.

3.4.1 Ninety-day rat study

Ma-Hock *et al (*2009)[97] studied the toxicity of MWCNTs administered by head/nose-only inhalation of generated aerosols 6h/day, 5 d/wk over 90 days (total 65 exposures) at three dose levels (0, 0.1, 0.5 & 2.5 mg/m^3). The study design was essentially that of OECD test guideline 413. It was claimed that the respirable material was generated from commercial MWCNTs using a proprietary brush generator which neither damaged the CNTs nor altered their ROS-generating surfaces. They used transmission electron microscopic (TEM) images to show that the MWCNTs were essentially the same in appearance before and after passing through the dust-generator, although scanning electron microscopy (SEM) images of material recovered from the test chambers suggested significant wool-like clumping had occurred, along with “hairy” surfaces on the CNTs.

The more important outcome of the study was that it revealed no systemic toxicity at any dose level, although the expected inflammatory lung toxicity was seen, characterised by increase lung weight, granulomatous inflammation, diffuse histiocytic and neutrophilic inflammation and intra-alveolar lipoproteinosis. The effects were dose-related, but since the lowest dose still showed minimal granulomatous inflammation, the study failed to demonstrate a clear NOAEL.

It is difficult to judge whether this study really contributes useful information for the HRA of MWCNTs or the development of exposure standards. The authors claimed that MWCNTs have low dust-forming potential and it is clear that they needed to extensively modify the MWCNT material in order to generate the respirable dusts.

3.4.2 Thirty- and 60-day mouse studies

A 30- and 60-day inhalational exposure study with MWCNT in mice was reported by Li *et al* (2009)[98]. However, since the study was only available in abstract form, with few details of the experimental design other than the exposure regimen (6 hr exposure to 32.6 mg/m^3 every second day), it is difficult to conclude anything about the results, which were reported to be severe pulmonary toxicity after 60 days, but not after 30 days.

3.4.3 Fourteen-day mouse study

Mitchell *et al* (2009)[99] studied the immunological responses in mice after exposure to MWCNTs in whole-body inhalation chambers. The dose rates were 0, 0.3 and 1 mg/m^3, 6 hr per day for 14 consecutive days. Mice exposed at 1 mg/m^3 had suppressed immune function associated with splenic activation of cyclooxygenases. This effect was partially reversible by treatment with ibuprofen, and involved signalling proteins released from the lung. Knockout mice with no splenic cyclooxygenase function were resistant to the effects of MWCNTs and did not respond immunologically to MWCNTs. The NOAEL dose was 0.3 mg/m^3 in this experiment.

3.5 Studies with fullerenes

Fujita *et al* (2009)[100] studied gene profile expression in rat lung after whole-body inhalational exposures to fullerenes NP aerosols (0.12 mg/m^3; 4.1 particles/cm^3; 96 nm diameter) 6 h/day; 5 days/week; 4 weeks. UF NiO particles were used as a positive control.

C60 NPs were localised in alveolar epithelial cells and engulfed by macrophages. Only some genes associated with immune system responses were up-regulated. In contrast, NiO upregulated genes across a broad spectrum, including chemokines, oxidative stress, suggesting that the inflammatory response with NiO was greater than that with C60.

The conclusion drawn from this study was that fullerenes have a relatively low potential to produce pulmonary inflammatory responses after repeated inhalational exposure, in comparison to metal oxide NPs.

3.6 Studies with nanosilver

3.6.1 Two-week inhalational toxicity study in mice

Lee *et al* (2009)[101] treated mice (n=7 per group) by nose-only inhalation 6 hr/day, 5 d/week for 2 weeks using silver NPs (1.91 particles/cm^3) prepared in a particle generator. The nanosilver was well characterised. The experimental design was based on a previous rodent studies which proposed the olfactory bulb as an important pathway for nanosilver intake into the CNS, leading to significant neurological damage. Half the treated mice were tested at the conclusion of 2 weeks exposure, while the remainder were allowed a 2-week recovery period.

An Affymetrix Mouse Genome Array assay was used to identify genes which were expressed in different parts of the brain. A total of 486 genes in cerebrum and 952 genes in the cerebellum were identified as nanosilver responsive, with genes associated with neurodegenerative disease and immune function being the most sensitive. However, there was no evidence of any histopathological changes in the brains of the mice studied. PCR on blood indicated a correlation between gene activation and neurotoxicity. No data were presented indicating whether or not the brain gene expression results were reversible in the recovery group.

3.6.2 Thirteen-week inhalational toxicity study in mice

Sung *et al* (2009)[102] exposed rats (n=10 per group) in whole-body inhalation chambers 6 hr/day, 5 d/week for 13 weeks using silver NPs (8-19 nm) at dose rates 0.6, 1.4 and 3 million particles/cm^3 (equivalent to 49, 133 and 515 $\mu g/m^3$). The toxicity target organs, consistent with measured silver deposition, were lungs and liver, with dose-related inflammatory cell infiltrates, chronic alveolar inflammation and small granulomatous lesions, and bile duct hyperplasia. While there was evidence of some dose-dependent silver accumulation in olfactory lobes, brain and kidneys, there was no apparent toxicity in these organs. The rounded estimate of the NOAEL established in this study was approximately 100 $\mu g/m^3$, which the authors noted was comparable to an established ACGIH TLV of 0.1 mg/m3 for silver dust.

Hussain & Schlager (2009)[103] editorialised on the importance of the Sung *et al* study, noting that while the exposure conditions were probably much higher than those likely to be encountered in workplaces where airborne nanosilver exposure might occur, the study represents a first step towards developing a health-based occupational exposure standard for nanosilver.

4. *In vivo* and *in vitro* toxicity studies

Many *in vivo* toxicity studies continue to use a single dose design, although toxicity assessment may be followed over longer periods of time.

There continues to be a substantial effort put into *in vitro* tests (exposing cell cultures of different types to NMs). This is despite the difficulties of relating the results of such tests to effects of the NMs *in vivo* and the acknowledgement that *in vitro* tests generally have a very limited role to play in assisting HRA (Park *et al* 2009)[85] and that they do not necessarily reflect the complexity of *in vivo* mechanisms (Jones & Grainger 2009)[104]. At best, the *in vitro* tests may be capable of providing some insight into relative cytotoxicity potential, although this does not necessarily reflect comparative *in vivo* toxicity potential. They may also be useful for elucidating whether generation of reactive oxygen species (ROS) plays an important mechanistic role, or whether other cytotoxic mechanisms may be equally, or more important (e.g. induction of apoptosis).

To illustrate the need to be wary of artefactual results in poorly conceived *in vitro* studies, Horie *et al* (2009)[105] described NP-induced cytotoxicity in cultured cells which could be attributed (at least in part) to depletion of proteins and Ca^{++} from incubation media by direct adsorption onto NP surfaces.

Differentiation of *in vitro* toxicity due to NPs and their solubilised components is rarely attempted. In the Park *et al* (2008)[106] study of CeO NP toxicity (see Section 4.2.1) one of the experiments with cultured A549 human epithelial cells compared the cytotoxicity of several metal oxide NPs (CuO, ZnO and TiO_2) with their soluble chloride salts. The results showed that the soluble metal salts were more cytotoxic than their NP counterparts (LC50s for the three NPs were 300, 375, and 1600 μM, while for the chloride salts the LD50s were 150, 100 and 600 μM respectively). Neither CeO NPs nor microparticulate CeO were cytotoxic at 1000 μM.

Haemolysis of human erythrocytes incubated with NPs *in vitro* has been suggested as a potentially sensitive method of assessing cytotoxicity, although in a study which used this method (Aisaka *et al* 2008)[107], it surprisingly found that micron-scale (<5000 nm) anatase-type TiO_2 was more potent than either amorphous (<50 nm) or rutile (<5000 nm) forms, and in turn the micron-scale anatase form was almost equipotent with the nanoscale TiO_2 (<25 nm). However, haemolytic effects were abolished by the presence of plasma, suggesting that this type of toxicity would be unlikely to occur *in vivo*.

