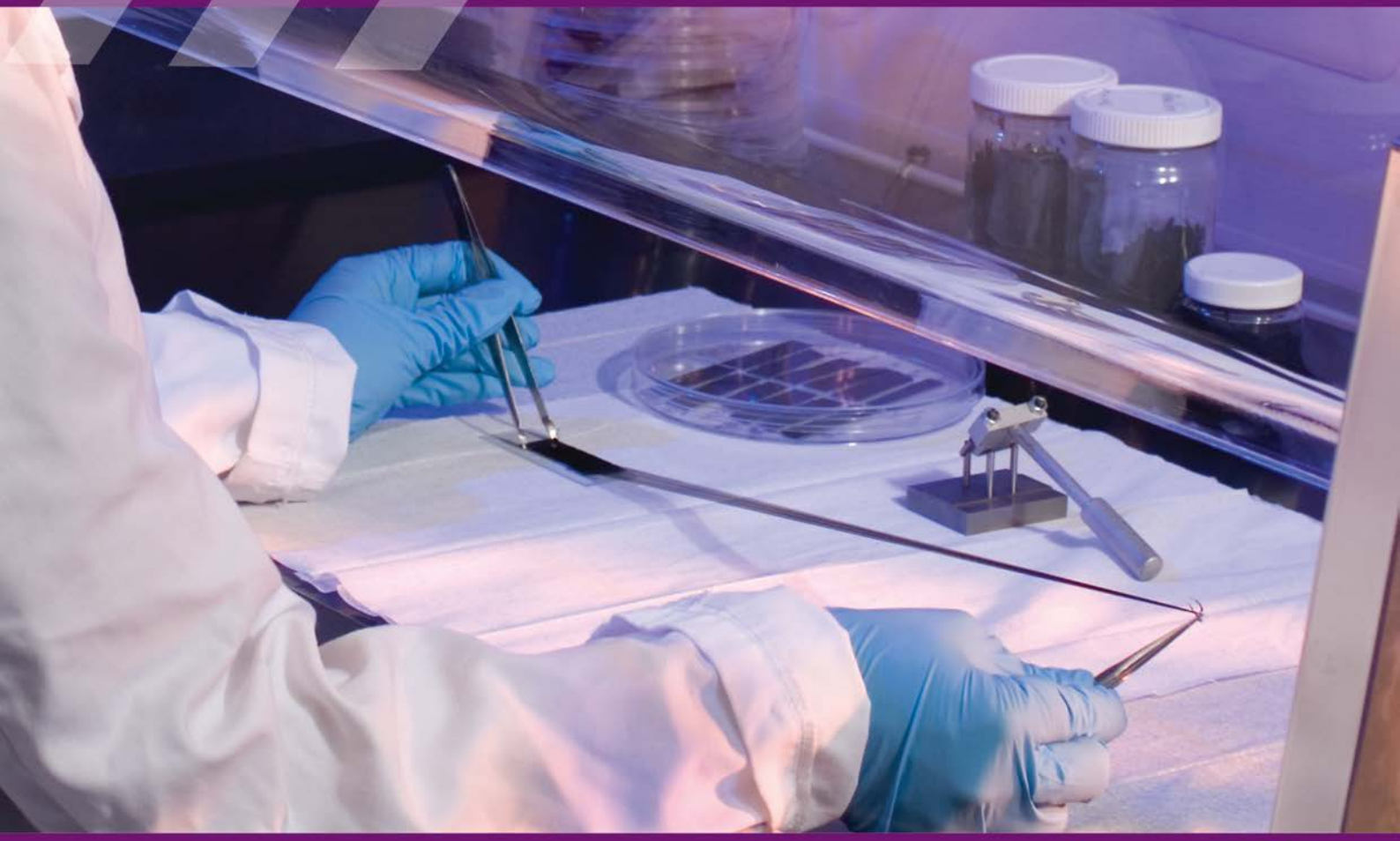
Engineered Nanomaterials: An Update on the Toxicology and Work Health Hazards



January 2015

Safe Work Australia commissioned ToxConsult Pty Ltd to undertake this project.
Funding for the project was provided by the Department of Industry and Science under the National Enabling Technologies Strategy.

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Disclaimer

Acknowledgement

The authors would like to thank Dr Vladimir Murashov (US National Institute for Occupational Safety and Health), Ms Shelley Rowett (SafeWork SA), Ms Sally North (WorkSafe WA), Mr Peter McGarry (Workplace Health and Safety Queensland) and Dr Jurg Schutz (CSIRO) for their review of the report. The authors would also like to thank Dr Howard Morris (Safe Work Australia) for his review comments and assistance with finalisation of the report.

Safe Work Australia commissioned Professor Brian Priestly (Priestly Consulting) to undertake a peer review of the report. Safe Work Australia would like to thank Professor Priestly for his contribution.

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ISBN 978-1-76028-042-0 [PDF]
ISBN 978-1-76028-043-7 [DOCX]

Executive Summary

In 2009 Safe Work Australia commissioned a report entitled "*Engineered Nanomaterials: A Review of the Toxicology and Health Effects"* (Safe Work Australia 2009a). This was a comprehensive review of the scientific literature from 2006 to early 2009. The 2009 report included detailed descriptions of the experimental methodologies underpinning the toxicological information. Recurrent themes throughout that review were the importance of appropriate characterisation of engineered nanomaterials (ENMs) in the experimental system, and how experimental protocols can profoundly influence the data and its interpretation/extrapolation to humans.

This current report is an update of the 2009 document for Safe Work Australia and canvasses the scientific literature and international agency deliberations on ENMs publically available between 2009 to early 2013. Less emphasis is placed on experimental methodologies but more on how the information pertains to hazard identification, exposures and risk management in the workplace (including research facilities). Nonetheless it is inevitable that in a review of this type there are large amounts of text describing individual studies. Early chapters of the report contain toxicological and health information for ENMs in general. In recognition of the plethora of publications on nanoparticulate toxicity, there are also chapters providing detailed information for carbon nanotubes (CNTs) and a number of nano-metal oxides.

With respect to some key conclusions in the 2009 review, it was concluded biopersistence was a critical ENM property for induction of particulate- and/or fibre-like responses in the lung; the current literature reinforces this view. Further information is now available that confirms long thin CNTs have potential fibrogenic activity, including ability to induce mesothelioma. The natural tendency of ENMs to agglomerate was considered a significant source of uncertainty in extrapolating *in vitro* and *in vivo* data to human exposure situations. While this is largely still the case there is now more appreciation by researchers that agglomeration can significantly influence toxicological responses of ENMs. This has resulted in experimental data being more useable for hazard identification, although this is not a universal phenomenon. In this regard a large amount of work has been published investigating the influence and role that protein binding to ENMs has on their cellular uptake, distribution and biological effects. In 2009 workplace exposure standards (WES) had not been specifically set for ENMs, however following the availability of rodent sub-chronic inhalation studies for some ENMs, WESs have now been established by authorities or industry for those materials. While the dearth of workplace exposure information noted in the 2009 review is starting to be addressed, publications researching ENM exposures markedly lag behind those related to possible ENM hazards. There is still much to do in the exposure arena.

Agency reviews:

Over 90 relevant reports/communications/reviews were identified and sourced from national and international agencies. Overall there is consensus that ENMs cannot be collectively or categorically considered either intrinsically benign or harmful. Rather, hazard and risk assessments should be conducted on a case-by-case basis. Many of the OECD test guidelines for physical and chemical properties, and associated hazards, for chemicals are regarded as not applicable for ENMs. The tests either need to be modified or new ones developed. On the other hand existing toxicology testing guidelines are generally considered applicable. However the physicochemical characteristics of ENMs present significant challenges in understanding the absorption, distribution, metabolism, and excretion of ENMs.

There is general agreement that the risk assessment paradigm for chemicals is appropriate guidance for assessing risk from exposure to ENMs. A central theme of the agency reviews remains the lack of sufficient or adequate data to allow for meaningful exposure assessment and risk characterisation, and the need for more information. Currently significant data gaps for most ENMs impart marked uncertainty to risk assessments, which in the opinion of some authors renders them unusable for regulatory decision making.

It is generally understood by agencies that existing WESs for bulk substances may not provide adequate health protection from nanoparticles made from that substance. Nonetheless in the literature there are many instances where the acceptability of workplace ENM exposure measurements has been done by comparison with a WES for the bulk material or for general dust. Such comparisons are not appropriate without very careful consideration and documentation of the circumstances and rationale.

Conclusions made from preliminary investigations of industry ENM workplaces by a European agency suggested risks from ENM exposure may not be adequately controlled. On the other hand, a UK agency found that research groups and university departments generally have adequate control of exposures to nanomaterials in their laboratories.

Occupational exposure and illness:

For a variety of reasons epidemiology studies exploring associations between occupational illness and exposure to ENMs are challenging to undertake, arguably more so than with traditional chemical exposures *per se*. Not the least of which is the number of workers exposed when making or using ENMs is currently small. This severely limits the statistical power of epidemiology studies traditionally used to identify health effects in humans.

While there are no medical tests that are specific for the effects of ENMs, there could be future benefit in monitoring the health of workers who may be substantially or repeatedly occupationally

exposed to ENMs, or at least collecting information on who may be exposed, the materials being used, the duration of use and potential or actual exposures. Furthermore, many of the medical tests for conventional chemical exposure, such as those investigating respiratory and cardiovascular endpoints, may also be appropriate for ENMs. Medical tests specified for bulk materials would be expected to also apply to nano-forms of the material.

A variety of commercial instruments are now available that facilitate measuring airborne ENMs in workplace air and breathing zones of workers. It is essential when undertaking such measurements that background particulate exposure for the specific exposure location be thoroughly characterised. Also noted is the difficulty of quantitatively generalising data across workplaces. This is due in part to different measurement techniques, reporting details, manufacturing/handling circumstances, and background sources of particles. When appropriate exposure controls are in place (usually engineering solutions) concentrations of ENMs in air are universally reported to be low.

Only a few medical cases involving exposure to nanoparticles in the workplace have been published in the medical literature. These have all involved very high exposures due to failure of engineering or hygiene controls. A feature of these cases has been the rapid onset of respiratory distress, rapid progression to serious clinical symptoms, marked difficulty to treat, and irreversible tissue damage that in some cases has been associated with death.

General toxicological considerations:

As observed in the Safe Work Australia (2009a) review of ENMs, the particle and fibre toxicity paradigms continue to underpin the majority of *in vitro* and *in vivo* toxicological investigations of these materials.

The ability of ENMs to produce oxidative stress and increases in inflammatory markers, in a wide range of cell types and using a variety of endpoints, is still the focus of many *in vitro* studies with ENMs.

Over the last few years a ‘Trojan horse’ mechanism has been developed for many nano-metal oxides whereby the ultimate toxic species, the metal ion, is produced within certain cell organelles after the nano-metal oxide has entered the cell.

When ENMs are absorbed into the body or into cells it is apparent they are able to be extensively and stably coated with a variety of macromolecules present in biological milieu in which they may be distributed. This forms a biological (protein) corona around the particle. This in large part determines how cells “see” nanomaterials. Although species of bound protein may be exchanged as the ‘protein’-ENM complex is distributed in the body and moves through cells, the complexes are stable and can be isolated for study. The corona affects their interaction with cells and hence their toxicity.

Agglomerated ENMs are less well taken up by cells than primary nanoparticles, and agglomerates can form when the nanoparticles are within the cell. Agglomerates within cells are essentially confined to the cytoplasm, whereas individual nanoparticles can be found in the nuclei and mitochondria. Agglomerated ENMs have lower *in vitro* cellular toxicity compared to the primary nanoparticle. Thus determining whether workers are exposed to individual nanoparticles or agglomerates is important when trying to match the measured exposure with a toxicological effect. The latter may have been determined with well dispersed nanoparticles. The United States National Institute for Occupational Safety and Health (NIOSH) has conducted field measurements which show the majority of ENM emissions tended to agglomerate and form aerosol structures that were larger than the nanoscale, but still would be considered nanomaterials.

The latest published information suggests concordance between *in vitro and in vivo* toxicological information is poor. But this may be improving with the development and validation of *in vitro* assays, the use of surface-area normalised response data, and the use of coculture systems; however the concordance is assay and ENM specific. In addition, it is not unusual for there to be differences in experimental outcome when the same *in vitro* or *in vivo* techniques are applied to a common ENM.

Inhaled ENMs do not appear to be readily absorbed through the lungs into the systemic circulation. While low concentrations, relative to those in the lung, can be found in pulmonary lymph nodes none, or very little appears to reach extrapulmonary tissues. The exception may be the spleen for certain ENMs after very high inhalation exposure. Nevertheless, distribution studies after parenteral administration show that if ENMs are absorbed into the systemic circulation they are widely distributed throughout the body. But primarily to tissues that have fixed phagocytic cells (e.g. the reticuloendothelial system of the liver, spleen and lymph system) where, due to the longevity of the phagocytosing cells, they may remain for a long time. The consequences of long term ENM retention in these tissues have not been studied. The half-life of an ENM in blood depends on uptake by the reticuloendothelial system rather than elimination from the body.

There are several common experimental design issues associated with kinetic/distribution studies undertaken with ENMs that should be considered when evaluating or using the data for human risk assessment:

- Administered doses are often unrealistically high relative to anticipated human exposures.
- Doses that exceed the capacity of the pulmonary macrophage system to sequester the ENMs cause pulmonary inflammation and subsequent changes in the pulmonary biokinetics of the ENM. Consequently such studies are not providing relevant information on the biokinetics, systemic absorption or hazard profile of the ENM.
- Because the capacity of macrophages to sequester ENMs is determined by the volume of the phagocytosing cell and the macrophage pool, the density and volume of ENM are important

parameters to consider when designing inhalation dosing regimes. Less dense ENMs occupy more volume than an equivalent mass of denser ENMs, or non-nanomaterial. Consequently pulmonary overload (i.e. exceedance of phagocytic activity) occurs at lower mass doses for less dense materials. Due to air spaces between packing particles, aggregation and agglomeration of ENMs effectively reduces the density of the resulting particle and hence the possibility of pulmonary overload.

- Due to the higher density of wet aerosol ENM preparations compared to dry particle preparations, the extent and relative regional deposition in the respiratory tract may be quite different.
- Characterisation of the nanomaterial is often in a medium different from that to which humans are exposed. Occupational exposure is invariably to agglomerates and not the pristine nanomaterial that has been carefully characterised and tested in biokinetic or toxicological studies.
- Quantification of tissue concentrations is frequently indirect, and not for the nanoparticle *per se*. For example for nano-metal oxides (nMeO), tissue measurements are frequently for the metal ion rather than the nanoparticle (NP). For carbon based ENMs (e.g. SWCNT and MWCNT) the tissue quantitation may be measurement of the metal catalyst (or an isotope thereof, e.g. ^{60}Co) used in its production. Unfortunately these metals are able to be leached from the ENM and so their detection and quantification in tissues is not necessarily a reflection to where translocation of the ENM may have occurred. At the present time the latter can only be reliably determined by transmission electron microscopy (TEM). Given that usually only a very small fraction of the administered NP reaches the systemic circulation (particularly after inhalation of concentrations relevant for human exposure in controlled work places), it is a very tedious, labour intensive and time consuming exercise to scan enough cells to detect the few NPs some of them may contain. It is literally equivalent to the proverbial ‘looking for a needle in a haystack’, indeed several haystacks.
- Mass balance calculations are often not undertaken. As a result the proportion of ENM exposure that may be retained by the respiratory system, or that may reach the systemic circulation, is not able to be quantitated. Without such measurements risk assessment for the ENM is compromised.
- The biokinetics of ENMs in *in vitro* cell culture test systems is often ignored. More often than not the concentration of ENM applied to the system is not the concentration at the cell surface. As the ENM acquires a protein corona and/or agglomerates it effectively becomes less dense. Consequently the time required for uniform distribution within the *in vitro* test system can be longer than the time of the actual test, particularly if ENM contact with cells adhered to the bottom of the incubation plate relies on gravitation.

Common biokinetic themes across ENMs:

- After inhalation of relevant concentrations of ENM, most is exhaled or swallowed as a result of being entrained in mucus or removed via the ciliary ladder in macrophages. The latter accounts for the initial rapid clearance from the lungs (half life ~ <1 -2 hr). Only a small fraction of the external exposure reaches alveolar cells and a very small fraction (of the order of <0.01%) may be absorbed. Nonetheless this small absorbed fraction may be a source of concern due to uncertain consequences of long term retention in the scavenging cells fixed in some tissues.
- Most of the absorbed ENM is translocated to the respiratory lymph system and not to the general circulation.
- If an ENM enters the systemic circulation a major determinant of ENM disposition is the degree of interaction with the reticuloendothelial (RE) cell system.
- Small particles evading the RE system may be excreted by the kidney.
- Larger particles and those with a compatible surface charge may get targeted to RE cells in the liver, spleen and other organs.
- The protein corona plays a large part in how avidly RE cells sequester ENMs.
- Most nanomaterial kinetics are characterised by relatively short blood half-lives reflecting tissue extraction from blood (i.e. distribution) and not clearance from the body.
- A common attribute of nanomaterial kinetics is retention of particles in the tissues that have sequestered them. This is probably a reflection of the turnover time of the phagocytic cells embedded in the tissue.
- ENMs may preferentially be transported in the body via the lymphatic system.

The fact that nanomaterials, after inhalation or other routes of exposure are distributed to lymph nodes suggests the possibility they could modulate immune responses to antigens on bacteria, viruses or foreign proteins. The limited number of studies reviewed in this report confirms this potential. Unfortunately the experimental designs are such that extrapolation to workers is not straightforward. There is however theoretical potential for immunosuppression to occur and be manifested by increased respiratory infections of workers inhalationally exposed, but the extent of exposures for this to be realised is unknown.

The weight of evidence indicates ENMs of various kinds do not penetrate through intact or mildly abraded skin into the live cell layers. The ENMs are confined to the non-viable stratum corneum layer. There are however a few studies suggesting nano-TiO_2 <10nm may cross into deeper layers of the skin and in certain animal models (e.g. nude mice) into the systemic circulation. The relevance of these studies to hazard identification and risk assessment for humans has been questioned by the Australian Therapeutic Goods Administration.

For a few specific ENMs (CNTs, nano-TiO_2 and nano-Ag) health based WESs have been established by authorities or industry. These are underpinned by sub-chronic (i.e. 90 day) repeat exposure inhalation toxicity data and are described in the sections dealing with the specific nanomaterial. The majority of ENMs do not have toxicological data on them that will allow a specific health based WES to be set. Instead authorities in Australia, Germany, the Netherlands and the UK are investigating/implementing control banding approaches.

The reality of workplace risk assessments is that they heavily rely on toxicological and physical hazard information in safety data sheets. It is apparent these workplace information sources are largely inadequate.

Carbon nanotubes:

Long thin single-walled or multi-walled CNTs (SWCNTs or MWCNTs) have fibrogenic toxicological properties similar to certain forms of asbestos. Short or tangled CNTs did not display these effects. The 2009 report for Safe Work Australia made a precautionary recommendation that unless shown otherwise, it would be prudent to assume CNTs of a size and shape similar[1] to other known fibrogenic fibres could also be fibrogenic, i.e. they may have the potential to cause pulmonary fibrosis and pulmonary mesothelioma with long term exposure and retention in the lung. It was emphasised that CNTs may have the capability of eliciting both a particulate and/or fibre-like pathogenic response in respiratory or mesothelial tissue. The biological responses to inhaled CNTs are complex and this precautionary advice is still applicable today.

Since the last review (Safe Work Australia 2009a) techniques have been developed that allow reliable generation of CNT aerosols for toxicity inhalation tests. These studies support the earlier findings from intratracheal or nasopharyngeal exposure procedures. The mesothelioma risk depends on the extent that CNTs of pathogenic fibre dimensions are in workplace air. The evidence to date is that most (maybe all) CNTs in workplace air are respirable agglomerates which are of sub- or low micron size. Studies were not located which addressed the question whether such agglomerates could dissociate into single fibres or fibre-like structures within the lung milieu.

It has been well demonstrated that CNTs are indirect genotoxins. They primarily cause DNA/chromosomal breaks via reactive oxygen formation. In addition CNTs can directly interact with the centrosome structure of dividing cells and induce DNA damage.

The primary properties of CNTs associated with their toxicological mode(s) of action currently being investigated, and which may help with predicting their toxicity, or workplace classification banding based on associations between the properties and biological responses are:

[1] It is fibres of high-aspect ratio and minimum length of approximately 15 - 20 µm that are of most concern.

- Propensity to induce sustained oxidative stress. However it is noted pulmonary inflammation *per se* is not necessarily an automatic indicator for adverse effects.
- Physical dimensions and tumorigenic potential.
- Physical interference with intercellular structures.
- Biopersistence. CNTs are potentially able to be degraded in simulated biological fluid or by peroxidase enzymes in neutrophils and other cells. These effects have the potential to help define what is meant by 'biopersistent'.
- CNT corona when in biological medium.

A number of organisations have recently established exposure standards for CNTs. Using different studies and different adjustment factors to account for uncertainty the derived standards for long term exposures range from 0.0003 – 0.034 mg/m^3. It is considered those based on sub-chronic inhalation exposure are more germane for possible adoption in Australia. NIOSH has undertaken the most in depth assessment of the literature and scholarly derivation of a WES. The value suggested by NIOSH is the limit of quantitation (0.001 mg/m^3) achievable by the recommended analytical method for elemental carbon (NIOSH method 5040). It is suggested this, and the analytical technique, be considered for adoption in Australia as an 8 hr WES for MWCNTs, SWCNTs and CNFs.

The CNTs and CNFs evaluated in animal and *in vitro* studies represent just a small fraction of the CNT/CNF materials that may, or will be in commerce. It is also likely there will be different toxicological potencies between them. However, until validated reliable *in vitro* tests or economic short term *in vivo* hazard identification tests with potency discrimination can be developed, it is prudent to treat all CNTs and CNFs in the work place as if they have the same potential for adverse effects. Thus it is recommended a single WES should apply to all.

Titanium dioxide (TiO_2):

Overall, since the last review, there have not been significant additional pivotal studies published which could potentially provide a further avenue for occupational risk assessment of TiO_2 NP exposures. The toxicological and distribution studies published since the last review provide a better understanding of the potential hazards of TiO_2 NPs, and supporting evidence for conclusions made in the last review.

Overall, the recent acute and repeat exposure inhalational studies support the conclusion that TiO_2 NPs have low potential to induce pulmonary irritation or inflammation, since often only mild, transient effects are seen at high concentrations. Reminiscent of ambient particulate matter pollution, after a single inhalation exposure of a few hours TiO_2 NPs seem capable of producing cardiac effects *in vivo* in the absence of pulmonary inflammation. However these studies are primarily designed to investigate possible modes of action and do not report dose response relationships. It is anticipated

additional information on the cardiopulmonary aspects of nano TiO_2 (and other ENMs) will be forthcoming.

A number of *in vitro* and *in vivo* investigations have shown nano-TiO_2 has genotoxic potential manifested primarily as DNA strand breaks in the Comet assay. This has been concluded to occur through secondary genotoxic mechanisms (oxidative stress) and not direct interaction with the genome. Information additional to the 2009 SafeWork review on inhalational carcinogenicity was not located. Current opinion remains that TiO_2 carcinogenicity is related to pulmonary overload. A two stage skin carcinogenicity assay showed nano-TiO_2 does not have tumour promotion potential.

A number of agencies have derived provisional health-based WESs (or similar standards) for TiO_2 NPs. They range in value from 0.017 – 0.3 mg/m^3. Since the NIOSH 2011 Recommended Exposure Limit of 0.3 mg/m^3 is based on chronic rat studies and contemporary risk assessment methodology it is suggested that this be adopted as a WES for Australia.

Zinc oxide (ZnO):

The toxicological database of nano-ZnO is not as extensive as some other nano-metal oxides (nMeOs). As with other nMeOs, nano-ZnO in *in vitro* cell culture systems is able to cause cytotoxicity and indirect DNA damage via oxidative stress. This appears to be mediated by zinc ions within the cell after the nano-oxide has been translocated from the media into the cell.

Use of acute inhalation toxicity information for workplace assessment is compromised by the very high concentrations that have been used in the experiments. At such concentrations the expected lung inflammation and cytotoxic responses are observed. While the available acute and sub-chronic oral studies with nano-ZnO show increased metal concentration in various tissues, plus or minus indications of tissue damage, their usefulness is diminished by lack of evidence that the nano-ZnO has actually been absorbed from the gastrointestinal tract. In this milieu nano-ZnO is likely to be extensively solubilised.

The use of nano-ZnO in sunscreens has prompted sophisticated human and murine studies to be conducted in Australia whereby very sensitive stable isotope techniques have been able to show small fractions (<0.001%) of the dermally applied zinc (either in nano or sub-micron form), are absorbed into blood. Furthermore the absorption continues for some days after the repeat applications stop and the skin has been washed. It cannot be determined whether the increased blood zinc is the result of zinc ion or nano-zinc absorption. Relative to normal circulating levels of zinc, the amount absorbed from nano-ZnO sunscreen is tiny and does not affect the homeostatic balance of zinc in tissues. No adverse effects are expected. The weight of literature evidence indicates nano-ZnO does not penetrate the skin. Other investigators may not have detected the very small absorption of metal because their analytical techniques were not sensitive enough.

Cerium oxide (CeO_2):

CeO_2 nanoparticles have low solubility and are potentially retained in the lungs. High inhalation exposures have resulted in pathology changes in the lung typical of particulates. Biokinetic studies have been peformed by measuring the fate of cerium, rather than the nanoparticle *per se*. However because they are poorly soluble, and stable, it is presumed by investigators that tissue cerium concentrations are associated with particulates. Soon after inhalation of moderate amounts of nanoceria approximately 25% is excreted in faeces, of this more than 90% is in the first 24 hours. This indicates the clearance from the lung is rapid (via the mucociliary ladder) and gastrointestinal absorption is limited. Once in the systemic circulation CeO_2 nanoparticles may be widely distributed with the highest tissue concentrations found in the reticuloendothelial system. Once there they are retained for a long time.

A mode of toxicological action has not yet been assigned to nanoceria. Nevertheless *in vitro* and high exposure *in vivo* inhalation and intratracheal investigations indicate a typical oxidative stress and inflammatory response associated with biopersistent particulates; including formation of pulmonary granulomas. Not unexpectantly for a particle that produces oxidative stress after entering cells, nanoceria can cause DNA strand breaks in *in vitro* systems.

Silver (Ag):

Intravenous studies with silver nanoparticles (Ag-NPs) show silver accumulating in the liver, spleen and kidneys but increased concentrations in other organs are also noted.

There is a growing body of evidence indicating the toxicological effects of Ag-NPs may be influenced more by silver ions than their nano-form. While there are many studies investigating *in vitro* cellular toxicity and genotoxicity there is a lack of *in vivo* toxicity information that allows correlations between *in vitro* and *in vivo* findings to be made. This is exacerbated by the excessively high particle concentrations frequently used in *in vitro* experiments. The *in vitro* work points to intracellular oxidative stress as being the principal, although perhaps not the sole mode of action as a number of esoteric *in vivo* effects have been observed that do not rely on oxidative stress to occur.

For Ag-NPs there are a number of short term (10, 28 and 90 day) repeat exposure inhalation studies available. Some of these have been conducted according to OECD inhalation guidelines designed to generate safety data for chemicals. While there are clear dose related increases in blood and tissue silver concentrations it appears significant effects (alveoli inflammation and alterations in lung function) only occur in the lungs, and then at high exposure concentrations. The bone marrow micronucleus test was negative after 90 days exposure to concentrations that had been shown to cause lung toxicity. Silver ions and Ag-NPs can form DNA adducts and micronuclei in a concentration-dependent manner, with silver ions being more potent.

Limited monitoring of workplace air at facilities making or handling Ag-NPs shows very small mass concentrations of silver in the air that are orders of magnitude lower than employed in toxicological studies. While it is acknowledged it is a complex question whether there is a higher risk associated with exposure to Ag-NPs compared to similar airborne concentrations of silver ions the available toxicological data point to silver ions as being the ultimate toxic entity of Ag-NPs. Furthermore there is some evidence indicating silver ions are toxicologically more potent than Ag-NPs. This suggests that perhaps the existing WES for soluble silver compounds may be suitable for Ag-NPs.

Contents

Glossary

In addition to the list provided below additional definitions may be obtained from the following.

Nanotechology Now:
http://www.nanotech-now.com/nanotechnology-glossary-A-C.htm

Institute of Nanotechnology, UK:
http://www.nano.org.uk/nano/glossary.htm

National Cancer Institute:
http://nano.cancer.gov/learn/understanding/nanotech_glossary.asp

Northwestern University:
http://www.discovernano.northwestern.edu/whatis/Glossary

National Nanotechnology Initiative:
http://www.nano.gov/about-nni/glossary

ADME: Absorption, Distribution, Metabolism and Excretion.

aerodynamic diameter: Diameter of a spherical particle with a density of 1000 kg/m^3, that has the same settling velocity as the particle under consideration; related to the inertial properties of aerosol particles.

adjuvant: Agents (e.g. chemicals, macromolecules, or cells) which enhance the immune response to an antigen.

Ag-NPs: Silver nanoparticles.

agglomerate: Group of particles held together by relatively weak forces, including van der Waals forces, electrostatic forces and surface tension.

aggregate: Heterogeneous particle in which various components are not easily broken apart.

anthropogenic: Of human origin; man-made.

apoptosis: A form of *regulated* cell death initially identified from pathology but fully characterised as a genetically controlled program most often seen in development (a.k.a. “programmed cell death”). It is usually characterised by the ordered disassembly of the cell’s contents, formation of smaller fragments known as “apoptotic bodies” and engulfment by neighbouring cells. Apoptosis, without secondary necrosis, is not inflammatory.

atherosclerosis: A form of vascular disease characterised by a fatty degeneration of the middle part of the artery wall.

autophagy: A physiological process of organelle degradation within the cell. When autophagy involves the total destruction of the cell it is called autophagic cell death and is a regulated process.

BALF: Bronchoalveolar lavage fluid.

blood-brain barrier: A CNS epithelial cell barrier that is impermeant to all except lipophilic molecules (such as oxygen, carbon dioxide, and ethanol) and those with specific transporters (such as sugars and some amino acids). Substances with a molecular weight higher than 500 Daltons generally cannot cross the blood-brain barrier (incl. viruses and most drugs).

carbon nanofibres: Cylindric nanostructures with graphene layers arranged as stacked cones, cups or plates. Carbon nanofibres with graphene layers wrapped into perfect cylinders are called carbon nanotubes. Abbreviation for carbon nanofibres is **CNFs**.

carbon nanotubes: Tiny tubes about 10,000 times thinner than a human hair -- consist of rolled up sheets of carbon hexagons. Abbreviation **CNTs**.

cytochrome P-450: Any of a large group of haem containing and electron-transferring enzymes that are involved in drug, steroid or chemical metabolism.

CNS: Central nervous system.

DNEL: Derived No Effect Levels. Term used by the European Commission to describe what is effectively equivalent to a workplace exposure standard with no regulatory status.

effective particle size: Measure of a particle that characterises its properties or behaviour in a specific system.

engineered nanoparticles: Nanoparticles between 1 nm and 100 nm manufactured to have specific properties or composition. Abbreviation **ENP**.

engineered nanomaterials: Nanomaterial intentionally produced for commercial purposes to have specific properties or specific composition. Abbreviation **ENM**.

epithelial: Relating to cells in close proximity and which line the surface of an organ or hollow internal structure without the need for connective tissue.

fibrosis: An abnormal (pathological) formation or development of excess fibrous connective tissue in an organ or tissue as a reparative or reactive process.

fullerene: An allotrope of carbon characterised by a closed cage structure consisting of an even number of three coordinate carbon atoms without hydrogen atoms. This class was originally limited to closed-cage structures with twelve isolated five- membered rings, the rest being six- membered rings.

glomerular: Relating to the capillary structures that form the filtering unit of the kidney.

granuloma: Small nodules usually consisting of epithelioid macrophages surrounded by lymphocytes. When necrosis is evident internally this is termed 'caseating granulomas' - especially as observed with tuberculosis.

graphene: Individual layers of carbon atoms arranged in a honeycomb-like lattice, found in graphite.

hepatocyte: The main non-connective cell of the liver (*adj*. **hepatocellular**).

homeostasis: The maintenance of the body's normal operating conditions.

hydrodynamic diameter: Effective diameter of a particle in a liquid environment.

hypertrophy: An abnormal increase in organ size which is not usually cancerous.

intraperitoneal: Within the membrane that lines the abdominal cavity (peritoneum).

ischaemia: A period of reduced or absent blood flow to a tissue which can be caused by many different factors.

keratinised: Regarding the protein comprising the surface layer of the skin.

LDH: Lactate dehydrogenase.

lysosomal: A cytoplasmic organelle containing hydrolytic ("degrading") enzymes and surrounded by a membrane.

MPS: Mononuclear phagocyte system. This is a part of the immune system constisting of phagocytic cells in reticular connective tissue. The cells are primarily monocytes and macrophages and accumulate in lymph nodes and the spleen.

multi-walled carbon nanotubes: Carbon nanotubes (q.v.) which consist of more than one nanotube completely contained within another. Abbreviation **MWCNTs**.

nano: Nanometer = 10^{-9} m or, alternatively, 0.000000001m

nanoaerosol: A collection of nanoparticles suspended in a gas.

nanocrystals: A nanocrystal typically has a diameter of between 1 and 10 nm and may contain as few as a hundred or as many as tens of thousands of atoms. Many fundamental properties of nanocrystals depend strongly on their size. Related term: **quantum dots.**

nanoengineering: The construction of nanostructures and their components.

nanofibre: Nano-object with two similar external dimensions in the nanoscale and the third dimension significantly larger.

nanomanufacturing: Is expected to be high- volume, high- rate, integrated assembly of nano-elements into commercial products. This involves controlling position, orientation, and interconnectivity of the nano- elements.

nanomaterials: Material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale.

nano-object: Material with one, two or three external dimensions in the nanoscale.

nanoparticle(s): An engineered form of matter having at least one dimension (length, breadth or width) in the nanometre scale (<100 nm). Nanoparticles are considered distinct from UFPs (q.v.) for the purposes of this report only insomuch that UFPs are derived from "accidental" sources (human or natural). Abbreviation: **NPs**.

nanophase: Discrete phase (i.e. material's physical state), within a material, which is at the nanoscale.

nanopowder: Dry nanoparticles.

nanoscale: 1 to 100 billionths of a metre (i.e. 1nm to 100 nm).

nanoscience: The study of phenomena and manipulation of materials at atomic, molecular and macromolecular scales, where properties differ significantly from those at a larger scale.

nanospheres: Spheres ideally completely spherical and homogeneous in size and at the nanoscale.

nanostructures: Composition of inter-related constituent parts, in which one or more of those parts is a nanoscale region. Chemically, nanostructures are molecular assemblies of atoms numbering from 10^3 to 10^9 and of molecular weights of 10^4 to 10^{10} Daltons. Thus, they are chemically large supramolecules. To molecular biologists, nanostructures have the size of objects such as proteins or

viruses and cellular organelles. Material scientists and electrical engineers view nanostructures as the current limit of nanofabrication.

nanotechnology: Application of scientific knowledge to manipulate and control matter in the nanoscale in order to make use of size- and structure-dependent properties and phenomena, as distinct from those associated with individual atoms or molecules or with bulk materials.

nanotoxicology: The study of the adverse effects of nanoparticles (NPs) on living organisms.

nanotubes: Nanometre-sized hollow nanofibres composed of various substances including carbon, boron nitride, or nickel vanadate. Carbon nanotubes were discovered in 1991 by Sumio Iijima and resemble rolled up graphite.

nanowires: Molecular wires millions of times smaller in diameter than a human hair.

necropsy: The procedure of postmortem examination.

necrosis: A form of cell death most often – but not entirely - occurring from acute cellular injury and generally considered to be unregulated ("*accidental cell death*"). It is usually characterised by a disruption of the cell's outer plasma membrane and release of internal contents which can then initiate inflammation.

nephropathy: Any damage or disease to the kidney.

neutrophil: A type of leucocyte or white blood cell.

NMeO: Nano-metal oxides.

NPs: Abbreviation for nanoparticles (q.v.), c.f. UFPs (q.v.).

OEL: Occupational exposure limit.

oligonucleotides: A string of up to approximately 30 DNA bases.

particle size: Size of a particle as determined by a specified measurement method.

permissible Exposure Limit (PEL): OSHA (USA) standard for maximum workplace exposure over an 8-hour time weighted average (TWA) exposure. Equivalent to Australian WES (Workplace Exposure Standard).

phagosomal: Relating to a specialised cellular structure formed during the internalisation of foreign particles by enclosing in the outer membrane. (*Verb*: **phagocytosis**).

proteolytically: Relating to the splitting of proteins or protein fragments by enzymes.

quantum dots: Nanometre sized fragments of semiconductor crystalline material.

reticuloendothelial: The widely diffused bodily system constituting all phagocytic cells except certain white blood cells.

ROS: Reactive oxygen species, i.e. non-organic molecules containing oxygen that cause oxidative effects.

sarcoma: A malignant tumour of non-epithelial tissue (e.g. connective tissue).

sequestration: The action or process of making unavailable without destroying or inactivating.

semiconductor: Material whose conductivity is normally in the range between that of metals and insulators and in which the electric charge carrier density can be changed by external means.

single-walled carbon nanotubes: Carbon nanotubes (q.v.) which do not contain any material internally. Abbreviation **SWCNTs**.

specific surface area: Ratio of the surface area to the mass of nanoparticles.

squamous cell: A morphologically thin and flattened cell of an epithelial layer.

TEM : Abbreviation for Transmission Electron Microscopy.

UFPs: Abbreviation for ultrafine particles (q.v.).

transcription factor: A protein which is involved in the control of new gene expression.

ultrafine particles: An anthropogenic or natural form of nanoparticle which is usually derived from combustion processes. UFPs are distinguished by large variations in size and composition.

WHS: Work health and safety.

workplace exposure standard (WES): Safe Work Australia standard for maximum workplace exposure over an 8-hour time weighted average (TWA) exposure. Equivalent to US PEL (Permissible Exposure Limit). This term has been used throughout the report when referring to occupational exposure limits from various countries.

xenobiotic: A chemical foreign to the body and is not normally produced or expected to be present in it.

1. Introduction & Scope

1.1 Safe Work Australia 2009a

This report is an update of the toxicology and health effects of engineered nanomaterials (ENMs) as the information pertains to hazard identification and risk management in the workplace. The previous report, "*Engineered Nanomaterials: A Review of the Toxicology and Health Effects"* (Safe Work Australia 2009a) was a comprehensive review which covered the literature from 2006 to 2008 with some important studies from early 2009, it was published in 2009. Overarching conclusions from that review were:

- Biopersistence is a critical ENM property for induction of particulate- and/or fibre-like responses in the lung.
- It was clear non-functionalised biopersistent carbon nanotubes (CNTs) of pathogenic fibre-like dimensions are potentially hazardous to health if inhaled in sufficient quantity. Consequently, manufacturing and handling procedures need to minimise workplace exposure to all respirable CNTs that physically resemble known fibrogenic materials.
- For the purposes of managing workplace exposure and minimising possible health effects, no distinction could be made between pathogenic fibre size single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) as to their potential hazards and risks. Further data was required.
- Consideration be given to establishing a workplace exposure standard (WES) for CNTs and non-functionalised nano-TiO_2.
- There was a significant need for chronic, or at least, repeat exposure inhalation studies of ENMs. One of the reasons why repeat inhalation toxicity studies had not been widely undertaken for many ENMs was the fact that ENMs in air readily agglomerate and it was difficult to generate aerosols of individual ENMs in sufficient quantity for such studies.
- Agglomeration also occurs in aqueous suspensions and while addition of lipoprotein, albumin, serum and/or surfactant lessens agglomeration there was uncertainty whether the nanomaterial being evaluated in intratracheal and/or aspiration toxicity studies reflected the physical state of the nanomaterial to which workers may be exposed.
- To promote the generation and use of experimental dose-effect information that would be useful for occupational risk assessment, there was an urgent need to characterise in detail the nature of airborne ENMs in workplace air and determine the dissolution behaviour of such ENMs in lung fluid.

The previous review included many descriptions of the experimental methodologies underpinning the toxicological information. A theme throughout that review was how experimental protocols can profoundly influence the data obtained and its interpretation. This current review is a broad appraisal

of the scientific literature and international agency deliberations on ENMs that was publically available between 2009 to early 2013. The literature search strategy is summarised in Appendix A. It differs from the previous review in that there is less emphasis on experimental details, except where it is necessary for data elucidation. This review attempts to identify key advances in knowledge in a manner that is understandable by persons who are not necessarily experts in toxicology. Nonetheless, since the majority of the available information is still being generated by test tube *in vitro* studies it explores the role of *in vitro* information in hazard and risk assessment.

Two of the themes that emerged from the 2009 review (Safe Work Australia 2009a) were the importance of appropriate characterisation of ENMs in the experimental system, and how understanding the experimental procedures enable interpretation of the data and extrapolation to humans. Most, but not all, studies in the last few years are now characterising ENMs in the presence of proteins or pulmonary surfactants. The influence of proteins and surfactants on the biological effects of ENMs is discussed in Section 3.2. As indicated this current review does not dwell on issues of experimental protocol unless there is significant effect on data interpretation. However the reader is directed to several recent publications in which the difficulties, nuances and pitfalls in conducting toxicological studies with ENMs are described. These publications cover issues such as genotoxicity testing, reliable nanoparticle (NP) aerosol generation, appropriate measurements for hazard identification of inhaled NPs, pathological assessment of NPs and test batteries for safety assessment (Hubbs et al. 2011; Kim et al. 2010a; Krug and Wick 2011; Gonzalez et al. 2011; Landsiedel et al. 2009, 2010a, 2012a, 2012b; Ma-Hock et al. 2009b; Maynard et al. 2011; Oberdorster 2010; Park et al. 2009a; Rivière 2009a; Sayes et al. 2011; Schmoll et al. 2009; Warheit and Donner 2010; Warheit 2010a, 2013).

In addition, some ENMs can interfere with techniques used for assessing cell toxic responses (Wang et al. 2012c, Holder et al. 2012, Jiang et al. 2013, Safe Work Australia 2013a, EC 2009b, Vankoningsloo et al. 2010, Landsiedel et al. 2010a, Monteiro-Riviere et al. 2010) and that agents used to disperse ENMs may break down during preparation of the ENM test solution or in *in vitro* systems, be toxic in their own right or alter the culture medium to the extent that it affects cells (Mahmoudi et al. 2010, Wang et al. 2012b).

This current review has greater emphasis on the implications the emerging toxicological data may have for hazard identification, exposures and risk assessments associated with ENM processes in the workplace (which includes reseach facilities). Some consideration is given to estimating/measuring airborne ENMs and establishing workplace exposure standards (WESs). Early chapters of the report contain toxicological and health information for ENMs in general. In recognition of the explosion of publications on nanoparticulate toxicity, there are chapters providing detailed information on carbon nanotubes (CNTs) and a number of nano-metal oxides.

1.2 Scope

Although it may differ from the perception[2] of reseachers in nanotechnology, in the workplace inhalation of airborne ENMs is the most important route of exposure. This review therefore concentrates on information relevant to inhalation exposure, in particular experiments in which there is repeated exposure attempting to identify intermediate or long term effects. The proportion of ENMs deposited from the lungs into the systemic circulation is likely to be small, nevertheless it is known that if ENMs reach the systemic circulation they are widely distributed throughout the body. Of concern therefore is the potential toxicological consequences of the distribution and long term tissue retention of ENMs.

An important issue for workplace exposure is the form of ENM exposure. Workers may be exposed to the primary ENM, agglomerates or aggregates. The review looks to see if there are differences in the toxicology and dose response of these particles.

Since certain carbon nanotubes (CNTs) have raised concern due to potential fibre-like responses in the lung, and because nanoforms of metal oxides (e.g. titanium dioxide, zinc oxide and cerium oxide) and silver are widely used, these ENMs are examined in detail.

Of obvious importance to the workplace are exposure limits for ENMs. Some organisations have proposed limits for certain ENMs. This review examines the toxicological evidence supporting the proposed exposure limits and the feasibility of setting workplace exposure standards (WES) for these nanomaterials.

In this regard a recent publication from the NIOSH Nanotechnology Research Center is a useful compilation of information (NIOSH 2012). This report summarises NIOSH nanotechnology research accomplishments from its inception (2004) through 2011 and provides direction on how the data are being used for improving workplace safety. The report contains analyses of progress made toward accomplishing the goals and objectives of the NIOSH Strategic Plan for Nanotechnology Research, as well as addressing the goals and research needs identified in the US National Nanotechnology Initiative Environmental, Health, and Safety (EH&S) research strategy. The report highlights more than 40 field assessments in nanomaterial manufacturer and user facilities; innovative sampling methods for ENMs; recommended exposure limits for titanium dioxide, CNTs, and carbon nanofibres; and guidance for safe practices for working with ENMs in research laboratories. Various sections of this review summarise pertinent information from NIOSH (2012).

[2] The British Health and Safety Executive found that the majority (52%) of researchers in university laboratories in the UK believed the main route of exposure to ENMs to be dermal, followed by inhalation (37%) (UK HSE 2013). This perception was linked to many researchers handling ENMs in solution rather than dry form, and coincided with the high tendency for glove usage during handling.

1.3 Overview of agency reviews

Over 90 relevant reports/communications/reviews were identified and sourced from national and international agency websites. A brief description of each is provided in Appendix B. The majority of the agencies still provide very general information on the health and safety aspects of ENMs. However, there is a consensus that ENMs cannot be collectively or categorically considered either intrinsically benign or harmful. Rather, hazard and risk assessments must be conducted on a case-by-case basis.

Agencies also concur that if existing test methods cannot be adequately adapted to cater for ENMs, new test methods should be developed. The OECD (2009d) reviewed the applicability of existing OECD test guidelines for ENMs. They concluded many of the guidelines relating to physical and chemical properties are not applicable to ENMs or require further modification before they can be used. For guideline protocols relating to toxicity (i.e. health effects) testing, OECD concluded in general they are applicable to ENMs. But with the important proviso that additional consideration needs to be given to the physicochemical characteristics of the material tested. In some cases, however, (e.g. inhalation and studies on absorption, distribution, metabolism, and excretion, i.e. ADME) the OECD guidelines require further modification (OECD 2009d). OECD (2012b) has since held an expert workshop specifically to discuss what modifications to the existing inhalational toxicity guidelines are required.

A central theme of the reviews remains the lack of sufficient or adequate data to allow for exposure assessment and risk characterisation, and the need for more information. There has been progress in that some agencies have funded, or are planning to fund, additional toxicity studies with the intention of providing clarifying information (e.g. BauA 2011a, 2011b; Hankin et al. 2008; Umweltbundesamt 2010). In an attempt to focus further research efforts, a few agencies and other organisations have published research strategy guidance documents (NNI 2011, NRC 2012, Tran et al. 2008, US EPA 2009a).

Participants from various international agencies at an OECD workshop on risk assessment of ENMs agreed that the risk assessment paradigm for chemicals will continue to guide approaches for risk assessment of ENMs (OECD 2010b, 2012d), however many of the assumptions and estimations embedded in risk assessments of chemicals or bulk materials[3] need to be reevaluated for ENMs. The participants of the workshop also concluded there does not seem to be a rationale for employing a nano-specific risk assessment uncertainty factor, nevertheless application of the standard risk assessment uncertainty factors should undergo validation. Participants concurred it is expected, at

[3] In this report a bulk material is considered one that does not meet the criteria for being nano-form, i.e. at least one dimension <100nm.

least for the near future, that risk assessments will continue to be reported in terms of mass based units. It was however recommended risk assessments should include a discussion of the limitations this metric may present (OECD 2010b, 2012d).

A few agencies have conducted preliminary generic quantitative hazard and risk assessments for ENMs in the workplace (e.g. EC 2009b, BauA 2010). Although the European Commission (EC 2007b) emphasise the preliminary nature and the high uncertainty in their assessment, they concluded that for most exposure scenarios the risks from ENM exposure do not appear to be adequately controlled in the workplace. Contrary to this, a report by the UK HSE (2013) found that research groups and university departments generally adequately controlled exposures to nanomaterials in their laboratories. In addition, regulators who visited Australian workplaces where ENMs are handled also found that in most instances exposures were well controlled [4].

Some agency reviews continue to express a need for adequate tools for ENM exposure assessment and mitigation in the workplace (e.g. OECD 2009a, b, c; EC 2009b), but many now provide practical risk management options (EASHW 2009, Environment Canada 2009, BauA and VCI 2012, NIOSH 2009a; Safe Work Australia 2011a, 2012c; UK HSE 2009, UKNSPG 2012, US EPA 2012), such as the use of HEPA filters, local exhaust ventilation, and PPE. Others have derived provisional workplace exposure standards (WESs) to assist in controlling exposures in the workplace; these are either health-based or non-health based (see Section 3.10.1). It is generally understood by agencies that existing WESs for bulk substances (e.g. graphite) may not provide adequate health protection from nanoparticles made from that substance (e.g. carbon nanotubes) (OSHA 2013, NIOSH 2013a, UKNSPG 2012, UK HSE 2009).

2. Occupational exposure and illness

2.1 Introduction

While a review of measurement and exposures to ENMs in the workplace is not part of the scope of this document it is nonetheless useful to be able to put into perspective the issues involved with gathering human health data on ENMs. Also to understand how the toxicological information may mutually inform workplace hazard and risk assessments, and consequently risk management.

Identification of illness in the workplace due to ENM exposure is challenging. Epidemiology investigations are problematic to design and exposure determinations are usually their Achilles heel. Even when traditional exposure estimations are well trialled (e.g. measurement of chemical in air,

[4] Personal communication with Safe Work Australia 02/09/2013.

biomarkers linked to air exposure, job description matrices, etc), it is daunting to adapt them to ENMs. Issues associated with exposure estimation of ENMs are canvassed later in this section.

Eisen et al. (2011) discuss some of the epidemiology issues for ENMs. Consistent with NIOSH (2009b), they maintain that to resolve some of the problems, and provide a basis for future health studies, hazard surveillance offers a framework for the systematic collection and analysis of exposure data. Anticipating the most likely adverse health effects is but one of the challenges. In this regard Eisen et al. (2011) draws on the epidemiology work with urban particular matter and ultrafine particulates to, not surprisingly, suggest health monitoring for ENMs should focus on respiratory (chronic obstructive pulmonary disease) and cardiovascular endpoints. Due to uncertainty in defining the hazards and possible health effects of ENMs, and practicality concerns, others suggest general medical screening with just a few tests targeted to some of the anticipated health outcomes (Bergamaschi 2009).

The NIOSH (2009a) interim guidance on hazard surveillance of ENMs addresses the question of whether specific medical screening is appropriate for workers potentially exposed to engineered nanoparticles who do not display symptoms of disease. NIOSH concluded there was insufficient scientific and medical evidence to recommend specific medical screening of workers potentially exposed to engineered nanoparticles. However it was suggested that where occupational medical screening recommendations exist for given chemicals or bulk materials, those recommendations would be applicable for workers exposed to engineered nanoparticles composed of those same chemicals or bulk materials. NIOSH stress hazard surveillance[5] for ENMs is an essential component of any occupational health monitoring effort and is used for defining the elements of the risk management program. A guidance document on the safe handling of CNTs, states with respect to health monitoring, there are no current tests specific to CNTs (Safe Work Australia 2012c). However, it was suggested a prudent approach would be to collect information about the materials being used, the duration of use and potential or actual exposures. The guidance emphasises that such information can assist in building up a risk profile which could be important if health effects are observed at a later date (Safe Work Australia 2012c).

The French Institute for Public Health Surveillance (L'Institut de veille sanitaire, i.e. InVS) was charged with developing a health monitoring protocol for workers with occupational exposures to ENMs. The first stage of the project involved general research into feasibility, delineation of first steps and defining (in general terms) the type of monitoring which could be done (InVS 2012). InVS concluded that while it seems very important to orient the monitoring system toward pulmonary and

[5] According to NIOSH (2009b) hazard surveillance involves identifying potentially hazardous practices or exposures in the workplace and assessing the extent to which they can be linked to workers, the effectiveness of controls, and the reliability of exposure measures. One component of a risk management program involves taking action to minimise exposure to potential hazards.

cardiovascular pathologies, it is essential that it maintains an unspecific feature to allow study of health events affecting other organs or systems, such as the liver, kidneys, CNS or reproductive system.

Lee et al. (2012a) undertook a health monitoring case study on workers who manufacture Ag-NPs. In this instance the 'monitoring' was limited to measurement of Ag-NPs in air, silver in blood and urine, and blood biochemistry and a haematology. Animal inhalation studies identify the lung and liver as potential toxicological targets (Section 8.4.2). Only two workers took part in this voluntary study and lung function tests were not undertaken, the workers had been exposed for 7 years. Their spot personal air exposure measurements (over 3 days) were markedly less than the ACGIH TLVs for silver dust or soluble silver (see Section 8.6), blood and urine silver concentrations were less than in the general population and blood biochemistry and haematology showed no significant findings.

Not much can be gleaned from the 'health monitoring' study of Lee et al. (2012a). It does however illustrate a major difficulty in undertaking occupational epidemiology studies for ENM exposure. Compared with conventional chemical industries the number of workplaces manufacturing or using nanomaterials is relatively small; obtaining sufficient personal exposure data and the matching health monitoring data for meaningful statistical analysis is therefore difficult. For example Curwin and Bertke (2011) investigated nano-metal oxide exposures in seven facilities, the number of employees specifically involved in producing and handling the nanomaterials in each facility was only one or two employees.

2.2 Exposure

2.2.1 Measurement/control

As indicated above it is not the intention of this review to document how ENMs are measured in the workplace or what concentrations are being recorded. Nevertheless the reader should be aware that a number of strategies for estimating ENM exposure in the workplace have been suggested (some are briefly described later in relation to exposure control). Within the European Union harmonised approaches for measurement strategy, data analysis and reporting are being developed (Brouwer et al. 2009, 2012). According to Brouwer et al. (2009), studies of workplace air measurements have generally been explorative by character and have focused on the potential for emission of ENMs. Safe Work Australia has recently published a research report on the measurements of particle emissions from nanotechnology processes in which measuring techniques and workplace controls were assessed (Safe Work Australia 2012e). It was found that it was essential for local background particle exposure to be accounted for when characterising the emission of particles and assessing exposure of nanotechnology workers. Indeed average particle concentrations from the nanotechnology processes assessed were the same order of magnitude as the local particle

background exposure. However peak concentrations were higher and it was suggested these may be better indicators for when a process required specific particle emission control measures. Plizko (2009) also stressed the importance of adequately characterising particles in ambient air when ENM processes were not being undertaken.

Considerable advancement has been made in analytical instrumentation and measurements. Area and personal exposures for a variety of ENMs are now being published (e.g. Liao et al. 2009, Methner et al. 2009a, 2009b; Huang et al. 2010, Kuhlbusch et al. 2011, Dahm et al. 2012, Lee et al. 2011, Leppänen et al. 2012) but it is problematic in extrapolating these from one situation to another, for example from a well characterised workplace to one that has no exposure information. According to Brouwer (2010) a representative estimate for the potential for worker exposure to a particular ENM, or agglomerate, during a particular step of the manufacturing process is currently not possible. It seems that bands of exposure connected to common job descriptions are likely an appropriate way forward (see later). A report commissioned by Safe Work Australia (2012e) suggests a complimentary set of instruments such as a P-Trak [i.e. a portable condensation particle counter], optical particle counter, and DustTrak can be used to gather both temporal and spatial data. If further information on the nature of the particles is required filter and electrostatic precipitator based samples can be collected for analysis by electron microscopy and energy-dispersive x-ray spectrometry.

Exposure measurements have been most often undertaken using static devices, these represent aerosol concentrations and size distributions at a fixed position, occasionally they have been positioned close to the emission source. These measurements however do not necessarily represent concentrations being inhaled by workers, nor, due to agglomeration, the character of ENMs used in toxicological studies to identify potential hazards (Brouwer et al. 2009). Indeed, due to agglomeration, exposure to primary nanoparticles in aerosols is expected to be low (Plitzko 2009).

NIOSH (2009a) consider personal sampling is preferred to ensure an accurate representation of the worker's exposure, whereas area sampling and real-time exposure measurements may be more useful for evaluating the need for improvement of engineering controls and/or work practices. A variety of exposure matrices (number, mass and surface area concentrations) should be reported to facilitate comparison between exposure studies (Bello et al. 2009a, Hameri et al. 2009). Recent studies have heeded this advice. For example, in a study conducted by Queensland University of Technology for Safe Work Australia, the characteristics and behaviour of particles arising from the operation of six nanotechnology processes were investigated using a combination of real-time measurement techniques of sub, and supermicrometre particle number, mass concentration and count median diameter (Safe Work Australia 2012e).

So that the information is useful in a wider context, Clark et al. (2012) propose types of information that should, at a minimum, be included when reporting the results of ENM exposure research. One of the key messages of this paper is that significantly more research is needed before comprehensive exposure scenarios and associated exposure estimates for ENMs can be developed for potentially predicting possible exposures in new facilities. A plea is also made to researchers that the advancement of the ENM exposure information to maximise the impact of their work could be achieved by better descriptions of the work. For example, including detailed descriptions of production processes, product uses, and sampling strategies. Lists of such details are provided.

As discussed in the Safe Work Australia (2009a) review, experimental toxicological studies should mimic the exposures that occur in the workplace. But, due to formation of larger agglomerates after release of the individual nanoparticles they may not. Curwin and Bertke (2011) found that in seven facilities manufacturing nano-metal oxides[6], the majority of the particles in workplace air were agglomerated, with the predominant particle size being between 0.1 and 1µm. Notwithstanding this they noted exposures were occurring at levels well below established limits[7].

Exposure 'targets' articulated as workplace exposure limits are often used to judge effectiveness of engineering controls and gauging the margin of safety for measurement of a given airborne ENM. This is further discussed in Section 3.10.1. Risk matrices are also being touted as effective exposure control tools. According to Brouwer (2012) the latter are risk-assessment/management approaches that apply the generally accepted risk paradigm of risk being a function of severity of impact (hazard) and the anticipated probability of that impact (exposure). In Brouwer (2012) both hazard and exposure are graded into 2 - 5 different levels referred to as bands. The two sets of bands are combined as a matrix that reveals the control, or risk bands. Brouwer (2012) describes six control banding tools, their uncertainty and how they address exposure and hazard uncertainty. It was concluded the value of the currently available control banding tools for nanomaterials can be enhanced by transparently elucidating the differences in the tools for user consideration during the selection of a tool for a specific scenario of application. The task based exposure sampling advocated by Ham et al. (2012) is an approach that could support broad application of the control banding approach.

[6] The facilities studied by Curwin and Bertke (2011) manufactured oxides of titanium, magnesium, yttrium, aluminium, calcium, and iron. There were two broad process categories—production and handling. The production process included spray drying, combustion reaction, and chemical reaction (observed in manufacturing facilities). Handling included weighing, mixing, pouring, and collecting (observed at both manufacturing and end use facilities).

[7] The reference limits used by Curwin and Bertke (2011) were the NIOSH (2011) recommended exposure limit (REL) of 2.4 mg/m^3 for fine TiO_2 (<2.5 µm diameter) and 0.3 mg/m^3 for ultrafine TiO_2 particles (<0.1 µm diameter) and the OSHA permissible exposure limit (PEL) of 5 mg/m^3 for the respirable fraction of particulates not otherwise regulated.

Kuempel et al. (2012a) describe a strategy for developing exposure control limits and bands incorporating risk-based estimates derived from comparative potency of a particular ENM to 'benchmark' ENMs. The strategy relies on a bank of nanomaterials which are representative of various modes of action classes, and for which hazard and risk evaluations have been done. These then act as 'benchmarks' for new nanomaterials which can be assigned a surrogate workplace exposure limit or a hazard/risk band (Figure 2.1). The apical information for such a scheme is toxicological data for a number of well-characterised ENMs. While the scheme is sensible and consistent with contemporary risk assessment methods for chemicals the toxicological updates for ENMs in this review suggests the data are not yet available for its wide spread implementation.

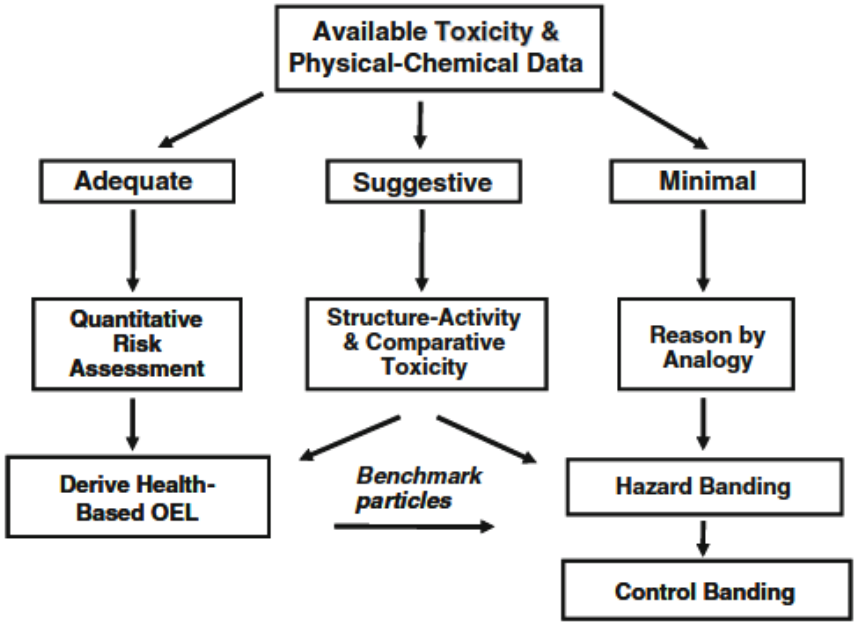

Figure 2.1: Strategy suggested by Kuempel et al. (2012a) for developing workplace exposure limits and hazard bands.

Ling et al. (2012a) propose three levels of ENM characterisation based on aspect identification (fibre like material), solubility tests, dermal absorption, and cytotoxic analyses (see also Section 4). From these determinations a workplace exposure to an ENM is placed into three risk management bands of escalating exposure protection. The use of *in vitro* cytotoxic assays in the scheme is novel but somewhat arbitrary. It appears a threshold IC_{50} of 100 µg ENM/mL in the culture medium for cell death has been set as a differentiator between the top two levels of protection. However there is no discussion how this value was decided.

Ramachandran et al. (2011) describe a strategy for assessing workplace exposure to ENMs based on the American Industrial Hygiene Association paradigm. The authors consider it to be a highly tailorable exposure assessment strategy for nanomaterials that enables effective and efficient

exposure management (i.e. it is a strategy that can identify jobs or tasks that have clearly unacceptable exposures), while simultaneously requiring only a modest level of resources to conduct.

Standard occupational hygiene control measures are important and effective in limiting exposures to ENMs. Good work practices can minimise worker exposures and well-designed exhaust ventilation systems with high-efficiency particulate air (HEPA) filters can effectively remove nanomaterials from the air (NIOSH 2009a). Indeed in Han et al. (2008) concentrations of MWCNT in a research facility were reduced by 3 – 4 orders of magnitude[8] after installation of simple ventilation fans and isolating the MWCNT blender. Plitzko (2009) reports airborne concentrations of ENMs in research laboratories and companies producing small amounts of ENM. It was concluded there was no significant increase of nanoparticle numbers provided proper handling was guaranteed (e.g. closed systems, extractor hoods).

In Australia, a sector that is handling small amounts of ENMs is research laboratories. In a research and development centre laboratory in the United States, Johnson et al. (2010) measured airborne nanomaterials as they were weighed, transferred to beakers filled with water and sonicated. It was found ENMs can become significantly airborne when mixed in solution by sonication, especially when they are functionalised or in water containing natural organic matter.

For assessing workplace exposures to airborne ENMs a three tiered approach has been suggested (Safe Work Australia 2012e):

- Tier 1: A standard industrial hygiene survey of the process area to gather qualitative information identifying likely points of particle emission relative to the background.
- Tier 2: Measure real-time local background particle exposure and from time-series plots determine average and peak concentrations, and repeat for the breathing zone of ENM process workers. The ratio of the measurements is compared to excursion guidance criteria. Further assessment maybe required if:
    - Short term emissions/exposures exceed three times the local particle reference value for more than a total of 30 minutes during a work day; and/or
    - if a single short term (peak) value exceeds five times the local particle reference value.
- Tier 3: Collect particles for characterisation. Detailed advice is provided on how this may be accomplished.

[8] In Han et al. (2008) the concentration in a blending laboratory ranged from 172.9 to 193.6 MWCNTs/cc before the control measures, and decreased to 0.018–0.05 MWCNTs/cc after the protective improvements.

2.2.2 General nanomaterial exposure

Sanding coated surfaces:

Theoretically, it is not only during manufacture and application of surface coatings (the latter being the case in Song et al. 2009 discussed below) that worker exposure to ENMs in these products may occur.

Koponen et al. (2011) examined the possibility that introduction of ENMs into traditional surface coatings (e.g. paints, lacquers, fillers etc) may result in nanoparticle exposures to workers and consumers when coated surfaces were sanded. The products tested contained nano-TiO_2, carbon black or silicon dioxide. The authors noted electric motors emit high numbers of particles in the nano-range (Szymczak et al. 2007) and that measurements were complicated as a result, and also the extent of pressure applied to the machine while sanding altered particulate release into the air. Koponen et al. (2011) found adding ENMs to the surface coatings only vaguely affected the geometric mean diameters of the particle modes in sanding dust when compared to their reference products, but there were differences in the number concentrations in the different size modes. Overall there was no clear effect of ENPs in applied surface coatings on the particle size of dust emissions when the surfaces were later sanded. It was found that the dominant source of particles smaller than 100nm was the sander motor. This reinforces the need for cognisance to be paid to concomitant 'background' sources when measuring airborne nanoparticles in the workplace (discussed in Brouwer et al. 2009, NIOSH 2009a, Kuhlbusch et al. 2011).

Gohler et al. (2010) designed an experimental system that controlled many of the variables influencing the data generated by Koponen et al. (2011). They showed there was considerable generation of nanoparticles during the sanding process. However, in agreement with Koponen et al. (2011) no significant difference was observed between coatings containing and not containing nanoparticle additives. A similar finding was made in a report prepared for Safe Work Australia (2013c) which found the overall mass of emissions from machining (including wet and dry cutting, drilling, sanding, grinding, and abrasion) composites reinforced with nanomaterials was in most instances not significantly different from machining composites not containing nanomaterials. Furthermore, the reinforcing nanoparticles were embedded within particles released from machining, with the number of free nanomaterials released being low.

Oil mists:

Wang et al. (2011b) determined concentrations of oil mist nanoparticles from mineral oil-based metal working fluids in a fastener manufacturing plant. They cite epidemiological and animal studies indicating oil mist exposures might result in laryngeal cancer, asthma, bronchial hyperresponsiveness, lipoid pneumonia, and lung cancer. Wang et al. (2011b) found oil mist nanoparticles were formed

mainly through evaporation and condensation processes. As such they are unlikely to act as delivery vehicles for metal fragments into the deep lung. While strictly not ENMs, the nano-nature of a proportion of oil mists from metal working fluids is a reminder that nanomaterial exposure is not necessarily only about the new phenomenon of ENMs, rather it has been in the work environment for a long time. In addition such sources of 'nanoparticles' form part of the background measurements in workplaces that do make or handle ENMs. Similarly workers in subways and metal refineries are exposed to nanomaterials (Midander et al. 2012, Miller et al. 2010).

Aerosol products:

Chen et al. (2010), from the United States National Institute for Occupational Safety and Health and the U.S. Consumer Product Safety Commission, have demonstrated that a commercially available aerosol consumer product creates nanoparticles. The product was marketed as containing nano-TiO_2 particles, and intended to be used as a bathroom cleaner/sanitiser. While aerosol droplets initially from the can were large with a count median diameter of 22 µm, the final aerosol contained primarily solid TiO_2 particles with a diameter of 75 nm. The size reduction was due to the surface deposition of large droplets and the rapid evaporation of the aerosol propellant. The authors calculated the worst-case lung burden for a human adult male after a 1-min spray indoors in a room with limited ventilation, was approximately 0.075 µg TiO_2 per m^2 alveolar epithelium. This was equivalent to a pulmonary dose of 0.03 µg TiO_2 in a rat. While the dose was low compared to those that induced systemic microvascular dysfunction in rodent studies the authors expressed concern for potential harmful exposure if repetitive sprays are conducted in a poorly ventilated environment. This concern would be higher if products such as these become commonly used by professional cleaners.

Information is provided in Section 8 on exposure to Ag-NPs from aerosol products.

It is interesting that nanoparticle containing aerosols are being explored as promising new therapeutic options for drug delivery (Froehlich et al. 2012c).

2.3 Respiratory effects

A paper by Song et al. (2009) was described in the 2009 review (Safe Work Australia 2009a). Exposure over 5 – 13 months to aerosols and/or fumes of a polyacrylic ester board coating, putatively containing nanosize particles, resulted in serious lung injury and extrapulmonary toxicity in seven Chinese females. Notably the exposures occurred because there was catastrophic failure of engineering exposure controls and other standard hygiene practices. The patients were admitted to hospital with shortness of breath, and common clinical findings of pleural effusion[9], progressive

[9] Pleural effusion is an abnormal amount of fluid in the pleural cavity between the lung and chest wall (ribs).

fibrosis, pleural damage and pericardial effusion[10]. Two of the women died, others suffered long term respiratory damage that developed very quickly or over a longer period after cessation of exposure. It was apparent their medical condition was not exacerbation of pre-existing illness. In addition these persons had intense itching on their faces, hands and forearms. This raises the spectre of immunological involvement. Sobering features of these clinical cases are the severity of the effects and the difficulties in diagnosis and treatment. The finding of nanoparticles in accumulated workplace dust and in the pleural effusion of some of the patients prompted the authors to suggest nanoparticles of the polyacrylic ester were responsible for the effects. Further work has since identified the nanoparticles in the tissues were silica (Song et al. 2011, Song and Tang 2011), this material is commonly used as filler for surface coatings.

It is well known long term exposure to silica dusts can result in, or contribute to severe respiratory diseases, including silicosis, interstitial fibrosis, industrial bronchitis, small airway disease, emphysema, and vascular diseases, as well as immunologic reactions. Interestingly in the early stages of the above cases (within 3 months of onset) the silica nanoparticles were located primarily in lung macrophages (cytoplasm, nuclei and organelles) and in pulmonary microvessels. Late in the disease (18 months, just prior to death), nanoparticles were observed in pulmonary cells and interstitial tissue, but few in macrophages (Song and Tang 2011). While the recent information suggests nanosized silica dust may have played a major part in the onset and rapid progression of the pulmonary symptoms, it cannot be dismissed that other types of nanoparticles could also have contributed. Although this recent evidence has strengthened a causative role for ENMs in these cases, the fact remains that they occurred as a result of gross over exposure due to extremely poor occupational hygiene.

From a pathology perspective Phillips et al. (2010) revisited a case described in 1994 (Rendall et al. 1994) in which a healthy male inhaled nanoparticles of nickel while spraying nickel onto bushes for turbine bearings using a metal arc process. While the exposure was not to ENMs *per se*, this case has disturbing parallels to the circumstances described by Song et al. (2009); these are a failure of good occupational hygiene practice resulting in very high exposure, the speed of onset of respiratory effects, and their seriousness. The worker died, the diagnosis was adult respiratory distress syndrome[11].

[10] Pericardial effusion is fluid around the heart.

[11] Adult respiratory distress syndrome (ARDS) is a life-threatening lung condition that prevents enough oxygen from getting to the lungs and into the blood. ARDS leads to a buildup of fluid in the air sacs (the alveoli) and prevents oxygen from passing into the bloodstream. The fluid buildup also makes the lungs heavy and stiff, and decreases the lungs' ability to expand. ARDS is different from the pleural effusion described by Song et al. (2009).

The occupational hygiene issues were:

- The process was new to the plant and the worker had no previous experience in its use.
- He was provided with a half face mask but was observed to remove it during the spraying process which lasted for approximately 90 min.
- On reenactment of the process (Rendall et al. 1994) the exposure was determined to be massive. The particulate nickel concentration in the vicinity of the operator was 382 mg/m^3. This concentration is well above the current workplace exposure standard for nickel metal in Australia of 1 mg/m^3 (Safe Work Australia 2014) and the US National Institute of Occupational Safety and Health Immediately Dangerous to Life (IDLH) concentration of 10 mg Ni/m^3 (NIOSH 1994). The majority of the particles were about 50 nm, and during the 90 min exposure the worker was estimated to have inhaled approximately 1 g of nickel nanoparticles.

The health effects were:

- Immediately after operating the process he complained of feeling unwell and went home.
- Visited doctor next day, complaining of cough, shortness of breath, and a tight chest. Prescribed anitbiotics.
- Four days later he went back to the doctor and was admitted to hospital.
- The worker died of respiratory failure 13 days after the exposure to nickel.
- Heart, brain and kidney pathology, together with high urine nickel, strongly suggested translocation of nickel across the lung into the systemic circulation[12].
- Nanoparticles (4 – 25nm) of nickel were observed in pulmonary macrophage lysosomes, but were not seen in other cells.

Journeay and Goldman (2014) describe a case study of a female chemist who developed throat irritation, nasal congestion, post nasal drip, facial flushing, and skin reactions to her earrings and belt buckle after working with nickel nanoparticle powder on a lab bench with no protective measures other than latex gloves while washing equipment. Her tasks included measuring out 1-2g quantities of dry powder, transferring the powder to a jar containing water or ethylene glycol, pipetting the resulting liquid into containers in which the nanoparticles were ‘ball milled’ (in closed containers), vacuum filtering the mixture (closed), as well as cleaning the equipment. She had previously not directly handled any type of nickel powder.

[12] Pathology showed diffuse alveolar injury throughout all lung lobes with alveolar haemorrhage. Alveoli contained oedema fluid, remnants of hyaline membranes, and macrophages. Heart and brain had focal areas of acute necrosis and small blood vessel lesions. There was haematuria, proteinurea, and histological evidence of tubular necrosis of the kidney. Unexposed individuals have urine nickel levels between 0.25 and 5.0 mg/L. The level of nickel in the urine of the subject was 780 mg/L.

She was found to test positively for nickel in a patch test (in addition to molds, cat dander, ragweed and fragrance mix paraben), and although her forced expiratory volume in one second (FEV_1) was within the normal range, it improved by 16% after use of bronchodilators. Her symptoms improved after she stopped handling the powder directly. Airborne nanoparticle sampling was not performed. Nickel is a common skin and respiratory sensitiser hence the putative sensitisation may not be a toxicological property of the nanoform *per se*, but rather an issue of increased potency. However, it is unknown if sensitisation would have occurred had she been exposed to nickel powder in bulk form. The authors discuss several aspects that may have influenced the appearance of symptoms, not the least of which is the increased dustiness of nanomaterials, and the patch testing. The case highlights the difficulty in obtaining conclusive human health information for nanoparticles.

Implementing appropriate engineering controls and maintaining them, using personal protective equipment, establishing safe handling procedures, training and informing workers are all strategic components of a risk management programme at facilities where there is potential for nanoparticle exposure. The publications of Song et al. (2009) and Phillips et al. (2010) illustrate that for ENMs the consequences can be debilitating lung damage and death if these elements are not promoted in the workplace.

2.4 Skin

In a case that echoes the difficulty observed by Song et al. (2009) in treating diseases potentially induced by ENMs (or their intermediates), Toyama et al. (2008) describe a case of severe dermatitis in a student who was synthesising nano-dendrimers in a laboratory. Within 1 -2 months of starting his experimental dendrimer synthesis he was diagnosed with erythema multiforme-like contact dermatitis on his hands. Oral and topical steroids, and anti-histamines failed to control the rash which progressed to erythematous lesions. Just 10 days after the initial contact dermatitis appeared he was admitted to hospital with fever, rash, bullae[13], and erosive blisters over his entire body except for the face. He also had burning sensations and pain. At this time his diagnosis was changed to toxic epidermal necrolysis-like dermatitis[14]. Symptoms could not be controlled by oral prednisolone and he received high dose intravenous steroids for three days followed by high dose oral steroids. He was discharged from hospital approximately 4 weeks after admission. It is reported he has frequent relapse of exudative erythema on his right hand each time he enters his office which is near the laboratory. Patch testing with the chemicals used to make the dendrimers was negative.

[13] Bullae are fluid-filled sacs or lesions that appear when fluid is trapped under a thin layer of skin. They are similar to blisters but are much smaller but nonetheless can fuse to create large water filled sacs (blisters).

[14] Toxic epidermal necrolysis-like dermatitis (scalded skin syndrome) is a variant of Stevens Johnson Syndrome, it is a rare, life-threatening condition usually caused by a reaction to drugs. It has auto-immune features. The top layer of skin detaches from the bottom layer and treatment is usually done in a burn unit or intensive care.

Toyama et al. (2008) do not describe the hygiene precautions, if any, in the student's laboratory. Whether this case of severe dermatitis was due to the dendrimers or one of the numerous intermediate reaction products, or whether it was unique to the immune response of the individual is moot. What is important is:

- Adverse reactions involving ENMs, while not common, are potentially very severe and difficult to treat.
- Inhalation and dermal exposure precautions are required in research laboratories as well as in industry. A recent investigation of research laboratories in the UK found that overall such precautions were already being taken (HSE 2013).