4.1 Studies with metal oxides

4.1.1 *In vivo* studies

Liang *et al* (2009)[108] demonstrated that a single intratracheal instillation of TiO_2 NPs (surface area SA 50 m^2/g and 250 m^2/g) at 0.5, 5 or 50 mg/kg bw produced no obvious hepatic or renal toxicity at 7 days. However, there were some relatively minor differences in toxicity of the high and low SA TiO_2 - lipid peroxidation, observed as elevated malondialdehyde (MDA), but only with the material having the higher SA.

In contrast to these results, Ma (2009)[109], who had previously reported liver damage in mice after administration of anatase TiO_2 NPs, found that repeated IP injections of anatase

TiO_2 NPs (5 nm; 5-150 mg/kg bw) in mice produced clear dose-dependent inflammatory lesions in the liver. There were elevations of plasma enzymes indicative of liver disease and evidence of apoptosis and histopathological changes as well. Similar toxicity, although less marked, was seen in mice injected with 150 mg/kg non-nano anatase TiO_2.

In a rare study which used an oral route of NP administration, Wang *et al* (2006)[110] compared the systemic and gastrointestinal toxicity of nanoscale ZnO (58 nm) compared to microscale ZnO (1080 nm). Both were administered as a single 5g Zn/kg bw dose by gavage, and the mice observed for 14 days. The doses used were remarkably high, and they produced significant hepatic and renal toxicity, with less marked gastrointestinal inflammation. There were two deaths associated caused by gastrointestinal obstruction with aggregated NPs. Given the very large doses used, and lack of discrimination between nano- and micro-scale ZnO, it is unlikely that this study adds information useful for HRA.

Finally, in a study which used an IV route of administration to bypass absorption barriers and enable tissue distribution to be studied, Fabian *et al* (2008)[111] showed that TiO_2 NPs (5 mg/kg bw IV) produced highest tissue accumulation in liver>spleen>lung>kidney, with no detectable accumulation in blood (cells or plasma), brain or lymph nodes. Importantly, this direct route of administration showed no inflammatory response or systemic toxicity.

Chen *et al* (2009)[112] reported a wide range of systemic toxic effects in mice after IP injection of TiO_2 NPs (~100nm). However, the doses used were huge (324-2592 mg/kg). While the TiO_2 was shown to distribute into several tissues (notably spleen, liver and lungs) and there were suggestions of substantial acute systemic toxicity, the utility of this study to inform HRA is limited.

4.1.2 *In vitro* studies

Hussain *et al* (2009)[113] studied the cytotoxic effects of two chemically distinct NPs, carbon black (CB) and titanium dioxide (TiO_2), on a human bronchial epithelial cell line. They reported that size differences of even few nanometres in primary particle size lead to significant changes in inflammatory and oxidative stress responses. Oxidative stress was well correlated with NP surface area and the extent of intracellular uptake of the NPs. Inflammatory effects were also related to SA and oxidative stress.

Similar findings were reported by Lin *et al* (2009)[114] using ZnO NPs (70-420nm) in A549 human bronchiolar carcinoma-derived epithelial cells. The toxicity was shown to be associated with oxidative stress, ameliorated with N-acetylcysteine, and not dependent on free Zn^{++} release.

4.1.3 Photogenotoxicity

Given that both ZnO and TiO_2 NPs are used in sunscreen preparations, it is of interest to know whether photoactivation with UVA light influences their toxicity.

While Lu *et al* (2009)[115] did not assess photogenotoxicity with TiO_2 NPs, they did demonstrate that, under UV irradiation, TiO_2 NPs (commercially available anatase and Degussa), both strongly potentiated tyrosine nitration of bovine serum albumin and mouse skin homogenates *in vitro*. Rutile NPs were much less potent. The implication was that two of the commercial TiO_2 NPs could be implicated in photosensitization reactions with commercial sunscreens. The authors conceded that more work needs to be done to verify this potential toxicity.

Photogenotoxicity, as well as cytotoxicity and skin/eye irritancy potential , was assessed by Park *et al* (2009)[116] for a TiO_2 NP modified by incorporating 0.7% manganese into the

crystalline lattice structure. It was developed as a commercial sunscreen ingredient (Optisol®). Cytotoxicity and phototoxicity were essentially negligible, and it compared favourably in such tests with other commercially available coated TiO_2 NPs.

Gopalan *et al* (2009)[117] used human sperm cells and lymphocytes and the Comet assay to investigate this phenomenon. They found evidence of photogenotoxicity, in the absence of frank cytotoxicity, for both NPs and that both pre- and concurrent- irradiation with UVA enhanced the reduction in DNA content compared to incubation in the dark. The effects were more marked with lymphocytes than with sperm.

Theogaraj *et al* (2007[118] compared the genotoxicity of 8 different classes of TiO_2 NPs in CHO cells, with and without UV light irradiation. None of the TiO_2 NPs produced any clastogenicity (chromosomal aberrations), irrespective of UV irradiation.

The endpoints (clastogenicity and DNA damage) are different in the above two studies, suggesting that if TiO_2 is photo-activated, the outcomes may be relatively specific.

4.2 Studies with CeO

CeO NP toxicity is probably mediated much like other metal oxides. Only two *in vitro* studies with CeO were identified as published during the period of this report.

Eom *et al* (2009)[119] showed that in cultured human bronchial epithelial cells, the effects of CeO NPs are mediated by ROS inducing p38-Nrf-2 signalling transcription pathways which activate haem oxygenase.

The other study (Gojova *et al* (2009)[120]) on cardiovascular cells is discussed in Section 9.2

4.2.1 HRA for CeO

The study by Park *et al* (2009)[106] is potentially more important, because it attempted to correlate *in vivo* and *in vitro* toxicity endpoints with estimates of likely human exposure associated with the use of CeO NPs as a diesel fuel additive. In other words, it was an attempt to carry out a classical HRA.

The study used UK air monitoring data, from cities where CeO had been introduced to diesel fuel of local buses, to estimate likely inhalational exposures. Modelled estimates were converted into estimated human equivalent internal doses for comparison with LC_{50} and NOAEL figures from *in vitro* toxicity studies. The CeO materials studied were characterised as NPs 9 nm mean particle size, BET surface area 94.7 m^2/g and these were compared with conventional CeO particles of size 320 nm and BET 2.74 m^2/g. Both CeO materials were assessed as non-irritant in an *in vitro* EpiDerm skin irritancy test, non-mutagenic in the Ames test, and non-cytotoxic to L929 cells. Cultured rat lung cells were exposed to CeO aerosols for 3 hr to assess cell viability and effects on cellular antioxidant and energy production functions. There was no significant toxicity with 140nm NP aggregates at exposure levels of 14, 76 or 157 mg/m^3. Similarly, there was no cytotoxicity observed with either CeO particulates at up to 100 $\mu g/mL$ in cultured A549 cells.

The overall conclusion was that the internal dose exposure estimates to human lung cells (from 1.26×10^{-4} to 3.8×10^{-7} cm^2/cm^2 based on NP surface area conversion) were well below the levels showing toxicity. The comparator was internal doses of 26.75 cm^2/cm^2 calculated from the highest NOAEL in the *in vitro* studies. This demonstrates acceptable safety for the specified use of CeO NPs. In fact, the authors suggested that addition of

CeO NPs to diesel fuel may reduce the level of airborne particulates associated with diesel exhaust, and possibly reduce overall health impacts of such pollution.

4.3 Studies with CNTs

4.3.1 *In vivo* studies

Tong *et al* (2009)[121] compared the cardiopulmonary toxicity of SWCNTs and UF CB in mice. The study used oropharyngeal administration of 20 or 40 μg of SWCNTs and UFCB, with and without acid functionalisation (AF) of the particle surfaces. Pulmonary inflammatory responses and cardiac toxicity was assessed 24 hours after dosing. Significant toxicity was observed only in the high dose groups, although sporadic clumps of particulate material were observed in the airways of all groups. AF increased the pulmonary toxicity of both SWCNTs and UFCB, but cardiac toxicity (ischemia/reperfusion injury and myocardial degeneration) was only seen with AF-UFCB. The authors were unable to conclude whether the differences in toxicity associated with AF were due to changes in translocation or reflect a novel systemic cardiovascular response.

Yang *et al* (2008)[122] studied the systemic toxicity of SWCNTs after IV injection in mice (40, 200 and 1000 μg/mouse). They used Raman spectroscopy and TEM to demonstrate long-term accumulation (up to 3 months) in various tissues (mainly liver, lung and spleen), but the level of induced toxicity was mild. There was some evidence of inflammatory cell infiltration in the lungs, but no evidence of apoptosis in any organ, and only mild indications of oxidative stress.

Deng *et al* (2009)[123] examined the potential splenic toxicity of MWCNTs. There were no functional changes (RES phagocytic activity, reduced glutathione, superoxide dismutase or malondialdehyde) or observable histopathological damage after administering 60 or 100 mg/kg bw IV MWCNTs. However, MWCNTs gradually transferred from red pulp to white pulp tissue within the spleen.

MWCNTs suppress T-cell related immune responses after inhalational exposure via indirect signals to T-cells in the spleen (Elder 2009)[65].

Dermal toxicity was the focus of a study by Murray *et al* (2009)[124], where SWCNTs were tested in two skin culture systems. The response in Epiderm engineered human skin was epidermal thickening, accumulation and activation of fibroblasts, with consequent collagen formation and release of pro-inflammatory cytokines. Cultured murine epidermal cells responded with dose-dependent hydroxyl radical formation and activation of cytokines AP-1 and NFκB. However, there was a difference in the cytokine response, with AP-1 not activated by purified SWCNTs (0.23% Fe), while NFκB occurred with both purified and unpurified (30% Fe) SWCNTs. In a repeated-exposure *in vivo* component to the study using hairless mice, daily application of 40, 80 or 160 μg for 5 days resulted in various changes indicative of oxidative stress.

With a warning that impurities in manufactured CNTs can influence the outcome of toxicity studies, Koyama *et al* (2009)[125] showed that subcutaneous implantation of purified MWCNTs in mice produced significantly less immunological toxicity and alopoecia than MWCNTs which had not been purified.

Ryman-Rasmussen *et al* (2009)[126] tested the hypothesis that inhalational exposure to MWCNTs could potentiate pulmonary responses (measured as airway fibrosis) in a murine

allergic asthma *in vivo* model. A single 6 hr exposure (100 mg/m^3) confirmed the hypothesis, with significant airway fibrosis observed at 14 days. There were differential results on the release of inflammatory mediators, with platelet-derived growth factor (a fibroblast mitogen) and TGF-β1 (a collagen stimulator) being required for maximal response.

Inoue *et al* (2009)[127] reported similar findings with intratracheally instilled MWCNTs (50 μg weekly for 6 weeks) in a murine allergic asthma model, although they measured different endpoints. They found MWCNTs aggravated the pulmonary inflammatory responses, as measured by infiltration of eosinophils, neutrophils and monocytes into lung tissues, along with an increase in goblet cells in the bronchial epithelium. MWCNTs amplified lung protein levels of Th cytokines and chemokines compared to allergen alone, and exhibited adjuvant activity for allergen-specific IgG1 and IgE. An allergen-induced increase in syngeneic T-cell proliferation in vitro was enhanced at low concentration in MWCNT-treated mice. The conclusion was that MWCNT can exacerbate murine allergic airway inflammation, at least partly, via the promotion of a Th-dominant milieu. In addition, the exacerbation may be partly through the inappropriate activation of antigen-presenting cells including DC.

Extrapolation of these findings to humans (if valid) would imply that individuals with pre-existing asthma may be more susceptible to pulmonary toxicity from inhaled MWCNTs. This could be consistent with epidemiological findings of correlations between asthma severity and UF air pollutants. This appears to be an important finding to follow up.

4.3.2 *In vitro* studies

Herzog *et al* (2009)[128] compared the ROS-generating effects of two types of SWCNTs, CB and crocidolite asbestos in human lung epithelial cells *in vitro*. They found that all three produced moderate to low levels of oxidative stress, but the responsiveness was markedly dependent on the type of dispersant used.

Witasp *et al* (2009)[129] studied the effects of SWCNTs on human monocyte-derived macrophages. The SWCNTs were not cytotoxic, although they suppressed macrophage chemotaxis. Moreover, they markedly impaired macropyga engulfment of apoptotic target cells.

While there has been no speculation that CNTs may be inherently neurotoxic, their potential biomedical applications caused Bardi *et al* (2009)[130] to investigate MWCNT toxicity to cultured mouse cerebral cortex. They could find no evidence of cytotoxicity using MWCNTs coated with Pluronic F127 surfactant. While the surfactant is itself relatively neurotoxic, binding it to MWCNTs exerts a protective effect.

4.4 Studies with fullerenes

Studies assessing the potential cardiovascular toxicity, inhalational toxicity and genotoxicity of fullerenes have been summarised elsewhere in this report (see Sections 2.1, 3.5 and 5.4).

4.4.1 *In vivo* studies

Fullerene injected directly into the brain of mice produced neurotoxicity in the form of increased locomotor activity and modified various brain neurotransmitters (Yamada *et al*

2008) [131]. The fact that no such findings occurred when C60 was injected IP suggests that the NP was unable to cross the blood-brain barrier.

A rather bizarre effect of fullerenes was reported by Zhou *et al* (2009)[132]. They found that fullerenes enhanced hair follicle numbers and promoted hair growth after topical application to shaved mouse skin. The effects were even more marked in genetically "bald" (SKH-1) mice. Similar hair follicle promotion was reported for cultured human skin. The biological mechanism remains obscure, although it could involve activation of cell signalling pathways which regulate hair follicle turnover.

The implications for HRA are also obscure, but there is an obvious potential for a therapeutic application.

4.4.2 *In vitro* studies

Since fullerenes suspended in tetrahydrofuran (THF) have generally been shown to be more toxic than native C60, Zhang *et al* (2009)[133] investigated THF formulation effects. They demonstrated the formation of a reactive peroxide which could account, at least in part, for the enhanced toxicity of THF/C60 compared to native C60 NPs. This further emphasises the potential for *in vitro* studies with NPs to result in toxicity which would not necessarily occur *in vivo*. Similar findings were reported by Spohn *et al* (2009)[134] using A549 cells and *Daphnia magna*.

4.5 Studies with nanosilver

The three reviews of nanosilver toxicity[39-41] all enunciate the enigma of whether the toxicity of silver NPs can be differentiated based on attribution of some effects to the release of soluble silver ions, or to the NP surface characteristics, or to a combination of the two.

Apart from inhalational toxicity studies described in Section 3.6, *in vitro* studies were the only other nanosilver toxicity identified as published during the period of this report.

The common effects of PVP-coated silver NPs (69 nm) and silver ions was demonstrated by Foldbjerg *et al* (2009)[135] in an *in vitro* study with THP-1 cells (a human acute monocytic leukaemia cell line). Both forms of silver induced apoptosis and necrosis via intracellular ROS generation and there was no basis for differentiating between the two forms, other than silver ions were slightly more potent.

Arora *et al* (2009)[136] and Asharani *et al* (2009)[137] both used TEM to demonstrate the entry of silver NPs into the cytoplasm and mitochondria of cultured cells. However, there were differences in the cytotoxic response, depending on what type of cell was challenged. Human lung fibroblasts responded primarily with ROS production, reduced ATP and DNA damage, while human glioblastoma (cancer) cells appeared to be slightly more susceptible to DNA damage. Liver cells were less sensitive than fibroblasts. Necrosis required quite high incubation concentrations (100-500 μg/mL), consistent with there being an enhancement of cellular antioxidants, although the threshold for inducing apoptosis (3-12 μg/mL) appeared to be lower than the IC50 for necrosis. Toxicity studies with human epidermal keratinocytes showed that lysozyme-stabilised silver NPs are not cytotoxic at concentrations (up to 25 μg/mL) which are clearly antimicrobial (Eby *et al* 2009)[138]. In contrast, nanosilver was shown to be cytotoxic to human mesenchymal stem cells >5 μg/mL, and induced proliferation, cytokine release and inhibited chemotaxis at 2.5 μg/mL (Greulich *et al* 2009)[139].