2.5 Summary and conclusions

- Markedly more publications are now available describing measurements of airborne ENMs in workplaces. It is however difficult to quantitatively generalise data across workplaces due to different measurement techniques, reporting detail and manufacturing/handling circumstances.
- When appropriate exposure controls are in place (usually engineering solutions) concentrations of ENMs in air are universally reported to be low. Usually a workplace exposure limit, not necessarily specific for the ENM[15], has been used to make this judgement. Extrapolating the workplace measurements to equivalent exposure concentrations in experimental animals is not typically done. This may be because there is still a dearth of appropriate repeat inhalation exposure experiments for ENMs.
- The number of workers exposed when making or using ENMs is small. This severely limits the statistical power of epidemiology studies traditionally used to identify health effects in humans. It is also noted that many facilities are appropriately curtailing exposure to ENMs in the workplace with engineering controls (e.g. closed systems and ventilation) and other work health and safety (WHS) techniques. It is therefore, hopefully, unlikely that exposures will be sufficient to initiate health effects that are amenable to study using conventional epidemiology methods. One wonders if identification of ENM health effects, whether acute or chronic, in humans will be limited to case studies arising from high short term exposures where exposure prevention has been unheeded. Some of these cases are described in this report.
- A striking feature of the few medical cases arising from nanoparticle exposure in the workplace, all be they high exposures due to hygiene control failure, is that the health effects are severe, difficult to treat and may have very serious consequences. Although there is only limited information, the pulmonary effects resulting from large exposures to airborne

[15] Because the workplace exposure limit, particularly the general dust limit, is not based on toxicological or health effects of the specific ENM it may not be sufficiently protective. Therefore a 'low' concentration relative to such exposure limits does not necessarily mean the concentration is not of concern regarding possible health effects.

nanoparticles appear more intense and progress more quickly to a severe level than would be expected from equivalent exposures to larger size (say 2.5 – 10 µm) particles.

- There is uncertainty regarding human health effects potentially associated with long term exposure to relatively low airborne concentrations of ENMs. To address this issue routine worker health monitoring is advocated by some authors. It is debatable as to what tests should be done; ideally they should be relevant to the hazards identified in animal inhalation studies. It would seem lung function and standard clinical chemistry would be minimum. However these may not be practical for some work situations. It is also arguable how workers will perceive such programs and whether they should be voluntary or compulsory. While pre-employment health evaluation will provide 'baseline' data for an individual, there are significant issues in interpreting a health parameter change being linked with ENM exposure.
- There are jobs where worker health has been historically compromised. In some of these (e.g. welding fumes, cutting oil mists) the exposure in some part is likely to have been via nano-aerosols (Antonini et al. 2011). In addition to animal toxicology studies and health impacts associated with ambient air pollution and diesel exhaust, these non-ENM exposures may provide clues for what health monitoring tests may be useful.
- Animal experiments provide the foundation data for delineation of ENM hazards. Most of these are performed with 'pristine' (i.e. individual) ENMs. However it appears that predominantly, workplace exposures may not be to the ENM nano-form *per se*, rather to agglomerates of the ENM. The agglomerates are nonetheless of respirable size and can reach the deep lung where they may or may not disperse.

3. General toxicological considerations

3.1 Mode of action

The 2009 review for Safe Work Australia contained information on the toxicological modes of action for pulmonary effects of ENMs. Central to the particulate and fibre toxicity paradigms was inappropriately sustained oxidative stress, mediated by components of the innate immune system, that lead to various tissue responses. These remain the working hypotheses underpinning the biochemical and toxicological endpoints measured in the vast majority of *in vitro* and *in vivo* toxicological investigations of ENMs published since the review.

The ability of ENMs to produce oxidative stress and increases in inflammatory markers, in a wide range of cell types and using a variety of endpoints, is still the focus of many, if not most, *in vitro* studies with ENMs (e.g. Arora et al. 2012, Asare et al. 2012, Beck-Speier et al. 2012, Berg et al. 2010, Burello and Worth 2011, Cho et al. 2012a, b; Corbalan et al. 2011, Foldbjerg et al. 2011, Prasad et al.

2013). In this nothing much has changed since the 2009 review (Safe Work Australia 2009a). The limited utility of these studies to contribute towards hazard identification in the workplace also remains the same (Section 3.6).

However Shvedova et al. (2012a) have posed the question of whether oxidative stress is a uniform and sensible paradigm for nanoparticle-induced cytotoxicity. While broadly supporting the oxidative-stress mode of action, these authors challenge the common belief that nanomaterials cause non-specific oxidative damage. Shvedova et al. (2012a) consider a much better appreciation for the biological/toxicological effects of nanoparticles will be obtained if greater attention was given to organelle-specific pathways of reactive oxygen species production and the ways in which nanoparticles may impinge upon these pathways. Thus to understand the effects of different ENMs it is not sufficient to simply measure gross indicators of oxidative stress. For SWCNT administered to mice evidence is presented for specific *in vivo* patterns of phospholipid peroxidation rather than that expected if the oxidative stress was non-specific (Tyurina et al. 2011).

The importance of the biological corona, i.e. the complex formed by the interaction of ENMs with various biological macromolecules, has come to light in recent investigations. These interactions may affect the eventual fate, and therefore potentially the toxicity, of the ENM in the body (Section 3.2).

The suggested mode of action for many of the metal oxide NPs is a 'Trojan horse' mechanism, in which the NP size and shape facilitate entry into a cell, where the metal oxides are subsequently dissolved to their corresponding metal ions which are ultimately responsible for the toxicity observed (Sections 6.2, 7.2, 8.2).

Investigations into the mechanism of toxicity by metal oxides which do not release metal ions, e.g. TiO_2, are surprisingly few. Nevertheless the available information suggests the NPs are not taken up by organelles within the cell and do not break down within the cell. Rather, after being taken up, the NPs themselves seem to cause a non-specific increase in intracellular reactive oxygen species (by various mechanisms) resulting in cellular apoptosis and/or initiation of an inflammatory response.

In the 2009 review, much evidence was found to suggest that NPs tend to be more bioreactive/toxic than larger particles of the same material. Although recent information for silver NPs supports this conclusion (Section 8.2), a recent paper by Karlsson et al. (2009) indicates this is not always the case. In this study, the micrometer TiO_2 particles caused more DNA damage in a human cell line compared to the nano-form, whereas for iron oxides (which exhibited low toxicity) no clear difference could be found between the different particle sizes.

3.2 Biological corona

The 2009 review (Safe Work Australia 2009a) did not comment on the biological corona, i.e. the complex formed by the interaction of ENMs with various biological macromolecules (Figure 3.1), on ENMs or its potential importance. However over the last five years there have been a number of publications that show ENMs are able to be extensively and stably coated with a variety of macromolecules present in biological milieu. This in large part determines how cells "see" nanomaterials. Not surprisingly the coronas of proteins and/or lipids vary depending on the biological compartment in which the ENM traverses and ultimately finds itself. The binding of a protein onto an ENM can change the protein's native conformation, thereby altering its biochemical function, it may also result in the presentation of novel epitopes to the adaptive immune system (Section 3.9). The adsorbed proteins give the nanoparticle its biological identity and can decrease or increase the interface with cells. Macromolecular binding and opsonisation[16] of the ENM surface creates a "molecular signature" which may affect the eventual fate of the ENM in the body and have implications for adverse effects.

[16] Opsonisation is the attachment of a molecule to the surface of a particle or pathogen that enhances phagocytosis by macrophages.

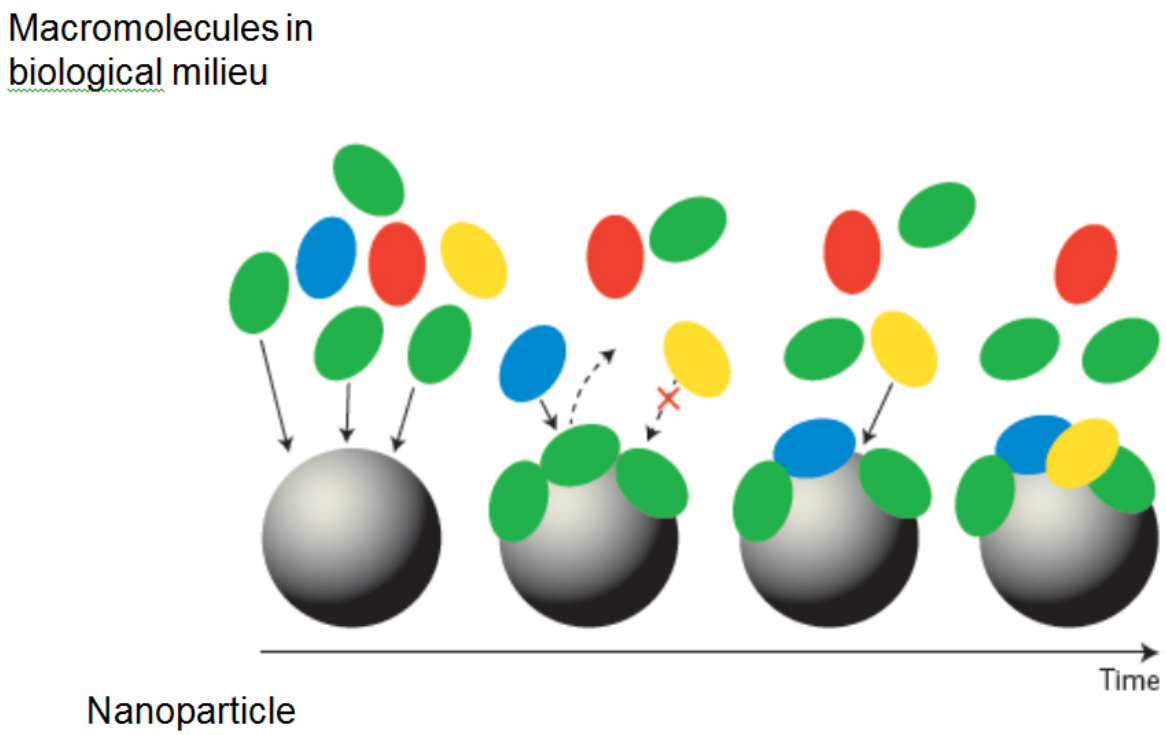

Figure 3.1: Diagram of nanoparticle-corona formation
(Adapted from Monopoli et al. 2012)

From left to right: An initial corona forms from those biomolecules (in green) that arrive first to the surface of the nanoparticle (these are typically highly abundant proteins). An initially adsorbed molecule with low affinity (in green) is subsequently displaced by a different molecule with higher affinity (in blue) arriving later. A third molecule (in yellow), that initially had low affinity for the bare nanoparticle surface now adsorbs owing to favourable interactions with the adsorbed (green and blue) biomolecules. A different biomolecule (in red) does not adsorb at all.

There have been numerous publications (some reviewed in Safe Work Australia 2009a) that promote the notion that the unique surface properties of a particular naked ENM markedly influence its biological fate and adverse effects. A logical extension is that each ENM will potentially need to be tested for its potential adverse effects. This dogma is being challenged by the demonstration that protein binding to ENMs is remarkably stable. Indeed to the extent that the 'protein'-ENM complexes can be isolated and studied (Walczyk et al. 2010, Walkey and Chang 2012).

Walczyk et al. (2010) make compelling and cogent arguments that due to the relatively long biomolecular residence time of proteins bound onto ENMs that the particle-bound proteins are the primary biologically relevant species. Indeed they may be the defining biological property of the ENM that determines the distribution of an ENM, its cellular uptake and toxicity. The concept of the ENM corona being more important, or at least as important, as the surface properties of the primary ENM is being actively promoted and pursued by major international research groups (e.g. Shvedova et al. 2012a, Johnston et al. 2012).

The binding of proteins onto ENMs appears non-specific (i.e. not associated with receptors) [17], nonetheless for some ENMs, size and surface modifications influence the extent of binding and subsequently the agglomeration of ENM-protein complexes in body fluids (e.g. that lining the lung and blood) (Table 3.1, Fertsch-Gapp et al. 2011). It should also be noted that protein binding to nanoparticles or agglomerates can influence the interpretation of *in vitro* cytotoxicity assays (Saathoff et al. 2011) [18].

Table 3.1: Mean diameter (nm) of nanoparticle agglomerates in various solutions [a] (data from Fertsch-Gapp et al. 2011)**.**

	Distilled water	PBS	BSA	Transferrin
PS-Plain	70	50	1,190	930
PS-COOH	110	110	110	110
PS-NH_2	170	110	90	90
Printex 90	50	>1,000	nd	nd
Printex G	260	>1,000	nd	nd

[a] The PS (polystyrene)-NPs were monodisperse with a mean diameter 50nm as determined by the manufacturer. PS-plain = no surface modifications; PS-COOH = Carboxyl surface modification; PH-NH_2 = Amino surface modification. Printex 90 and Printex G are carbon black polydisperse industrial NP's with primary particle size of 14 and 50 nm respectively.

Johnston et al. (2012) have investigated the relationship between nanomaterial hazard and physicochemical properties and concluded:

- The composition of the protein corona is likely to be dictated by their route of entry into the body.
- The translocation of ENMs to secondary target sites may influence the composition of the protein corona and subsequent evolution of the protein corona over time.
- The physicochemical characteristics of ENMs influences protein binding specificity and affinity and therefore dictates the composition of the protein corona hence the toxicity of ENMs.
- ENMs can affect secondary target sites that vary according to delivery route and corona composition.

[17] Cai et al. (2012) used stable isotope labeling of amino acids in cell culture to characterise the binding of human cellular proteins to MWCNTs and carbon black (CB). The relative binding efficiency of over 750 proteins was measured. The data indicate that MWCNTs and CB bind to vastly different sets of proteins. CNTs were able to selectively enrich (by more than 10 fold) some specific proteins from protein mixtures. There was a higher level of protein binding by larger MWCNTs and effective protein binding was possible only if the MWCNT exceeded a certain size. The authors suggest the protein-CNT interaction depended on the 3- dimensional arrangement of carbon atoms in CNTs, and not on the chemical properties of carbon itself.

[18] Saathoff et al. (2011) found the pro-inflammatory mediator IL-8 release was significantly decreased by hydroxylated fullerenes relative to controls. The authors speculated most of the IL-8 released into the media was probably adsorbed by large fullerenol agglomerates which were formed at this concentration.

Thus ENMs attain a corona of proteins from biological media in which they find themselves. The fact that this affects their interaction with cells, and hence their toxicity, is demonstrated by recent studies with CNTs.

- Binding of blood proteins to CNT strongly reduces their *in vitro* cytotoxicity[19] (Ge et al. 2011b).
- MWCNT were one of several ENMs investigated *in vitro*[20] by Sund et al. (2011) for their ability to bind various proteins. Plasma proteins adsorbed onto MWCNT are involved in cellular internalisation. According to the authors the proteins covered the nanomaterials independently of their surface properties. However the binding efficiency to cell lysate proteins (intracellular proteins) was influenced by the characteristics of the nanomaterial surface. Pulmonary surfactant components significantly reduced the overall protein adsorption on the surface of MWCNTs. This may have implications for judging the concordance between *in vitro* and *in vivo* studies for respiratory toxicity.
- The pattern of adsorbed proteins and pulmonary surfactants may influence significantly the interaction and subsequent effects of the MWCNT on biological systems (Gasser[21] et al. 2010; Schleh et al. 2011).

The above suggests the characterisation of ENMs should be multifactorial. The following is suggested (Johnston et al. 2012, Casals and Puntes 2012):

- Physicochemical characteristics, including impurities and defects, of pristine particles as supplied by the manufacturer. This was often the only characterisation undertaken in the mid 2000's.
- Determination of the nature of the ENMs in the experimental media, e.g. state and characteristics of agglomerates (recommended in Safe Work Australia 2009a).
- Characteristics of the ENM once in contact with the tissue or biological system, i.e. ENM characteristics with its relevant protein corona(s).

Silver nanoparticles (Ag-NPs)[22] show significant agglomeration in the presence of proteins (e.g. those in saliva) but after gastric digestion the number of individual NPs returns to values measured in the

[19] The cell lines used by Ge et al. (2011b) were a human acute monocytic leukemia cell line (THP-1) and human umbilical vein endothelial cells. They found fibrinogen, immunoglobulin, albumin, and transferrin ameliorated the cytotoxicity of uncoated SWCNT (diam <2 nm & 5-30 μm in bundle length). Fibrinogen was particularly effective.

[20] Sund et al. (2011) used human lung epithelial cells and human monocyte-derived macrophages.

[21] In a cell free system Gasser et al. (2010) showed that surfactant lipids bind unspecifically to different functionalised MWCNTs, in contrast to blood plasma proteins which showed characteristic binding patterns. Patterns of bound surfactant lipids were altered after a subsequent incubation in blood plasma, and *vice versa* bound plasma protein patterns were altered when MWCNTs were previously coated with pulmonary surfactant.

[22] The Ag-NPs used by Walczak et al. (2012) were 60 nm dispersed in 2mM citrate buffer.

absence of protein (Walczak et al. 2012). The authors concluded that the Ag-NPs investigated under physiological conditions can reach the intestinal wall in their initial size and composition.

The influence of agglomeration on the cellular uptake, intracellular distribution and cytotoxicity of Ag- or TiO_2-NPs was investigated *in vitro* by Lankoff et al. (2012)[23]. Although dependent on the type of cell, generally the agglomerated ENMs were less well taken up by cells, and were essentially confined to the cytoplasm, whereas individual nanoparticles were found in the nuclei and mitochondria. Although both non-agglomerated and agglomerated particles caused cell death, the agglomerated particles were significantly less potent. Ge et al. (2011b) demonstrated the toxicity of SWCNT was markedly decreased after the binding of various serum proteins.

3.3 Toxicokinetics and distribution

Overview:

Toxicokinetics refers to the fate of a foreign material, not intended for therapy, in an organism and is a sequential process of absorption, distribution, metabolism and excretion (ADME).

There are several common experimental design issues associated with kinetic/distribution studies with ENMs that should to be considered when evaluating or using the data for human risk assessment:

- Administered doses are often unrealistically high relative to anticipated human exposures.
- Doses that exceed the capacity of the pulmonary macrophage system to sequester the ENMs cause pulmonary inflammation and subsequent changes in the pulmonary biokinetics. Consequently such studies are not providing relevant information on the biokinetics, systemic absorption or hazard profile of the ENM.
- Because the capacity of macrophages to sequester ENMs is determined by the volume of the phagocytosing cell and the macrophage pool, the density and volume of ENM are important parameters to consider when designing inhalation dosing regimes. Less dense ENMs occupy more volume than an equivalent mass of denser ENM, or non-nanomaterial. Consequently pulmonary overload (i.e. exceedance of phagocytic activity) occurs at lower mass doses for less dense materials. Due to air spaces between packing particles, aggregation of ENMs effectively reduces the density of the resulting particle and hence the possibility of pulmonary overload.

[23] The ENMs in Lankoff et al. (2012) were Ag-NP (non-agglommerated 20nm or 200nm, agglomerated approximately 80 – 600nm and 300 – 400nm respectively) and TiO_2-NP (non-agglommerated 21nm, agglomerated 130 – 1,200nm). It is noted the Ag-NPs with a primary particle size of 200 nm would not be considered nanoparticles using the commonly accepted definition of a nanoparticle (<100µm). Cultered cell lines used in Lankoff et al. (2012) were human epithelial (A549), human hepatic (HepG2) and human undifferentiated monocytes (THP-1).

- Due to the higher density of wet aerosol ENM preparations compared to dry particle preparations, the extent and relative regional deposition in the respiratory tract may be quite different.
- Characterisation of the nanomaterial is often in a medium quite different from that to which humans are exposed. Occupational exposure is invariably to agglomerates of ENMs and not the pristine NM carefully characterised and tested in biokinetic or toxicological studies.
- Quantification of tissue concentrations is frequently indirect, and not for the nano-particle *per se*. For example for nano-metal oxides (nMeO), tissue measurements are frequently for the metal ion rather than the NP. For carbon based ENMs (e.g. SWCNT and MWCNT) the tissue quantitation may be measurement of the metal catalyst (or an isotope thereof, e.g. ^{60}Co) used in its production. Unfortunately these metals are able to be leached from the ENM and so their detection and quantification in tissues is not necessarily a reflection to where translocation of the ENM may have occurred. At the present time the latter can only be reliably determined by transition electron microscopy (TEM). Given that usually only a very small fraction of the administered NP reaches the systemic circulation (particularly after inhalation of concentrations relevant for human exposure in controlled work places), it is a very tedious, labour intensive and time consuming exercise to scan enough cells to detect the few NPs some of them may contain. It is literally equivalent to the proverbial 'looking for a needle in a haystack', indeed several haystacks.
- Mass balance calculations are often not undertaken. As a result the proportion of ENM exposure that may be retained by the respiratory system, or that may reach the systemic circulation, is not able to be quantitated. Without such measurements risk assessment for the ENM is compromised.
- The biokinetics of ENMs in *in vitro* cell culture test systems is often ignored. More often than not the concentration of ENM applied to the system is not the concentration at the cell surface. As the ENM acquires a protein corona and/or aggregates it effectively becomes less dense. Consequently the time required for uniform distribution within the test system can be longer than the time of the actual test, particularly if contact with cells adhered to the bottom of the plate relies on gravitation.

Common biokinetic themes across ENMs deduced by Riviere (2009b) and others:

- After inhalation of relevant concentrations of ENMs, most is exhaled or swallowed as a result of being entrained in mucous or removed via the ciliary ladder in macrophages. The latter accounts for the initial rapid clearance from the lungs (half life ~ <1 -2 hr). Only a small fraction of the external exposure reaches alveolar cells and a very small fraction (of the order of <0.01%) may be absorbed.

- Most of the absorbed ENM is translocated to the respiratory lymph system and not to the general circulation.
- If an ENM enters the systemic circulation a major determinant of ENM disposition is the degree of interaction with the reticuloendothelial (RE) cell system.
- Small particles evading the RE system may be excreted by the kidney.
- Larger particles and those with a compatible surface charge may get targeted to RE cells in the liver, spleen and other organs.
- The protein corona plays a large part in how avidly RE cells sequester ENMs.
- Most nanomaterial kinetics are characterised by relatively short blood half-lives reflecting tissue extraction from blood (i.e. distribution) and not by clearance from the body.
- A common attribute of nanomaterial kinetics is retention of particles in the tissues that have sequestered them. This is probably a reflection of the turnover time of the phagocytic cells embedded in the tissue.
- ENMs may preferentially be transported in the body via the lymphatic system.

Some specific studies:

There are distinguishing factors of nanomaterials (NMs) which strongly influence the kinetics in an organism compared to the behaviour of larger-sized materials of identical chemical composition. Such factors include their size (primary particle and agglomeration), their surface charge, and protein binding (Landsiedel et al. 2012a). For example, in comparative studies on the influence of NM size on their biodistribution, it was observed that after intravenous administration the smaller-sized NM had wider biodistribution compared with larger-sized NMs which were principally confined to the liver and spleen (De Jong et al. 2008, Sonavane et al. 2008). This distribution is a reflection of the large ENMs being taken up by the RES but the smaller sizes (~<10 – 15nm) are not as readily sequestered by these phagocytic cells, as a result they have wider distribution.

In contrast to inhaled particles larger than 10 µm in diameter which are trapped on the mucous membrane of the nose and, if this fails, deposited on airway mucus and are propelled toward the mouth by cilia (i.e. the mucociliary escalator), NPs tend to remain suspended in the inhaled air. Hence they are able to be deposited throughout the entire respiratory tract, nevertheless their relative deposition in each region still depends on their particle size. According to the mathematical model of ICRP (1994) 90% of deposited 1-nm particles occur in the nasopharyngeal compartment, only about 10% in the tracheobronchial region with almost none in the alveoli. Particles 5-nm in diameter are deposited equally at about 30% in the three regions, and 20-nm particles have their highest deposition in the alveolar region (~50%). Aggregates have larger particle sizes and therefore different deposition ratios than the pristine well dispersed ENMs which are usually subject to characterisation techniques (Landsiedel et al. 2012a). From the alveoli, the NPs are removed by macrophages and neutrophils which internalise particles, degrade them or carry them to the mucociliary escalator.

Recent studies indicate many ENMs aggregate or agglomerate in aerosols and do not appear to disaggregate in the lung and become systemically available (Landsiedel et al. 2012a).

Klein et al. (2012) reviewed the pulmonary burden of a number of ENMs that had been tested in the short-term rat inhalation study (STIS)[24] protocol developed for the OECD (see also Section 3.10.2). They found ENM lung burden increased with increasing air concentration but not linearly. While for different ENMs much lower concentrations than in the lung were found in mediastinal lymph nodes, they were rarely found in extra-pulmonary organs. When they were it was in the spleen and after high exposure (10 mg/m^3) of a polymer-coated synthetic amorphous silica.

If absorbed, ENMs may reach the blood stream and/or the lymphatic system and become widely distributed to many tissues.

Gold has been used on numerous occasions to assess the biodistribution and cellular uptake of NPs following exposure. Once in the systemic circulation the primary site of ENM particulate accumulation has been consistently demonstrated to be the endothelial reticulation system of the liver and spleen (Austin et al. 2012, Dziendzikowska et al. 2012, Glazer et al. 2011, Johnston et al. 2010b, Landsiedel et al. 2012a, Semmler-Behnke et al. 2008). These organs contain high numbers of macrophages, which are part of the mononuclear phagocyte system (MPS)[25]. Many NMs are prone to lymphatic transport and many are taken up by the MPS which may act as a depot for the ENM. Their half-life in blood depends on their uptake by MPS rather than their elimination from the body (Landsiedel et al. 2012a). Binding of some plasma proteins, the opsonins, to the NM increases their uptake by MPS. A hydrophilic or neutral surface of NMs disfavours binding of opsonins (Landsiedel et al. 2012a). Uptake by MPS is also strongly influenced by the size of the NM. Generally, NPs of about 100 nm in diameter (compared with <50 nm or >250 nm) show a low rate of uptake by MPS and therefore a prolonged half-life in the blood. For the liver, a diameter <50nm (i.e. smaller than the pore size of liver endothelia of around 100 nm) often leads to higher uptake (Landsiedel et al. 2012a).

An important implication of their lymphatic transport is that via this route NMs have the potential to interact with the immune system (Riviere 2009b, Section 3.9).
NMs reaching the gastrointestinal tract (either through the oral uptake of food or through the mucociliary escalator after inhalation) are excreted with the faeces, but low levels of some NMs are

[24] The STIS protocol for nanomaterials is 5 day inhalation (6hr/d) exposure to 3 concentrations (one of which is usually very high) with 28 day follow up. Part of the evaluation procedure is measurement of ENM burden in the lungs and other tissues immediately after the last exposure and again after 28 days.

[25] An older name for the mononuclear phagocyte system is the reticuloendothelial system. Apart from circulating monocytes and macrophages which tend to accumulate in the spleen and lymph nodes, included are specialised endothelial cells lining the sinusoids of the liver (Kupffer cells), spleen, and bone marrow, and histocytic cells of lymphatic tissue and bone marrow (fibroblasts). The all have in common an ability to take up and sequester bacteria, viruses, inert particles and certain dyes.

also absorbed (e.g. gold, polystyrene NPs) and may become systemically available (Landsiedel et al. 2012a).

Most NMs are either not seen in the brain or poorly enter the brain (Boyes et al. 2012 and references therein; Hardas 2010), however some NMs can cross the blood-brain barrier, probably due to the interaction between some plasma proteins (e.g. albumin, ApoE, Apo C-II, and immunoglobulin G) adsorbed onto the NM surface and CNS blood capillary endothelial cells (Kao et al. 2012a, Landsiedel et al. 2012a, Sonavane et al. 2008, Zensi et al. 2009). Uptake into the brain is favoured by negative surface charge (Landsiedel et al. 2012a).

One study (Yamashita et al. 2011), and subsequent commentaries and reviews of the subject (Keelan 2011, Kulvietis et al. 2011) suggest size may be an important determinant for placental uptake. Silica and TiO_2 NPs (primary particle size 70 nm and 35 nm, hydrodynamic diameter (HD) 65 nm and 217 nm, respectively) injected intravenously into pregnant mice crossed the placenta barrier and were found in the placenta, foetal liver and foetal brain. Larger silica particles (primary particle size 300 nm and 1000 nm, HD 322 nm and 1140 nm, respectively) neither accumulated in the placenta nor reached the foetus (Yamashita et al. 2011). Overall the reviews indicate experimental information in placental uptake of ENMs is scarce (Kullvietis et al. 2011, Menezes et al. 2011).

Nanomaterials (e.g. fullerenes, silica NPs, SWCNTs) are typically not metabolised, however surface coatings of some NMs may be cleaved off and degraded separately (Deshmukh et al. 2012, Landsiedel et al. 2012a). NMs may be cleared directly from the port of entry, excreted by the kidney/urine (e.g. some SWCNTs and MWCNTs) as well as the liver/bile route (e.g. polystyrene NPs, some quantum dots) (Landsiedel et al. 2012a and citations therein). One study found some quantum dots remain in the body, but appear to slowly degrade to their constituent metal ions which may then accumulate in the kidneys, liver and spleen (Ye et al. 2012).

Since many ENPs are being developed for *in vivo* medical applications there is a growing body of studies that have investigated the fate of ENPs once they reach the systemic circulation. Riviere (2009b) reviewed the literature on the systemic pharmacokinetics of nanomaterials to assess whether any overarching findings can be defined. The review was confined to carbon based nanomaterials and quantum dots that had been administered parenterally. Given the difficulty in measuring CNTs in biological matrices it was noted that most such studies rely on indirect measurements of concentration in tissues. These rely on radiolabel or fluorescent tags to remain attached to the nanomaterial as it is distributed through the body, as well as not to impart any different physiochemical properties that would alter disposition. In many instances the disposition studies of ENMs were in fact studies of ENM-protein disposition, particularly for CNTs (see also Section 3.2). Because it is relatively easy to observe by electron microscopy, gold has been used on numerous

occasions to assess the biodistribution and cellular uptake of NPs following exposure (Johnston et al 2010b, Khlebtsov and Dykman 2011, Lipka et al. 2010, Peckys and de Jonge 2011, Sonavane et al. 2008, Trickler et al. 2011).

3.4 Intracellular location

Recent studies reported that endosomes and lysosomes are the most common compartments for nanomaterial localisation (Bhattacharya et al. 2009, Dragoni et al. 2012, Hussain et al. 2009, Maurer-Jones et al. 2010, Peckys and de Jonge 2011, Zhang and Monteiro-Riviere 2009), however some nanomaterials localise in mitochondria and the nucleus (Hackenberg et al. 2011b, Johnston et al. 2010c, Kim et al. 2009, Raoof et al. 2012) or endoplasmic reticulum (Wei et al. 2010). Others are confined to the cytoplasm (Mu et al. 2012).

Intracellular location seems dependent on the type of ENM and degree of agglomeration. More detailed information on intracellular location of ENMs is provided in sections of this report that deal with specific ENMs.

3.5 In vivo effects

An area in which there has been limited study is the time effect. Most data on reactions of biological systems exposed to nanoparticles are based on acute experiments. As with chemicals it is normal for a cell or tissue to react to nanoparticle exposure; how and to what extent will depend on dose and the nanoparticle. Whether this response is a simple defence mechanism or an adverse effect can most relevantly be determined by observing the organism over a longer period of time (e.g. the reaction may well be reversible after cessation of exposure). Chronic studies are thus critical to the understanding whether an acute response is part of, or leads to longer term toxicity, or is more simply a manifestation of a reversible adaptive response. Unfortunately, as reported in the 2009 review (Safe Work Australia 2009a), there still remains a dearth of chronic repeat dose toxicity studies for ENMs.

3.5.1 Dermal

Dermal penentration studies of various ENMs have mostly been done *in vitro* with animal or human skin preparations. A cross-section of human skin is depicted in Figure 3.2(A). The outermost layer, the stratum-corneum is the non-viable protective barrier of skin. A histologic image showing the epidermis in more detail is provided in Figure 3.2(B).

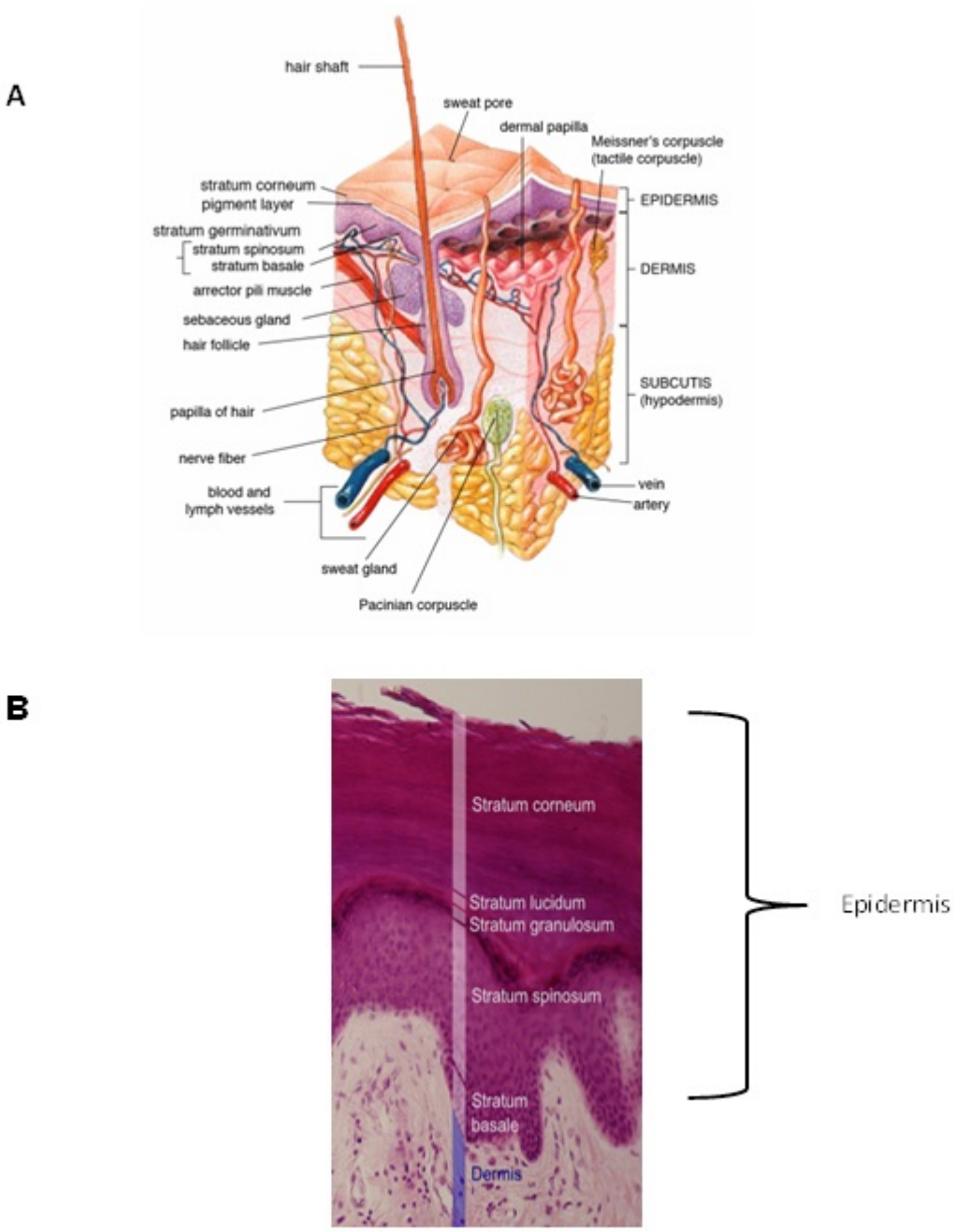

Figure 3.2: **A) Diagram of cross section of human skin** (US Gov 2009), **B) Histologic image of epidermis** (Häggström 2010)

In human and animal skin irritation and sensitisation tests published since the 2009 review, CNTs and fullerenes were minimally or non-irritating and not sensitisers (Aoshima et al. 2009, Ema et al. 2011, Ema et al. 2013).

Dermal penetration – in vitro studies

Regarding the potential for dermal penetration there are many *in vitro* studies[26] using animal or human skin that show:

- For intact skin a variety of nanoparticles do not pass the stratum corneum. Occasionally there is limited penetration further than the stratum corneum but not into the dermis (Zhang and Monteiro-Riviere 2008, Zhang et al. 2008, US EPA 2010a, Prow et al. 2012, Ryman-Rasmussen et al. 2006, Adachi et al. 2010, Nohynek and Dufour 2012, Inman et al. 2010, Furukawa et al. 2011, Sadrieh et al. 2010, Tyner et al. 2011, Schilling et al. 2010, Larese Filon et al. 2011, 2012; Campbell et al. 2012, Kimura et al. 2012, Cohen et al. 2013).
- Disturbance or removal of the stratum corneum such as with non-vigorous tape stripping, depilitating cream or in dermal disease (psoriasis) may increase infiltration further into the stratum, but rarely through to the viable dermis layers (Pinheiro et al. 2007, Zhang and Monteiro-Riviere 2008, Ravichandran et al. 2011, Larese Filon et al. 2011, 2102: Prow et al. 2012). However this is not always observed (Campbell et al. 2012, Kimura et al. 2012).
- On the other hand with abraded skin, where the layers of the stratum have been markedly compromised or completely removed, applied nanomaterials can reach the dermis (Zhang and Monteiro-Riviere 2008, Kimura et al. 2012).
- Mechanical flexing of intact skin increases diffusion of nano-particles into (Rouse et al. 2007, Zhang and Monteiro-Riviere 2008), and occasionally through the strateum corneum. This has also been observed for larger particles (0.5 -1 µm) (Tinkle et al. 2003).

[26] Not all the studies listed in these dot points are described below. The discussion on nano-TiO_2 in this section provides details of some experiments with this material.

- In Zhang and Monteiro-Riviere (2008) rat skin (after shaving with scalpel blade) was used intact, stripped 10 times with tape (Scotch Magic TM) to remove the stratum corneum, or abraded 60 times with sand paper until the skin was bright red but not bleeding and blood vessels could be seen on the surface, this process also removed hairs. Intact skin was flexed for 60 min being placed onto the flow-through diffusion cells. The ENMs investigated were QD565 and QD655 and visualisation in the skin was by confocal microscopic imaging.
- Zhang et al. (2008) used a flow-through diffusion system with weanling porcine skin and QD-621, a nail-shaped QD with PEG coating.
- Prow et al. (2012) used human abdominal and breast skin obtained from volunteers undergoing elective surgery. There was negligible penetration of QDs (PEG, PEG-NH2 and COOH coated) in intact skin. In tape stripped skin, all QDs at all pHs tested (7.0, 8.3 and 9.0) entered the upper layers of the viable epidermis.
- Ravichandran et al. (2011) used QDs (5.8 nm) with excised human skin (abdominal or breast from patients undergoing abdominoplasty or mammoplasty and used within 6 hours with fat and dermal layer thinned leaving the epidermis intact). Skin was depilitated with commercial cream or taped stripped. Both treatments increased penetration into the stratum corneum, the latter more so, and tape stripping also facilitated small appearance of QD in the dermis.
- Kimura et al. (2012) used insoluble fluorescent polystyrene latex microspheres (Fluoresbrite®, 50 nm) and soluble fluorescein isothiocyanate-dextrans (FDs, 3-42 nms) with excised hairless rat skin that was either intact or damaged. No permeation into the viable dermis or epidermis was observed through intact, tape-stripped, or razor-treated skin, but was observed through needle-punctured skin.

Dermal penetration in vivo studies

In Nabeshi et al. (2011b) nano-Si (70 nm) and QD (~35 nm) were suspended in 10% isopropyl myristate and applied to the inside of both ears of mice each day for 28 days. Twenty-four hours after the last application both QD and nano-Si were detected in the skin, lymph nodes, liver, cerebral cortex and hippocampus, imlpying they had potentially penetrated through the skin. It is not mentioned by Nabeshi et al. (2011b) whether the mice were individually or group housed during treatment. In either case, given the length of treatment it is a real possibility that self, or communal grooming lead to ingestion of some of the applied nanomaterial.

Additional information on *in vivo* dermal absorption studies of ENMs is provided in discussions on nano-TiO_2 and ZnO below.

In vitro cytotoxicity assays with skin cells

For a variety of ENMs *in vitro* cytotoxicity assays with cultured human skin epithelial cells often report adverse effects (Saathoff et al. 2011, Gao et al. 2010, Monteiro-Riviere et al. 2010, Nabeshi et al. 2011a, b; Jebali et al. 2013). In such experiments if the concentration of ENM is high enough, cytotoxicity, usually accompanied with signs of oxidative stress, is inevitable. The effects are also influenced by agglomeration (e.g. Vankoningsloo et al. 2010) and are not unique to skin cells. Since the *in vitro* experiments were often conducted at high concentrations which are likely to be outside any potentially achievable biological concentrations that viable cells in the epidermis may experience, the application of the data to *in vivo* conditions and human risk assessment is limited.

Information for TiO_2 and ZnO

In the 2009 review, it was stated that reviews of dermal absorption studies for nanoscale TiO_2 consistently conclude that TiO_2 is not absorbed through intact skin. However it was noted dermal penetration had not been studied in damaged or compromised skin. The same was concluded by US EPA (2010a).

A number of animal, human, and *in vitro* studies published since the US EPA (2010a) review provide additional evidence that TiO_2 NPs neither penetrate into viable cell layers nor cause local toxicity after application (exposed for 4 hours up to 20 weeks) to intact or partially damaged skin, including that from sunburn (Pinheiro et al. 2007, Adachi et al. 2010, Nohynek and Dufour 2012, Inman et al. 2010, Furukawa et al. 2011, Sadrieh et al. 2010, Tyner et al. 2011, Schilling et al. 2010) [27]. However

[27] Adachi et al. (2010) exposed hairless rats to water/oil emulsions of 10% uncoated anatase TiO_2 NPs (primary NPs = average of 27 nm, aggregates ~390nm; most of the particles were present as primary particles and were well dispersed) for 4, 24, 72 and 168 hours. Electron microscopy revealed that most TiO_2 particles were localised in the interfollicular stratum disjunctum and the keratinised layer of follicular infundibulum, with no TiO_2 particles detected in the viable skin. This was confirmed by energy-dispersive X-ray spectrometry.

diffusion into the stratum may be higher with concomitant exposure to UV (Bennet et al. 2012)[28] or if skin was experimentally damaged by UV (Inman et al. 2010).

In contrast, one study (Wu et al. 2009) found that 5% uncoated TiO_2 NPs (4 and 60 nm; anatase, rutile, or a combination of the two) penetrated through the stratum corneum into the basal cell layer of the skin (but not the dermis) after application onto pigs' ears for 30 days[29]. The penetration was size-dependent, only particles of 4nm passed the stratum corneum. The treatments caused neither dermal irritation, nor any clinical signs or behavioural changes in pigs. Pathological changes in the skin (e.g. extended intercellular space, vacuoles emerged around nucleus) were observed. In contrast, exposure of isolated porcine skin to the TiO_2 NPs for 24 hours gave different results; the NPs remained in the outermost surface of the stratum corneum. The difference between the *in vivo* data showing penetration through the stratum corneum and the *in vitro* results suggesting the stratum corneum is an efficient barrier is not expected since there are a host of *in vitro* studies indicating *in vivo* penetration was unlikely.

Nohynek and Dufour (2012) reviewed various studies on the dermal penetration and toxicity of TiO_2 and ZnO NPs in sunscreens which showed that they do not penetrate human skin beyond the superficial layers of the stratum corneum and are non-toxic *in vivo*.

Inman et al. (2010) dosed UVB-damaged and normal weanling pig skin (occluded) *in vivo* with four sunscreen formulations: 10% coated TiO_2 in oil/water (o/w); 10% coated TiO_2 in water/oil (w/o); 5% coated ZnO in o/w; and 5% uncoated ZnO in o/w. TiO_2 was 10 x 50 nm (mean agglomerates 200 nm), and ZnO had a mean size of 140nm (range ca. 60-200 nm). Pigs were redosed after 24 hours and terminated after 48 hours. Skin was harvested and processed for TEM, SEM and Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS). Both the Ti and Zn NPs were localised to the stratum corneum in both normal and UVB-damaged skin, although they penetrated into deeper layers of the SC in UVB-damaged skin.

Furukawa et al. (2011) examined the post-initiation carcinogenic potential of coated and uncoated TiO_2 NPs using a mouse medium-term skin carcinogenesis bioassay. The NPs were applied to mouse skin at 5, 10, and 20 mg/animal doses in the post-initiation phase (up to 20 weeks) in a two-stage carcinogenesis model, 7,12-dimethylbenz[a]anthracene (DMBA) and 12-o-tetradecanoylphorbol 13-acetate (TPA) were used as the initiator and a positive control promoter, respectively. No changes in survival rate, general condition, body weight, or development of nodules was observed in the study.

Sadrieh et al. (2010) conducted a dermal penetration study in minipigs using three different TiO_2 particles applied 5% by weight in a sunscreen. These and control formulations were applied topically at 2 mg cream/cm^2 skin (4 applications/day, 5 days/week, 4 weeks). No significant penetration of TiO_2 NPs through the intact epidermis was found.

Tyner et al. (2011) found no enhanced penetration of sodium fluorescein of normal artificial skin models treated with any of the three TiO_2 suspensions for 24 hours tested over the control. Skin models challenged with UV exposure prior to the addition of sunscreen did not show enhanced permeability.

[28] The hydrodynamic diameter of the TiO_2 aggregates in Bennett et al. (2012) reduced from ~250 nm to ~160 nm after 30 minutes of light exposure due to photo-induced disaggregation of the NPs. After a 90 minute exposure to sunlight, the mass of TiO_2 NPs that travelled through the entire dermal profile of the skin was determined to be 0.1% of the applied amount. In control samples, i.e. dark conditions, TiO_2 was only found on the surface of the stratum corneum.

[29] Treatment was 24 mg of 5% nano-TiO_2 over 3 cm^2 each day for 30 days (i.e. 1.2 mg TiO_2/d). Transmission electron microscopy was used by Wu et al. (2009) to detect nano-TiO_2 in punch biopsies of treated ears.

In the same paper, after 60 days dermal exposure (covered non-occlusively) in hairless mice, TiO_2 NPs also penetrated through the skin and were found in various organs (i.e. skin, liver, heart, lung, spleen), but were not detected in the blood (Wu et al. 2009). The results of the mouse study are less applicable to humans, as the stratum corneum of the hairless mouse is less than half as thick as a human's (Simon and Maibach 1998, Haigh and Smith 1994). Hence, the penetration of NPs is expected to be greater in hairless mice. It is unclear whether the mice were housed individually [30].

In a recent comprehensive review by the Australian Therapeutic Goods Administration (TGA 2013), it was concluded that the weight of evidence from *in vitro* and *in vivo* studies using both animal and human skin have shown that TiO_2 NPs do not penetrate past the stratum corneum of the skin, making systemic absorption unlikely. The few *in vivo* studies suggesting deeper penetration into skin, in the opinion of TGA, had limited application to human risk assessment.

The overwhelming majority of studies have also shown minimal or no dermal penetration past the stratum corneum and no toxicity *in vivo* or *in vitro* for a variety of ZnO NPs applied to intact, damaged, psoriatic, or dermatitic skin (Leite-Silva et al. 2013, Gulson et al. 2010, Lin et al. 2011b, Filipe et al. 2009, Durand et al. 2009, Inman et al. 2010, Prow et al. 2011)[31]. In a recent review on the risk of

[30] In Wu et al. (2009) the treatment skin area for the mice was a circle of approximately $3cm^2$ and was treated with 24mg of the nano test emulsion. The treatment area was protected by a selfmade plastic collar which fitted on the neck and remained uncovered. 3 h later the collar was removed and the skin washed with lukewarm water and dried. The extent of removal of NPs from the surface of the skin was not determined. The treatment regime suggests animals were housed individually for 3 hours but perhaps in a group after that time. Although steps were taken to minimise ingestion of TiO_2, exposure by ingestion could still have occurred. Particularly if there was group grooming. The distribution of nano TiO_2 was primarily by atomic absorption spectroscopy (i.e. of Ti ions) but presence of NPs in liver (not other organs) was shown by TEM.

[31] Leite-Silva et al. (2013) used multiphoton tomography with fluorescence lifetime imaging microscopy (FLIM) to observe ZnO NP (on average ~60-90 nm) penetration and potential metabolic changes within the viable undamaged epidermis of human volunteers after topical application of various ZnO NP products (2 mg/cm^2). For the duration of the 6-hour experiment, coated and uncoated ZnO NPs remained in the superficial layers of the stratum corneum and in the skin furrows. Limited penetration into the stratum granulosum was observed for coated ZnO NPs dispersed in a water-in-oil emulsion formulation, predominantly localised adjacent to the skin furrow. However, the latter did not alter the metabolic state or morphology of cells, as indicated by their fluorescence lifetimes. The authors concluded that some limited penetration of coated and uncoated ZnO NPs may occur into viable stratum granulosum epidermis adjacent to furrow but that the extent is not sufficient to affect the redox state of those viable cells.

Gulson et al. (2010) exposed human volunteers to sunscreens containing ZnO NPs for five consecutive days outdoors, and collected venous and urine samples 8 days prior, twice daily during, and 6 days post-exposure. After a 30-minute UV exposure following a 30-minute equilibration of each sunscreen application, the subjects were free to undergo normal activities at a beach. No effort was made to control perspiration or movement resulting in skin flexing. Stable isotope tracing was used where ZnO was enriched to >99% with the stable isotope ^{68}Zn; this allowed dermally absorbed Zn to be distinguished from naturally occurring Zn. Compared with the amounts of 'natural' Zn normally present in the human body, small amounts of the tracer were absorbed and detected in blood and urine. The amount of ^{68}Zn tracer detected in blood represents <0.001% of the applied dose. It is unknown whether ^{68}Zn was absorbed as ZnO NPs or soluble Zn ions or both.

nanoparticles in sunscreens and cosmetics, Nohynek and Dufour (2012) indicated *in vitro* data should be interpreted with caution, and concluded that the weight of scientific evidence suggests insoluble NPs used in sunscreens pose no or negligible risk to human health. A similar conclusion was made in another review by Schilling et al. (2010). The TGA (2013) also concluded ZnO NPs do not penetrate the underlying layers of the skin, and therefore systemic absorption is unlikely. A similar conclusion was reached by the Scientific Committee on Consumer Safety (SCCS 2012) for nano-ZnO.

3.5.2 Eye Effects

In the 2009 review, it was stated the available hazard data, albeit limited, indicate most ENMs are unlikely to produce adverse eye effects with workplace exposures.

Only a few studies have been published since the 2009 review investigating the toxicity of NPs delivered to the eye. Collectively they support the conclusions made in the 2009 review, and suggest minimal or no eye irritation is expected from common NPs encountered in the workplace.

In an acute eye irritation study (conducted in accordance with OECD Guideline 405) with two SWCNTs and two MWCNTs in rabbits, the maximum allowable concentration for administration (0.1 mL of 0.1-1% CNTs)[32] did not result in eye irritation, apart from one MWCNT product which caused minimal conjunctival redness and blood vessel hyperemia at 1 hour post exposure, but which resolved by 24 hours (Ema et al. 2011). In a follow-on study the same minimal, reversible effects were observed with C_{60} fullerenes (0.1 g/eye, eyes rinsed with saline after 1 hour) (Ema et al. 2013).

Aoshima et al. (2009) conducted an eye irritation test using highly purified fullerenes in rabbits. They observed conjunctival redness and corneal epithelial defects in all three animals (where eyes were not rinsed) at 1 and 24 hours after instillation of 0.1 g of fullerenes into the eye[33], which disappeared by 48 hours. The authors concluded the fullerenes were “minimally irritating” to the eye, and hypothesised the minimal effects observed were due to the physical effects of the fullerene powder.

Lin et al (2011b) used fluorescence lifetime imaging microscopy to detect the dermal penetration and NAD(P)H changes as a result of topically applying ZnO NPs on to human subjects’ intact, tape-stripped, psoriatic or dermatitic skin. The authors detected no ZnO NP penetration past the stratum corneum in any group after 2 or 4 hours of treatment, albeit the concentrations that penetrated into the SC were higher for psoriatic/dermatitic skin. The ZnO NPs did not have a detectable effect on NAD(P)H in the viable epidermis of treated volunteers.

Filipe et al. (2009) used nuclear microscopy techniques to examine skin penetration of Ti and Zn NPs after application of NP-containing sunscreens to volunteers with intact, tape stripped, and psoriatic skin. The skin to which the sunscreen was applied (3-mm diameter) was biopsied after a 2-hour or 48-hour (under occlusion) exposure period and analysed in microscopy mounting medium in the laboratory. Detectable amounts of TiO_2 and ZnO NPs were only present on the skin surface and the uppermost stratum corneum in all groups.

[32] CNTs were mixed with a minimum amount of olive oil to prepare a test material in liquid form which resulted in good contact and uniform distribution to the eye, without resulting in overflow when instilled into the conjunctival sac.

[33] The authors indicate fullerenes were instilled as a powder (average particle size = 100-300 µm).

Kim et al. (2012a) also observed no significant acute irritation or corrosion after instilling 100 mg of Ag NPs (10nm) dispersed in 1% citric acid to rabbit eyes according to a standardised protocol (OECD 405). Conjunctival redness, oedema and discharge were observed 1 hour after application, which cleared by 24 hours. The authors classed the NPs as “non-irritating.

In a modified Draize test, Nagarwal et al. (2012) tested for eye irritation by instilling 50 µL of chitosan coated sodium alginate-chitosan NPs either loaded or not loaded with 5-fluorouracil (a drug used for chemotherapy). No signs of ocular irritation were observed for either NP at 2, 4, 8 and 12 hours after application.

As indicated in the 2009 review, the route of administration used in other studies (i.e. subretinal injection) is unlikely to be relevant for the most common workplace exposures. Nevertheless, Ding et al. (2009b) observed no signs of inflammatory responses after subretinal injection of DNA compacted polyethylene glycol-substituted polylysine nanoparticles in adult mice (1 µL at 0.3, 1.0 and 3.0 µg/µL). Retinas were examined for signs of inflammation at 1, 2, 4 and 7 days post-injection.

Sanders et al. (2012) investigated the phototoxicity of six different types of titanium dioxide NPs in a human-derived line of retinal pigment epithelial cells. Cells were treated with 0, 0.3, 1, 3, 10, 30, or 100 µg/mL nano-TiO_2 in media for 24 hours, then exposed to UVA (2 hrs, 7.53 J/cm^2) or kept in the dark. Viability of cells was assessed 24 hours after the end of UVA exposure. Exposure to higher concentrations of nano-TiO_2 in combination with UVA lowered cell viability, and cytotoxicity varied between different TiO_2 types. As discussed elsewhere, *in vitro* cytotoxicity test results are of limited usefulness for determining the hazard potential of ENMs (Section 3.6).

3.5.3 Reproductive effects

As indicated in Section 3.3, the limited information on ENM uptake in the placenta suggests ENMs of a specific size might be able to cross from the maternal systemic circulation into foetal blood.

Yamashita et al. (2011) intravenously injected fluorescent labelled silica-NPs (70nm) or TiO_2-NPs (35 nm) to pregnant mice on gestation days 16 and 17. Mice were killed on gestation day 18 and the NPs were found in the placenta, and foetal liver and brain. There was increased embryo toxicity (increased resorptions) and retarded foetal growth. It is unlikely there were direct effects on the foetus as maternal weight was lower than controls and uterine weights were significantly lower. The authors concluded there were structural and functional changes in the placenta. The relevance of this study to the workplace is obscure since the exposure was high (0.8 mg/mouse) and intravenous. It is notable however that the effects were abolished when the surfaces of the silica nanoparticles were modified with carboxyl and amine groups.

Other studies have shown that subcutaneous exposure of pregnant mice to TiO_2-NPs resulted in TiO_2 aggregates in foetal brain and testis, dose-dependent effects on testicular morphology, and brain changes including increased apoptosis, altered gene expression, and increased dopamine levels (Shimizu et al. 2009; Takahashi et al. 2010; Takeda et al. 2009). The non-physiological routes of exposure and high doses create uncertainty regarding how this information may be used to inform workplace hazard and risk assessment.

Inhalation exposure of pregnant mice to TiO_2-NP has been reported to produce slight neurobehavioural changes in offspring. However the exposures in this study were very high[34] and no increase in levels of titanium in pups was seen; concentrations were below the limit of detection and controls were at or about the limit of detection (Hougaard et al. 2010). Until neurobehavioural effects have been substantiated by other investigators this study is of doubtful significance for hazard identification of TiO_2-NPs.

3.6 In vitro and in vivo concordance

It was mentioned in the previous Safe Work Australia (2009a) review that, where meaningfully investigated, studies have shown low concordance between *in vitro* and *in vivo* toxicity. The latest published information suggests *in vitro – in vivo* concordance is still poor but may be improving with the development and validation of *in vitro* assays, the use of surface-area normalised response data, and the use of coculture systems; however the concordance is assay and ENM specific. In addition, differences between the same *in vitro* or *in vivo* studies are also common. This can probably be explained by differences in experimental design, particle size and shape of the ENM studied, functionalisation methods, cell types, particle doses, animal types, administration methods, etc. (Khlebtsov and Dykman 2011, Sayes et al. 2009, Landsiedel et al. 2010a). Although some assays are useful for providing rapid inexpensive information on possible acute toxicity and mechanistic aspects of ENMs, *in vitro* experiments remain less useful for delineating potential chronic toxicity. The conclusion made in the 2009 review still holds true that overall, *in vitro* cellular systems need to be further developed, standardised, and validated (relative to *in vivo* effects) in order to provide useful screening data on the potential effects of inhaled ENPs of unknown toxicity.

In a comprehensive review of the health and environmental safety of four classes of nanomaterials (fullerenes, carbon nanotubes, metals and metal oxides), the European Commission (EC 2009b) concluded that the *in vitro* response is often representative of the *in vivo* response, but stress that careful comparisons and controls are required to ensure the relevance of *in vitro* studies. *In vitro*

[34] Hougaard et al. (2010) exposed pregnant mice via inhalation 1h/day to 42 mg TiO_2/m^3 aerosolised powder ($1.7x10^6$ particles/cm^3; peak-size: 97 nm) on gestation days 8-18. According to the authors the dose from this one hour exposure corresponds approximately to the 8-hr time weighted average (TWA) workplace exposure limit for titanium dioxide (as Ti, not specifically NPs) in Danish Regulations (the latter is $6mg/m^3$ according to DWEA 2007). Not surprisingly long term lung inflammation was observed in dams.

cytotoxicity studies are considered to be of limited relevance for risk assessment, since very few particle-induced diseases are associated with acute immediate cell death. Instead, sub-lethal effects measured *in vitro* have more potential to be useful for risk assessment.

Several research groups have investigated the concordance between *in vitro* and *in vivo* studies. Examples of concordance are:

- Rushton et al. (2010) investigated the capacity of two cell-free and two cell-based *in vitro* assays for predicting the *in vivo* acute pulmonary inflammatory responses in rats following intratracheal instillation of eight NP types with different physicochemical characteristics (i.e. elemental carbon, three different TiO_2, Cu NPs, Ag NPs, Au NPs, and aminated polystyrene). When the responses were expressed as per unit particle surface area, reliable correlations between the *in vivo* and *in vitro* results were found. When the values were instead normalised per unit mass, the correlation coefficients were reduced but still significant; however this was largely driven by the results of a single NM (Cu NPs). When the authors reanalysed the data from a previous study (Sayes et al. 2007) which had found poor correlations between the measured highest responses seen *in vitro* and *in vivo* using mass doses, their correlations using surface-area-normalised responses were significant (R^2 = 0.92 or 0.97) (Rushton et al. 2010).
- Al-Hallak et al. (2010) demonstrated collapse pressure of a model lung surfactant film *in vitro* correlated well with observed *in vivo* pulmonary toxicity of polysorbate-80-coated NPs.
- Dragoni et al. (2012) noted the absence of acute toxicity of gold NPs (5 nm, stabilised with PVP) to hepatocytes *in vitro* and confirmed these results *in vivo* in rats after administering the NPs by intraperitoneal injection.
- Han et al. (2012) found a good correlation between *in vitro* lactate dehydrogenase (LDH) release and protein oxidation induction in an alveolar epithelial cell line and *in vivo* polymorphonucelar neutrophils (PMNs) in brochoalveolar lavage fluid of rats (intratracheal instillation) as a result of exposure to TiO_2 NPs.
- Horie et al. (2011a) observed concordance between oxidative stress-related endpoints measured *in vitro* and *in vivo* (via intratracheal instillation).
- Jun et al. (2011) found that the results of an *in vitro* assay which showed human platelet aggregation induction and procoagulant activation (both contributors to thrombotic diseases) after exposure to Ag NPs lined up well with results for enhanced venous thrombus formation and platelet aggregation *in vivo* (via intravenous or intratracheal instillation).
- Kato et al. (2013) found MWCNTs to be genotoxic in both *in vitro* micronucleus and sister chromatid exchange tests as well as short-term *in vivo* comet assay, DNA adduct formation and mutation assay.

- Naya et al. (2011) found SWCNTs were not genotoxic under the conditions of an *in vitro* bacterial reverse mutation assay, *in vitro* chromosomal aberration assay, and an *in vivo* bone marrow micronucleus test. Hence the *in vitro* tests provided good predictability of the *in vivo* results.
- Poland et al. (2012) explored whether an *in vitro* system in which nickel nanowires were incubated with macrophages could be used to predict their behaviour *in vivo*. The results showed internalisation of the shorter fibres and similar protrusions of the long fibres out of the cells as seen *in vivo* in a peritoneal mouse model.
- Tosuka et al. (2009) examined genotoxic effects of C_{60} (fullerenes), carbon black and kaolin in an *in vitro* micronuclei test using a human lung cancer cell line and an *in vivo* comet assay in mice (intratracheal instillation). All three NPs resulted in increased micronuclei *in vitro* and induced DNA damage in the lungs of mice *in vivo*. Hence the test responses correlated with each other.
- Although more relevant to therapeutic than occupational exposure, several studies reviewed by Dobrovolskaia and McNeil (2013) have reported a good correlation between the results of *in vitro* immunotoxicity assays and *in vivo* toxicity studies in which haemolysis, complement activation, opsonisation and phagocytosis, and cytokine secretion were investigated. Other immunotoxicity assays for thrombogenicity and immunosuppression showed a weaker correlation, but were still predictive (e.g. CFU-GM and leukocyte proliferation, platelet aggregation, various plasma coagulation tests).

On the other hand:

- Sayes et al. (2009a) investigated the ability for three *in vitro* systems to predict the *in vivo* inflammation and cytotoxicity response of rats instilled with carbonyl iron, crystalline silica, amorphous silica, nanoscale ZnO, or fine ZnO. The demonstration of cytotoxic or inflammatory effects *in vitro* did not correlate well with the measured *in vivo* effects to the same particle-types. The *in vitro* assays were also unable to predict biologically important mechanisms of recovery after exposure which occurred with amorphous silica *in vivo*. Moreover, the responses of the cells in culture were not consistent among the various exposure groups and/or cell types.
- Cho et al. (2012a) showed that zinc ions can produce false-positive pro-inflammatory effects *in vitro* that are not seen *in vivo*.
- Kan et al. (2012) found that an *in vitro* study with cardiac myocytes was unable to predict adverse cardiac effects (i.e. increased phosphorylation levels of p38 MAPK and cTnI in the heart) observed *in vivo* as a result of ultrafine (~21 nm) TiO_2 exposure via inhalation. The latter suggests that translocation of ultrafine TiO_2 from pulmonary deposition sites into the

circulatory system and direct interaction with cardiac myocytes is not the mechanism of action for the induced biological changes observed in the heart *in vivo*.

- Khlebtsov and Dykman (2011) reviewed available *in vivo* biodistribution and *in vitro* cytotoxicity studies for gold NPs, and found there was generally a lack of correlation between the two.
- Although several *in vitro* studies have suggested nanosized TiO_2 is genotoxic (Falck et al. 2009, Barillet et al. 2010, Osman et al. 2010, Shukla et al. 2013, Roller 2011), a recent *in vivo* 5-day inhalation study in mice found no significant effect on the level of DNA damage in lung epithelial cells or micronuclei in blood polychromatic erythrocytes, suggesting no genotoxic effects (Lindberg et al. 2012).
- Singh et al. (2012) studied the functionalisation density dependence of MWCNTs' toxicity to macrophages and correlated the results with previously reported *in vivo* findings. An opposite trend was observed *in vitro* as to that *in vivo*. The authors attributed the differences observed to different experimental conditions in the tests, and the differences between administered and actual doses associated with cells.
- Stoeger et al. (2009) assessed whether the *in vivo* inflammatory response of mice to combustion-derived nanoparticles can be predicted *in vitro* by a cell-free ascorbate (i.e. Vitamin C) assay for surface reactivity. For six types of NPs with varying particle diameter, organic content, and surface area, oxidative potency in the *in vitro* assay correlated well with *in vivo* inflammatory response (i.e. PMN influx after intratracheal instillation). However, this was not the case for NPs with high organic content.
- Warheit et al. (2009a) compared the results of short-term *in vivo* intratracheal instillation and inhalation studies in rats with nanoscale or fine ZnO to the results of *in vitro* studies using three different cell culture conditions. One of these included an alveolar macrophage-lung epithelial cell coculture. *In vivo* exposures produced transient, short-term lung inflammatory or cytotoxic responses, whereas *in vitro* exposures produced minor cytotoxic responses only in cocultures and only at the highest dose (equivalent to the particle overload concentration). This led the authors to conclude the *in vitro* results showed little concordance with what was observed *in vivo*.

Rapid, high throughput *in vitro* screening assays for ENM induced cell toxicity are being developed and commercialised. For example, the stable reporter cell line AREc32 derived from human mammary MCF7 cells and containing a luciferase reporter gene has been adapted to screen and rank ENMs for their ability to induce oxidative stress in the cells. It is claimed to be an effective predictor in measuring oxidative stress potential when cytotoxicity is taken into account to successfully show a clear difference between well documented high-toxicity NPs and relatively low toxicity ones (Duffin et al. 2013). While being able to possibly rank ENMs for oxidative stress potential, *in vitro* systems such

as these still lack predictive concordance between the *in vitro* phenomenon of cellular oxidative stress and potency for inducing a relevant *in vivo* toxicological outcome.

Several researchers (Monteiro-Riviere et al. 2013, Horie et al. 2009, Monopoli et al. 2011b) have shown that the association of NPs with different serum proteins, i.e. protein coronas, affect the uptake of NPs *in vitro* compared to native (i.e. non-bound) NPs (see also Section 3.2). This may have significant implications for relating *in vitro* assay information where *in vivo* proteins are absent, to the behaviour of NP-protein coronas *in vivo.* Perhaps this could (at least) partially explain the disconcordance of some *in vitro-in vivo* studies in the literature. Similarly, the traditional monoculture *in vitro* systems have also been suggested to be partially responsible for the observed lack of concordance, and suggestions have been made to develop and validate coculture systems which would be more reflective of cell-cell interactions and hence biological effects observed *in vivo* (Snyder-Talkington et al. 2012).

Regardless of concordance, it is recognised to be important not to exclusively use *in vitro* screening approaches to evaluate the potential hazards of ENMs (Donaldson et al. 2009, Snyder-Talkington et al. 2012, Warheit 2010b). Suggestions of alternative approaches to screening the hazard potential of NMs have also been made (Lai 2012), which consist of a combination of high-throughput *in vitro* screening and mechanism-based short term *in vitro* assays and comparing data with those of reference materials of specific NM classes, keeping in mind that data for reference materials need to be generated using short-term subchronic *in vivo* studies. Computational toxicogenomics coupled with coculture systems *in vitro* have also been proposed as a possible future investment of resources for hazard identification purposes (Snyder-Talkington et al. 2012).

In discussing the OECD integrated test strategy for NMs, which notionally includes *in vitro* tests for biological effects (Figure 3.3), Klein et al. (2012) notes for several effects of NMs, there are currently no validated and accepted *in vitro* methods available. Hence, *in vivo* tests are still needed for reliable and relevant hazard identification. Logically the *in vivo* data should be the reference data for the design and validation of *in vitro* methods.

3.7 Genotoxicity

Genotoxicity potentially lends itself to rapid throughput screening for ENMs that have potential to interact with the genome.

Researchers at Flinders University, in a report to Safe Work Australia (Safe Work Australia 2013a), describe the development of an automated high-throughput screening procedure for assessing the genotoxicity of ENMs. The genotoxicity endpoint is production of micronuclei. The concept has been

tested in lymphocyte cell lines and primary cultures[35] using silver nanoparticles (Ag-NPs) of different size (10 and 70nm) and surface properties (citrate treated and PVP capped). Providing appropriate attention is paid to the conditions of the assay the authors demonstrated that protocols enabling automated high throughput genotoxicity screening of ENMs can be developed. However, as was alerted in the previous review (Safe Work Australia 2009a), cell cytotoxicity[36] can significantly interfere with quantitating micronuclei. An appreciable amount of developmental work was necessary to identify the working conditions of the assay. It is uncertain whether the conditions developed for Ag-NPs could be simply carried over to assess other ENMs. It is likely the assay will need to be optimised for each ENM. Recognising that the data in the report are preliminary a material difference in size or surface coating of Ag-NPs was not apparent, although there are suggestions that size and surface coating modulated the genotoxicity. The highest genotoxicity was seen for particles that also have the highest cytotoxicity.

In the 2009 review prepared for Safe Work Australia (2009a) it was remarked that the genotoxicity of ENMs appeared to be secondary to production of reactive oxygen species and that the available tests were not assessing innate genotoxic potential of ENMs. It appears this is also the situation of the high throughput methodology of Safe Work Australia (2013a). Thus, as with other genotoxicity assays being applied to ENMs, care is required in its interpretation with respect to identification and classification of a workplace hazard. As is noted elsewhere in this review, and in Safe Work Australia (2009a), experimental designs should also include the 'bulk material' counter part of the ENM to help identify whether the observed effects are unique for the nano-nature of the material or are reflecting greater potency for production of an effect of the bulk material. It is noted that bulk material (i.e. Ag^{+}) was not included by Safe Work Australia (2013a) in their 'proof-of-principle'. Prasad et al. (2013) and Jiang et al. (2013) are examples in which inclusion of the 'bulk material' has assisted delineation of the silver species potentially responsible for the observed oxidative stress effects and consequential genotoxicity.

3.8 Carcinogenicity

As has been noted previously chronic animal inhalation studies with ENMs conducive for the detection of cancer are not available. The longest repeat exposures so far published to date are 90 day experiments for CNTs, TiO_2 and Ag-NP (Sections 4, 5 and 8). Nevertheless cancer screening assays used to identify fibrogenicity are considered to be sufficiently predictive to conclude that CNTs of certain size and shape are likely to be carcinogenic (Section 4.6). It would also not be

[35] The cell lines used in Safe Work Australia (2013a) were HR1K (human B lymphocyte cell line), Jurkat (human T lymphocytes cell line) and WIL2-NS (Human B lymphocyte cell line).

[36] Cytotoxicity results in cells with various stages of death. High cytotoxicity may mean there are insufficient viable binucleated cells to assess for the presence of micronuclei. At lower cytotoxic concentrations of the test substance there may be cellular and nuclear debris that are unable to be differentiated from micronuclei.

unreasonable, unless there is evidence to the contrary, to assume that if a bulk material was an animal or human carcinogen that the nano-form would also likely be carcinogenic. Similarly, if an ENM was shown to be directly genotoxic (i.e. not acting through a secondary mechanism such as production of reactive oxygen species) it should signal possible carcinogenicity and warrant special care with respect to controlling particle emissions when handling or manufacturing the material (Roller 2009, Tsuda et al. 2009).

3.9 Immunotoxicity

Many NMs are preferentially taken up by lymph which makes the toxicokinetics different from that of most small molecular mass xenobiotics (Riviere 2009b, Landsiedel et al. 2012a; Section 3.3). Immunotoxicity studies with CNTs and CNFs are discussed in detail in Section 4.8.

In a collaborative effort between European laboratories, existing *in vitro* immunological assays were tested and compared for their suitability to assess the effects of NMs on immune responses using metal oxide NPs (Oostingh et al. 2011). Several problems and challenges were encountered during assay validation, ranging from particle agglomeration in biological media and optical interference with assay systems, to chemical immunotoxicity of solvents and contamination with endotoxin. The Oostingh et al. (2011) investigation highlights caution must be practiced when interpreting *in vitro* immunotoxicity data generated with NMs. Nevertheless, as indicated in Section 3.6, Dobrovolskaia and McNeil (2013) have shown some *in vitro* immunoassays show promise in terms of their predictability with *in vivo* outcomes.

NPs can interact with components of the immune system, leading to enhanced release of both inflammatory and proinflammatory cytokines, but mechanistic information on how NPs modulate the immune system and the potential for diseases resulting from this imbalance remain unclear (Chang 2010, Dwivedi et al. 2009, Hussain et al. 2012c).

Ban et al. (2012) administered sub-micron and nano-iron oxide particles to mice by either a single intratracheal instillation at different concentrations (250, 375, or 500 µg/mouse) or four repeated instillations at 500 µg/mouse each (Ban et al. 2012). These are high intratracheal doses. Both types of particles induced lung inflammation associated with increased cytokine productions in lymph node cell cultures and decreased pulmonary immune responses against sheep erythrocytes[37]. That is there

[37] The pulmonary immune response to sheep red blood cells (SRBC) was assessed by administering SRBC to anaesthetised mice through a small slit in the trachea. The lung-associated lymph nodes were removed and cultured. SRBC were introduced into the culture and the number of plaque forming cells counted. If lymphocytes in the *in situ* lymph nodes were making antibodies to the SRBC then in the culture those antibodies will lyse the SRBC and around the antibody producing cells a clear area (a plaque) against a red background (the SRBCs) will be formed.

was immunosuppression. For the same dose, FeO NPs produced higher levels of inflammation and immunosuppression than their submicron sized counterparts (Ban et al. 2012).

In another study, where Fe_2O_3 NPs or sub-micron particles were intratracheally administered to OVA-sensitised mice [38], the NPs at the high and intermediate dose significantly inhibited the OVA-induced allergic Th2-response (Ban et al. 2013). The low dose of sub-micron particles had no significant effect on the OVA allergic response, whereas the low dose of NPs had an adjuvant effect (i.e. enhancing the allergic response).

The influence of Cu NP exposure on lung infection of mice by *Klebsiella pneumonia (K.p.)* was investigated by Kim et al. (2011a). Exposures were either via sub-acute inhalation (4 hr/d, 5 d/week for 2 weeks, 3.5 mg/m^3) or intratracheal instillation (24 hr post-exposure, 3, 35 and 100 µg/mouse). Pulmonary responses were evaluated by lung histopathology, differential cell counts, total protein LDH activity and inflammatory cytokines in BAL fluid. Cu NP exposure increased recruitment of total cells and neutrophils to the lung as well as increasing total protein and LDH activity in BAL fluid. Both inhalation and instillation exposure of the NPs significantly decreased the pulmonary clearance of *K.p.*- mice compared to sham-exposed mice. That is the bacterial infection of Cu NP exposed mice was worse than the control mice. The authors concluded that Cu NP exposure impaired host defense against bacterial lung infections and may therefore increase the risk of pulmonary infection (Kim et al. 2011a).

Adjuvants are agents (e.g. chemicals, macromolecules, or cells) which enhance the immune response to an antigen. Several studies have suggested NPs may exhibit adjuvant activity and may increase the potency of known sensitisers. However, in these studies NPs were intradermally or intraperitoneally injected. The relevance of these results with respect to exposures in the workplace is debatable.

- Hirai et al. (2012) investigated the adjuvant activity of amorphous silica particles (dispersed, not aggregated) of different sizes on atopic dermatitis induced by intradermal injected mite antigen in mice. Injections (10 µL) were made on the ventral side of both ears with antigen alone or antigen plus silica particles in saline on days 1, 3, 5, 7, 9, 11, 13, 16, and 18. Ear thickness measurements and histopathological analysis (day 0 and 19) showed that a combined injection of amorphous silica particles and mite antigen caused aggravation of atopic dermatitis in a size-dependent manner compared to the mite antigen alone. Particularly, aggravation was stronger in nano-silica particle injected groups ($\leq$100nm). The effects in the nano-silica plus antigen groups were correlated with the higher induction of total IgE and a

[38] For each particle type, different intratracheal doses, namely 4x100, 4x250 or 4x500 µg/mouse, were used and for each dose there were four groups of mice, i.e. treated with (1) saline solution, (2) OVA , (3) particles and (4) OVA plus particles (Ban et al. 2013).

stronger systemic Th2 response than in the antigen-only group, as demonstrated by IL-18 induction and production of thymic stromal lymphopoietin (TSLP) in the skin lesions.

- In another study, sub-cutaneous injections of TiO_2 NPs increased lymph node proliferation induced by dermal dinitrochlorobenzene (DNCB) sensitisation in a variant local lymph node (LLN) assay (Hussain et al. 2012a). Sub-cutaneous TiO_2 NP pre-treatment did not alter the lymphocyte subpopulations, but significantly increased IL-4 and decreased IL-10 production in DNCB treated animals, demonstrating a Th2 response.
- Larsen et al. (2010) investigated whether photocatalytic TiO_2 NPs have adjuvant effect, when administered in combination with ovalbumin (OVA) in mice. Mice were immunised via intraperitoneal injections of OVA, OVA + TiO_2 or OVA + $Al(OH)_3$ and challenged with aerosols of OVA. At the end of the study, serum was analysed for content of OVA-specific IgE, IgG1 and IgG2a antibodies, and the bronchoalveolar lavage fluid (BALF) was analysed for content of inflammatory cells and levels of interleukin (IL)-4, IL-5, IL-10 and interferon-γ. The TiO_2 NPs promoted a Th2 dominant immune response with high levels of OVA-specific IgE and IgG1 in serum and influx of eosinophils, neutrophils and lymphocytes in BALF. The NPs induced a significantly higher level of OVA-specific IgE than the standard adjuvant $Al(OH)_3$. However, the two substances were comparable regarding the level of eosinophilic inflammation and interleukins present in BALF.

3.10 Workplace implications

3.10.1 Limiting workplace exposure

Workplace exposure standards (WESs) are a traditional regulatory tool for assisting industry to protect their workers[39]. In the main these are based on toxicological or health information that is specific for the particular substance and are generally regarded as being health based. However, what is reasonably achievable in practice and measurement capability may also be factors in deriving a WES. A variety of WESs have been proposed by international agencies and industry. An alternative approach to limiting workplace exposures is the establishment of procedures that qualitatively assign risk ranking to substances depending on the ENM hazard and likely exposure. Information for related bulk materials may also inform the derivation of a WES for a nanomaterial. For example, WESs have been established for fumed silica and a number of forms of carbon black; these materials may be largely composed of nanoparticles. In this report the term ‘health-based’ is used for WESs derived from toxicological or health information for the particular nanomaterial.

[39] In Australia, one of the requirements in the Workplace Health and Safety Regulations (WHS Regulations 2011, amended 2014) is to manage risks from airborne contaminants by ensuring workplace exposure standards for substances and mixtures are not exceeded.