Kim *et al* (2009)[140] compared the cytotoxicity of silver ions (Ag^+) and Ag^+-free silver NPs in cultured human hepatoma cells. While both produced intracellular oxidative stress and were comparably cytotoxic, NP-induced oxidative stress and DNA damage could be reduced by antioxidants. The authors concluded that Ag NP-induced oxidative stress is largely independent of free Ag^+ release.

5. Studies of NP genotoxicity

Because of substantial evidence that the toxicity of NPs is at least partly mediated by ROS generation, as well as studies which infer a mesothelial carcinogenic comparability between CNTs and asbestos, there has been substantial interest shown in assessing the genotoxic potential of a range of ENPs. There have been general reviews of NP genotoxicity (Doak *et al* 2009[67], Singh *et al* 2009[68]), as well as studies on individual NP types and a critical review of the methodologies for assessing the genotoxicity of NPs (Landsiedel *et al* 2009[141]).

In a study which compared the cytotoxicity and genotoxicity of several NPs (CB, SWCNTs, silicon dioxide, and ZnO) in relation to their size and shape, Yang *et al* (2009)[142] concluded that their relative cytotoxicity was more influenced by the NP size and composition, but that NP shape was a more important determinant of genotoxicity. ROS formation was confirmed as a key step influencing both types of toxicity.

5.1 Studies with ZnO and TiO_2

Bhattacharya *et al* (2009)[143] compared the *in vitro* cytotoxicity and genotoxicity of TiO_2 and FeO NPs in cultured human lung fibroblasts and bronchial epithelial cells. Both types of NPs were shown to translocate into the cytoplasm, but not into the cell nucleus. Generation of ROS was clearly demonstrated and, in the case of fibroblasts incubated with TiO_2, DNA damage was shown in the form of 8-OHdG adducts. Bronchial epithelial cells were more resistant to NP-induced DNA damage. In contrast to TiO_2, FeO induced DNA breakage (clastogenicity), but only when presented in a reducing environment.

Sharma *et al* (2009)[144] used the Comet assay to demonstrate that even relatively low concentrations (0.8 μg/mL) of ZnO NPs with 6 hr culture of human epidermal cells may have genotoxic potential. They also showed significant oxidative stress at the same concentration, as well as tenfold lower. Their conclusion that such genotoxicity could be associated with *in vivo* dermal exposures (such as use in sunscreens) was probably overstated.

Kang *et al* (2008)[145] also used the Comet assay, as well as the cytokinesis-blocked micronucleus assay with human lymphocytes *in vitro* to show that TiO_2 NPs induce DNA damage. However, they showed that TiO_2 does not stimulate transactivational activity of p53-derived proteins, but the ROS activated by the NPs activate p53-mediated DNA damage checkpoint signals.

5.2 Studies with CeO

Auffan *et al* 2009[146] found nanoscale CeO NPs (7 nm) to be both cytotoxic and genotoxic in human dermal fibroblasts. DNA damage and chromosomal changes were presumably associated with ROS formation.

5.3 Studies with CNTs

Di Sotto *et al* (2009)[147] and Wirnitzer *et al* (2009)[148] found no evidence of genotoxicity with MWCNTs, using a conventional battery of bacterial reverse mutation assays and chromosomal aberration tests in V79 cells.

Lindberg *et al* (2009)[149] found that NPs containing >50% SWCNTs and graphite nanofibres both caused dose-dependent DNA damage in cultured human lung epithelial cells, using a Comet assay and micronucleus assay. However, the authors were unable to determine the extent to which catalyst metals present in the NP samples may have contributed to the results.

5.4 Studies with fullerenes

Liang *et al* (2009)[150] demonstrated an interesting effect of C60 fullerene on DNA replication, which may be of some use in improving the efficiency or real-time polymerase chain reaction techniques. They suggested that fullerenes could bind to DNA and alter DNA replication by changing the conformation of DNA templates.

Song *et al* (2009)[151] reported that fullerenes solubilised using surface modification with mono- and bis-methanophosphonate groups could inhibit DNA restrictive endonucleases. The effects were not reversed by ROS scavengers.

5.4.1 Studies with fullerenes and other NPs

Folkmann *et al* (2009)[152] compared the genotoxicity of SWCNTs and C60 fullerenes after administration of 0.064 or 0.64 mg/kg bw by intragastric intubation in rats. Genotoxicity was assessed by determining the levels of premutagenic 8-oxo-7,8-dihydro-2'-deoxyguanosine adducts in the colonic mucosa, liver and lungs. Both NPs increased DNA adducts in liver and lung, although with C60, only the high dose increased adducts in lung. No adducts were found in colonic mucosal cells. While the authors concluded that the genotoxic effect was direct, rather than via inhibition of DNA repair, the implications for HRA are not clear.

Xu *et al* (2009)[153] identified peroxynitrate anions as important mediators of genotoxicity in a genetically modified mouse model (gpt delta transgenic mouse primary fibroblasts). They studied both unmodified fullerenes and TiO_2 NPs.

Essentially, these findings demonstrate a potential for fullerenes to interact with DNA under some experimental conditions, but they are probably of limited significance with regard to HRA.

5.5 Studies with nanosilver

Despite the known ability of silver NPs to produce ROS, there appears to have been little interest in studying its potential genotoxicity. Only one study was identified in the period covered by this report, and the technique used to assess genotoxicity was of dubious validity.

Chi *et al* (2009)[154] showed that nanosilver by itself is weakly genotoxic (if at all), but combining it with a detergent (cetylpyridinium bromide) markedly enhanced the response, as measured by resonance light scattering.

6. Dermal absorption and skin penetration

The extent of dermal penetration of NPs is of interest in relation to OHS investigations and also in relation to the deliberate application of some NPs to skin in the form of sunscreens and cosmetics[49, 50]. Previous studies and reviews suggest that skin penetration through intact skin may be minimal for most NPs. However, like most other chemicals, the penetration of NPs into the skin is likely to be enhanced when the skin is broken or damaged (Kezic and Nielsen 2009[155]).

6.1 Metal oxides (ZnO and TiO_2)

Kiss *et al* (2008)[156] studied penetration of TiO_2 NPs (anatase 9 nm) in an immunocompromised mouse model with grafted human skin. They confirmed that there was no penetration of the intact epidermal layer, using nuclear microprobe analysis. However, when cultured human keratinocytes were exposed to TiO_2 NPs *in vitro*, there were effects on a range of cell functions, including proliferation, apoptosis, differentiation and viability. This suggests that TiO_2 NPs are likely to be more toxic to damaged skin, as well as having greater skin penetration.

Kuo *et al* (2009)[157] used photoluminescence techniques to study skin penetration of ZnO NPs in cultured skin from nude mice. They demonstrated that ethanol, oleic acid and ethanol/oleic acid combinations all enhanced skin penetration, by increasing the fluidity of the *stratum corneum*.

Zvyagin *et al* (2008)[158] used a combination of SEM, TEM and multiphoton microscopy to investigate the transport of nano ZnO (26-30 nm) into excised human skin and after topical application *in vivo*. They showed that nano ZnO does not penetrate past the upper layers of the *stratum corneum*, remaining within skin folds and hair follicles. This study confirms previous assumptions that the nano ZnO preparations used in sunscreens are unlikely to present a systemic toxicity hazard. These conclusions were confirmed by even more extensive investigations by Gontier *et al* (2008)[159] using a combination of TEM, STIM, Rutherford Backscattering Microscopy, and Particle-induced X-ray emission to study the penetration ZnO NPs through human and porcine, as well as a human skin grafted mouse model *in vivo.* Shulz *et al* (2002)[160] had previously confirmed the lack of penetration of TiO_2 and ZnO NPs into human skin, irrespective of particle size or shape variability (study not included in other reviews).