3.10.1.1 Health based WES

Health based WES for ultrafine TiO_2, Ag-NPs, SWCNT and MWCNT, and fullerenes have been set by various authorities. These are summarised in Table C1 of Appendix C. More detailed descriptions of some of these WESs are contained in the relevant chapters on the particular ENM. They all follow a basic paradigm that has been used for many years for establishing workplace exposure limits for chemicals. This is essentially identifying a toxicological exposure to which safety factors (uncertainty factors) are applied to reach a human exposure that will be protective of nearly all exposed workers. The toxicological exposure, called a 'point of departure' in risk assessment jargon, can be a concentration at which no adverse effects are observed (NOAEL), the lowest exposure at which adverse effects are observed (LOAEL), or a mathematically modelled point of departure determined from the overall exposure-response information. The applied uncertainty factors differ according to the study, point of departure, and policy aspects of the jurisdiction undertaking the assessment. Thus for the same ENM there may be a number of different values for its WES.

3.10.1.2 Other WESs

The 'control-banding' method is applied at workplaces to assess and control occupational exposure in case of missing occupational limits or missing exposure measurements (EASHW 2009). Chemical substances and exposure to them are typically grouped in categories of toxicity (i.e. hazard bands) and exposure (bands) in a qualitative fashion. Initiatives have been undertaken to adapt the principle to nanomaterials (Schulte et al. 2008, Paik et al. 2008, Kulinowski and Lippy 2011). A team of experts at Lawrence Livermore National Laboratory created the 'CB Nanotool' which incorporates a risk level that is a combination of a severity score and a probability score in a standard 4x4 risk matrix (Kulinowski and Lippy 2011). This model has been validated against recommendations of industrial hygiene experts, and was demonstrated to have a high level of consistency and tended towards over-control rather than under-control. The risk level calculated by the tool defines the appropriate control strategy (e.g. general ventilation, fume hood, containment, or seeking advice from a specialist).

The German IFA (2012) has determined that size and density of nanoparticles should be employed as classification criteria for derivation of recommended exposure limits. Based on a similar 'control banding' method as above, they proposed the following recommended benchmark levels as increases over the background exposure during a worker's shift (8 hours) for monitoring the effectiveness of protective measures[40] (i.e. engineering or PPE controls):

- For metals, metal oxides and other biopersistent NMs with a density of >6,000 kg/m^3, a particle number concentration of 20,000 particles/cm^3 in the range of measurement between 1-100 nm should not be exceeded.

[40] Such efforts may be somewhat impeded by the difficulty and/or cost of using the type of equipment capable of measuring particles in the nanoscale.

- For biopersistent granular NMs with a density <6,000 kg/m^3, a particle number concentration of 40,000 particles/cm^3 in the measured range between 1-100 nm should not be exceeded.
- Owing to the mounting evidence that biopersistent CNTs which satisfy the WHO fibre definition or have similar dimensions may harbour effects similar to those of asbestos, IFA (2012) recommended only CNTs be used that have been tested for this end point and do not exhibit these properties. For CNTs for which no such manufacturer's declaration is available, a provisional fibre concentration of 10,000 fibres/m^3 was proposed for assessment, based on the unit risk ratio for asbestos[41].

The agency states that these benchmarks are not substantiated toxicologically, and even where they are observed, a health risk may still exist for employees.

The Dutch parliament requested the Knowledge and Information Centre Risks of Nanotechnology (KIR-nano), in cooperation with the National Institute for Public Health and Environment (RIVM), with the task of examining whether provisional reference values could be derived for ENMs. The experts concluded that at present, they were not aware of any method better than that of the German IFA (2012) described above. Nano-reference values (NRVs) based upon the IFA concept were adopted by the Dutch government for 23 most commonly used ENMs until such a time that health-based limit values are defined (van Broeckhuizen et al. 2012; RIVM 2010). The NRVs were as follows:

- 20,000 particles/cm^3 for nano- Ag, Fe, Au, Pb, La, TiO_2, CeO_2, ZnO, SiO_2, Al_2O_3, Fe_xO_y, SnO_2, CoO and nanoclay.
- 40,000 particles/cm^3 for C_{60}, carbon black, TiN, Sb_2O_5, polymers, polystyrene, dendrimers and carbon nanotubes for which effects comparable to the effects of asbestos can be excluded.
- 0.01 fibres/cm^3 for carbon nanotubes for which effects comparable to the effects of asbestos cannot be excluded.

These NRVs are intended to be warning levels; when exceeded, exposure control measures should be taken. They are 8-hour time-weighted average (TWA) exposure levels corrected for background particle numbers. RIVM (2010) emphasised the NRVs should under no circumstances be regarded as safe WESs. Employers should always attempt to minimise their employee's exposure to ENMs as much as possible, even if this means exposure is far below the provisional NRVs.

[41] IFI (2012) converted the US EPA IRIS unit risk of 2.3 x 10^{-1} per F/mL for asbestos to a unit risk specific for workplace exposures of 0.43 x 10^{-1} per F/mL, and subsequently calculated the 'tolerable' exposure corresponding to a 4:1,000 cancer risk and the 'acceptable' exposure corresponding to a 4:10,000 cancer risk. Since 0.1 F/mL is equal to 100,000 fibres/m^3, the 'tolerable' and 'acceptable' exposure concentrations are approximately equal to 100,000 fibres/m^3 and 10,000 fibres/m^3, respectively. The latter was provisionally recommended for assessment of CNTs.

British Standard BSi PD 6699-2:2007 (BSI 2007) adopts a pragmatic approach, proposing 'benchmark exposure levels' (BELs) for nanomaterials. They proposed these BELs to be calculated by multiplying the workplace exposure limit values for the conventional substance (non-nano form) by a specific value (0.066 for insoluble NMs, 0.5 for soluble NMs, 0.1 for [CMAR][42] NMs). RIVM (2010) critiqued this approach and considered it difficult to measure the benchmark exposure levels proposed by BSI with the currently available instruments and methods, also commenting that the proposed correction factors are not scientifically substantiated. RIVM (2010) also stated it is unclear how the BSI approach should be applied to coated nanomaterials. As an alternative, BSI (2007) also presented benchmarks based on particle number concentrations, similar to the IFA (2012) 'control banding' approach described above. In the UK urban pollution is in the range of 20,000 to 50,000 particles/cm^3. The lower limit of 20,000 particles/cm^3 was proposed as a possible benchmark for insoluble nanomaterials. The authors probably wanted to apply this to a diameter range for which this maximum concentration should apply, but did not provide such a range in the document. This particle number concentration is the same as the German IFA (2012) value for metals, metal oxides and other biopersistent ENMs with a density of >6,000 kg/m^3. BSI (2007) also proposed a BEL of 0.01 fibres/cm^3 for fibrous NMs with an aspect ratio greater than 3:1 and length greater than 5,000 nm. This level represents the clearance limit in asbestos removal activities in the UK.

In a report prepared for Safe Work Australia Australia (2010a) the applicability of 'control banding' methods for exposure management to ENMs in Australia was investigated. It was concluded that such methods are likely to be a suitable risk control approach in many situations, especially when combined with BELs. However the following comments were made specifically with respect to the BSI (2007) BELs:

- The BEL for fibrous NMs should be modified to 0.1 fibres/cm^3 rather than 0.01 fibres/cm^3, as there is no evidence that these NMs are more toxic on a fibre-by-fibre basis than asbestos.
- There is currently limited scientific evidence to support the quantitative BEL for CMAR nanomaterials (i.e. 0.1 x WEL) proposed by BSI (2007).
- There is toxicological evidence to support the BSI (2007) recommendation of 0.066 x WEL for insoluble NMs similar to TiO_2, but there is a lack of quantitative evidence for most other insoluble NMs.
- Although there is also insufficient evidence to support the quantitative BEL of 0.5 x WEL for soluble NMs, this may be prudent due to the possibility that size, shape and surface chemistry may lead to increased dose rates.

[42] Carcinogenic/mutagenic/asthmagenic/reproductive toxin

3.10.2 Risk assessments

The EU Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR 2007) indicates current methodologies are generally likely to be able to identify the hazards associated with the use of nanomaterials, but modifications are required for the guidance on the assessment of risks. A group of researchers have followed the traditional risk assessment paradigm (Figure 3.3) to assess occupational and consumer risks to a number of ENMs; Ag-NPs, CNTs and metal oxides (Christensen et al. 2010, 2011; Aschberger et al. 2010, 2011). These are discussed in detail in the relevant sections of this report. The approach taken was to estimate occupational and consumer exposure levels and to derive no-effect levels to be compared with the exposures in a risk characterisation. This is the same method recently recommended to be used by agencies in Australia for assessing human health risks from environmental hazards (enHealth 2012).

However applying this methodology to open literature information for Ag-NPs, Christensen et al. (2010) concluded there were significant information gaps for hazard and exposure that precluded making meaningful regulatory decisions other than repeated inhalation exposure in the workplace and possibly consumer inhalation may be associated with adverse risk. Similarly Aschberger et al. (2011) concluded the currently available database on ENMs for both hazard and exposure is limited and as a result there are marked uncertainties in any conclusion on possible risk. The authors indicated results of the risk assessment should not be used for regulatory decision making.

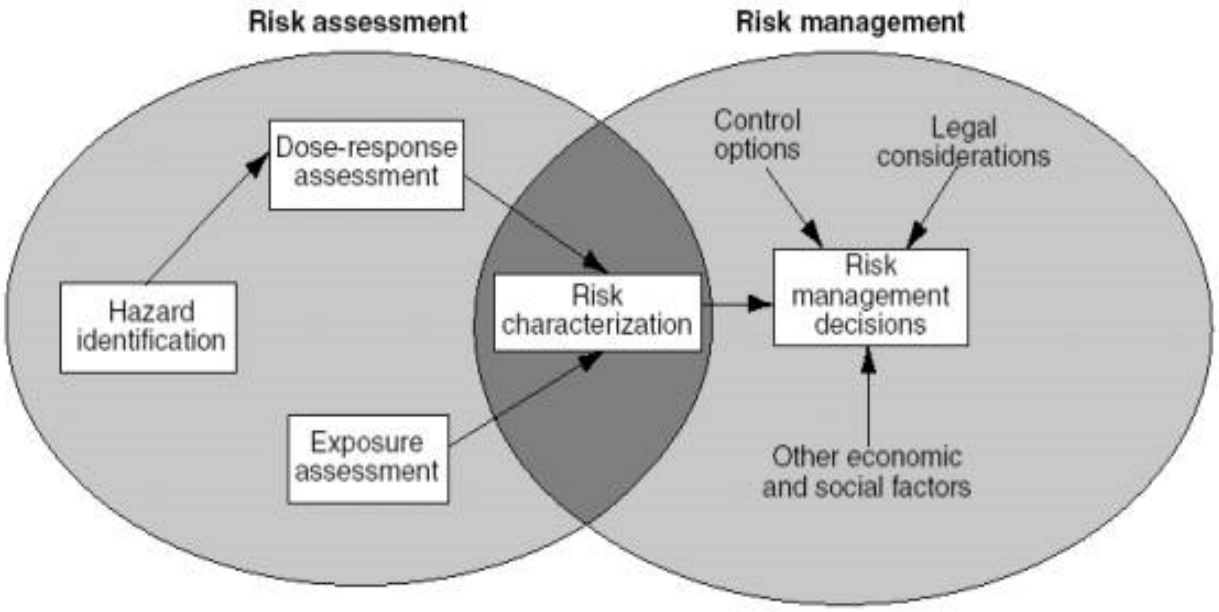

Figure 3.3: Traditional risk assessment paradigm (US EPA 2014))

A short-term rat inhalation study (STIS)[43] protocol for nanomaterials was developed within the European FP6 project NanoSafe 2 and the German BMBF project nanoCare (Ma-Hock et al. 2009b, Klein et al. 2012). The protocol requires three concentrations to be tested, and examines effects in the lung, persistence, progression or regression of effects over 28 days post-application (currently not obligatory in OECD inhalation tests), effects outside the lung, and lung burden and potential translocation to other tissues (also not required in current OECD test protocols). More than 20 nanomaterials have been tested in research projects. Klein et al. (2012) consider the STIS method has proven to be a valuable tool for comparing nanomaterial toxicity for prioritising further toxicity testing and/or further ENM development, as well as defining groups of ENMs with similar toxicity based on their biological activity rather than just on material properties. It was found the ranking of different NMs to induce adverse effects and the ranking of the respective NOAEC was the same in the STIS data as corresponding subchronic and chronic studies of the ENM or bulk material.

The STIS is central to the first tier of unified testing strategy proposed by OECD for NMs (Figure 3.4). If the tested nanomaterial does not show any biological effects, it is unlikely to have long-term effects *in vivo*, and further toxicity testing may be considered unnecessary (bioaccumulation testing might, however, be needed). If the STIS when reliably conducted does not indicate NM translocation, systemic effects are considered unlikely, and further studies (if required) may be limited to delineating local pulmonary effects.

[43] The rat STIS is 28-day study with 5-day inhalation exposure (head-nose, 6 hr/d) in male Wistar rats and a three weeks postexposure period. End points examined are histological examination of the respiratory tract and brain with olfactory bulb (n = 3/concentration, immediately after exposure and at termination of the study), bronchoalveolar lavage with standard parameter analysis (n = 5 per concentration, 3 days after exposure and at termination of the study). Haematology and clinical chemistry in lavaged animals. Determination of ENM lung burden and clearance, burden in lung, mediastinal lymph nodes, liver, kidney, heart, spleen and brain (n = 3/concentration, immediately after exposure and at termination of the study).

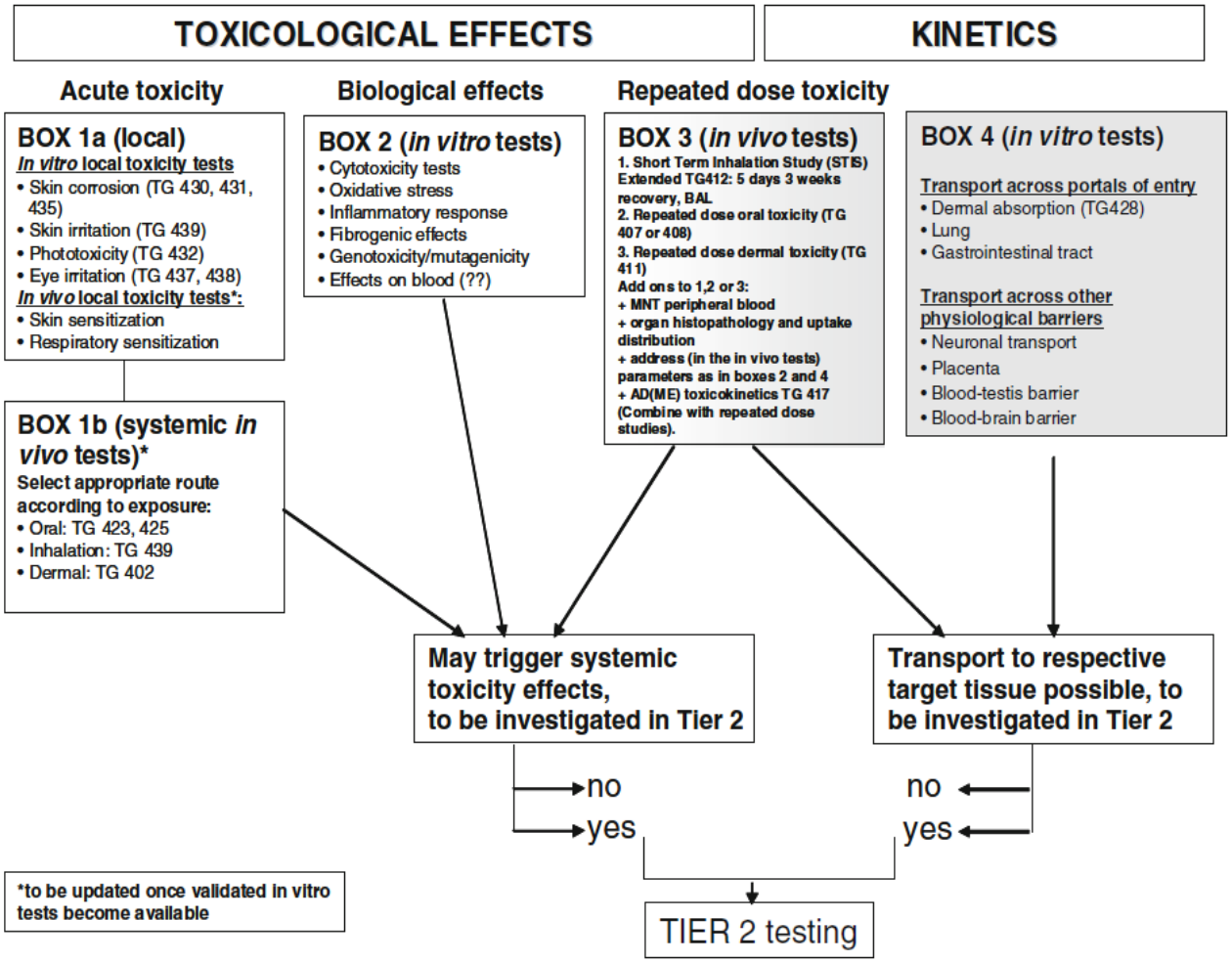

Figure 3.4: The position of the rodent short term inhalation study (STIS), Box 3, in Tier 1 integrated testing of nanomaterials (From Klein et al. 2012)

Risk assessments in the workplace rely heavily on safety data sheets (SDS) for identification of possible hazards, usually associated with over exposure or physicochemical properties that may confer fire or explosion hazards, and initial advice on controlling exposures, particularly WES and spill advice. Lee et al. (2013) undertook an evaluation of SDSs according to the criteria of GHS (Globally Harmonized System of Classification and Labelling of Chemicals). They found most of the SDSs did not include sufficient information on the safety of nanomaterials, such as their toxicity and physicochemical properties. A similar finding was made in a previous Safe Work Australia (2010c) report, where 84% of the SDSs evaluated did not provide sufficiently adequate or accurate information. Lee et al. (2013) reference an International Standards technical report (ISO TC 229) on the requirements for information that should be included in a SDS. Usually SDSs are prepared if a substance has been classified as hazardous according to GHS criteria. In recognising the dearth of appropriate available information, Lee et al. (2013) indicate that ISO TC 229 recommends SDSs be prepared for all nanomaterials, unless there is evidence that they are not hazardous. It is noted the Australian Code of Practice for *Preparation of Safety Data Sheets for Hazardous Chemicals* (Safe

Work Australia 2011d) also makes this recommendation. Lee et al. (2013) also recommends nanomaterial-related SDSs should be prepared on a precautionary approach in terms of the toxicity and other risks associated with the nanomaterial. If this advice is followed SDSs are likely to exaggerate hazards and risks. While this is not necessarily a bad thing for the workplace as it will encourage control of particle emissions in the ENM processes, it may on the other hand raise unwarranted consumer concern.

3.11 Summary and conclusions

As observed in the Safe Work Australia (2009a) review of ENMs, the particle and fibre toxicity paradigms continue to underpin the majority of *in vitro* and *in vivo* toxicological investigations of these materials. The ability of ENMs to produce oxidative stress and increases in inflammatory markers, in a wide range of cell types and using a variety of endpoints, is still the focus of many in *vitro* studies with ENMs. There does however appear to be subtle shifts in the appreciation of the role of oxidative stress in ENM toxicity, away from reporting non-specific oxidative damage stress towards investigating organelle-specific pathways of reactive oxygen species production and the ways in which nanoparticles may impinge upon these pathways. Consistent with this idea is the 'Trojan horse' mechanism proposed for many nano-metal oxides whereby the ultimate toxic species, the metal ion, is produced within certain cell organelles after the nano-metal oxide has been taken up by a cell.

The 2009 review for Safe Work Australia (2009a) did not comment on the biological corona, i.e. the complex formed by the interaction of ENMs with various biological macromolecules. However it is apparent ENMs are able to be extensively and stably coated with a variety of macromolecules present in biological milieu in which they may be distributed. This in large part determines how cells "see" nanomaterials. Although bound protein may be exchanged as the 'protein'-ENM complex is distributed in the body and moves through cells, the complexes are stable and can be isolated for study. The corona affects their interaction with cells and hence their toxicity. Overall the data indicate different proteins have different affinities for ENM binding and generally protein binding ameliorates toxicity (at least in *in vitro* systems) but the degree of alteration depends on the proteins. The size of the complex may play a role.

Consistent with this idea is demonstration that agglomerated ENMs are less well taken up by cells than primary nanoparticles, and agglomerates can form when the nanoparticles are within the cell. No doubt the protein corona has a role in the intracellular agglomeration process, but this has not yet been studied. Agglomerates within cells are essentially confined to the cytoplasm, whereas individual nanoparticles can be found in the nuclei and mitochondria. Agglomerated NPs have lower *in vitro* cellular toxicity compared to the primary nanoparticle.

Inhaled ENMs do not appear to be readily absorbed through the lungs and into the systemic circulation. While low concentrations, relative to those in the lung, can be found in pulmonary lymph nodes none, or very little appears to reach extrapulmonary tissues. The exception may be the spleen for certain ENMs after very high inhalation exposure. Nevertheless, distribution studies after parenteral administration show that if ENMs are absorbed into the systemic circulation they are widely distributed throughout the body. ENMs are primarily distributed to tissues that have fixed phagocytic cells (e.g. the reticuloendothelial (RE) system of the liver, spleen and lymph system) where, due to the longevity of the cells, they may remain for a long time. The effects of long term ENMs in these tissues have not been studied. The half-life of an ENM in blood depends on the uptake by the reticuloendothelial system rather than elimination from the body.

Apart from metal ENMs where the metal can be directly measured in tissue, if exposure has been high enough and the technique sensitive enough, most distribution studies of ENMs rely on measuring radiolabel or fluorescent tags that have been placed on the ENM.

Common themes of ENM disposition across all particle types are:

- Small particles evading the RE system may be excreted by the kidney.
- Larger particles and those with a compatible surface charge may get targeted to RE system cells in the liver, spleen and other organs.
- Most nanomaterial kinetics are characterised by relatively short blood half-lives reflecting tissue extraction and not clearance from the body.
- A common attribute of nanomaterial kinetics is retention of particles in the body (i.e. the RE system).
- ENMs may preferentially be transported in the body via the lymphatic system.

The weight of evidence indicates ENMs of various kinds do not penetrate through intact or mildly abraded skin into the live cell layers. While skin flexing may increase the amount of NP that may get into the stratum corneum it does not appear to facilitate diffusion into the dermis. ENMs when applied to the skin are essentially confined to the non-viable stratum corneum layer. There are however a few studies suggesting nano-TiO_2 <10nm may cross into deeper layers of the skin and in certain animal models (e.g. nude mice) into the systemic circulation. The relevance of these studies to hazard identification and risk assessment for humans has been questioned by the Australian Therapeutic Goods Administration. ENMs applied to severly damaged skin (e.g. complete removal of all stratum layers) may penetrate well into the dermis.

Most ENMs are unlikely to produce adverse eye effects with workplace exposures. If effects occur they are likely to be mild and self limited.

The latest published information suggests *in vitro – in vivo* concordance is poor but may be improving with the development and validation of *in vitro* assays, the use of surface-area normalised response data, and the use of coculture systems; however the concordance is assay and ENM specific. In addition, differences between studies using the same *in vitro* or *in vivo* protocol for the same ENM are also common. This can probably be explained by differences in experimental design, particle size and shape of the ENM studied, functionalisation methods, cell types, particle doses, animal types, administration methods, etc. (Khlebtsov and Dykman 2011, Sayes et al. 2009, Landsiedel et al. 2010a). Although some *in vitro* assays are useful for providing rapid inexpensive information on possible acute toxicity and mechanistic aspects of ENMs, *in vitro* experiments remain less useful for delineating potential chronic toxicity. The conclusion made in the Safe Work Australia (2009a) review still holds true that overall, *in vitro* cellular systems need to be further developed, standardised, and validated (relative to *in vivo* effects) in order to provide useful screening data on the potential effects of inhaled ENMs of unknown toxicity. Nevertheless as demonstrated in many places in this review *in vitro* tests can be pivotal for differentiating between similar ENMs for commercial development, for determining ameliorating effects of surface coatings, and investigating/developing mode of action hypotheses.

The fact that nanomaterials, after inhalation[44] or other routes of exposure are distributed to lymph nodes suggests the possibility they could modulate immune responses to antigens on bacteria, viruses or foreign proteins. The limited number of studies reviewed in this report confirms this potential. Unfortunately the experimental designs are such that extrapolation to workers is not straightforward. There is however a theoretical potential for immunosuppression which may result in increased respiratory infections of workers inhalationally exposed, but the extent of exposures for this to be realised is unknown.

There is also the theoretical possibility that if ENMs penetrated the skin and reached the dermis and interacted with proteins in such a way that antigenic haptens were formed, then dendritic cells processing and delivering the hapten to lymphocytes in the local lymph node could result in dermal sensitisation to the ENM. While this is proposed as a viable mechanism for beryllium nanoparticles (noting that beryllium itself is a known sensitiser in humans), sensitisation studies for other ENMs were not located.

For a few specific ENMs (CNTs, nano-TiO_2 and nano-Ag) health based WESs have been established by authorities or industry. These are underpinned by sub-chronic (i.e. 90 day) repeat exposure inhalation toxicity data and are described in the sections dealing with the specific nanomaterial. The majority of ENMs do not have toxicological data on them that will allow specific health based WES to

[44] As noted in Section 3.3, only a small fraction of the external inhaled exposure reaches alveolar cells and a very small fraction (of the order of <0.01%) may be absorbed.

be set. Instead authorities in Australia, Germany, the Netherlands and the UK are investigating/implementing a control banding approach.

While the traditional risk assessment paradigm is considered appropriate for ENMs its application to specific ENMs is hampered by hazard and exposure data gaps that will likely take some time to be filled. Nevertheless there are a number of initiatives in place aimed towards generating minimal data sets that will facilitate screening risk assessments for ENMs. These data sets are aimed at identifying ENMs which require more intensive investigation and perhaps generation of chronic toxicity information.

The reality of workplace risk assessments is that they rely on toxicological and physical hazard information in safety data sheets. It is apparent these workplace information sources are largely inadequate.

Measurement of ENMs in workplace air and beathing zones of workers has, and is becoming easier and more efficient. Practical advice on interpretation of measurements against background airborne nanoparticle concentrations, i.e. when ENM handling or manufacturing processes are not operating, is provided by Safe Work Australia and others. A pragmatic and precautionary approach is to eliminate or minimise particle emissions wherever possible.

4. Carbon nanotubes (CNTs)

4.1 Overview

Carbon nanotubes (CNTs) are manufactured as single-walled (SWCNTs) or multi-walled (MWCNTs). They have different lengths, widths, shapes, purities, surface modifications, and aggregation states. At the time of the completion of the previous review for Safe Work Australia (2009a) there was emerging evidence that long thin SWCNTs or MWCNTs had fibrogenic toxicological properties similar to certain forms of asbestos. Short or tangled CNTs did not display these effects. There was limited evidence from animal experiments designed to screen for fibrogenic activity that CNTs could induce pulmonary inflammation and if this was sustained pulmonary fibrosis. Further, as a result of their fibre like appearance, if they came into contact with mesothelial tissue they could induce mesotheliomas. The evidence for these effects was obtained in rodents after intra-peritoneal (injection into the abdominal cavity), intra-tracheal (bolus instillation into the upper bronchi of the lungs), intra-scrotal (injection into the scrotum), or pharyngeal aspiration (breathing in a single exposure of CNT aerosol after a suspension is placed on the back of the tongue of mice). Since the majority of these experiments were conducted with high single exposures and non-physiological exposure routes it was difficult to extrapolate what the information may mean for workers potentially exposed. The high doses were employed to artificially create a situation in which the tissues had prolonged contact with

the CNTs. While this mimicked the necessary bio-persistence and long tissue retention of known fibrogenic fibres it was uncertain whether the high experimental exposures compromised the usual defence mechanisms of the lung. Repeat exposure chronic inhalation studies for CNTs were not available when the first review for Safe Work Australia (2009a) was undertaken. A precautionary recommendation was made that unless shown otherwise, it would be prudent to assume CNTs of a size and shape similar[45] to other known fibrogenic fibres could also be fibrogenic, i.e. they may have the potential to cause pulmonary fibrosis and pulmonary mesothelioma with long term exposure and retention in the lung.

The 2009 review emphasised the fact that CNTs may have the capability of eliciting both a particulate and/or fibre-like pathogenic response in respiratory or mesothelial tissue. However fibrogenic CNTs may agglomerate and hence change their nature from being fibre-like to being particulate, and as with other ENMs they may gather a corona of proteins, lipids, and other biomolecules on their surface (Monopoli et al. 2011, 2012). Recent work described in Section 4.3 shows some CNTs are subject to biodegradation by biological fluids and/or enzyme activity. Important questions are whether these events change the biological activity of the original CNT, and should they influence the perception of CNT hazards in the workplace.

Raffa et al. (2010) have reviewed the properties of CNTs that influence their cellular uptake. According to available evidence, the degree of dispersion, the formation of supramolecular complexes (i.e. corona) and the nanotube length are important factors that determine the mode of cellular uptake. They concluded:

- phagocytosis appears to be the internalisation pathway for CNT aggregates, bundles, cluster or single dispersed nanotubes that are 1 µm or more in length,
- endocytosis is the internalisation mechanism for nanotubes forming supramolecular structures, and
- diffusion is the internalisation mechanism for submicron CNTs that do not form supramolecular complexes.

4.2 Mode of Action

Given the role that the fibrogenic nature of a CNT may have in determining its toxicity it is arguably more important to understand their mode(s) of action, and the physical attributes that contribute to the mode of action, than perhaps with other ENMs. This understanding will underpin the ultimate objective of being able to predict the hazard, and hence the workplace risk, without necessarily having to undertake extensive *in vivo* testing on each variant of CNT.

[45] It is fibres of high-aspect ratio and minimum length of approximately 15 - 20 µm that are of fibrogenic concern.

The primary properties of CNTs associated with their mode(s) of action currently being investigated, and which may help with predicting their toxicity, or workplace classification banding based on associations between the properties and biological responses are:

- Propensity to induce sustained oxidative stress.
- Physical dimensions and tumorigenic potential.
- Physical interference with intercellular structures.
- Biopersistence (discussed in Section 4.3).
- CNT corona when in biological medium (Section 3.2).

Bhattacharya et al. (2013) has reviewed the mechanisms of CNT-induced toxicity with a focus on the key event of pulmonary inflammation. The review provides a summary of many studies that have investigated the release and role of a variety of inflammatory mediators. They note the 'two-edged sword' of the inflammatory response. It being a normal reaction to infection or cell injury which occurs to restore normal functional homeostasis to the tissue. However if the inflammation is prolonged (such as may occur with 'frustrated phagocytosis' of fibre-like material) the adaptive changes may become detrimental. Thus it is important to distinguish between transient, protective responses versus chronic and maladaptive ones; not only for CNTs, but for all ENMs. Pulmonary inflammation *per se* is not necessarily an automatic indicator for adverse effects.

As with other ENMs, numerous *in vitro* experiments, some quite elegant, investigating the mechanistic detail of various aspects of the inflammatory response have been published. For example, consistent with the fibre toxicity paradigm, He et al. (2011) showed in cultured human lung cells that MWCNTs induced a fibrotic response by stimulating ROS production, they also induced the production of profibrogenic growth factors from macrophages that function as paracrine signals to promote the transformation of lung fibroblasts into myofibroblasts, a key step in the development of fibrosis. The data shows MWCNTs elicit multiple and intertwining signaling events involving oxidative damage, inflammatory cytokine production, and myofibroblast transformation. Katwa et al. (2012) have shown *in vivo* in mice that macrophages are mediators and modulators of the pulmonary and cardio-toxicity of MWCNTs.

While noting these *in vitro* studies are important for unravelling the molecular mechanisms of ENM induced toxicity, at this time they are of limited value for identifying and managing ENM risks in the workplace. Such investigations are therefore not described in detail in this review.

In relation to a pathogenic fibre response and CNT physical dimensions:

- The work of Osmond- McLeod et al. (2011) supports the current view that CNTs of high aspect ratio and length greater than ~20µm are associated with induction of fibre-like responses in the lung and mesothelial tissue.

- It has been shown *in vitro* that long, needle-like MWCNTs can activate the inflammasome[46] in a similar manner as does asbestos. This is dependent on production of reactive oxygen species (ROS) and activation of certain kinases (Palomäki et al. 2011).
- The diameter/rigidity may be critical for the lung toxicity and mesothelial cell damage and mesothelioma induction (Fenoglio[47] et al. 2012).
- Nagai et al. (2011) indicates MWCNTs directly pierced mesothelial plasma and nuclear membranes, whereas asbestos fibres were internalised by mesothelial cells via encapsulation by vesicular membranous structures. Thus, although MWCNTs and asbestos both share needle-like structures and have high durability, they do not necessarily enter mesothelial cells in the same way. The ability of MWCNTs to pierce cell membranes depended on the thickness of the fibre. Thin and rigid MWCNTs (diameter ~ 50 nm) showed mesothelial cell membrane piercing and cytotoxicity *in vitro* and subsequent inflammation and mesothelioma induction *in vivo*[48]. In contrast, thick (diameter ~ 150 nm) or tangled (diameter ~ 2–20 nm) MWCNTs caused less inflammation and a lower carcinogenic response. Thin and thick MWCNTs similarly affected macrophages. Interestingly, once inside the mesothelial cell MWCNTs produced a similar genetic alteration profile as is observed in asbestos-induced mesothelioma in mice and in human mesothelioma (Nagai et al. 2011, Jean et al. 2011).
- The lung inflammation, granulomas and fibrotic reactions and epithelial cell mutations appears to depend on, or be enhanced by the presence of defects on the CNTs (Muller et al. 2005, 2008a, b; Fenoglio et al. 2008).
- Despite causing lung inflammation, granulomas and fibrotic reactions and mutations, such CNT fibres that are <1µm long do not elicit a strong inflammatory response nor mesothelioma in the rat peritoneal screening assay (Muller et al. 2009, discussed in Safe Work Australia 2009a).

[46] Inflammasomes are large multiprotein cytoplasmic complexes that assemble inside a cell in response to a variety of infectious and noxious insults. Several distinct inflammasomes have been described, each of which contains a unique sensor protein for bacteria that initiates the assembling of the inflammasome, once formed it starts the inflammatory cascade. Inflammasomes are part of the innate immune response. They function to link the sensing of microbial products and metabolic stress to the proteolytic activation of the proinflammatory cytokines IL-1beta and IL-18.

[47] Fenoglio et al. (2012) tested two samples of MWCNTs (each <5µm but 9.4 or 70nm thick) *in vitro* (with murine macrophages and ROS production & cytotoxicity measurements) and *in vivo* (rat intratracheal doses of 2mg/rat & measurement of LDH & protein in lavage fluid). Both samples formed stable aggregates <10µm. Thin MWCNTs appeared significantly more toxic than the thicker ones both *in vitro* and *in vivo* when compared on a mass−dose basis.

[48]The *in vivo* tests for inflammation and mesothelioma induction by Nagai et al. (2011) was the rat interperitoneal screening assay described in Safe Work Australia (2009a). For assessing inflammation rats were injected with 1 or 5 mg CNT per animal and evaluated after 1 month. For induction of mesothelial tumours young rats were injected with 1 or 10 mg CNT and assessed after 1 yr. It should be noted that in the tumour inducing experiments that nearly half the rats at the top dose died with bloody ascites. The length of the various CNTs was ~5µm.

- Murphy et al. (2012a, b) have demonstrated an inflammatory response in the pleural cavity to long fibres but not short. Phagocytosis of particulates by macrophages activates a pro-inflammatory response in co-cultured endothelial or epithelial cells (Jimenez et al. 2002, Shaw et al. 2011). Similiarly it appears the long CNTs elicit an inflammatory response in macrophages via frustrated phagocytosis and the cytokines released stimulate a much amplified pro-inflammatory cytokine response in cultured mesothelial cells. It is hypothesised that these are the key sequence of events for production of mesothelioma in the pleural cavity. The authors also note the *in vitro* experimental system demonstrating indirect stimulation of mesothelial cells may be used to screen CNTs for potential for inducing mesothelioma.
- In addition to macrophage stimulation of pro-inflammatory cytokine release dispersed SWCNTs can directly stimulate cultured fibroblasts into collagen production (Wang et al. 2010a).
- In relation to physical shape and size it is interesting to note that spherical Ag-NP (30 nm) and silver microparticle powder (<45 µm) had no effect on cultured A549 alveolar epithelial cells. Whereas silver wire (length: 1.5 – 25 µm; diameter 100 – 160 nm) induced significant cytotoxicity and decreased cell viability, according to the authors this was not due to release of silver ions (Stoehr et al. 2011).

Physical interference of important cellular structures by CNTs has been described by:

- Sargent et al. (2012), where SWCNTs were incorporated into the centrosome structure and induced DNA damage[49] at *in vitro* doses which according to the authors were compatible with exposures expected in an occupational setting[50].
- Holt et al. (2010), who found the intracellular presence of SWCNTs altered the assembly and function of actin cytoskeleton filaments.
- Zuo et al. (2010), who showed SWCNTs can occupy the hydrophobic core of proteins to form stable complexes, leading to the disruption of ligand binding and loss of protein function.

While not within the scope of this review it is nonetheless worth noting that system-based identification of toxicity pathways associated with CNTs induced pathological responses is being explored (e.g. Alazzam et al. 2010, Pacurari et al. 2011a, Guo et al. 2012, Snyder-Talkington et al.

[49] The DNA damage observed in Sargent et al. (2012) was fragmented centrosomes, disrupted mitotic spindles and aneuploidy chromosome number.

[50] Sargent et al. (2012) cite Shvedova et al. (2008a) who calculated that 5 mg/m^3, 5 h, 4 days to mice resulted in a lung burden of 5 µg per mouse, they also showed a single nasopharyngeal aspiration exposure of 20 µg was associated with epithelial cell proliferation and abnormal nuclei in mice. Sargent et al. (2012) divided the 20 µg/mouse *in vivo* dose by the alveolar surface area of 500 cm^2/mouse lung to give the equivalent *in vitro* dose of 0.04 µg/cm^2. According to Sargent et al. (2012) the doses 0.024, 0.24, 2.4 or 24 µg/cm^2 of SWCNTs applied to their lung epithelial cell cultures were equivalent to worker exposure of 40 hr/week for 20 weeks at the OSHA particle exposure limit of 5 mg/m^3. How this was exactly calculated is not provided.

2013). These approaches to understanding the toxicity of CNTs use 'omic' technology that in the future perhaps holds promise for screening CNTs and predicting likely toxicological outcomes. The techniques are also being used for other ENMs (e.g. Ag-NP, Foldbjerg et al. 2012). At the current state of knowledge however it is somewhat obscure how they contribute to identification of workplace hazards.

4.3 CNT Biopersistence/kinetics

While the dogma of sustained 'frustrated phagocytosis' by macrophages is a well-entrenched mode of action for biopersistent fibres, Shvedova et al. (2012a) and Bhattacharya et al. (2013) point out that some of the recruited inflammatory cells (neutrophils and eosinophils) are capable of enzymatically degrading CNTs. Due to their multilayer structure MWCNTs seem to be more resistant to degradation by enzymatic catalysis than SWCNTs. Furthermore enzymatic degradation of SWCNTs and MWCNTs proceeds to a high extent if pristine nanotubes have been functionalised or otherwise treated to create defect sites (e.g. carboxylic groups, phenolic groups, heteroatoms etc) on their surface (Vlasova et al. 2012).