However, on a more cautious note, Yanigasawa *et al* (2009)[161] studied the ability of intradermally injected (20 μg) TiO_2 NPs (15, 50 and 100 nm; SA 110, 20-25 and 10-15 m^2/g respectively) to reveal toxic effects when applied to damaged skin. They used a mouse model with atopic dermatitis induced by treatment with mites. The aggravation of the mite-induced atopic dermatitis was TiO_2 NP size-related, although the differences were relatively small. Immunological studies showed the effects to be mediated by T-helper type cytokine responses. While TiO_2 NPs alone produced no obvious skin changes, there were some increases in histamine plasma levels and interleukin-13 expression.

Wu *et al* (2009)[162] used two different approaches to studying the absorption of nano-TiO_2 after dermal application. The materials studied were commercially obtained nano-TiO_2 (anatase 4 and 10 nm; rutile 25, 60 and 90 nm; anatase/rutile 21 nm). Like others, they

showed minimal penetration of NPs through intact porcine skin *in vitro* over 24 h using AAS analysis of receptor fluid and tape stripping of the skin fragments. The NPs (5%) were suspended in a 20% caprylic/capric triglyceride vehicle containing 1% Tween 80. However, when topically applied daily to porcine ears *in vivo* over 30 days (24 mg of 4 and 60 nm Nano-TiO_2 at 5% in an aqueous 2% carbopol/triethanolamine vehicle) there was substantial movement of NP through the horny layer into the deeper epidermis. When applied to the skin of hairless mice daily over 60 days (same vehicle with five of the NPs 10-90 nm; 24 mg emulsion equal to 400 $\mu gTiO_2/cm^2$), there was substantial systemic absorption and translocation into tissues (mainly skin, subcutaneous muscle, spleen heart and liver). Although one of the NPs (21nm anatase/rutile) was shown to penetrate into brain tissue, the amounts of TiO_2 deposited in various tissues showed substantial variability, and with no clear pattern associated with NP size. The liver and skin were the main sites of systemic toxicity, with evidence of oxidative stress-mediated effects.

6.2 CeO

No studies of the dermal penetration of CeO NPs were identified during the 20 month period of interest for this review.

6.3 CNTs

No studies of the dermal penetration of CNTs were identified during the 20 month period of interest for this review.

6.4 Fullerenes

Only one study relating to the dermal penetration of fullerenes was identified during the 20 month period of interest for this review.

Kato *et al* (2009)[163] studied human skin permeability and consequent toxicity of fullerenes dissolved in squalene (LipoFullerene). They found that C60 could permeate into the epidermis, but could not cross the basement membrane into the dermis. The same material was negative for cytotoxicity, phototoxicity and mutagenicity in relevant *in vitro* tests.

6.5 Nanosilver

Only one study relating to the dermal penetration of nanosilver was identified during the 20 month period of interest for this review.

Larese *et al* (2009)[164] studied the absorption of nanosilver through intact and damaged human skin in a Franz diffusion cell. NPs passed through intact skin at a rate of 0.46 ng/cm^2 in 24 hours, but in damaged skin, the penetration was approximately five-fold higher.

7. Worker exposure studies

It is generally acknowledged that making reliable measurements of NP exposure in the workplace presents challenges in formulating appropriate OHS strategies. A significant part of this challenge is differentiating ENP exposure associated with manufacturing activity from the background exposure to incidental NPs and UFPs of anthropomorphic and non-anthropomorphic origin (Ono-Ogasawara *et al* 2009[165], Peters *et al* 2009[166]).

Plitzko (2009)[167] has summarised concentrations and size distributions of NPs (TiO_2, nanofibres, ceramic NMs and other nanostructured materials) in German productions plants. While the levels were generally low when effective exhaust-hood conditions were in place, occasional measurements of NP agglomerate were reported. Plitzko also emphasised the importance of differentiating workplace NPs sources from incidental background levels, and proposed the combined use of electron microscopy techniques along with chemical composition and particle number, in order to characterise agglomerates.

Schulte *et al* (2009)[27] reviewed the literature on workplace exposure to NPs and defined twelve key issues which need to be addressed in developing epidemiological studies of possible health impacts on NPs. Of these, the most important factors were understanding the nature of NP heterogeneity, the temporal aspects of exposures, characterization of exposures, defining appropriate disease endpoints and characterizing the study populations.

While it did not address NP exposures directly, a review by Shaffer & Rengsamy (2009)[28] did address the efficacy of different types of respirator filters to protect workers against inhaling airborne NPs.

7.1 TiO_2

Liao *et al* (2009, 2008)[168,169] set out to estimate potential worker exposure to TiO_2 NPs using published data on NP airborne concentrations and size distribution across a range of industries. Based on these data, and using a physiological model of lung deposition, they estimated the phase-specific NP cell burdens on target dermal and lung cells. They compared these estimates of cellular burden with cytotoxicity ED_{50}s derived from *in vitro* studies using human dermal cells and inflammatory ED_{50}s for human epithelial lung cells. They concluded that packers and surface treatment workers at TiO_2 production plants were unlikely to have exposures resulting in acute inflammatory lung disease, but because the dermal cytotoxicity ED_{50}s are at least an order of magnitude lower, there may be a risk of skin effects associated with relatively high airborne levels of anatase TiO_2 NPs (20-30 nm).

Robichaud *et al* (2009)[170] tried a different approach to estimating TiO_2 exposure. They surveyed evolving manufacturing data and market trends to estimate possible upper bounds of environmental outputs. However, while the methodology may have some utility in measuring global environmental outputs, it has no utility in predicting individual workplace exposures.

7.2 CNTs

Tantra & Cumpson (2007)[171] reviewed the microscopic and spectroscopic techniques for detecting & quantitating CNTs in the workplace. It was clear that there are many challenges to be met in accurately quantitating worker exposures to CNTs and no 'perfect' technique exists. Ways to improve current technologies or develop/explore future technology for CNT detection are still very much needed.

Tsai *et al* (2009)[172] have published some workplace field monitoring data from premises where SWCNTs and MWCNTs have been manufactured. They considered some of the factors which could result in lowering airborne emissions.

7.3 Nanosilver

Park *et al* (2009)[173] measured worker exposure to nanosilver in a Korean production facility. While they found that the liquid-phase process minimises inhalational exposure compared to a gas-phase process, there was still significant exposure potential associated with release of nanosilver and formation of airborne aggregates from the liquid-phase reactors. They also showed that nanosilver exposures could be significant during drying and grinding processes, even when efficient ventilation practices were in place.

Similarly, Tsai *et al* (2009)[174] found that significant airborne nanosilver and nanoalumina release could occur in Japanese production facilities even when processes were handled in fume hoods, but especially when the processes involved dry powders. They made recommendations relating to hood design and work practices to minimise this exposure potential.

7.4 Chinese workers

In the only reasonably well documented case of NP worker exposure (Song *et al*[175]), seven female workers in a Chinese print facility were diagnosed with pulmonary inflammation, fibrosis and granulomatous foreign bodies in the pleura, having presented to hospital with a history of shortness of breath and pleural effusions. One male worker from the same facility was asymptomatic. The workers had all been exposed for between 5-13 months to fumes from the spray application of a polyacrylic ester paste to polystyrene boards. The presence of NPs (<30 nm) in pulmonary epithelial and mesothelial cells was demonstrated by TEM, and *post-hoc* sampling of the workplace also showed substantial NP deposition. However, there was no conclusive proof that the NPs caused the disease, since the workers were likely to have been exposed to other toxic materials.

While it was clear that the occupational hygiene practices in the workplace were far from ideal[176], the cases attracted quite a lot of media attention because this was the first human linkage of NP exposure to an adverse health effect consistent with those seen in animal studies and which could therefore be expected to be an outcome associated with long-term NP inhalational exposure. Furthermore, two of the seven cases died as a result of progressive lung disease.

8. Special issues relating to carbon nanotubes

8.1 Mesothelioma potential

Ever since Poland *et al* published their 2008 study (reviewed in the Toxikos report) showing the size-dependent effects of asbestos and MWCNTs to induce a pathogenic response indicative of mesothelioma, there has been substantial interest in further exploration of this toxic effect. See also, reviews by Jaurand *et al* (2009)[177] , Kostelaros (2008)[178] and Sanchez *et al* (2009)[179].

8.1.1 Two-year rat study

Muller *et al* (2009)[180] reported a 2 year carcinogenicity study with MWCNTs designed to establish the comparative potency of MWCNTs and asbestos (crocidolite fibres) to cause peritoneal mesotheliomas in Wistar rats (n=50 per group). Rats were given a single IP injection of MWCNT with (2 or 20 mg/rat) and without (20 mg/rat) structural defects thought to enhance the surface area and pathogenicity of the fibres. The incidence of tumour formation was followed with serial sacrifices over two years. Negative and positive controls (n=26 per group) received either vehicle or 2 mg/rat crocidolite.

The key findings of this study were that crocidolite increased the incidence of peritoneal mesotheliomas (34.6%) over negative controls (3.8%), while neither the modified nor the unmodified MWCNTs increased this response (4, 0 and 6%). The rat IP methodology has been previously used to clearly demonstrate a carcinogenic response (mesothelioma) to asbestos and some other fibrogenic materials.

The authors cautioned against interpretation of the negative carcinogenicity response, since the length of the MWCNTs used (<1000 nm) may have been inappropriate and there was an absence of a sustained inflammatory response.

8.1.2 Rat peritoneal mesothelioma study

In a study with a rather unusual design, Sakamoto *et al* (2009)[181] investigated the ability of MWCNTs instilled as a single dose (1 mg/kg bw) into the scrotal sac of male rats (n=7) to induce peritoneal mesotheliomas. The negative and positive controls received vehicle (n=5) or crocidolite asbestos (2 mg/kg bw). The authors proposed that the rat scrotal sac has direct contact with the peritoneum, with materials released from the scrotum with a “depot-like effect”.

Only one rat in the MWCNT group survived for 52 weeks, with most developing invasive mesotheliomas in weeks 37-45 and succumbing. The surviving rat had clear mesothelial hyperplasia. In contrast, all the control and asbestos-treated rats survived for the full 52 weeks, and the incidence of mesothelioma and mesothelial hyperplasia was zero in both groups. There was some evidence of asbestos fibre deposition in the peritoneum, but no obvious pathological sequelae.

The interpretation of this study is difficult because of the low numbers of test animals used and the unusual experimental design. However, the comparative toxicity between asbestos and MWCNTs was stark. It would not be surprising if this study receives publicity as yet another indication that MWCNTs have pathogenic properties at least comparable to, if not greater than, asbestos.

8.2 Neurotoxicity

Xu *et al* (2009)[182] studied the effects of MWCNTs on membrane ionic channels using whole-cell patch clamp electrophysiology in undifferentiated phaeochromocytoma cells *in vitro*. While CNTs have not been specifically implicated as being neurotoxic, the authors were able to demonstrate that three types of K^+ channel ionic functions were inhibited by carboxyl-terminated MWCNTs at concentrations which did not alter ROS expression or change intracellular Ca^{++}.

8.3 Other studies

Buckypaper is an innovative material made from MWCNTs with interesting physical/chemical properties and possible usefulness in pharmacological and prosthetic materials. Because of the general interest in the carcinogenic potential of MWCNTS, Bellucci *et al* (2009)[183] assessed the toxicity of buckypaper carbon nanotubes, which have a structural resemblance to asbestos by testing their effects on cancer and primary cell lines *in vitro* and *in vivo* in rats. They found buckypaper decreased proliferation of human colorectal, breast and leukemic cancer cell lines *in vitro*. However, there was no effect on the proliferation and viability of normal human arterial smooth muscle cells and human dermal fibroblasts *in vitro*. There was a moderate inflammatory reaction after *in vivo* administration but no mutagenic effects. After implantation the rats showed an inflammatory reaction followed 2 weeks later by a cicatrization reaction with the organization and fibrosis of the scar.

While the results with this novel form of MWCNTs indicate some of the expected inflammatory reactions and the in vitro proliferation of some cells (including cancer cells) was inhibited, it would be premature to draw any conclusions about the carcinogenic risks of buckypaper.

9. Special issues relating to metal oxides

9.1 Neurotoxicity

Ever since Oberdorster's group demonstrated a potentially direct uptake pathway for NPs into brain via neurones of the olfactory system, thus circumventing the blood-barrier, there has been speculation about the potential CNS neurotoxicity of NPs. Oberdorster *et al* (2009)[184] have followed this topic up with a recent review. However, they concede that the current evidence is insufficient to conclude that either ENPs or UF air pollutants have significant neurotoxic effects.

This section reviews some recent studies where the neurotoxic potential of metal oxide NPs has been assessed *in vivo* and *in vitro*.

9.1.1 *In vivo* studies

Even though Wu *et al* (2009)[162] demonstrated distribution of TiO_2 NPs to the brain of hairless mice after 60 days dermal application, there was no evidence of neurotoxicity (see also Section 6.1).

Shimizu *et al* (2009)[185]demonstrated that maternal exposure to TiO_2 NPs (100μg SC) during pregnancy days 6,9,12 & 15 in mice caused several genes associated with apoptosis to be over-expressed in the brains on neonates, along with alterations in genes involved in early development, neurotransmitter function and some psychiatric diseases. While this study falls short of proving developmental neurotoxicity as a risk associated with TiO_2 NPs, it demonstrates that the foetus is accessible to such NPs and posts a warning sign.

Similar findings were reported by Takeda *et al* (2009)[186], who showed that the offspring of pregnant mice injected SC with 100μg anatase TiO_2 NPs (25-70 nm; 20-25 m^2/g) on postcoital days 3, 7, 10 and 14, showed neurotoxic damage to their genital and cranial nerves. TiO_2 NPs were detected in brain and testes of the newborn pups, indicating that the NPs crossed the placental barrier.

The most compelling evidence for TiO_2 induced neurotoxic potential comes from the studies of Wang *et al* (2008, 2008)[187,188]. They demonstrated direct uptake into brain via the olfactory lobes after intranasal instillation, with the hippocampal region being a primary target for accumulation and toxicity. Surprisingly, larger (155 nm) anatase TiO_2 NPs were more toxic than smaller (85 nm) rutile TiO_2 NPs. The authors suggested that this may somehow be associated with differences in crystalline structure. Confirmation that crystalline structure influences TiO_2 cytotoxicity may be found in the review by Braydiche-Stolle *et al* (2009)[189].

9.1.2 *In vitro* studies

Long *et al* (2009)[190] proposed that, as brain microglia are susceptible to oxidant stress, they may be a suitable model for studying TiO_2 NP neurotoxicity. They incubated brain microglia with TiO_2 NPs *in vitro*, and were able to demonstrate a rapid ROS burst, followed by sustained release ROS release, possibly mediated by interference with mitochondrial energy production. The importance of this study was that it demonstrated ROS release at non-cytotoxic concentrations 2.5 – 120 ppm). The implication that the ROS bursts did not trigger apoptosis in these susceptible cells is inconsistent with their pilot

data showing TiO_2- inducing apoptosis at >20 ppm. Alternatively, the study was simply not long enough to reveal the eventual neurotoxic cell damage.

Deng *et al* (2009)[191] studied the effects of ZnO NPs on cultured murine neural stem cells. While they were able to demonstrate induction of apoptosis and dose-related decreased cell viability, the fact that they were unable to differentiate between different NP sizes and surface activities, led them to conclude that the neurotoxicity was probably entirely attributable to dissolved Zn ions in the media.

In contrast, D'Angelo *et al* (2009)[192], investigating the potential for antioxidant effects of CeO NP to have useful therapeutic properties in the treatment of neurodegenerative diseases (Alzheimer's Disease) showed that SH-SY5Y neuroblastoma cells incubated with CeO NPs have beneficial effects as ROS scavengers, as well as influencing neuroprotective cell signalling pathways.

9.2 Cardiovascular toxicity

Since cardiovascular (CV) toxicity is one of the key health effects associated with UF air pollutants, there has been significant interest in whether manufactured NPs interact specifically with the CV system. This form of potential toxicity has been mentioned in reviews[57, 193] and there have been a number of recent studies addressing this potential toxicity for selected NPs.