Several studies have shown SWCNTs are susceptible to biodegradation.

- *In vitro* studies with horseradish peroxidase (Allen et al. 2008, 2009; Kotchy et al. 2011, Russier et al. 2011) show CNTs are decreased in length, with surface carboxylation increasing degradation (Allen et al. 2009, Zhao et al. 2011c).
- Carboxylation also increased the non-enzymatic degradation of MWCNTs by phagolysosomal simulant fluid containing hydrogen peroxide at a typical physiological concentration of 1 mM. Unmodified, ozone treated, and aryl-sulphonated tubes did not degrade under these conditions (Liu et al. 2010d).
- While the *in vitro* biodegradation of SWCNTs occurs over weeks or months, degradation by inflammatory cells (neutrophils) is much quicker, it is also more efficient if the SWCNTs are coated with immunoglobulins or are pegylated (Kagan et al. 2010, Vlasova et al. 2012). This occurs under the action of myeloperoxidase [51].
- In myeloperoxidase deficient mice clearance of SWCNTs from the lungs was markedly less than in wild-type animals, and the inflammatory response much stronger, thereby providing

[51] Myeloperoxidase activity is very important in ameliorating respiratory infections. Its microbiocidal action is the result of catalysing the hydrogen peroxide-mediated oxidation of halide ions to hypochlorous acid. It is the myeloperoxidase in recruited neutrophils that imparts a greenish colour to the nasal and pulmonary secretions in upper respiratory tract infections. It is likely that two major pathways – hypochlorite generated by the enzyme and reactive intermediates of myeloperoxidase – are both involved in the oxidative biodegradation process (Bianco et al. 2011; Shvedova et al. 2012b; Vlasova et al. 2012).

compelling evidence that myeloperoxidase can significantly influence pulmonary response to SWCNTs[52] (Shvedova et al. 2012b).

- The peroxidase enzyme in eosin cells (eosinophil peroxidase) can also degrade SWCNTs (Andon et al. 2013).

In demonstrating that CNTs are potentially able to be degraded in simulated biological fluid or by peroxidase enzymes in neutrophils and other cells, the above studies also stress that defects on the CNTs are an important factor for such degradation. Studies such as these have the potential to help define what is meant by 'biopersistent' in relation to CNT production of fibrogenic/mesothelial responses.

In a study commissioned by Safe Work Australia as part of its Nanotechnology Work Health and Safety Program, Osmond-McLeod et al. (2011) investigated the relative durability of four types of CNTs and their subsequent ability to elicit an inflammatory response *in vivo*. The four types of CNTs included one SWCNT and three MWCNTs ['spinnable' (CNT_{SPIN}); 'long' (CNT_{LONG1}); and 'tangled' (CNT_{TANG2})]. Acting as negative and positive controls were a glass wool fibre and two types of asbestos fibres (long fibre amosite and long fibre chrysotile). Durability was tested in simulated biological fluid (Gambles solution) with loss of mass used as a measure of durability after various times of incubation over 24 weeks. The effect of durability on the ability to elicit fibre-like pathogenicity was assessed[53] after injection into the peritoneal cavities of mice.

- The non-durable glass fibre lost 60% of its weight and the durable asbestos fibre 25%. The CNT_{LONG1} lost 30% of its weight but the others did not lose any weight. The results suggest they were durable in this test. Nevertheless after 10 weeks incubation the proportion of long fibres (>15 µm) in CNT_{LONG1} was markedly reduced.
- When CNTs were injected into the abdominal cavities of mice as long discrete fibres, or as fibre-like structures, an inflammatory and fibrotic response was induced. However, where no or relatively few long fibres were present, as in the case of CNT_{LONG1} after 10 weeks incubation in Gambles solution, the inflammatory response was minimal.

[52] In Shvedova et al. (2012b) knock out and wild type mice were exposed to SWCNTs by pharyngeal aspiration (40 µg/mouse suspended in PBS). Inflammation was evaluated by total cell counts, cell differentials, and accumulation of cytokines in the BAL fluid. Fibrogenic responses were assessed by morphometric measurements and collagen deposition. At day 1 inflammatory responses to the SWCNTs were the same in both strains of mouse. At day 28 knock-out mice had significantly more SWCNTs retained in the lungs and significantly more collagen deposition. There was evidence of 'cutting' of SWCNT aggregates by myeloperoxidase.

[53] The intraperitoneal test employed by Osmond- McLeod et al. (2011) is described in Safe Work Australia (2009a), it is a well validated screening assay used to identify fibres with asbestos-like biological activity. Not all the fibres were assessed; the glass wool fibre, the asbestos fibres, SWCNTs and CNT_{LONG1} were tested in the mouse screening assay before and after they went through the durability test (10 weeks).

- In the case of SWCNTs, where the fibrous shape of individual tubes was masked by tight bundling, the inflammatory response was also minimal.

The authors concluded the results supported the view that CNTs are generally durable but may be subject to bio-modification in a sample-specific manner. They also suggested that pristine CNTs, either individually or in rope-like aggregates of sufficient length and aspect ratio, can induce asbestos-like responses in mice, but that this effect may be mitigated for certain types that are less durable in biological systems. Results indicate that durable CNTs that are either short or form tightly bundled aggregates with no isolated long fibres are less able to produce inflammation in fibre-specific assays. The data suggest that the pathogenicity of CNTs is not absolutely dependent upon the presence of defects on the CNT as may be suggested by other investigators (Muller et al. 2005, 2008a, b; Fenoglio et al. 2008). It could be that defects on the CNT and/or the presence of metal impurities enhance the inflammatory response of pristine CNTs initiated by their 'fibrogenic' dimensions.

The information in Osmond- McLeod et al. (2011) is consistent with previous results from that laboratory (Poland et al. 2008) and the long – short fibre hypothesis described in Safe Work Australia (2009a). It is noted the durability test fluid while adjusted to the pH of a lysosome was an electrolyte solution that did not include myeloperoxidase or any neutrophils. It is therefore uncertain how well this test reflects CNT durability *in vivo.* Nevertheless the results support the continuing pursuit of biopersistence characterisation of CNTs as a potential predictor of fibrogenic activity. Patently more work is required to develop a reliable and predictive durability test.

Of course the biopersistence of CNTs is not only determined by their durability but also by how well they are cleared from the lung by physiological processes. To date there has been little published information on lung clearance.

4.4 Effects of aggregation/agglomeration

Being highly hydrophobic, CNTs are known to agglomerate/aggregate in air and aqueous solution and the degree of agglomeration/aggregation has been reported to modulate CNT uptake and toxicity.

Shvedova et al. (2008b) indicate aerosolisation of SWCNTs for studying the effects of inhalation exposures is very difficult because of their hydrophobicity and tendency to agglomerate yielding large entangled structures, often "bird's nest"-like, of micrometer-size. Since these physical forms of CNTs do not appear to be strongly associated with lung fibrogenicity (Section 4.2) it is a pressing question to determine the form of airborne CNTs in the workplace. Otherwise it is very difficult to extrapolate hazards identified in inhalation studies conducted with well dispersed 'single' CNTs to potential workplace hazard and risk.

The United States National Institute for Occupational Safety and Health (NIOSH) has conducted field studies at a number of sites using the Nanoparticle Emission Assessment Technique (NEAT) to characterise emissions during processes where engineered nanomaterials were produced or used. With respect to particle size, shape, and degree of agglomeration, the technique provided visual evidence that the majority of emissions of ENMs tended to agglomerate and form aerosol structures that were larger than the nanoscale (i.e. < 100nm) but still would be considered nanomaterials because the international definition of a nanomaterial is one made of, or containing nanoparticles (Methner 2008, Methner et al. 2007, 2009a, b).

In contrast to asbestos, the SWCNTs tested for their pulmonary inflammatory potential by Murray et al. (2012) were agglomerated in aqueous media and in the lung. Consistent with previous work (Shvedova et al. 2005) these produced a granulatomous response 28 days after nasopharyngeal aspiration (40 µg) by mice. This is characteristic of a particulate response rather than fibres. On the other hand carbon nanofibres (CNF), probably by virtue of their more rigid structure, elicited an inflammatory and short term pathological response (thickening of alveolar wall) reminiscent of asbestos fibres.

Pauluhn (2010a) undertook a 13 week inhalation study in rats with MWCNTs (Baytubes) using four different aerosol concentrations. The no observed adverse effect level was 0.1 mg/m^3. The test substance was agglomerated and appeared to resemble low-density spheres of packed, tangled, and intertwined MWCNTs. It was found the toxic effects (inflammation and granulomatous changes) were determined by the density of agglomerate structures, not fibrillar structures. The study demonstrated that the induced pathological changes were consistent with overload related phenomena, also observed with carbon black. The issue of particulate pulmonary overload in rodent studies and how this affects data interpretation is discussed in detail in Safe Work Australia (2009a).

4.5 *In vivo* toxicity studies

NIOSH (2013a) has undertaken a comprehensive review of the animal toxicology literature that was not bounded by date of publication. It is beyond the scope and resources of this current review to match the in depth work of NIOSH. Indeed, nor is it necessary given the inclusiveness of the NIOSH work and the fact that it has undergone peer review (NIOSH 2013b).

NIOSH (2013a) systematically reviewed 54 laboratory animal studies (exposure by inhalation, intratracheal instillation, or pharyngeal aspiration), many of which indicated that CNT/CNF could cause adverse pulmonary effects including inflammation (44/54), granulomas (27/54), and pulmonary fibrosis (25/54). In addition reduced particle lung clearance has been observed in mice or rats exposed to relatively low-mass concentrations of CNTs. These non-cancer effects are similar to what is observed in animal studies and workers who have been chronically exposed to high airborne

concentrations of particulates. Pulmonary responses were qualitatively similar across the various types of CNTs (single-walled and multi-walled) and CNFs, purified, or unpurified with various metal content, pristine or with defects, and different dimensions. In addition there is evidence from studies where CNTs were compared with other known fibrogenic materials (e.g. silica, asbestos, ultrafine carbon black), that the CNTs were of similar or greater potency and the effects, including fibrosis, developed soon after exposure (within a few weeks) and persisted during follow up periods of approximately 1 – 6 months.

The extent to which the early inflammatory and slight fibrosis (alveolar wall thickening and increased collagen content) observed in animals may predict clinically significant lung effects in workers is not known. However it is standard toxicological and risk assessment practice to assume humans may respond in a similar manner at similar exposures. Apart from the issue of pulmonary particulate overload in rats discussed in Safe Work Australia (2009a), there is no compelling information to suggest that human susceptibility, or responsiveness, to CNTs or CNFs would be different from experimental animals.

The pivotal studies identified by NIOSH for establishing a WES are the 90 day inhalation investigations of Ma-Hock et al. (2009a) and Pauluhn (2010a) with MWCNTs.

- Ma-Hock et al. (2009a) are from BASF in Germany. They exposed rats head-nose only for 6 h/day, 5 days/week, 13 weeks, for a total of 65 exposures to MWCNT concentrations of 0, 0.1, 0.5, or 2.5 mg /m^3 (n = 10/group/sex) according to OECD test guideline 413. The test substance was a commercial thin (~ 60 nm diam) MWCNT (Nanocyl NC 7000) of low dustiness manufactured in Belgium. The purity was 90% carbon and 10% metal oxide. Generated aerosols for the experiment had an aerodynamic diameter of ~2µm.

  Systemic toxicity was not observed. The important lung effects were increased lung weights and pronounced multifocal granulomatous inflammation in lung and lung-associated lymph nodes at 0.5 and 2.5 mg/m^3. The incidence and severity were exposure concentration related. At 0.1 mg/m^3, there was still minimal granulomatous inflammation in the lung and lymph nodes. Thus this concentration was taken as the LOAEL.
- Pauluhn (2010a) is a study from Bayer Schering Pharma in Germany. Rats were nose only exposed to MWCNTs for 6 h/day, 5 days per week for 13 consecutive weeks to 0, 0.1, 0.4, 1.5, and 6 mg/m^3 (n = 50 male/group; 10 female/group). The test substance was a commercial MWCNT (Baytubes) of low dustiness and present in the exposure aerosols as agglomerates of spherical assemblages (~1 – 20 µm) of intertwined tubes with frayed surfaces. Based on their low specific density relative to carbon black and consequential higher displacement volume in alveolar macrophages (ILSI 2000) it was estimated 0.1 mg/m^3

would be in the non-overload range, 0.4 mg/m^3 to be at a transitional level where lung overload may occur and frank lung overload with partial or inhibited clearance would occur at 1.5 and 6 mg/m^3.

At the top two doses there was marked reduction in particle clearance from the lungs, and significantly increased lung and local lymph node weights. Histopathology revealed exposure-related lesions at 0.4 mg/m^3 and above in the upper respiratory tract (goblet cell hyper- and/or metaplasia, eosinophilic globules, and focal turbinate remodeling) and the lower respiratory tract (inflammatory changes in the bronchioloalveolar region and increased interstitial collagen staining). Granulomatous changes and a time-dependent increase of a bronchioloalveolar hyperplasia occurred at 6 mg/m^3. No effects occurred at 0.1 mg/m^3.

These studies were used as described in Section 4.9.1.1 to establish WESs.

4.6 Carcinogenicity

In Safe Work Australia (2009a) it was concluded MWCNTs of fibrogenic dimensions present a mesothelioma hazard if sufficient number are in contact with mesothelial tissue. This was based on preliminary data which showed that after pharyngeal or inhalation exposure MWCNTs can penetrate through to the pleural mesothelial tissue and cause lesions, and in the peritoneal mesothelioma screening assay long, but not short or tangled MWCNTs initiated inflammation and mesothelioma.

Since that time it has been confirmed that after peritoneal injection, the ensuing pleural inflammation and mesothelioma are related to the thin diameter and rigid structure of MWCNTs (Nagai et al. 2011, Murphy et al. 2012b). Long and thick MWCNTs, but not short and thin MWCNTs, caused DNA damage[54] *in vitro* in A549 cells (human alveolar carcinoma epithelial cells) and THP-1 cells (human monocytic cells) and severe inflammatory effects after intraperitoneal injection of 50 µg to mice (Yamashita et al. 2010).

It has further been demonstated:

- Exposure of rats to MWCNTs or crocidolite by intrapulmonary spraying (total dose over 9 days was 1,250 µg) produced inflammation in the lungs and pleural cavity and mesothelial proliferative lesions. Importantly the fibers were found in macrophages within the pleural cavity but not in the lesions themselves. Because conditioned cell culture media from macrophages treated with MWCNTs and crocidolite, and the supernatants of pleural cavity lavage fluid from the dosed rats, increased mesothelial cell proliferation *in vitro*, the authors

[54] In Yamashita et al (2010) the MWCNTs investigated were M1: length 5–15 µm, diameter 20–60 nm; M2: length 1–2 µm, diameter 60–100 nm and M3: length 1–2 µm, diameter <10 nm. The DNA damage was strand breaks and alkaline labile sites measured by the Comet assay.

concluded the mesothelial proliferative lesions were induced by inflammatory events in the lung and pleural cavity, and likely mediated by macrophages (Xu et al. 2012c).

- Inhalation to MWCNTs (5 mg/m^3, 5 hours/day, 5 days/week) for a total of 15 days can promote the growth of lung tumours initiated by intraperitoneal injection of methylcholanthrene (Sargent et al. 2013).
- SWCNTs or MWCNTs can transform cultured lung epithelial cells such that when subcutaneously injected into the flanks of immunodeficient nude mice, small tumors are observed one week post-injection (Wang et al. 2011c).

These latter data suggest that the CNTs may not have to be in contact with the mesothelial cells in the pleural cavity in order to exert carcinogenic influence.

4.7 Genotoxicity

It was previously indicated in Safe Work Australia (2009a) that the majority of genotoxic effects demonstrated with ENMs, particularly *in vitro,* were the result of secondary ROS production, and not a reflection of an intrinsic toxicological hazard of the ENM. CNTs however have been shown to have direct (physical interaction with chromosomes) as well as indirect genotoxicity (due to reactive oxygen formation) (van Berlo et al. 2012) (Section 4.2). Nevertheless it appears that much of the genotoxic potential of CNTs is driven by structural defects or the presence of metal impurities facilitating ROS formation. Based on the spectrum of genotoxic effects, van Berlo et al. (2012) drew strong parallels with the genotoxicity of asbestos. Indeed Nagai et al. (2011) have identified a specific mutation to tumour suppressor genes in the mesotheliomas produced in the rat peritoneum by long MWCNTs, this is similar to mutations observed in asbestos-associated mesotheliomas.

CNTs can damage DNA *in vitro* and *in vivo* however the majority of evidence is from *in vitro* studies (Table 4.1), the preponderance of this data is for DNA/chromosomal breaks[55] rather than the mutations more frequently seen with genotoxic chemicals. Perhaps not unexpectantly, the genotoxic potential of different CNT samples varies considerably.

Physical interaction with genetic material and consequential damage has also been demonstrated. Using immortalised human airway epithelial cells, Sargent et al. (2012) have shown SWCNTs can be incorporated into the centrosome structure of dividing cells and induce DNA damage at tissue doses

[55] DNA/chromosomal strand breaks are measured as breakage (Comet assay), micronuclei and chromosomal aberrations.

claimed to be comparable[56] with those expected in an occupational setting. According to the authors disruption of the centrosome is common in many solid tumours including lung cancer. The resulting aneuploidy is an early event in the progression of many cancers, suggesting that it may play a role in both tumourigenesis and tumour progression.

Table 4.1: CNT-induced genotoxic effects (Adapted from van Berlo et al. 2012).

Endpoint	Test cells	SWCT	MWCNT
DNA strand breakage (comet assay)	A549 human lung epithelial		Karlsson et al. 2008 Yamashita et al. 2010 Ursini et al. 2012 Cavallo et al. 2012
	RAW264.7 mouse macrophages	Di Giorgio et al. 2011	Di Giorgio et al. 2011
	NHDF human dermal fibroblasts		Patlolla et al. 2010a
	Rat lung epithelial cells evaluated at 0 & 30 post inhalation exposure		Kim et al. 2012b
	Murine lung tissue		Kato et al. 2013
	Normal &malignant human mesothelial cells	Pacurari et al. 2008	
	Primary mouse embryo fibroblast	Yang et al. 2009	
	V79 Chinese hamster lung fibroblasts	Kisin et al. 2011	
	Murine leukocytes	Patlolla et al. 2010b	
DNA base oxidation	RAW264.7 mouse macrophages	Migliore et al. 2010	Migliore et al., 2010
	Murine lung tissue		Kato et al. 2013
Micronuclei	A549 human lung epithelial		Kato et al. 2013
	RAW264.7 mouse macrophages	Di Giorgio et al. 2011	Di Giorgio et al. 2011 Migliore et al. 2010
	MCF-7 human lung epithelial		Muller et al. 2008b
	Rat type II lung epithelial		
	RLE rat lung epithelial		
	Normal human dermal fibroblasts	Cveticanin et al. 2010	Cveticanin et al. 2010
	BEAS-2B human bronchial epithelial	Lindberg et al. 2009 Manshian et al. 2013	
	V79 Chinese hamster lung fibroblasts	Kisin et al. 2011	
	Murine bone marrow	Patlolla et al. 2010b	
Chromosomal aberrations	RAW264.7 mouse macrophages	Di Giorgio et al. 2011	Di Giorgio et al. 2011
	CHO AA8 Chinese hamster ovary		Kato et al. 2013
	BEAS-2B human bronchial epithelial	Sargent et al. 2012	
	Human airway epithelial	Sargent et al. 2009; 2012	
	Murine bone marrow	Patlolla et al. 2010b	
Formation of gH2AX foci [a]	Normal human dermal fibroblasts	Cveticanin et al. 2010	Cveticanin et al. 2010
	Human umbilical vein endothelial		Guo et al. 2011
	Normal and malignant human	Pacurari et al. 2008	

[56] The *in vitro* exposures at the occupational exposure in Sargent et al. (2012) were calculated as a mass per surface area of cells as follows. The dose of SWCNTs was based on *in vivo* exposures that demonstrated epithelial cell proliferation and abnormal nuclei at 20 µg/mouse, and is equivalent to an exposure predicted in workers of 40 h per week for 20 weeks at the OSHA particle exposure limit (PEL) of 5 mg/m^3 for particles less than 5 µm in diameter (Shvedova et al. 2008b). The 20 µg/mouse *in vivo* dose was adjusted to the alveolar surface area of a 500 cm^2/mouse lung (Stone et al. 1992). The adjusted dose for *in vitro* exposure was 0.02–0.08 µg/cm^2 of culture surface area.

Endpoint	Test cells	SWCT	MWCNT
	mesothelial		
	Mouse embryonic stem		Zhu et al. 2007
Mutant frequencies	Mouse embryonic stem		Zhu et al. 2007
	Lung tissue gpt delta transgenic mice		Kato et al. 2013
	BEAS-2B human bronchial epithelial	Manshian et al. 2012	
	Murine lung tissue	Shvedova et al. 2008b	

[a] gH2AX foci are a marker for double DNA strand breaks.

4.8 Immunotoxicity

In relation to immunotoxic effects it must be noted that the effects observed in the lungs of experimental animals is the result of CNTs (and other ENMs) interacting with the innate immune system (i.e. the interaction with local macrophages). There have been limited investigations on whether inhalation of CNTs may influence the systemic function of the adaptive immune system.

When given concurrently with intratracheal ovalbumin to mice, MWCNTs (50 µg/animal) acted as an adjuvant and amplified the immune response thereby aggravating the allergen induced airway inflammation (Inoue et al. 2009). The authors speculated the exacerbation may be partly through the inappropriate activation of antigen-presenting cells. Similarly Nygaard et al. (2009) found high doses[57] of MWCNTs acted as an adjuvant in mice when given intranasally with ovalbumin and the animals were boosted with subsequent exposures of ovalbumin. The MWCNTs strongly increased serum levels of OVA-specific IgE.

On the other hand, Mitchell et al. (2007) reported short term inhalation of MWCNTs [58] could cause systemic immunosuppression. In a follow up study Mitchell et al. (2009) exposed mice to 0, 0.3 or 1 mg/m^3 for 6 h per day for 14 consecutive days. Only animals exposed to 1 µg/m^3 had suppressed immune function[59]. This effect appeared dependent upon activation of cyclooxygenase enzymes in the spleen in response to a signal from the lungs. This was deduced because spleen cells from exposed animals partially recovered their immune function when treated with ibuprofen, a drug that inhibits cyclooxygenase enzymes. In addition knockout mice without cyclooxygenase enzymes were

[57] The actual dose given to mice in the Nygaard et al. (2009) study is difficult to determine. Sedimentation of the suspension with ovalbumin in Hank's solution occurred and the supernatant fraction was used to dose mice. Nominally the high dose, without aggregation and sedimentation, was 133 µg/animal per day for 3 days.

[58] The MWCNTs used by Mitchell et al. (2007) had diameters of 10 to 20 nm, lengths of 5 to 15 µm and were respirable aggregates.

[59] Immune function was assessed in Mitchell et al. (2009) by testing harvested spleen cells in the sheep red blood cell plaque forming assay.

not affected when exposed to MWCNTs. The authors suggest signals from the lung (proposed to be TGFβ) can activate signals in the spleen to suppress immune function.

Earlier reports indicated SWCNTs could increase the severity of *Listeria monocytogenes* infection *in vivo* (Shvedova et al. 2008a) which is consistent with an immunosuppressive effect. More recently Tkach et al. (2011) demonstrated that the local pulmonary inflammatory response induced *in vivo* by SWCNTs was accompanied by modified systemic immunity as measured by decreased proliferation of splenic T cells on stimulation with concavalin A. Preincubation of naïve T cells *in vitro* with SWCNT-treated dendritic cells reduced proliferation of T cells which suggested to the authors the *in vivo* systemic immunosuppression in the spleen may be due to SWCNTs[60] affecting dendritic cells that had infiltrated into the lungs (Tkach et al. 2011).

In contrast, Swedin et al. (2012) did not find pre-exposure to SWCNTs enhanced or suppressed the early immune response to the parasite *Toxoplasma gondii* in mice. Mice were pre-exposed by pharyngeal administration with SWCNTs (80 µg/mouse/d) for two consecutive days followed by intravenous injection with parasite tachyzoites. The distribution of parasite and the spleen cell proliferation response was not affected. This suggests the immunosuppression observed as decreased spleen cell proliferation with chemical stimulation in Tkach et al. (2011) did not compromise the immune system's ability to mount a response to a systemic infection.

Clearly the effects of CNTs on the functions of the immune system are complex and additional investigations are required to determine if workers making or handling CNTs are more at risk of developing upper respiratory tract infections, or clinical illness as a result of CNTs modulating immune responses. Many of the studies described above were designed to acutely place a burden of CNTs into the lungs of mice that was considered equivalent to pulmonary burden that may be acquired by a worker over many (20+) years of exposures similar to those measured by NIOSH (2010, 2013a). This assumed negligible clearance of the CNTs from the lungs, an assumption based on high dose rat studies (e.g Pauluhn 2010a) in which clearance was only markedly compromised in situations of pulmonary overload. Since the effects appear to be dose related and primarily observed with the high exposures, it is somewhat uncertain to extrapolate the animal immunotoxicity studies to workers at this time.

4.9 Workplace implications

The effects observed in animals when administered CNTs and the physical similarity of some CNTs with fibrogenic asbestos raises concern for the health of persons working with these materials. While NIOSH reports it is not aware of adverse health effects in workers using or producing CNTs or CNFs,

[60] In Tkach et al. (2011) the mean diameter of the SWCNTs was 1 – 4 nm and length 1 – 3 µm, they were administered by pharyngeal aspiration (40, 80 or 120 µg/mouse) with animals evaluated 1 and 7d post exposure.

it should also be recognised that some of the potential effects may take many years to develop. Consequently NIOSH (2013a) has recommended an exposure limit for CNTs and CNFs that is at the limit of quantification of the most sensitive and reliable method for measurement of elemental carbon (NIOSH method 5040).

In addition NIOSH (2013a) has recommended it is prudent for employers to institute medical surveillance and screening programs for workers who are exposed to CNTs and CNFs for the purpose of possibly detecting early signs of adverse pulmonary effects including fibrosis. This is made on the evidence for early pulmonary fibrosis from animal studies and the fact that this effect can be detected by medical tests[61]. The role of medical monitoring/surveillance is generally discussed in Section 2.1.

While long term repeat exposure inhalation studies in animals have not yet been conducted NIOSH has used short term (90 day) inhalation studies (Ma-Hock et al. 2009a, Pauluhn 2010a) to inform the development of a workplace exposure limit; the exposure limit is based on the limit of quantitation with consideration of residual health risk at that concentration (Section 4.9.1). The observed pulmonary effects in these short term studies are supported by several intratracheal or nasophyangeal exposure investigations (Lam et al. 2004, Muller et al. 2005, Shvedova et al. 2005, 2008b; Mercer et al. 2011). NIOSH (2013a) estimate the risk of developing early-stage[62] (slight or mild) lung effects over a working lifetime if exposed to CNTs at the analytical limit of quantification (NIOSH Method 5040) of 1 $\mu g/m^3$ (8-hr time-weighted average [TWA] as respirable elemental carbon) is approximately 0.5% to 16% (upper confidence limit estimates).

In a report prepared for Safe Work Australia (2012b), the National Industrial Chemicals Notification and Assessment Scheme (NICNAS) concluded that based on the information from these 90-day studies, CNTs should be provisionally classified as specific target organ toxicants (Category 2) and carcinogens (Category 2) according to the *Globally Harmonized System for Classification and Labeling of Chemicals* (GHS).

As with exposure to other ENMs, peak airborne concentrations of CNTs or CNFs are associated with workplace activities such as weighing, transferring, mixing, blending, or sonication (Castranova et al. 2012). However the exposure appears to be primarily to respirable agglomerates (see Section 4.4).

[61] Apart from an occupational and medical history, NIOSH (2013a) recommends assessing respiratory symptoms by use of a standardised questionnaire, baseline spirometry test, and chest X-ray. Periodic evaluation is at the discretion of the medical practitioner and should be guided by symptomology, manufacturing process changes and incident. However it is suggested spirometry not be conducted more frequently than 3 years. X-ray films should be interpreted by certified person using the standard International Classification of Radiographs of Pneumoconioses as a guide.

[62] These early-stage changes are minimal granulomatous inflammation or alveolar septal thickening/fibrosis, grade 1 or higher.

Presumably these may disperse into smaller or even single entities with the aid of pulmonary surfactant; this however has not been conclusively demonstrated in animal experiments. Patently the extent of airborne exposure will be dependent upon a range of workplace specific factors and exposures in one facility are unlikely to be readily transferable to another workplace. NIOSH has undertaken exposure studies at research laboratories, pilot plants and research facilities and while doing so has assessed the capability of various measurement techniques for quantitating CNTs and CNFs (NIOSH 2013a). These data should serve as indicators for the extent of potential exposure in Australian facilities handling CNTs.

4.9.1 Workplace Exposure Standards

A number of agencies and manufacturing companies have derived provisional workplace exposure standards (WESs) for CNTs. The WESs are shown in Table 4.2; their derivation is briefly described in the subsequent sections.

Table 4.2: WESs for carbon nanotubes and fullerenes

Nanomaterial	WES	Reference
CNT/ CNF (SWCNT & MWCNT)	0.001 mg/m^3 8-hour TWA, 45 working years	NIOSH 2013a (USA)
SWCNT & MWCNT	0.03 mg/m^3 Period-limited (15 years)	Nakanishi et al. 2011 (Japan)
CNT	Short-term exposure: Long term: 0.2 mg/m^3 or [a] 0.034 mg/m^3 or [a] 0.004 mg/m^3 0.0007 mg/m^3	EC 2009b
MWCNT (Baytubes®)	0.05 mg/m^3 8-hour TWA	Pauluhn 2010b (Germany – industry)
MWCNT	0.0025 mg/m^3 8-hour TWA	Nanocyl 2009 (Belgium – industry)
MWCNT	0.002 mg/m^3 or [b] 0.001 mg/m^3 8-hour TWA	Aschberger et al. 2010 (Europe)
Fullerenes	Short term exposure: Long term: 0.044 mg/m^3 0.0003 mg/m^3	EC 2009b (Europe)
C_{60} fullerenes	0.39 mg/m^3 Period-limited (15 years)	Shinohara et al. 2011 (Japan)

WES = workplace exposure standard; SWCNT = single-walled carbon nanotubes; MWCNT = multi-walled carbon nanotubes; TWA = time-weighted average; DNEL = derived no-effect level.

[a] The two occupational exposure limits are based on different toxicological end points in the same study (Mitchell et al. 2007). It is usual to base exposure guidelines on the most sensitive effect.

[b] The two limits proposed by Aschberger et al. (2010) are from two different 90 day inhalation studies (Pauluhn et al. 2010a, Ma-Hock et al. 2009a).

4.9.1.1 MWCNT

Germany:

Given the absence of a government promulgated workplace standard, private companies have begun to set 'in house' exposure standards for their products. Pauluhn (2010b), from Bayer Shering Pharmaceuticals, proposed a WES of 0.05 mg/m^3 (8-hour TWA) for a specific Bayer product, i.e. Baytubes®, a MWCNT type with the tendency to form assemblages. This was derived[63] from a

[63] The adjustment factor for deposited dose in humans relative to rats was calculated as follows:

$$AF_{deposited\ dose} = \frac{Ventilation_{human} \times Pulm.Deposition_{human}}{Ventilation_{rat} \times Pulm.Deposition_{rat}}$$

Where:
$Ventilation_{human}$ = The ventilation per human working exposure day (10m^3/70 kg = 0.14 m^3/kg-day-human)
$Ventilation_{rat}$ = The ventilation per rat exposure day (0.29 m^3/kg-day-rat)
$Pulm.Deposition_{human}$ = Pulmonary deposition in humans (11.8)
$Pulm.Deposition_{rat}$ = Pulmonary deposition in rats (5.7).

Substituting the parameters into the equation gives an adjustment factor of 1.

NOAEL of 0.1 mg/m^3 for lung and nasal inflammatory responses from a 13-week inhalational rat study with Baytubes® (Pauluhn 2010a). The NOAEL was adjusted by a factor of 2 for worker exposure day, air intake, deposition, and clearance kinetics to derive a WES of 0.05 mg/m^3. No uncertainty factors were applied.

Belgium:

Nanocyl (2009), a producer of CNTs, briefly presented the derivation of a WES for one of their MWCNT products, Nanocyl NC 7000, at the European Responsible Care Conference. They used a LOAEL of 0.1 mg/m^3 for minimal granulomatous inflammation in the lung from a 90-day inhalational rat study (Ma-Hock et al. 2009a). An uncertainty factor of 40 was applied (details not provided) to derive a WES of 0.0025 mg/m^3 as an 8-hour time-weighted average.
The LOAEL obtained in this study is for minimal effects which may in fact be closer to a NOAEL.

Europe:

- Aschberger et al. (2010) adjusted the NOAEL/LOAEL of 0.1 mg/m^3 for MWCNTs from Pauluhn et al. (2010a) and Ma-Hock et al. (2009a) to a human equivalent concentration of 0.05 mg/m^3 (0.1 mg/m^3 x 6h/8h x 6.7m^3/10m^3). They then applied an uncertainty factor of 25 (2 for using a LOAEL for minimal effects, 2.5 for interspecies differences, and 5 for worker variability) or 50 (extrapolation from subchronic to chronic exposure in Ma-Hock et al [2009a] study)[64] to give proposed WESs of 0.002 or 0.001 mg/m^3 from the two studies.

- The European Commission (EC 2009b) has also undertaken a risk assessment for CNTs. They calculated 'Derived No Effect Levels' (DNELs) for worker exposure using NOAEL or LOAEL for two different toxicological endpoints; lung inflammation or immunological effects in mice after inhalation (14 days at 6 hr/d) (Mitchell et al. 2007).

The adjustment factor for the retained dose was calculated as follows:

$$AF_{\text{retained dose}} = AF_{\text{deposited dose}} \times \frac{AF_{\text{kinetics}}}{AF_{\text{AM volume}}} = 1 \times \frac{10}{1} \times \frac{8.7 \times 10^{10}}{5 \times 10^{11}}$$

$$= 1 \times 10 \times 0.17 = \sim 2.$$

Where:

AF_{kinetics} = Interspecies difference in retention kinetics (i.e. lung burden of rat exposed for 3 months compared with humans exposed long enough to attain steady state lung burden). This is estimated to be a factor of 10.

$AF_{\text{AM volume}}$ = The volume of alveolar macrophages of humans divided by that of rats, to account for differences in anticipated toxicity in humans due to higher number of macrophages in lungs when compared to rats (i.e. $5 \times 10^{11}/8.7 \times 10^{10} = 5.7$).

[64] The additional uncertainty factor of 2 was not applied to the Pauluhn et al. (2010a) NOAEL, since there was a post-exposure period of 6 months after the 90-day exposure period, and it was considered an extrapolation for duration was not needed when using this study.

For the first endpoint, they used a NOAEL of 5 mg/m^3 (highest concentration) for MWCNTs for the absence of lung inflammation or tissue damage in a 14 day mouse (whole-body) inhalation study by Mitchell et al. (2007). The experimental NOAEL was converted to a worker NOAEL (6h/8h x 6.7 m^3/10m^3) of 2.5 mg/m^3. Assessment factors of 12 (short term exposure) or 75 (long term exposure) were applied, consisting of 2.5 (interspecies variation), 5 (intraspecies) and 6 (only for long term exposure) to give a $DNEL_{inhalation,\ scenario1,\ short\ term}$ of 0.2 mg/m^3 and a $DNEL_{inhalation,\ scenario1,\ long\ term}$ of 0.034 mg/m^3.

For the second endpoint, a LOAEL of 0.3 mg/m^3 for MWCNTs for systemic immune effects in the spleen (but not lung) from the same study was used. This was converted to a worker LOAEL (6h/8h x 6.7 m^3/10 m^3) of 0.15 mg/m^3. Assessment factors of 37.5 (short term exposure) or 225 (long term exposure) were applied, consisting of 3 (extrapolation from LOAEL to NOAEL), 2.5 (interspecies variation), 5 (intraspecies), and 6 (only for long term exposure) to give a $DNEL_{inhalation,\ scenario2,\ short\ term}$ of 0.004 mg/m^3 and a $DNEL_{inhalation,\ scenario2,\ long\ term}$ of 0.0007 mg/m^3.

USA:

In a draft document, NIOSH (2010) recommended limiting the concentration of CNTs in workplaces to below 0.007 mg/m^3, measured in terms of elemental carbon in accordance with NIOSH method 5040. This recommended exposure limit (REL) was revised down to 0.001 mg/m^3 in the final document (NIOSH 2013a), to correspond with methodology improvements which improved laboratory limit of quantification (LoQ) to 0.001 mg/m^3.

NIOSH (2010, 2013) determined the best data to use for evaluating the basis of an REL for CNTs and CNFs are the non-malignant pulmonary data from CNT animal studies. They deemed the data on cancer and cardiovascular effects inadequate at the time of consideration.

Given that the animal effects are relevant for humans, the REL was derived using data for early stage fibrotic and inflammatory lung responses in short-term and subchronic animal studies. Critical effect levels for the noncancerous lung effects were estimated from the animal dose-response data (e.g. BMD and BMDL) and NOAELs or LOAELs reported in Ma-Hock et al. (2009a) and Pauluhn (2010a) were also used. These doses were extrapolated to humans by accounting for factors influencing pulmonary dose in each species. Working-lifetime exposure concentrations were calculated based on estimates of the deposited (i.e. assuming no clearance) or retained (i.e. normal clearance) alveolar lung dose of CNTs for an 8-hour TWA exposure during a 40-hour work week, 50 weeks per year, for 45 years.

- Based on the BMD modelling of the subchronic animal inhalation studies with MWCNTs (Ma-Hock et al. 2009a, Pauluhn 2010a), a working lifetime exposure of 0.2-2 µg/m^3 (8-hour TWA) was estimated to be associated with a 10% excess risk of early-stage adverse lung effects (95% lower confidence limit estimates).
- Using the NOAELs or LOAELs from the studies, the estimated human equivalent working lifetime concentrations were approximately 4-18 µg/m^3 (8-hour TWA). Dividing these estimates by data-suitable uncertainty factors (of 20-60), and assuming a threshold model, the estimated zero risk levels were 0.07-0.8 µg/m^3 (8-hour TWA over working lifetime).

In view of the current limit of quantitation of 1 µg/m^3 elemental carbon by NIOSH method 5040, NIOSH (2013a) recommended the REL be set at this concentration. Residual risks at 1 µg/m^3:

- The 45-year working lifetime excess risk estimates of minimal (grade 1 or greater) lung effects in rats at 1 µg/m^3 (8-hour TWA) ranged from 2.4-33% (maximum likelihood estimates, MLE) and 5.3-54% (95% upper confidence limit estimates, 95% UCL).
- For slight/mild (grade 2) lung effects, these risks are 0.23-10% (MLE) and 0.53-16% (95% UCL).

Because of the above residual risk of adverse lung effects at the REL, NIOSH (2013a) recommended efforts should be made to reduce airborne concentrations to CNTs and CNFs as low as possible below the REL, and indicated it would be prudent for medical surveillance of exposed workers.

Japan:

Japanese researchers also proposed provisional WESs for carbon nanotubes (Nakanishi et al. 2011). They determined a NOAEL of 0.13 mg/m^3 and 0.37 mg/m^3 for SWCNTs and MWCNTs, respectively, for 'lung inflammation' from 4-week inhalation experiments (3 month observation period). An uncertainty factor of 2 was applied to convert these into sub-chronic NOAELs of 0.065 and 0.185 mg/m^3. The adjusted NOAELs were then converted to human equivalent NOAELs of 0.09 and 0.24 mg/m^3 for SWCNTs and MWCNTs, respectively[65]. An uncertainty factor of 3 (for toxicokinetic

[65] Extrapolation was done using the following equation:

$$NOAEL_H = NOAEL_R \times \frac{t_r}{t_H} \times \frac{Q_R}{Q_H} \times \frac{day_r}{day_H} \times \frac{DF_r}{DF_H} \times \frac{BW_H}{BW_R}$$

Where:
$NOAEL_H$ = NOAEL in humans
$NOAEL_R$ = NOAEL from rat study (0.065 or 0.185 mg/m^3)
Q = respiratory minute volume (m^3/min). This can be in turn determined by the following: $Q = 0.499 \times BW^{0.809}$. For rats, this was 0.189×10^{-3} m^3/min; for humans this was 0.025 m^3/min.
T_R = exposure time in experiment (360 min/day)
T_H = exposure time for worker (480 min/day)
DF_R = deposition fraction of CNT in lungs of rats (unitless) (0.15)
DF_H = deposition fraction of CNT in lungs of humans during light work (unitless). This was modelled and found to be similar to rats, so it was assumed to be the same (0.15)
BW_R = body weight of experimental animal (0.3 kg)
BW_H = body weight of human (73 kg)

differences between animals and humans) was applied to derive provisional WESs of 0.03 and 0.08 mg/m^3 for SWCNTs and MWCNTs, respectively.

Nakanishi et al. (2011) proposed the use of a period-limited[66] WES (i.e. WES [PL]) of 0.03 mg/m^3 for SWCNTs (1000 m^2/g) as a WES for CNTs in general, and recommended that if this value is applied to MWCNTs with substantially lower specific surface areas, this would be considered safe.

4.9.1.2 Fullerenes

Europe*:*

The EC (2009b) carried out a risk assessment exercise for fullerenes with the caveat that it has no regulatory relevance because there are too many uncertainties in the data. Derived No Effect Levels (DNELs) were calculated for worker exposure via inhalation as follows:

- For short term exposure, a NOAEC of 2.22 mg/m^3 (only dose) for absence of lung inflammatory related effects in rats from a 10-day inhalation exposure with C_{60} fullerene nanoparticles (55 nm diameter, Baker et al. 2008) was used. This was converted to a worker NAEC of 0.55 mg/m^3 (NOEC x 3h/8h x 6.7 m^3/10 m^3). An assessment factor of 12.5 (2.5 for interspecies variation and 5 for intraspecies variability) was applied to give a $DNEL_{inhalation,\ acute}$ of 0.044 mg/m^3.

- For long term exposure, a LOAEC of 0.12 mg/m^3 for up-regulation of genes involved in the inflammatory response from a subacute inhalation study (6h/d, 5d/week for 4 weeks) (Fujita et al. 2009) was used. This was converted to a worker LAEC of 0.06 mg/m^3 (LOAEC x 6h/8h x 6.7 m^3/10 m^3). An assessment factor of 75 (3 for use of LAEC, 2.5 for interspecies variation, and 5 for intraspecies variability) was applied to give a $DNEL_{inhalation,\ chronic}$ of 0.0003 mg/m^3. It is noted the latter effect was very weak, and may not be considered adverse by most jurisdictions.

Japan:

For fullerenes (C_{60}), the same group of Japanese researchers observed no sustained inflammation or changes in inflammation-related markers in rats exposed to 0.12 mg/m^3 fullerenes for 6 hours/d, 5 days/week for 4 weeks (3 month observation period) (Shinohara et al. 2011). Because longer studies were not available, lung retention of C_{60} was calculated at 90 days using clearance rate and deposition fraction information. This was calculated to be 0.019 mg/lung at 90 days after exposure (0.013 mg/lung at 28 days). In an intratracheal instillation test (Morimoto et al. 2010a), the lung retention was much higher (0.1 – 0.7 mg/lung) and no sustained adverse effects were observed for 6

[66] According to Nakanishi (2011), they have limited the exposure period for which the period-limited WES can be applied to approximately 10-15 years or so, with the premise of reviewing the result within approximately 10 years using new test results obtained in the meantime.

months. Based on this information, Shinohara et al. (2011) concluded there are no adverse effects even if exposure to 0.12 mg/m^3 of C_{60} had continued for 90 days in the inhalation study. Because the NOAEL from inhalation exposure tests was considered too conservative, the results of the intratracheal instillation tests (which did show slight but significant increases of neutrophilic cells at higher concentrations) were used to obtain a NOAEL which would be equivalent to the results of a 90-day inhalation exposure test. Shinohara et al. (2011) converted the NOAEL of 0.7 mg/lung from intratracheal studies (Sayes et al. 2007b, Morimoto et al. 2010a) to an air concentration of 3.1 mg/m^3 using information for clearance, lung retention and physiology of the rat. A human equivalent NOAEL of 3.5 mg/m^3 was calculated.[67] An uncertainty factor of 9 (3 for methodological considerations, 3 for extrapolating results from intratracheal test) was applied to derive a provisional period-limited WES of 0.39 mg/m^3 for C_{60} fullerenes (geometric average of 96nm) (Shinohara et al. 2011). Because the alveolar deposition of fullerenes may vary with particle size, a particle-size specific WES may be derived using the modelling and equations provide in Shinohara et al. (2011).

4.10 Summary and conclusions

Since the last review for Safe Work Australia (2009a) techniques have been developed that allow reliable generation of CNT aerosols for toxicity inhalation tests. These studies support the earlier findings from intratracheal or nasopharyngeal exposure procedures. It is apparent that CNTs have potential to produce two primary types of pulmonary toxicity. One is particle-like, and characterised by sustained inflammation, fibrosis and a granulomatous reaction. The formation of granulomas is a 'protective' attempt to wall off foreign bodies. The other type of toxicity is associated with long, thin rigid fibre-like CNTs and is akin to the carcinogenic effects of asbestos. The mesothelioma risk depends on the extent that CNTs of pathogenic fibre dimensions are in workplace air. The evidence to date is that most (maybe all) CNTs in workplace air are respirable agglomerates which are of sub- or low micron size. Studies were not located which addressed the question whether such agglomerates could dissociate into single fibres within the lung milieu.

The biological responses to inhaled CNTs are complex. In accordance with the toxicological fibre paradigm, information published since the last review (Safe Work Australia 2009a) has confirmed

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[67] $NOAEL_{human\text{-}work} = NOAEL_{rat} \times \frac{t_r}{t_{work}} \times \frac{day_r}{day_{work}} \times \frac{f_r}{f_{h\_work}} \times \frac{q_r}{q_{work}} \times \frac{BW_h}{BW_r}$

Where:
$NOAEL_{rat}$ = 3.1 mg/m^3
t_r = exposure hours per day in the test, 6 hrs
t_{work} = exposure time per day at work, 8 hrs
day_r = exposure days per week in test, 5 d
day_{work} = exposure days per week at work, 5 d
f_r =deposition fraction in rat alveolar region (0.0758)
f_{h_work} =deposition fraction in human alveolar region during light work (0.0913)
q_r = respiratory volume of rats (0.27 m^3/d)
q_{work} = respiratory volume of humans during light work (36 m^3/d)
BW_h = human body weight (73 kg)
BW_r = rat body weight (0.3 kg)

phagocytosis of CNTs by macrophages generates ROS and a range of pro-inflammatory cytokines that induce inflammation and fibrosis. However it has also become apparent that CNTs can directly stimulate fibroblast proliferation and collagen production in the absence of macrophages. In addition agglomerates of CNTs within the lung provide a large surface area upon which protein and lipids can bind and therefore provide a scaffold for facilitating growth and proliferation of fibroblasts during granuloma formation. MWCNTs also have the ability to promote growth of lung tumours that are either spontaneously initiated or initiated by other agents, and transform (*in vitro* at least) lung epithelial cells to potentially proliferate into tumours.

Not all effects are located within the lung. The fact that MWCNTs can enter the pleural space adds to the concern that CNTs with pathogenic fibre dimensions may cause mesothelioma. Frustrated phagocytosis releases mediators that stimulate mesothelial cell proliferation. The emerging data suggests MWCNTs may not have to be in direct contact with the mesothelial cells in the pleural cavity in order to exert carcinogenic influence. While perhaps it is not surprising that CNTs may alter the pulmonary responses to infective agents, making the organism more susceptible, it also appears the inflammation generated in the lung may modulate the systemic adaptive immune system. However more information is needed before implications for human health can be made.

Layered on the above is the fact that it has been well demonstrated that CNTs are indirect genotoxins. They primarily cause DNA/chromosomal breaks via reactive oxygen formation. In addition CNTs can directly interact with the centrosome structure of dividing cells and induce DNA damage.

Many of the *in vitro* studies have used exposures that the authors claim, on a mass/surface area of cultered cells, is similar to the OSHA non-specific particulate workplace exposure standard of 5 mg/m^3 or with the amount of CNT lung burden that might be accumulated by a worker. Many of the *in vivo* studies also suggest the doses used for nasal-pharyngeal administration or in short term inhalation studies are within the range of occupational exposures. However these studies are acutely delivering an assumed lung burden that may be acquired by a worker over many years, they also assume (or result in) no or minimal clearance from the lungs.

A number of organisations have recently established exposure standards for CNTs. The derived standards for long term exposures range from 0.0003 – 0.034 mg/m^3. It is considered those based on sub-chronic inhalation exposure are more germane for possible adoption in Australia. These have utilised the MWCNT 90 day studies of Pauluhn (2010b) or Ma-Hock et al. (2009a) which are briefly described in Section 4.5. NIOSH (2013a) has undertaken the most in depth assessment of the literature and scholarly derivation of an exposure standard. Although the derived limit was lower, the value suggested by NIOSH is the limit of quantitation (0.001 mg/m^3) achievable by the recommended analytical method for elemental carbon (NIOSH method 5040). It is suggested this, with the attending

analytical technique, be considered for adoption in Australia as an 8 hr WES for MWCNTs, SWCNTs and CNFs.

Based on the 90-day *in vivo* inhalation studies NICNAS provisionally classified CNTs as specific target organ toxicants (Category 2) and carcinogens (Category 2) according to the *Globally Harmonized System for Classification and Labeling of Chemicals* (GHS) (Safe Work Australia 2012b).

The CNTs and CNFs evaluated in animal and *in vitro* studies represent just a small fraction of the CNT/CNF materials that may, or will be in commerce. It is also likely there will be different toxicological potencies between them. However, until validated reliable *in vitro* tests or economic short term *in vivo* hazard identification tests with potency discrimination can be developed, it is prudent to treat all CNTs and CNFs in the workplace as if they have the same potential for adverse effects. Thus a single WES should apply to all.

5. Titanium dioxide nanoparticles

As indicated in the previous review (Safe Work Australia 2009a), titanium dioxide (TiO_2) NPs are probably the ENM for which the health hazards of exposure are most understood due to their long-term use, and the wealth of standard and special toxicity/safety evaluation tests conducted with these NPs. Since the 2009 review, several national and international agencies have published literature reviews specifically concerning the health hazards of TiO_2 NPs (Hanai et al. 2009, NIOSH 2011, Ogura et al. 2011, TGA 2009, US EPA 2010a, NICNAS 2012a, b, c; TGA 2013). TiO_2 NPs are also one of the only ENMs for which health-based workplace exposure standards have been proposed (Section 5.7.1).

The most recent health hazard review (reviewing literature up to July 2012) was published by the National Industrial Chemicals Notification and Assessment Scheme, and is provided as a brief fact sheet (NICNAS 2013a), a more detailed technical information sheet (NICNAS 2013b), and an appendix with the corresponding references for the technical information (NICNAS 2013c). NICNAS (2013a, b, c) concluded:

- Data indicate TiO_2 NPs do not cause adverse effects if accidentally swallowed, however ingestion of very high amounts (5,000 mg/kg bw) may cause adverse effects (which are not nano-specific).
- Although no acute dermal toxicity studies are available, due to limited or no dermal penetration, it is expected to be low. Available studies indicate TiO_2 NPs do not cause skin irritation or allergies.
- The available data indicate low acute inhalation toxicity in mice for particles of 5 and 21 nm (LC_{50} > 7.35 mg/m^3/4 h).

- TiO_2 NPs cause mild eye irritation in rodents.
- The systemic toxicity following repeated dose exposure to TiO_2 NPs appears to be limited to organs where the particles accumulate over time.
- There are no repeated dose studies demonstrating a dose-response relationship or providing a No Observed Adverse Effect Level (NOAEL) relevant to human exposure.
- Reported effects on reproductive organs are limited and inconclusive.
- Genotoxic effects secondary to inflammatory response and/or oxidative stress have been observed in both *in vitro* and *in vivo* studies.

It is already well understood that different TiO_2 compositions may have variable toxicity potencies, depending on crystal structure, particle size, particle surface characteristics (e.g. shape) and surface coatings (Warheit 2013). This has been confirmed by many studies published between 2009-2013. Therefore, for hazard and risk assessment, it will not be possible to group all TiO_2 NPs into one category.

5.1 Distribution

Several studies have been published since the 2009 review which have investigated the intracellular distribution of TiO_2 NPs. An *in vitro* study investigating the uptake and genotoxicity of TiO_2 NPs (91 nm) by human lung fibroblasts and bronchial epithelial cells found the NPs were taken up by cells but remained in the cytosol near the nucleus, and were not found inside the nucleus, mitochondria or ribosomes (Bhattacharya et al. 2009). Another study also found TiO_2 NPs[68] to be localised within endosomes or free in the cytoplasm (Hussain et al. 2009). Pan et al. (2009b) also showed that individual rutile or anatase TiO_2 NPs can penetrate easily through the cell membrane of dermal fibroblasts in the absence of endocytosis, while some endocytosis is observed for larger particle clusters. Once inside, the particles are sequestered in vesicles, which continue to fill up until they rupture. Modification with a functionalised polymer coating prevented adherence of the NPs to the cell membrane and hence their penetration into the cells (Pan et al. 2009b).

Biodistribution of TiO_2 NPs (uncoated, anatase and rutile) after inhalation (head-nose exposure) of 100 mg/m^3 for 6 hours/day for 5 consecutive days in rats was investigated by van Ravenzwaay et al. (2009). The majority of the inhaled NPs (20-30 nm) were deposited in the lung, with some translocation to the lymph nodes, although interestingly in smaller amounts than following inhalation of pigmentary TiO_2 (rutile, 200-250 nm). Concentrations of TiO_2 in liver, kidney, spleen, and basal brain were all below the detection limit. Changes in bronchoaleveolar lavage (BAL) fluid composition and histological examination indicated mild neutrophilic inflammation and activation of macrophages

[68] The TiO_2 NPs had primary particle sizes of 15 or 50 nm but formed aggregates of 86, 356 or 243 nm (Hussain et al. 2009).

in the lung. The effects were reversible for nano- and pigmentary TiO_2, but progressive for quartz (315 nm) which was also tested. Intravenous injection of the NPs in the same experiment resulted in systemic distribution into the liver and spleen, whereas another short-term inhalation study also observed negligible translocation of TiO_2 NPs to the liver (Hougaard et al. 2010).

Other biodistribution studies published since the 2009 review prepared for Safe Work Australia (2009a) have used intravenous, intra-abdominal, or subcutaneous injections to deliver the NPs. These studies have found TiO_2 NPs distribute into and accumulate in various parts of the body, including the placenta. The relevance of the observed distribution to workers is questionable.

- After injection of nano-anatase TiO_2 (5 nm) into the abdominal cavity of mice once a day for 14 days, titanium was found to have accumulated in the liver, kidneys, spleen, lung, brain and heart (Liu et al. 2009). Low doses (5 and 10 mg/kg bw) had no effects on serum biochemical parameters, but high doses (50, 100, and 150 mg/kg bw) resulted in changes of various biochemical indicators pointing to liver and kidney toxicity, and myocardial dysfunction. The observed toxicity was linked to an inflammatory response as a result of NP accumulation in the various organs (Liu et al. 2009, 2010b; Ma et al. 2009).
- TiO_2 NPs (35 nm)[69] intravenously injected into pregnant mice [on gestational day (GD) 16 and 17] were found by TEM in the placenta, foetal liver and foetal brain at gestational day 18 (Yamashita et al. 2011). Maternal liver and kidney damage was not observed, however increased foetal resorptions and foetal growth restriction compared to the control group were observed. Maternal body weight and uterine weight decreased at GD 17 and GD 18. In contrast, transfer of coated 21 nm rutile across the placenta was very low after repeated inhalation exposure to 42 mg/m^3 for 1 hr/day on GD 8-18 (Hougaard et al. 2010). Apart from the maternal lung weight, there were no differences between treated mice and controls in fertility rates or offspring organ weights.
- Tissue distribution and histopathology effects of TiO_2 NPs (120 nm) in mice were investigated after intravenous (56 or 560 mg/kg) or subcutaneous (560 or 5600 mg/kg) injection on two consecutive days by Umbreit et al. (2011). Animals were examined 1 and 3 days, and 2, 4, 12 and 26 weeks after the final injection. Particle agglomerates were observed in the major organs: liver, lung and spleen, following intravenous injection. Particles were still observed 26 weeks after injection, indicating tissue clearance was limited. After subcutaneous injection, NP agglomerates were found mainly in the local lymph nodes, followed to a lesser extent by the liver, spleen and lung. With the exception of an increased number of macrophages in the lung and liver, there were no histopathological findings of tissue damage at any time point.

[69] Hydrodynamic diameter of TiO_2 NPs was found to be 217 nm by the authors (Yamashita et al. 2011).

Animal, human, and *in vitro* studies published since the 2009 review provide additional supporting evidence that TiO_2 NPs neither penetrate into viable cell layers nor cause toxicity after application (exposed for 4 hours up to 20 weeks) to intact or partially damaged skin, including from sunburn (Adachi et al. 2010, Nohynek and Dufour 2012, Inman et al. 2010, Furukawa et al. 2011, Sadrieh et al. 2010, Tyner et al. 2011, Schilling et al. 2010).

NICNAS (2013a, b, c) came to a similar conclusion.

5.2 Mode of action

The suggested mode of action of other metal oxide NPs discussed in this report is a 'Trojan horse' mechanism, where the NP size and shape facilitate entry into a cell, the metal oxides are subsequently partially dissolved to their corresponding metal ions which are ultimately responsible for the toxicity observed. This does not seem to occur with TiO_2. Although the number of studies investigating the specific cellular mechanistic aspects of TiO_2 is surprisingly few, the available information suggests the NPs are not taken up by organelles within the cell (Hussain et al. 2009, Bhattacharya et al. 2009, Pan et al. 2009b) and do not break down within the cell (Horie et al. 2010). Rather, after being taken up, the NPs themselves seem to cause a non-specific increase in intracellular reactive oxygen species (by various mechanisms)[70] resulting in cellular apoptosis and/or initiation of an inflammatory response (Horie et al. 2010, Nurkiewicz et al. 2009, Yazdi et al. 2010, Winter et al. 2011). Future mechanistic studies specifically investigating the 'Trojan horse' phenomenon in relation to TiO_2 NPs may shed more light on this theory.

Three studies suggest the interaction of TiO_2 NPs with cellular proteins may lead to activation of inflammasomes, subsequently resulting in release of inflammatory interleukins (Yazdi et al. 2010, Winter et al. 2011, Hamilton et al. 2009). The concept and significance of protein interactions with NPs in biological media is further discussed in Section 3.2.

5.3 In vitro toxicity

Numerous *in vitro* cytotoxicity experiments using various different cell types (e.g. immune cells, alveolar and bronchial epithelial cells, liver cells, kidney cells, intestinal cells, vascular endothelial cells, keratinocytes, lymphocytes, cardiomyocytes, glioma cells, retinal pigment epithelial cells) or experiments investigating the mechanistic detail of various aspects of TiO_2 NP toxicity have been

[70] This may be coupled with altered calcium homeostasis which is not necessarily associated with cell death (Simon et al. 2011, Chen et al. 2012c).

published since the 2009 review[71]. While noting these studies are important for unravelling the molecular mechanisms of ENM induced toxicity and providing insight into the relative toxic potencies of different TiO_2 NP types, they are still of limited value for identifying and managing risks in the workplace. Such investigations are therefore not described in detail in this review.

Interesting findings/conclusions from some of these *in vitro* experiments include:

- TiO_2 NPs (<75 nm, anatase/rutile) can directly stimulate mucin secretion from human bronchial epithelial cells via a Ca^{2+} signalling mediated pathway (Chen et al. 2011).
- TiO_2 NPs (6-100 nm, anatase/rutile) increased both histamine secretion and cytosolic Ca^{2+} concentration (via membrane disruption) in a dose-dependent manner in rat mast cells (Chen et al. 2012c).
- TiO_2 NPs (26 nm, anatase) both directly and indirectly induced insulin resistance in liver-derived rat cells (Gurevitch et al. 2012).
- Not surprisingly, alteration of anatase TiO_2 NMs into long fibre-like structures ('nanobelts', >15 µm) creates particles which are highly toxic *in vitro*, causing frustrated phagocytosis in alveolar macrophages, initiating an inflammatory response and activating inflammasomes and the release of inflammatory cytokines reminiscent of CNT (Hamilton et al. 2009).
- The cytotoxicity of different TiO_2 types to human lung epithelial cells was in the following order: amorphous (most toxic) > anatase > anatase/rutile (Hsiao and Huang 2011).
- The phototoxicity of ultraviolet A irradiation was enhanced by TiO_2 NPs (anatase/rutile, 20 nm), to a greater extent than by bulk TiO_2 powder (rutile, 1 µm) (Kang et al. 2011b).
- For one out of three cell types studied[72], the cellular binding and uptake of TiO_2 NPs was greater for smaller agglomerates (~130 nm) than larger ones (~1,000 nm) (Lankoff et al. 2012). No significant differences were observed for the other two cell types. Within the cells, TiO_2 NPs formed large aggregates/clusters, which were localised in vacuoles. However, no cell death was observed for either group of NPs, probably due to the low toxicity of TiO_2 NPs. For Ag NPs tested in the same experiment, the authors observed differences in the resultant cytotoxicity, and concluded the least agglomerated particles are more potent (Lankoff et al. 2012).

[71] e.g. Andersson-Willman et al. 2012, Barillet et al. 2010, Bhattacharya et al. 2009, Catalan et al. 2012; Chen et al. 2011, 2012c; Cho et al. 2012b, Ekstrand-Hammarstroem et al. 2012, Falck et al. 2009, Gerloff et al. 2009, Gurevitch et al. 2012, Hackenberg et al. 2011a, Hamilton et al. 2009, Han et al. 2013, Horie et al. 2010, Hsiao and Huang 2011; Hussain et al. 2009, 2010; Jaeger et al. 2012, Jawad et al. 2011, Jin et al. 2011, Jomini et al. 2012, Jugan et al. 2012, Kan et al. 2012, Kang et al. 2011b, Kitchin et al. 2011, Kocbek et al. 2010, Kroll et al. 2011, Landsiedel et al. 2010b, Lankoff et al. 2012, Lanone et al. 2009, Lindberg et al. 2012, Magdolenova et al. 2012b, Marquez-Ramirez et al. 2012, Muller et al. 2010, Otero-Gonzalez et al. 2012, Petkovic et al. 2011, Pujalte et al. 2011, Rotoli et al. 2012, Rushton et al. 2010, Sanders et al. 2012, Schanen et al. 2009, Scherbart et al. 2011, Shi et al. 2010a, Shukla et al. 2013, Simon et al. 2011, Srivastava et al. 2011b, Zhang et al. 2011c.

[72] Hepatocytes (HepG2), but not alveolar epithelial cells (A549) or leukaemic monocytes (THP-1).

- In one study, an anatase/rutile TiO_2 NP dispersion with large agglomerates (~800 nm) induced DNA damage in a modified comet assay in three cell lines, whereas the TiO_2 NP dispersion with smaller agglomerates (~100 nm) was not genotoxic (Magdolenova et al. 2012b).
- In cytotoxicity experiments comparing the potency of various metal oxides or other ENMs, TiO_2 NPs are typically one of the least potent (e.g. Rushton et al. 2010, Rotoli et al. 2012, Otero-Gonzalez et al. 2012, Pujalte et al. 2011).

One interesting and potentially important *ex vivo* test system was employed by Stampfl et al. (2011) to investigate the cardiovascular effects of TiO_2 NPs. The investigators established an isolated beating heart (Langendorff heart) as a model system, which enables observation and analysis of electrophysiological parameters over a minimal time period of 4 hours without influence by systemic effects. It also allows the determination of stimulated release of substances as a result of ENMs. The authors found a significant dose and material dependent increase in heart rate, accompanied by arrhythmia, evoked by anatase TiO_2 (20 nm)[73], SiO_2, flame soot (Printex 90), and spark discharge soot, but not with flame derived SiO_2 (Aerosil) or monodisperse polystyrene lattices. The differences observed in response may be due to different surface properties (e.g. hydrophobicity) or shape.

5.4 In vivo toxicity

5.4.1 Acute inhalation or instillation

Because inhalation exposures to particulate matter have been associated with cardiovascular disease, Kan et al. (2012) investigated the cardiac effects of inhaled TiO_2 NPs[74] in rats. Rats were exposed to aerosols of TiO_2 NPs at 6 mg/m^3 for 4 hours and were examined at 0 and 24 hours post-exposure. The authors state that previous studies had shown this exposure scheme produces a pulmonary deposition of 10 µg TiO_2 NPs in the rat, which, according to the authors, is equivalent to a worker who is exposed at 0.1 mg/m^3 for 27 workdays in a typical occupational environment. Exposure increased the phosphorylation levels of p38 mitogen-activated protein kinase (MAPK) and cardiac troponin I, but not Akt in the heart[75], and substance P synthesis in nodose ganglia[76]. Circulatory levels of pro-

[73] The ENMs were suspended in 5 mL of Krebs-Henseleit buffer (KHB) containing various essential salts and minerals, glucose and bovine serum albumin. The particle suspension was sonicated twice for 1 minute to deagglomerate the particles, and sequentially filtered through a 100 nm pore size. Size of agglomerates or tendency to agglomerate were not reported in the paper (Stampfl et al. 2011).

[74] The primary particle diameter of the TiO_2 NPs is given as ~21 nm, but characterisation of agglomerate and aggregate formation in the aerosols was not provided (Kan et al. 2012).

[75] MAPK and cardiac troponin I are proteins integral to the regulation of cardiac function, and are often used as indicators of damage to the heart muscle. Akt is also known as protein kinase B and plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration.

[76] Substance P is a neuropeptide and has an important role in pain perception. The nodose ganglia are involved in the integration and control of lung and heart function by receiving primary sensory fibres from the lung and transmitting this information to the brainstem to regulate autonomic efferent neuron activity (Kan et al. 2012).

inflammatory cytokines were not significantly changed after exposure, suggesting the cardiac responses are independent of systemic inflammation (Kan et al. 2012).

LeBlanc et al. (2009) also investigated cardiovascular effects after exposure of rats to 6 mg/m^3 powder aerosolised TiO_2 NPs (anatase/rutile, 21 nm) for 4 hours. NP exposure did not cause pulmonary inflammation but significantly impaired endothelium-dependent vasodilation in subepicardial arterioles. Such disturbances in coronary microvascular function are consistent with cardiac events associated with particle pollution exposure (LeBlanc et al. 2009).

Leppanen et al. (2011) investigated respiratory tract effects caused by nanosized TiO_2 (anatase/brookite, primary particle size 20 nm)[77] in acute (single 0.5 h exposure)[78] and repeat (total of 16 hours, 1 h/d, 4 d/week for 4 weeks)[79] inhalation exposures in mice. TiO_2 caused airflow limitation in the conducting airways at each studied exposure concentration (8, 20 and 30 mg/m^3) in acute exposures, shown as a reduction in expiratory flow (Vd) (being at the lowest 73% of baseline). However, the response was not dose dependent. During the recovery period, Vd returned to normal. Repeat exposures (30 mg/m^3) caused an airflow limitation effect similar in intensity to the single exposures, and the extent of the responses stayed about the same along the exposure days. All mice recovered at the end of the exposure period. Sensory and pulmonary irritation was also observed in acute and repeated exposures, but because these effects were also seen in the control groups, they may have been due to reaction by-products in the filtered air rather than the TiO_2 NPs. TiO_2 accumulated primarily in pulmonary macrophages, and did not cause nasal or pulmonary inflammation. It is also noted the inhalation concentrations were very high. The authors concluded the irritation and inflammation potencies of the studied TiO_2 NPs were low (Leppanen et al. 2011).

The influence of the size of agglomerates on pulmonary toxicity was investigated by Noel et al. (2012b). They produced aerosolised TiO_2 NPs (anatase/rutile, primary particle size 5 nm) showing two different agglomeration states: 1) small agglomerates (<100 nm) and 2) large agglomerates (>100 nm). The methods used to generate the agglomerates are described in Noel et al. (2012a). Rats were nose-only exposed to mass concentrations of 2 or 7 mg/m^3 of each test material for 6 hours. The median aerodynamic diameters were 30 and 185 nm at 2 mg/m^3, and 31 and 194 nm at 7 mg/m^3 for

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[77] TiO_2 formed agglomerates in gas-phase aerosol with geometric mean diameters of 91, 113, and 130 nm at mass concentrations of 8, 20 and 30 mg/m^3, respectively (Leppanen et al. 2011).

[78] This consisted of a 15 minute control period when only filtered air was led into the exposure chamber, followed by a 30 minute exposure to the TiO_2, and finally a 15 minute recovery period (filtered air). Control experiments were performed with filtered air to inspect the effect of reaction by-products, mainly CO, NO_2, and propene (C_3H_6).

[79] Only two groups of mice were used for repeat exposure experiments. One group was exposed to the highest concentration used in the single exposure experiment (30 mg/m^3), the other group was a control group breathing filtered air (Leppanen et al. 2011).

the small and large agglomerates, respectively. Exposure to the 2 mg/m^3 mass concentrations had no effect on pulmonary parameters. The authors found a statistically significant 2.1- fold increase in the number of neutrophils in BALF for the group exposed to the 7 mg/m^3 large agglomerate nano-aerosol suggesting a mild inflammatory response. Rats exposed to the 7 mg/m^3 small agglomerate nano-aerosol showed a 1.8- fold increase in LDH activity and 8-isoprostane concentration in BALF, providing evidence for cytotoxicity and oxidative stress. Thus the mild pulmonary response observed for the two agglomerates seems to have been induced by different mechanisms. The authors concluded the results indicate that biological responses to NPs may depend on the dimension and concentration of NP agglomerates (Noel et al. 2012b).

Unsurprisingly, TiO_2 NP shape was found to alter pulmonary response of mice exposed to 0-30 µg/mouse via pharyngeal aspiration of nanospheres, short nanobelts or long nanobelts and monitored for 112 days post-exposure (Porter et al. 2013). Histopathological analyses at 112 days post exposure determined no interstitial fibrosis in any nanosphere-exposed mice, an increased incidence in 30 µg short nanobelt-exposed mice, and significant interstitial fibrosis in 30 µg long nanobelt-exposed mice (Porter et al. 2013). It is perhaps not surprising that long nanobelts with higher aspect ratios, reminiscent of the shapes of carbon nanotubes, are more toxic than the spherical shape of the same NM.

In an intratracheal instillation study, in contrast to other metal oxide NMs (e.g. CeO_2 NPs, NiO NPs, ZnO NPs, and CuO NPs), TiO_2 NPs (primary particle size 30-40 nm, forming aggregates of ~120 nm) were not inflammogenic to the lungs as evaluated 24 hours and 4 weeks after instillation in rats (Cho et al. 2010). Similar results were found for nano and fine TiO_2 in other instillation studies (Horie et al. 2012c, Husain et al. 2013). Repeated exposure to nano TiO_2 (primarily rutile, UV-Titan L181, 21 nm)[80] was associated with modest plaque progression in mice, but there were no associations between TiO_2 exposure and inflammation or vasodilatory function (Mikkelsen et al. 2011). In another intratracheal instillation experiment, a high dose (1 mg/rat), but not the low dose (100 µg/rat) of non-dispersed TiO_2 nanorods (rutile, 10 x 40 nm)[81] was found to generate transient and reversible pneumotoxic effects, and to not markedly alter pulmonary immune function (Roberts et al. 2011).

The pulmonary and hepatic toxicity resulting from a single intratracheal instillation in mice of sanding dust from nano-TiO_2 paint was compared to the dust from paint without nano TiO_2 (Saber et al. 2012b). Effects were evaluated 1, 3, and 28 days post-exposure. Pulmonary inflammation and DNA

[80] The NPs used in this study were UV-titan L181 enriched with rutile and modified with amounts of zirconium, silicon, aluminium, and coated with polyalcohol. NPs formed agglomerates of ~520 nm in the intratracheal instillation exposure dispersion (Mikkelsen et al. 2011).

[81] Nanorods formed agglomerates of 500 – 2,800 nm in size in phosphate-buffered saline used as the exposure vehicle (Roberts et al. 2011).

damage and hepatic histopathology were unchanged after exposure to the TiO_2 NPs (rutile, UV-Titan L181, coated with Si, Al, Zr and polyalcohol, 21 nm forming $\geq$ 100 nm aggregates in suspension). However, pure nano TiO_2 by itself caused greater inflammation than when it was embedded in the paint matrix, suggesting its toxicity was reduced (Saber et al. 2012b).

5.4.2 Acute oral

In the previous review, it was concluded very high acute oral doses of nano-TiO_2 given to mice suggest the potential for liver and kidney damage, however at the doses used the same effects are observed with fine particulate TiO_2 so it is difficult to ascribe this as a nano-size effect. This conclusion is further supported by a study published since the 2009 review, which also found liver function impairments and nephrotoxicity in mice treated via oral gavage with very high concentrations of TiO_2 NPs (5,000 mg/kg bw) of 50 or 120 nm in size (Zhang et al. 2010). It is noted that in this study tissue Ti content was measured and presence of nano-TiO_2 was not confirmed by TEM.

5.4.3 Repeat inhalation or instillation

Boisen et al. (2012) exposed pregnant mice by whole-body inhalation to aerosolised powder nano-TiO_2 UV-Titan L181 (rutile, 21 nm)[82] (~42.4 mg/m^3) one hour per day on gestation days 8-18. Note this exposure concentration is very high and the regimen allows oral exposure via grooming. Female F1 offspring were raised to maturity and mated with unexposed male mice. The F2 generation was examined for expanded simple tandem repeat (ESTR) germline mutation rates. ESTR mutation rates in UV-Titan exposed F2 offspring were not statistically different from those of F2 controls, suggesting the material is not genotoxic. In a paper describing the same study, UV-Titan exposure did not induce DNA strand breaks in time-mated mice or their offspring (Jackson et al. 2013). Other effects evaluated in the study included maternal lung inflammation, gestational and litter parameters, offspring neurofunction (using a neurobehavioural test battery) and fertility. Inhalation of nano-sized UV Titan dust induced long term inflammation in time-mated adult female mice, and gestationally exposed offspring showed moderate neurobehavioural alterations (e.g. avoidance of central zone of an open field and enhanced prepulse inhibition). This study is of limited usefulness for risk assessment, since exposure occurred at a single high concentration, and there is therefore no indication of a dose response for the neurobehavioural changes.

Neurological effects (i.e. morphological changes of neurons in the cerebral cortex and a disturbance of neurotransmitter levels) were also found in another study (Zhang et al. 2011c). In this study mice were intranasally instilled with four different types of TiO_2 particles (i.e. two hydrophobic particles in micro- and nano-size without coating and two types of water-soluble hydrophilic nano-sized particles

[82] Aggregates and agglomerates formed upon generation of the aerosol for inhalation. They were equidimensional to needle-shaped with diameters from <10 nm to >100 nm (50% <97 nm) (Jackson et al. 2013). The TiO_2 NPs were modified with Al, Si and Zr, and coated with polyalcohols.

with silica surface coating) at 500 µg/mouse every other day for 30 days. This is a high exposure regimen. Although the effects observed occurred with all four particle types, the changes were more obvious with hydrophilic NPs. The authors concluded size, shape and surface modification of NMs are important for evaluating neurological effects (Zhang et al. 2011c). However, the intranasal instillation route does not provide information which can be related readily to exposure in the workplace.

Ma-Hock et al. (2009b) exposed male rats to dust aerosols of 0, 2, 10 or 50 mg/m^3 TiO_2 NPs[83] by inhalation (head-nose exposure) for 6 h/day for 5 days. Necropsies were performed either immediately after the last exposure or 3 and 16 days post exposure. Treatment with TiO_2 NPs resulted in morphological changes in the lung with the highest concentration producing an increase in lung weight. Lung inflammation was associated with dose-dependent increases in BALF total cell and neutrophil counts, total protein content, enzyme activities and levels of a number of cell mediators. No indications of systemic effects could be found. Overall, the authors concluded the pulmonary effects observed in this short-term study are comparable to those previously reported in subchronic inhalation studies, and that such short-term inhalation studies could be used to provide early evidence of respiratory tract effects which may occur from long-term exposures (Ma-Hock et al. 2009b). A NOAEC could not be established.

Morimoto et al. (2011a) exposed rats to TiO_2 NPs (rutile, primary particle size of 35 nm, 51 $\pm$ 9 nm in aerosol) via inhalation for 4 weeks (6 hours/day). The average particle number concentration in the chamber was 2.8 x 10^5 particles/cm^3. Four days, 1 and 3 months after the end of exposure, the rats were sacrificed and expressions of genes related to inflammation and fibrosis were measured. No inflammation was observed in the lungs of rats treated with TiO_2 NPs, and no changes were found in the expression of the genes investigated. Conversely, in a study with mice exposed via inhalation for 1 h/day for 11 consecutive days to 42.4 $\pm$ 2.9 mg/m^3 surface-coated TiO_2 NPs (rutile, 20 nm)[84], changes in the expression of genes associated with acute phase inflammation and immune response were observed 5 days post exposure with concomitant changes in several microRNAs (Halappanavar et al. 2011).

Oosthuizen et al. (2012) exposed mice via inhalation (whole body) to two different concentrations (1 or 10 mg/m^3) of spherical (25 nm) or rod-shaped (bundle diameter 202 nm, length 1.1 µm) TiO_2 NPs (anatase/rutile) for 1 h/day, 4 d/week for 6 weeks. The presence of lung inflammation post-exposure was evaluated using light and transmission electron microscopy. No inter-particle comparison was

[83] Uncoated anatase/rutile TiO_2 with a primary particle size of 25 nm.

[84] The NPs used in this study were UV-titan L181 enriched with rutile and modified with amounts of zirconium, silicon, aluminium, and coated with polyalcohol. Powder aerosols consisted of aggregates and agglomerates of equidimensional to needle-shaped TiO_2 crystals. The major particle sizes in the aerosol were ~100 nm and 4 µm. About 80% of the particles by number were between 40 and 200 nm.

done or retention investigated. Histological and ultrastructural changes, typical of an inflammatory response, were noted in the lungs of exposed animals.