Gojova *et al* (2009)[120] showed that CeO NPs caused very little inflammatory response when incubated with human aortic epithelial cells at high doses (up to 50 μg/mL), while ZnO and Y_2O_3 NPs were somewhat more inflammatory. They concluded that dissolution characteristics and pH changes associated with dissolution of the metal oxides could explain the toxicity differences.

10. Special issues relating to nanosilver

10.1 Neurotoxicity

Sharma *et al* (2009)[194] speculated that brain neurotoxic potential for metal NPs could be quite dependent on the type of metals, the route of administration and the species used. They assessed blood-brain barrier permeability and specific forms of neural cell damage after relatively larges doses of copper, aluminium and silver NPs (50-60 nm) by IP, IV, intracarotid and intracerebroventricular injection. While the patterns of neural injury may not reflect exposure conditions normally expected from occupational exposures, in general terms, copper and silver NPs were more neurotoxic than aluminium NPs.

11. Other special issues

11.1 Effects of NP size and surface modification

Although it did little more than reinforce the fact that NP size influences cytotoxicity, Karlsson *et a*l (2009)[195] showed that nano- and micro-scale metal oxides have differential toxicity towards cell viability, ROS formation and DNA damage in cultured A549 cells. They attributed some of the difference to the intrinsic toxicity of the metal ions (see also Section 4).

Jiang *et al* (2008)[196] considered the effects of surface coating of NPs. They showed that coating gold and silver NPs with antibodies can modify the regulation of processes of membrane receptor internalization. They also showed that binding and activation of membrane receptors and subsequent protein expression is strongly depend on NP size, with 40-50 nm-sized NPs having the greatest effect on cell signalling processes within the range 2-100 nm size range.

Travan *et al* (2009)[197] also reported that coating nanosilver with polysaccharides reduces cytotoxicity without affecting antibacterial effects.

The ability of surface charge modification to alter solubility and the stability of NP aggregates has also been investigated for fullerenes (Chang & Vikesland 2009[198]). Fullerenes with a negative surface charge tend to be more stable in aqueous solution.

11.2 Structure-activity relationships (SAR)

Only four studies were found where there was a specific attempt to correlate SAR with NP toxicity. Two were *in vitro* studies with fullerenes; one used SWCNTs and the other MWCNTs.

Lao *et al* (2009)[199] used fullerenes functionalised by hydroxylation ($C_{60}OH_{22}$) or malonic acid derivatisation $C_{60}(C(COOH)_2)_2$. They showed that both derivatised fullerenes prevented sodium nitroprusside-induced nitric oxide-mediated apoptosis and loss of cell viability in cultured primary rat brain cerebral microvessel epithelial cells.

An antioxidant-like effect of native C60 on reducing apoptosis associated with nitric oxide formation was also shown by Misirkic *et al* (2009)[200]. This protective effect, mediated by prevention of mitochondrial depolarisation, caspase activation cell, exposure of cell membrane phosphatidylcholine and DNA fragmentation, was not thought to be a direct interaction with NO, but via neutralization of NO-induced hydroxyl radical production in mitochondria.

Martin *et al* (2009)[201] studied water-soluble carboxyfullerenes substituted with 6 or 12 carboxyl groups and synthesised from commercial C60. These two derivatives were compared with C60 fully caged with cyclodextrin, which enables the hydrophobic C60 to be solubilised and delivered to the cell surface. Dispersion and aggregation characteristics were correlated with their surface charge and redox potential, but there were also some significant differences in *in vitro* toxicity in human monocytic THP1 cells. While none of the derivatised fullerenes was particularly cytotoxic, functionalisation did influence their ability to elicit caspase protease responses, and to some extent, their ability to induce intracellular oxidative stress, necrosis and apoptosis. Carboxylate derivatised C60 was to

some extent protective against apoptosis, as opposed to cyclodextrin-caged C60, which was more capable of inducing apoptosis.

Lucente-Schulze *et al* (2009)[202] showed that derivatisation of SWCNTs with the phenolic antioxidant butylated hydroxytoluene (BHT), increased ROS quenching capacity to around 40 times that of fullerenes. However, increasing the extent of BHT loading both increased and decreased the ROS quenching effect, depending on whether the substitution was covalent and whether it was on the SWCNT sidewall. Both functionalized and non-functionalized SWCNTs were non-cytotoxic in this experiment.

Shen *et al* (2009)[203] showed that derivatisation of MWCNTs with polyethylenimine and subsequent charge modification with acids could modify their water solubility and functional stability. The resultant neutral and negatively charged NPs were non-cytotoxic to a human epithelial carcinoma cell line (at up to 100 μg/mL), while positively charged NPs were at least 10 fold more cytotoxic.

12. Conclusions and remaining gaps in information

There continues to be a rapid expansion of the literature base describing the toxicity of NMs. This report identifies several general reviews which have been published in the past 20 months, as well as a large number of experimental studies on the six NMs of interest to NICNAS (ZnO, TiO_2, CeO, CNTs, fullerenes and nanosilver).

In addition to summarising individual reports and information relating to the toxicity of the six NMs, attempts have been made to assess the extent to which the literature informs HRA and safety assessment. In this context, the plethora of *in vitro* studies probably add little more than identifying cellular mechanisms involved in NP toxicity, and possibly where modification of their size and surface structure may influence relative cytotoxicity.

A possible exception is the HRA case study on CeO described in Section 4.2.1. While there were no *in vivo* experimental studies described in this paper, attempts were made to interpret *in vitro* toxicity test data in terms of comparative estimates of human lung cell deposition (human equivalent doses) and estimates of CeO NP exposure from diesel fumes following their use as a fuel additive.

The Riviere (2009) review[204] emphasises the importance of understanding NP pharmacokinetics in framing methodologies and policies for HRA. It noted the common theme across all particle types is that a major determinant of nanomaterial disposition is the degree of interaction with the reticuloendothelial (RE) cell system. Small water-soluble particles evading this system may be excreted by the kidney. Larger particles and those with the proper surface charge may get targeted to RE cells in the liver, spleen and other organs. Another common attribute to nanomaterial kinetics is retention of particles in the body. Most NPs have relatively short half-lives, reflecting clearance by tissue sequestration rather than excretion. Finally, unlike many small organic drugs, nanomaterials may preferentially be trafficked in the body via the lymphatic system. Such a clearance process has immunological implications.

In relation to *in vivo* studies, there has been a strong emphasis on understanding potential toxic effects on the lung, respiratory and cardiovascular systems after inhalational exposure. All these endpoints have been identified as potential targets for adverse effects associated with UF air pollutants. The inhalational route also represents a major pathway for exposure of workers manufacturing or handling NPs. It is regrettable that the majority of these studies have limited utility for HRA since they were either not specifically designed to demonstrate a NOAEL, or they used a single dose exposure paradigm. Single dose studies often used intratracheal instillation to deliver NPs to the respiratory system, and this method is unfortunately prone to artefactual outcomes (as well described in the Toxikos review).

In summary, the literature over the past 20 months has offered some advances in knowledge, although there remain substantial gaps. The main areas of advancement have been in relation to *in vitro* mechanistic studies and sub-chronic inhalational toxicity studies in rodents, which have demonstrated:

- Biopersistance (long-term retention of NPs in target tissues) is a factor quite critical in determining whether NPs penetrate cells and are retained long enough to provide the continuing inflammatory signals and other biological stimuli which lead to pathological changes

- Most biopersistent NPs induce adverse cellular oxidative stress events dependent on reactive oxygen species, but that initiation of apoptosis can be a further factor inducing cell necrosis. Furthermore, biological signals indicative of apoptosis can occur at dose levels lower than those producing frank cytotoxicity. This in turn suggests that cellular antioxidant mechanisms are partially protective against cytotoxicity
- Modification of surface activity (particularly that leading to volatilization of otherwise hydrophobic NPs) modifies the toxicity potential of NPs. However, as yet, there is no clear pattern of structure activity relationships (SAR) which would permit predictability of NP toxicity
- Formal HRA is still hampered by a lack of useful chronic exposure studies, especially those which clearly demonstrate dose-response relationships, or provide for clear NOAELs. The innovative approach to HRA exemplified by the case study relating exposure to CeO NPs used as a diesel fuel additive and comparing estimated human lung internal doses with *in vitro* toxicity data is a fresh ray of sunlight in an otherwise dim scenario, and
- Characterization of NPs administered in either *in vitro* or *in vivo* experimental studies remains critical to the interpretation of the outcomes. Most studies now report the characteristics of the NPs they use, including the critical surface area in terms of Brunauer-Emmett-Teller values (an algorithm describing surface area of NPs).

One aspect of NP toxicity which has received specific attention has been follow-up studies with MWCNTs to assess their comparative toxicity with asbestos in relation to induction of mesothelioma. These studies had several flaws which limit their utility in informing a HRA of MWCNTs.

The Ma-Hock (2009)[97] 90-d rat inhalational study recently published online (see Section 3.4.1) failed to demonstrate a NOAEL, with evidence of lung granulomatous inflammation at the lowest dose level (0.1 mg/m^3). Mesothelioma was not reported among the adverse effects seen, but it may be that the duration of the study was not long enough. There were also issues around whether the equipment used to generate respirable MWCNT dusts reflect exposure conditions likely to be encountered in the workplace. Evidence of minimal-moderate lympho-reticulocellular hyperplasia in the mediastinal lymph nodes of most HD rats (but not LD) suggests translocation of the MWCNTs via lymph. Furthermore, there was suggestive evidence (but not confirmation) of intracellular penetration of submucosal epithelial cells. However, there was no descriptive evidence that the MWCNTs had reached mesothelial cells.

The Muller *et al* (2009)[181] 2-yr rat study (see Section 8.1.1) used only the intraperitoneal route for a single administration of two dose levels of MWCNTs. While the study methodology showed a clearly positive peritoneal mesothelioma response to asbestos (and other fibrous materials in other reports), it failed to show any mesothelioma response to MWCNTs. In direct contrast, the other study reported in Section 8.1.2 (Sakamoto *et al* 2009[130]) showed a paradoxical severe peritoneal mesothelioma response to MWCNTs instilled in the scrotal sac of rats, but failed to show a similar response to asbestos.

The conclusion at this time must be that the jury is still out on mesothelioma potential for MWCNTs.

12.1 Additional data gaps and further knowledge requirements

The Wijnhoven (2009)[41] review of nanosilver made attempts to outline the knowledge gaps critical to further understanding of the health risks of nanosilver. The review addressed the theories and studies which propose that much of the mammalian toxicity of nanosilver (as opposed to antibacterial effects) may be attributable to the release of soluble silver ions. Since human exposures to nanosilver can include its use for therapeutic purposes, there are likely to be significant sources of human data which can be used to complement animal and *in vitro* tests and assist with their interpretation.

The Hussain & Schlager (2009)[103] editorial on the significance of a rat inhalational toxicity study with nanosilver which demonstrated a clear NOAEL outlined the next steps to developing an occupational exposure standard – namely the benchmarking of lung nanosilver deposition levels in the rat model against intakes levels and measurement of actual workplace exposure levels.

In this context, the approach taken by Park *et al* (2008)[106] (see Section 4.2.1 and above discussion) to use a combination of *in vitro* toxicity data, exposure estimates and conversion to human equivalent lung NP burden estimates, may be useful in addressing worker HRAs for other NPs.

12.2 Data gaps identified by NICNAS

The following specific issues were nominated by NICNAS as requiring attention in this report:

- Chronic inhalation toxicity studies of these six nanomaterials;

  These have been addressed in Section 3 of this report.

- Dermal absorption/penetration information on these six nanomaterials;

  The information on dermal penetration through healthy skin is still quite limited. The scant information relating to TiO_2, fullerenes and nanosilver is summarized in Section 6 of this report

- Potential structure activity relationships that govern dermal penetration of nano particles;

  No studies were identified which could shed light on SARs which may determine skin absorption. Only one study with fullerenes attempted to show how surface structural modification could alter cytotoxicity potential (discussed in Section 11.2 of this report).

- How modified surface properties of fullerene, zinc oxide and titanium oxide affect penetration through membranes/skin;

  This is essentially the same point as above, and the available information is equally deficient. However, the review article by Nel *et al* (2009)[6] includes some useful detail on the types of SAR which can modify cell surfaces and alter biological interactions, including toxicity modification (see Section 2)

- Any worker exposure data on these six nanomaterials;

  Some limited information on worker exposure to nanoTiO_2 and nanosilver was identified, and discussed in Section 7. In addition, this Section notes the recent report of significant toxicity (include death) in Chinese workers allegedly

exposed to NPs. This Chinese report has attracted media attention because it highlights the human toxicity potential of NPs when attention to proper industrial hygiene practices is less than rigorous.

- Following information on CNTs: systemic distribution after inhalation, chronic repeat dose studies using any relevant human exposure route (oral, dermal or inhalation), 'Peritoneal fibre' screening assay for single walled CNTs with fibre properties;

  Most of the useful studies on CNTs have been limited to those with MWCNTs. Only two single dose *in vivo* studies using SWCNTs were identified (see Section 4.3.1). The inhalational and other repeated-dose toxicity studies with MWCNTs have been discussed in Sections 3.4.1 and 8. While these studies focused primarily on the potential for MWCNTs to induce mesothelioma, in comparison with asbestos, they really only demonstrated pathogenic precursors to mesothelioma, primarily because the studies were of relatively short exposure duration.

- Any studies addressing whether fibre like CNTs when inhaled can reach mesothelial lining;

  See Section 3.4.1 and discussion in Section 12. It remains to be determined whether CNT fibres can reach the mesothelial lining of lungs after inhalation. In any case, the literature surveyed in this report only refers to studies with MWCNTs,

- Dose response analysis of non-functional CNTs - NOAEL established;

  The one mid-term (90d) inhalational study conducted with MWCNTs described in Section 3.4.1 failed to demonstrate a NOAEL. A 14d inhalational study (Section 3.4.1) did demonstrate a NOAEL at 0.3 mg/m^3. However, taken together, these studies have a limited capacity to inform a HRA for CNTs.

- Accumulation of nano particles in organs after exposure via oral, dermal or inhalation route;

  This is quite a critical factor, especially if there is to be a bridge between toxicity outcomes in *in vitro* studies and prediction of target tissue doses which might inform HRA. Unfortunately, there are still few studies where meaningful data on tissue concentrations are reported. In most cases, the objective is simply to demonstrate that extracellular (or preferably intracellular) accumulation can occur from the chosen route of exposure.

  Because of earlier concerns (mainly raised by Oberdorster's group) that NPs can cross the blood-brain barrier under some circumstances, and induce neurotoxic responses, there has been some attention to this toxic outcome (see Sections 9.1 and 10.1). While there have been some studies addressing the potential neurotoxicity of metal oxides and nanosilver in the past 20 months, the issue of NP-induced neurotoxicity remains unresolved.

- Whether poorly soluble nanomaterials behave differently to other insoluble fine particles with respect to causing DNA damage;

  This may be answered indirectly by reference to the studies described in Sections 11.1 and 11.2. Solubilisation of hydrophobic NPs can modify cytotoxicity, but it appears the direction of change is not easily predictable. The

one fact which seems to stand out is that genotoxicity appears to be directly related to the ability of the NP to induce ROS formation.

- Studies on interactions between nanoparticles and allergens;

  Findings that MWCNTs exacerbate pulmonary responses in an allergic asthma model (see Section 4.3) suggest this is a finding which should be followed up with other NPs.

- Any toxicological/health information on nano cerium oxide and nano zinc oxide.

  There were relatively few *in vivo* studies found with either of these NPs, but they continue to be NPs studied more extensively in *in vitro* systems (see Sections 4.1, 4.2, 5.1 and 5.2). The use of *in vitro* tests to inform a HRA for CeO used as a diesel fuel additive has been described in more detail in Section 4.2.1.

13. References

The literature search identified some 264 relevant papers, most of which have been discussed in this report.

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