Another set of authors investigated the modulatory effects of TiO_2 particle exposure in an experimental allergic asthma model (Rossi et al. 2010b). Non-allergic and ovalbumin (OVA)-sensitised mice were exposed via inhalation to either TiO_2 NPs (rutile, silica-coated, 40 nm)[85] or fine TiO_2 (rutile, <5 µm) for 2 h/day, 3 d/week for four weeks at a concentration of 10 mg/m^3. Elicitation of pulmonary neutrophilia accompanied by significantly increased chemokine (CXCL5) expression was observed in healthy mice exposed to TiO_2 NPs, but not fine TiO_2. Surprisingly, allergic pulmonary inflammation was suppressed in the asthmatic model mice that were exposed to nano or fine TiO_2, i.e. the levels of leukocytes, cytokines, chemokines and antibodies characteristic to allergic asthma were substantially decreased. Exposure of OVA sensitised and metacholine challenged mice to TiO_2 NPs reduced airway hyperreactivity to the level of healthy mice. On the contrary, exposure to fine TiO_2 slightly increased the reactivity of the lungs, showing modest exacerbation of asthmatic symptoms. The authors concluded that particle exposure can modulate airway inflammation and airway hyperreactivity in very different ways depending on the immune status of the animals, and that the anti-inflammatory Th2 response caused by allergen sensitisation may be suppressed by the competing proinflammatory response elicited by TiO_2 NP exposure (Rossi et al. 2010b).
The potential for increased vulnerability of young animals to the development of asthma as a result of inhalation exposure to TiO_2 NPs was investigated by Scuri et al. (2010). The authors exposed newborn (2-days old), weanling (2-week old), and adult (12 weeks old) rats to 12 mg/m^3 of aggregated TiO_2 NPs (anatase/rutile, 21 nm)[86] for 5.6 hours/day for 3 days. The exposures produced an upregulation of lung neurotrophins[87] in weanling and newborn, but not in adult rats compared to controls. This effect was associated with increased airway responsiveness and upregulation of growth-related oncogene/keratine-derived chemokine in BALF. The latter is the rat equivalent of human IL-8, which is an indicator of inflammation. The authors concluded the results support the presence of a critical window of vulnerability in earlier stages of lung development compared to later in life (Scuri et al 2010).

[85] Aerosolised NPs occurred mostly as aggregates of 100 nm, whereas fine TiO_2 occurred as agglomerates of 1 µm.

[86] The TiO_2 aerosols had a mass median aerodynamic diameter of 1.6 µm, while the count mean aerodynamic diameter was 138 nm (Scuri et al. 2010).

[87] Lung neurotrophins are key regulatory elements of neuronal development and responsiveness that play an important role in the pathophysiology of childhood asthma (Scuri et al. 2010).

5.4.4 Repeat oral

Repeat doses of 0, 160, 400 and 1,000 mg/kg bw of TiO_2 NPs (anatase/rutile, <50 nm) were administered to rats via gavage once a day for 14 consecutive days (Bu et al. 2010). This resulted in changes to the energy and amino acid metabolism and gut microflora environment, which the authors postulated may be due to slight injury to the liver and heart (Bu et al. 2010).

In a long-term oral toxicity study, mice were administered 0 or 10 mg/kg bw TiO_2 NP (anatase, 5-6 nm)[88] suspensions (in 0.5% w/v hydroxypropylmethylcellulose K4M solvent) intragrastically every day for 90 days. The exposure resulted in titanium accumulation in mouse liver tissue (TiO_2 NP aggregation in hepatocyte cytoplasm was demonstrated by TEM), liver inflammation and hepatocyte apoptosis, coupled with increases in levels of biomarkers of liver damage (Cui et al. 2012).

5.5 Genotoxicity

Studies published since the 2009 review investigating the genotoxicity of TiO_2 NPs *in vitro* support an indirect genotoxic mode of action secondary to initiation of the inflammatory response, rather than a direct genotoxic effect (Bhattacharya et al. 2009, Catalan et al. 2012, Jomini et al. 2012, Sycheva et al. 2011, Trouiller et al. 2009).

However, in agreement with the NICNAS (2013a, b, c) review, there is inconsistency in the results from genotoxicity tests with TiO_2 NPs.

Although many *in vitro* studies have suggested nanosized TiO_2 is genotoxic (Falck et al. 2009, Barillet et al. 2010, Osman et al. 2010, Shukla et al. 2013, Roller 2011, Jaeger et al. 2012, Jugan et al. 2012, Kitchin et al. 2011), other *in vitro* studies do not (Hackenberg et al. 2011a, Kang et al. 2011b, Landsiedel et al. 2010b, Shi et al. 2010b, Saber et al. 2012b). Additionally, a recent *in vivo* 5-day inhalation study in mice found no significant effect on the level of DNA damage in lung epithelial cells or micronuclei in blood polychromatic erythrocytes, suggesting no genotoxic effects (Lindberg et al. 2012). Landsiedel et al. (2010b) used a standard battery of tests to assess the genotoxicity of TiO_2 but included improvements/adaptations; they observed no genotoxicity *in vitro* (Ames and micronucleus tests) or *in vivo* (comet and micronucleus test)[89]. Naya et al. (2012b) conducted an *in*

[88] The mean hydrodynamic diameter of TiO_2 NPs in the solvent ranged from 208 to 330 nm (Cui et al. 2012).

[89] In Landseidal et al. (2010b) the micronucleus test was in mouse bone marrow 24 hr after intraperitoneal administration of 15, 30 or 60 mg/kg of Z-COTE HP 1 (a nano TiO_2 preparation, primary particle size 30 – 300 nm, with triethoxycaprylylsilane coating used in cosmetics). The Comet assay was performed on isolated rat lung cells 23d after head-nose inhalation exposure (6 hr/d for 5d) to 10 mg/m^3 of T-LiteTM SF (titanium dioxide (and) aluminium hydroxide (and) dimethicone/methicone copolymer, mean aggregate size ~200nm, used in cosmetics).

vivo comet assay after single or repeat intratracheal instillation exposure of anatase TiO_2 NPs in rats, and found them to be non-genotoxic[90].

Jomini et al. (2012) commented that when the Ames test is used conventionally, it is not suitable to assess NP genotoxicity. This is because the medium used during exposure prevents electrostatic interactions between bacterial cells and nanoparticles, leading to false negative responses. Using an improved assay, involving preincuabtion of the bacteria in a low ionic strength solution, the authors found three TiO_2 NPs[91] returned positive results. They suggested this was due to a secondary genotoxicity mechanism (oxidative stress).

Two *in vivo* studies which evaluated genotoxicity of TiO_2 NPs by the comet assay after repeat administration to mice via oral gavage or drinking water observed genotoxicity, but also stated this was probably due to the secondary oxidative stress mechanism (Sycheva et al. 2011, Trouiller et al. 2009). Sycheva et al. (2011) investigated genotoxicity (Comet assay and histological nuclear abnormalities) in a wide range of tissues from mice treated by gavage with 40, 200 and 1000 mg/kg/d for seven days of nano-TiO_2 (33nm) or 'microsized' TiO_2 (140nm). While a number of positive results were found with the high dose, there was no material difference between the nano and micro forms of TiO_2. The Trouiller et al. (2009) study was with nano-TiO_2 in drinking water[92].

It seems genotoxicity, as with any other ENM toxicity being investigated, is dependent on a number of particle-specific factors. For example, it is possible that NPs with very active surface chemistry, which produce more reactive oxygen species (ROS) or a large inflammatory response, could induce genotoxicity (Boisen et al. 2012). The induction of DNA damage by TiO_2 NPs in one *in vitro* study depended on whether the Comet assay was processed in the dark or under standard laboratory lights (Gerloff et al. 2009). In a study designed to investigate whether dispersion procedures influence NP cytotoxicity and genotoxicity, Magdolenova et al. (2012b) found that a TiO_2 NP dispersion with large agglomerates and no foetal calf serum induced DNA damage in all cell lines studied (lymphoblasts, embryonic epithelial cells, kidney fibroblasts). On the other hand the TiO_2 NPs dispersed with agglomerates <200 nm in size and with foetal serum were not genotoxic. In another *in vitro* study, TiO_2 (anatase, <25 nm), but not rutile (<100 nm) caused a persistent increase in DNA strand breaks

[90] Naya et al (2012b) instilled 1.0 or 5.0 mg/kg body weight (single instillation group) and 0.2 or 1.0 mg/kg body weight once a week for 5 weeks (repeated instillation group) into rats. The comet assay was run on suspended lung cells prepared after homogenisation of the lung 3 or 24 hours after the last exposure.

[91] The NPs were 1) anatase/rutile, 25 nm, which agglomerated to sizes of 60-80 nm, 2) anatase/brookite 5.7 nm, remained well dispersed at 5-10 nm in size, or 3) rutile, 5-10 nm cross-section per 50-200 nm length, formed large clusters with average sizes of 200 nm (Jomini et al. 2012).

[92] Male mice were given nano-TiO_2 (primary particle size 21 nm, aggregate size in water 21 – 1,446 nm with ~70% at 160 nm) in drinking water for 5 days. Based on average water consumption and average mouse weight cumulative doses were calculated as 50, 10, 250 and 500 mg/kg.

(comet assay) and oxidised purines in cultured human hepatoma HepG2 cells (Petkovic et al. 2011). Thus showing the genotoxic potential of TiO_2 varied with crystalline structure.

5.6 Carcinogenicity

As discussed in the previous review (Safe Work Australia 2009a), the mode of action of lung cancer induced by poorly soluble particles with no specific toxicity is believed to be particle deposition in respiratory epithelium, decreased lung clearance (to the degree of overload), persistent inflammation, cellular injury and persistent cell proliferation, fibrosis, and secondary genotoxicity (mutation) in the lung cells. TiO_2 is traditionally considered chemically inert and falls into the category of poorly soluble particles with no specific toxicity. When dose-response is expressed as surface area (dose) to tumour proportion (response), TiO_2, nano-TiO_2, and other poorly soluble particles with no specific toxicity appear to share the same dose-response curve (US EPA 2010a). However when expressed on a mass basis, the concentration of TiO_2 NPs needed to elicit a response is obviously lower, due at least in part to pulmonary overload occurring at lower mass doses (see also Section 3.3). This is reflected by the different workplace exposure standards (WESs) proposed by NIOSH (2011) for nano- and fine TiO_2 (Appendix C). The NIOSH (2011) WESs are based on the induction of lung tumours in rats where particle overload was achieved. Similarly, based on lung tumours in rats, the International Agency for Research on Cancer (IARC 2010) has classified titanium dioxide as *possibly carcinogenic to humans* (Group 2B). Warheit (2013) has commented on the inappropriateness of using rat lung tumour data for human risk assessment due to the unique sensitivity of the rat for developing particle-overload-related lung tumours. It was noted the published epidemiological studies on workers with the greatest exposures to TiO_2 particles are negative for lung cancer risks as well as other non-malignant respiratory diseases (Ellis et al. 2010, 2013), albeit these did not look specifically at nano-sized TiO_2.

No additional inhalational carcinogenicity studies with TiO_2 were found since the 2009 review.

Since the 2009 review, an *in vivo* study has been published investigating the carcinogenicity of topically applied TiO_2 NPs in the mouse two stage cancer bioassay (Furukawa et al. 2011). Coated or uncoated TiO_2 NPs (spindle shape, long axis of 50-100 nm, short axis of 10-20 nm) were applied at 5, 10 and 20 mg/animal doses to mouse skin in the post-initiation phase[93] (up to 20 weeks). No changes in survival rate, general condition, body weight, or development of skin nodules was observed in the study. Positive controls gave the expected response. The authors concluded nano-TiO_2 does not possess tumour promotion potential in the mouse skin carcinogenesis assay.

[93] 7,12-dimethylbenz[a]anthracene (DMBA) and 12-o-tetradecanoylphorbol 13-acetate (TPA) were used as the initiator and positive control promoter, respectively (Furukawa et al. 2011).

5.7 Workplace implications

5.7.1 Workplace exposure limits

A number of agencies have derived provisional health-based workplace exposure standards (WESs) for TiO_2 NPs. The WESs are shown in Table 5.1; their derivation is briefly described in the table and in subsequent sections.

Table 5.1: WESs for TiO_2 NPs

WES (mg/m^3)	Basis	Reference
0.3 mg/m^3 ultrafine (<100 nm) 40 hours/week, 45 working years	Applied BMD modelling (using various models) to lung cancer risk in rat studies, converted BMDs and BMDLs to human equivalent doses using known species differences in lung surface area, and subsequently estimated working lifetime airborne mass concentrations using human lung dosimetry models. The WES represents the combined model averages (rounded) of BMDL estimated airborne mass concentrations of ultrafine TiO_2 associated with a 1 in 1,000 excess risk of lung cancer in humans after a 45-year working lifetime. No uncertainty factors were applied. A WES for fine TiO_2 of 2.4 mg/m^3 was also derived in the same document.	NIOSH 2011 (USA)
0.6 mg/m^3 Period-limited (15 years)	It was considered prevention of lung inflammation would also prevent cancer. NOAEL (lung inflammation) for TiO_2 was determined to be 2 mg/m^3 from a 4-week inhalation rat study with 3 month observation. This was extrapolated to a human equivalent air concentration of 1.82 mg/m^3. An uncertainty factor of 3 (for selection of dose metric) was applied to derive a WES of 0.61 mg/m^3. This was denoted a 'period-limited' WES with an exposure time of 15 years (to be revised when more knowledge is available).	Ogura et al. 2011 and Nakanishi 2011 (Japan)
(DNEL) 0.017 mg/m^3 8-hour TWA	A NOAEC of 0.5 mg/m^3 from a 13-week inhalation study in rats (6 hrs/d, 5d/week, 21nm particles) was adjusted to 0.25 mg/m^3 for worker exposure (0.5 mg/m^3 x 6h/8h x $6.7m^3/10m^3$). An uncertainty factor of 15 (see text) was applied to obtain a DNEL of 0.017 mg/m^3.	EC 2009b (Europe)

WES = workplace exposure standard; BMD = benchmark dose; BMDL = 95% lower confidence limit on benchmark dose; TWA = time-weighted average; DNEL = derived no-effect level; UF = uncertainty factor.

United States:

The US National Institute for Occupational Safety and Health (NIOSH 2011) recommended exposure limits of 2.4 mg/m^3 and 0.3 mg/m^3 for fine (<10 µm) and ultrafine (<100 nm) TiO_2, respectively. The recommended exposure level (REL) for ultrafine TiO_2 includes exposure to engineered nano-TiO_2. NIOSH (2011) used dose-response data from chronic inhalation studies for non-nano TiO_2 in rats exposed to TiO_2 to estimate working lifetime exposure and lung cancer risk in humans. The principal

supporting studies were Lee et al. (1985) and Muhle et al. (1991) for fine (pigment-grade) rutile TiO_2 and Heinrich et al. (1995) for ultrafine[94] TiO_2. The doses were 5 mg/m^3 (Muhle et al. 1991); 10, 50 and 250 mg/m^3 (Lee et al. 1985); and 10 mg/m^3 (Heinrich et al. 1995). Statistically significant increases in lung tumours were observed at the highest dose of fine TiO_2 (250 mg/m^3) or ultrafine TiO_2 (10 mg/m^3). NIOSH (2011) conducted benchmark dose (BMD) modelling with the rat tumour data. Risk estimates were based on the combined male and female rat lung tumours, excluding the squamous cell keratinising cystic tumours[95].The estimated particle surface area doses (BMD and BMDL) associated with a 1 in 1000 excess risk of lung tumour varied considerably depending on the shape of the mathematical data fitting model used in the low-dose region. The model-based estimates generated by NIOSH (2011) were summarised using an averaging technique, which weighted the various models based on model fit. Estimated BMDs and BMDLs of TiO_2 surface area doses in rat lungs associated with a 1 in 1,000 excess risk of lung cancer ranged from 0.0075-0.286 and 0.0058-0.082 m^2/g-lung, respectively. These critical doses were extrapolated to humans by adjusting for species differences in lung surface area[96]. The calculated human equivalent doses were then converted to estimated working lifetime airborne mass concentrations using human lung dosimetry models[97]. The combined model averages (rounded) of the 95% lower confidence limit estimated airborne mass concentrations of fine and ultrafine TiO_2 associated with a 1 in 1,000 excess risk of lung cancer in humans after a 45-year working lifetime were 2.4 and 0.3 mg/m^3, respectively. Cancer risks greater than 1 in 1,000 are considered significant and worthy of intervention by the Occupational Safety and Health Administration of the United States (OSHA), and for this reason, NIOSH (2011) also uses this risk level in their deliberations. NIOSH (2011) states that agglomerated ultrafine particle exposures should be controlled to the ultrafine REL.

Japan:

The Japanese Research Institute for Safety and Sustainability proposed a 'period-limited' WES for TiO_2 (Hanai et al. 2009, Ogura et al. 2011) derived using the methodology detailed in Nakanishi (2011). Since knowledge regarding toxicity of ENMs after long-term exposure is limited, the assumed exposure time for the WESs is 15 years, on the premise that these will be reviewed in ten years when further knowledge is available. In their deliberations for titanium dioxide, the Japanese researchers

[94] The primary particle size in Heinrich et al. (1995) was 15 – 40nm, but dry aerosol aggregates were 0.8 µm (GSD 1.8). There is only one exposure concentration because diesel exhaust rather than TiO_2 was the focus of the investigation.

[95] Since many pathologists consider the rat lung squamous cell keratinising cystic tumour to be irrelevant to human lung pathology, all lung tumour risk estimates from NIOSH (2011) excluded these tumours. Inclusion of these tumours in the analyses resulted in slightly higher excess risk estimates in females, but not in males.

[96] Assumed by NIOSH (2011) to be 0.41 m^2 for Fisher 344 rats, 0.4 m^2 for Sprague-Dawley rats and 102.2 m^2 for humans.

[97] NIOSH (2011) assumed 17.5 breaths per minute, a tidal volume of 1,143 mL, with exposures of 8 hours per day, 5 days per week for a working lifetime of 45 years.

concluded tumours are likely to occur due to inflammation resulting from high dose exposures, and that the dose-response relationship has a threshold value (Nakanishi 2011). They used the results from inhalational rat studies (4-weeks with 3 month observation), supported by intratracheal instillation rat studies (single dose, up to 2 years observation) to form the basis of a provisional workplace exposure limit for humans. 'Lung inflammation' was considered the endpoint of concern, as it was also observed at the lowest concentrations among exposure effects on lungs with nano-TiO_2. It was considered that prevention of inflammation would also prevent cancer (Nakanishi 2011). The NOAEL for TiO_2 (80% anatase/ 20% rutile, primary particle size of 21 nm) from these experiments and collective literature information (for rats and mice) was determined to be 2 mg/m^3 stemming from a 13-week inhalational study[98] (Ogura et al. 2011). This concentration was extrapolated to a human equivalent air concentration[99] of 1.82 mg/m^3. An uncertainty factor of 3 (for selection of dose metric) was applied to derive a workplace exposure limit of 0.61 mg/m^3 (Ogura et al. 2011).

Europe:

The European Commission (EC 2009b) quantitated inhalation risks of nano-TiO_2. In the process they calculated 'Derived No Effect Levels' (DNELs) for long-term worker exposure. A NOAEC of 0.5 mg/m^3 from a 13-week inhalation study in rats (6 hrs/d, 5d/week, 21nm particles) (Bermudez et al. 2004) was adjusted to 0.25 mg/m^3 for worker exposure time and inhalation rate (0.5 mg/m^3 x 6h/8h x 6.7m^3/10m^3). An uncertainty factor of 15 (1.5 for interspecies extrapolation, 5 for intraspecies variability, 2 for use of sub-chronic study) was applied to obtain a DNEL of 0.017 mg/m^3 (8-hour TWA). Although both Europe and Japan used the same study to derive their WES or DNEL (Bermudez et al. 2004), the two values used a different departure point (i.e. different NOAELs).

Bermudez et al. (2004) exposed female rats, mice and hamsters to aerosol concentrations of 0.5, 2 or 10 mg/m^3 of ultrafine TiO_2 particles for 13 weeks and evaluated a variety of lung parameters over several post exposure recovery time points up to 1 year. Following the end of exposures, mice and

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[98] The NOAEL was determined by Ogura et al. (2011), using data from the Bermudez et al. (2004) study.

[99] Extrapolation was done using the following equation:

$$NOAEL_H = NOAEL_R \times \frac{(RMV_R \times T_R \times DF_R)/BW_R}{RMV_H \times T_H \times DF_H)/BW_H}$$

Where:
$NOAEL_H$ = NOAEL in humans
$NOAEL_R$ = NOAEL from rat study (2 mg/m^3)
RMV = respiratory minute volume (m^3/min). This can be in turn determined by the following: $RMV = 0.499 \times BW^{0.809}$. For Rats, this was 0.123×10^{-3} m^3/min; for humans this was 0.025 m^3/min.
T_R = exposure time in experiment (257 min/day)
T_H = exposure time for worker (343 min/day)
DF_R = deposition fraction of TiO_2 particles in lungs of rats (unitless) (0.066)
DF_H = deposition fraction of TiO_2 particles in lungs of humans during light work (unitless) (0.11)
BW_R = body weight of experimental animal (0.1773 kg)
BW_H = body weight of human (73 kg)

rats had similar retained ultrafine TiO_2 lung burdens, while hamsters had reduced lung burdens due to faster particle clearance. Measurements of slow particle clearance from the lungs of mice and rats at the high dose (10 mg/m^3) provide evidence that particle overload had been achieved in these species, but not in hamsters. Assessment of BALF endpoints demonstrated increased lung inflammation and cytotoxicity biomarkers vs. corresponding controls in rats and mice in the high dose group (10 mg/m^3), but not in hamsters exposed to 10 mg/m^3 ultrafine TiO_2. Morphological observations revealed progressive lung epithelial and fibroproliferative changes, characterised by foci of alveolar epithelial cell proliferation in association with interstitial particle accumulation and progressive alveolar septal fibrosis in rats exposed to the high dose (10 mg/m^3), but not the lower doses (0.5 and 2 mg/m^3). In contrast to the rats, epithelial, metaplastic and fibroproliferative changes were not observed in the lungs of mice or hamsters. Overall, no significant histopathological and lung inflammatory changes were observed at the two lowest test concentrations (0.5 or 2 mg/m^3). It is clear from the above information that the appropriate NOAEC from this study is 2 mg/m^3 as was used by Japan, rather than 0.5 mg/m^3 used by Europe. However, the relevance of using endpoints for human risk assessment resulting from achieving particle overload in rodents, which is not as easily achievable in humans, is questionable (Warheit 2013).

5.7.2 Exposure

Considerable advancement has been made in analytical instrumentation and measurement of exposure to ENMs (Section 2.2.1). Since the 2009 review, a few authors have specifically investigated the extent of workplace exposures to TiO_2 NPs.

Huang et al. (2010) compared dust and NP concentrations, particle size distribution and metal content measured by different sampling devices at a TiO_2 pigment factory. The sampling device used in the study included a cyclone, a Multi-orifice Uniform Deposit Impactor, and a Fast Mobility Particle Sizer. They found the particle size distribution at this factory generally fell in the range of 1-10 µm, with only a very small proportion of nanoparticles. This suggests exposure to NPs *per se* at conventional facilities which are not intentionally manufacturing them may not be significant.

Lee et al. (2011) estimated potential exposure of workers manufacturing nano-TiO_2 in Korea through the use of personal sampling, area monitoring and real-time monitoring using a scanning mobility particle sizer (SMPS) and dust monitor. Gravimetric concentrations of TiO_2 ranged from 0.1 to 4.99 mg/m^3. Although the authors commented these concentrations are well below the American Conference of Industrial Hygienists' WES of 10 mg/m^3 for TiO_2, this WES is not nano-specific. From the repeat inhalation exposure studies conducted in animals, it seems evident adverse effects could potentially occur at concentrations lower than this 'bulk' TiO_2 WES. Particle numbers in the Lee et al. (2011) study ranged from 11,418 to 45,889 particles/cm^3 with a size range of 15 – 710.5 nm during the reaction, but decreased to 14,000 particles/cm^3 when the reaction was stopped.

The mass concentrations measured in the Lee et al. (2011) study at times exceeded the US WESs for ultrafine (<100 nm) and fine (<10 µm) TiO_2 of 0.3 and 2.4 mg/m^3, respectively. These WESs are associated with an estimated 1 in 1,000 excess risk of lung cancer in humans after a 45-year working lifetime (NIOSH 2011, Section 5.7.1), albeit this is based on information for rats exhibiting pulmonary overload. However, the Japanese proposed WES of 0.6 mg/m^3 (Ogura et al. 2011) and the European derived no effect level (DNEL) of 0.017 mg/m^3 (EC 2009b) were also exceeded. The Japanese WES is based on lung inflammation effects which were observed only in those animals exposed to high concentrations (10 mg/m^3) of ultrafine TiO_2 in the Bermudez et al. (2004) study. Therefore it is considered the workplace controls in this manufacturing facility were likely inappropriate or insufficient to control the risk of pulmonary inflammation.

The particle numbers in Lee et al. (2011) were also frequently above the German IFA (2012) proposed benchmark of 20,000 particles/cm^3 for metal oxides (Appendix C), albeit the latter is not based on toxicological evidence. Thus in this case occupational exposure to TiO_2 NPs may not be adequately controlled.

One set of authors simulated the use of a commercially available antimicrobial spray product containing TiO_2 NPs used to disinfect surfaces (Chen et al. 2010), and subsequently tested for health effects in rats at such exposure concentrations (McKinney et al. 2012). The TiO_2 particulate levels in the breathing zone of a person applying the spray to a vertical surface had a mass concentration of 3.4 mg/m^3 (or 1.6 x 10^5 particles/cm^3), with most of these particles less than 110 nm in diameter (Chen et al. 2010). McKinney et al. (2012) exposed rats by inhalation (whole body) to the spray product at three exposure levels: 1) 2.62 mg/m^3 for 2 hours (1 day) (half a can used; low dose), 2) 1.72 mg/m^3 for 4 h/day for 2 days (2 cans used; middle dose), 3) 3.79 mg/m^3 for 4 h/day for 4 days (8 cans used; high dose). These exposures were intended to mimic occasional consumer use and more frequent worker use (e.g. disinfection of surfaces by hospital personnel). The authors estimated that for the low, middle and high dose used in the study, equivalent human lung burdens would be achieved after application of the spray for 2 hours, 5.5 hours and 24 hours, respectively. Pulmonary (breathing rate, specific airway resistance, inflammation, and lung damage) and cardiovascular (responsiveness of tail artery to constrictor or dilatory agents) endpoints were monitored up to 24 hours post-exposure. No significant pulmonary or cardiovascular changes were noted at the low and middle doses. However the high dose caused significant increases in breathing rate, pulmonary inflammation (evinced by lavageable PMNs), and lung cell injury (increased LDH in BALF). Specific airway resistance and responsiveness of the tail artery to vasoconstriction or redilation were not significantly affected after any of the exposure levels. The authors concluded that occasional consumer use of the spray product is probably not hazardous. However, extended exposure of

workers routinely applying this product to surfaces should be avoided, and during application care should be taken to minimise worker exposure (McKinney et al. 2012).

5.7.3 Hazards

Overall, the acute and repeat exposure inhalational studies published since the 2009 review support the conclusion that TiO_2 NPs have low potential to induce pulmonary irritation or inflammation, since often only mild, transient effects are seen at high concentrations. Reminiscent of ambient particulate matter pollution, TiO_2 NPs seem capable of producing cardiac effects *in vivo* in the absence of pulmonary inflammation.

Data from a repeat oral toxicity study indicates long-term oral exposure to TiO_2 NPs may result in liver toxicity, however as exposure was only evaluated for a single very high dose, a no observed effect level could not be elucidated.

5.7.4 Risk assessment

Warheit et al. (2009b) outlines a framework for gathering the necessary information to evaluate nanomaterial risks (the Nano Risk Framework). This framework consists of conducting a base set of hazard tests on NMs. These are acute pulmonary and oral toxicity studies, skin irritation and sensitisation studies, an ocular irritation test, *in vitro* genotoxicity studies, and screening aquatic toxicity assays. The framework has already been applied to a newly developed, well-characterised, ultrafine rutile TiO_2, for which most test results indicated low toxicity in mammals or aquatic species following acute exposure. Warheit et al. (2009b) advocates the framework is a good start for risk assessment considerations of ENMs.

As discussed in Section 5.7.1, US, Japanese, and European authorities have utilised either acceptable risk levels or no observed adverse effect levels after intermediate or chronic inhalation of TiO_2 NPs in rats to derive workplace exposure limits or ‘derived no effect levels’ for worker exposure. However, the relevance of using endpoints for human risk assessment resulting from rodent studies in which particle overload was a major factor in the responses is questionable.

5.8 Summary and conclusions

Overall, since the last review, there have not been significant additional pivotal studies published which could potentially provide a further avenue for occupational risk assessment of TiO_2 NP exposures.

The toxicological and distribution studies published since the last review do however provide a better understanding of the potential hazards of TiO_2 NPs, and provide supporting evidence for conclusions made in the last review:

- The majority of inhaled NPs are deposited in the lung, with some translocation to the lymph nodes, but negligible translocation to the liver or other organs. The latter biodistribution is also evident when considering the absence of liver effects in acute and repeat exposure inhalation toxicity experiments.
- Biodistribution studies using an intravenous, intra-abdominal, or subcutaneous injection route to deliver the NPs have found TiO_2 NPs distribute into and accumulate in various parts of the body, including the placenta. However, the relevance of the observed distribution after injection exposures to workers is questionable.
- The available information suggests TiO_2 NPs are not taken up by intracellular organelles within the cell and do not break down within the cell.
- Animal, human, and *in vitro* studies published since the 2009 review provide additional supporting evidence that TiO_2 NPs neither penetrate into viable cell layers nor cause toxicity after application to intact or partially damaged skin, including that from sunburn.
- Although a vast array of *in vitro* cytotoxicity and mechanistic studies have been published since the 2009 review, they are still of limited value for identifying and managing risks of TiO_2 NPs in the workplace.
- The acute and repeat exposure inhalational studies published since the 2009 review support the conclusion that TiO_2 NPs have low potential to induce pulmonary irritation or inflammation, since often only mild, transient effects are seen at high exposure concentrations.
- Reminiscent of ambient particulate matter pollution, TiO_2 NPs seem capable of producing cardiac effects *in vivo* in the absence of pulmonary inflammation.
- Data were not located for this review that enabled identification of no observed effect levels associated with long term repeat inhalation studies.
- There is inconsistency in the results from genotoxicity tests with TiO_2 NPs. However studies with positive results support an indirect genotoxic mode of action secondary to oxidative stress rather than a direct interaction with DNA.
- The US proposed workplace exposure limits for ultrafine and fine TiO_2 are based on the induction of lung tumours in rats where particle overload was achieved. Nonetheless because the NIOSH exposure limit is based on chronic rat studies it is suggested it be adopted for Australia as a WES. The Japanese workplace exposure limit is based on lung inflammation, rather than cancer, but also stems from particle overload data in rats. We note the relevance of using endpoints for human risk assessment resulting from pulmonary particle overload in rodents, which is not as easily achievable in humans, is questionable.
- In contrast to the rat data, the published epidemiological studies in workers with greatest exposures to TiO_2 particles are negative for lung cancer risks as well as other non-malignant respiratory diseases, albeit these did not look specifically at nano-sized TiO_2.

6. Zinc oxide nanoparticles

Zinc oxide NPs are widely used as additives in sunscreens, with further anticipated applications such as photovoltaic devices and nanomechanical components. They have also been used as antibacterial agents in textiles, food packaging, baby powders, shampoos, bandage tapes and antiseptic ointments (Jachak et al. 2012).

6.1 Distribution

Only small amounts of fluorescently labelled zinc oxide NPs were found to move through undiluted human mucus (Jachak et al. 2012)[100]. In contrast, CeO_2 and ZrO_2 NPs and SWCNT were effectively immobilised by mucus therefore negligible fractions of these NMs are expected to penetrate to the tracheobronchial epithelium. Although there are a number of experimental issues[101] associated with this study, the results are consistent with the intuitive notion that mucous should significantly protect tracheal epithelial cells by retarding NM translocation to the cells.

Zinc from zinc oxide NPs made from zinc oxide vapour, has been found in olfactory bulbs and the brain of rats after nasal exposure[102] to airborne NPs (Kao et al. 2012a). The authors postulated an olfactory bulb-brain pathway of zinc oxide NP uptake involving endocytosis.

After orally administering a very high dose of uncoated zinc oxide NPs (~90 nm) or micro zinc oxide (1.2 µm) (2.5 g/kg body weight) to mice via gavage, zinc ions were rapidly absorbed into the circulation (within 0.5-2 hours) and biodistributed to the liver, spleen and kidney (Li et al. 2012a).

[100] In Jachak et al. (2012) cervicovaginal mucus was used as a surrogate for respiratory tract mucus. The nano metal oxides (nMeO) were functionalized to make them fluorescent. Fluorescent nMeOs were synthesized by the addition of primary amines, using aminopropyltriethoxysilane to attach alkoxy silane groups to the surface hydroxyl groups. Fluorescein isothiocyanate was then attached to the amines as the fluorescent tag, ZnO NPs had a measured average diameter of 116 $\pm$ 10 nm. The exposure concentration used in the experiment was 8 µg/cm^2, calculated using the US workplace exposure limit for ZnO (non-nano form) of 5 mg/m^3. Assuming an 8 hour workday, a breathing rate of 4.8 m^3/h (healthy male working adults) and a 10% deposition fraction, the authors calculated a mass dose of ZnO NPs deposited in the tracheobronchial airways of 19 mg. Using the surface area of the tracheobronchial region (2471 cm^2), the dose per area was calculated. It was estimated 2-3% of ZnO NPs would be able to penetrate the mucus layer within one hour of exposure.

[101] The nanometal oxides (nMeOs) were characterised and added to human mucus in phosphate buffered saline. It is therefore very likely they were significantly aggregated. Indeed size characterisation showed sizes all above 100nm and markedly more than the size nominated by the commercial suppliers. Workers are exposed to dry nanoparticle aggregates rather than wet ones. Since the density of dry particles is somewhat less than for the wet, the data for wet nMeOs are unlikely to reflect the kinetic behaviour of dry aggregates. Hence the propensity for translocation of nZnO could be overestimated. After adjusting from pH approximately 4 to 7 with sodium hydroxide fresh cervicovaginal mucus was used as a surrogate for respiratory tract mucus. Although the authors cite a reference indicating the mucus is similar to that in the respiratory tract, the effect of sodium hydroxide on the vaginal mucus proteins was not considered. Although the authors argue that the surface modification of the nMeO and fluorescent tagging is unlikely to have affected their interactions with mucus it is unknown whether these changes may have altered the behaviour of the nMeOs in the mucus.

[102] Kao et al (2012a) exposed rats nose only for a single 6 hour exposure to ZnO fumes (2.1×10^6 particles/cm^3; 38 nm particle size, sacrificed 2 hr after exposure) and measured the presence of Zn^{2+} with fluorescent dye (Newport Green DCF) that binds to Zn^{2+} but not to Ca^{2+}. Nanoparticles of ZnO were located in olfactory bulbs and brain by transmission electron microscope after 4 hr exposure on 3 consecutive days to 2.0×10^6, 3.4×10^6, and 6.6×10^6 particles/cm^3; 12–14 nm particle size.

While the rate of absorption appears the same for both forms of zinc oxide the extent of absorption was slightly greater for the NPs than for the zinc oxide microparticles. This study illustrates several common issues associated with kinetic/distribution studies with nano metal oxides.

- Administered doses are unrealistically high relative to anticipated human exposures.
- Tissue concentration quantitation is via measurement of the metal ion rather than of the NP, at the present time the latter can only be reliably determined by transition electron microscopy (TEM).
- Given that usually only a very small fraction of the administered NP reaches the systemic circulation (particularly after inhalation of concentrations relevant for human exposure in controlled work places) it is a very tedious, labour intensive and time consuming exercise to scan enough cells to detect the few NPs some of them may contain.
- Characterisation of the nanomaterial was in a medium quite different from that of the gastrointestinal milieu. It is certain that in that environment the nano-ZnO would have been present as aggregates and not NPs.
- Mass balance calculations were not undertaken.

Using highly sensitive stable zinc isotopes as tracers, Gulson et al. (2010) demonstrated that small amounts of zinc from ZnO particles in sunscreens when applied to humans can pass through the protective layers of skin exposed to the sun in a real-life environment and be detected in blood and urine. An independent laboratory confirmed these results (Larner et al. 2014). Although zinc from both bulk ZnO (114nm, range 25 – 384nm) and nano-ZnO (3nm, maximum 60nm) was absorbed into blood from application of the sunscreens to humans, more was absorbed from nano-ZnO than from the bulk material (3.2% vs 1.5%); the rate was also faster (0.17% per day vs. 0.10% per day) (Larner et al. 2014). The effect was larger for females than males. The authors are clear that it is not possible to determine whether it was Zn^{2+} that was absorbed or the nano-ZnO. However since blood zinc was increased with the bulk material and that at the skin pH in the presence of sweat ZnO can solubilise, it seems more likely the increase represents zinc ion rather than ZnO particles. It may be that other studies with nano-metal oxides applied to skin do not report translocation of the metal because their analytical sensitivity is lower than the very sensitive isotope ratio method used by Gulson et al. (2010). The authors point out approximately 15 µg of zinc tracer was absorbed into blood from the nano-ZnO sunscreen formulation and that compared with circulating levels of zinc (~12 mg) and the recommended dietary intake of 8 mg, this is an inconsequential amount (i.e. ~1/1,000th that of total Zn in blood compartment). Nevertheless the application period was only for 5 days, when in Australia sunscreens may be used for substantially longer periods.

Of interest is that blood concentrations continued to rise during the 6 day post observation period (Gulson et al. 2010, 2012; Larner et al. 2014), suggesting a tissue depot, zinc tissue turnover, that

could be slowly releasing zinc back into the blood. Lademann et al. (2006) have shown a ten fold longer storage of NPs (300 – 400nm) in hair follicles compared with the stratum corneum.

Osmond-McLeod et al. (2013) have applied the same sunscreen formulations containing isotopically enriched zinc to the backs of virgin and pregnant hairless mice. The authors point out that due to the substantial difference between the skin of hairless mice and humans that the zinc absorption data in this animal model cannot be directly extrapolated to humans. Increased concentrations of the zinc tracer were detected in internal organs of mice receiving topical applications of ZnO (nano-sized and larger particles), as well as in foetal livers from treated dams, compared with controls. However the concentrations were higher for the sunscreen containing nano-ZnO. No ZnO-mediated change in total zinc concentration in any of the major organs was observed thus zinc homeostasis was largely maintained. Furthermore an adverse biological response in the mice following short-term topical applications of these preparations was not observed as judged by serum amyloid A2 concentrations (a biomarker for acute phase inflammation) and whole-genome transcriptional profiling on livers.

6.2 Mode of action

Zinc oxide NPs follow a Trojan horse type mechanism of toxicity in which once taken up by cells, they release intracellular Zn^{2+} (Gilbert et al. 2012; Kao et al. 2012a, 2012b) (Section 3.1). This mode of action is supported by a number of mechanistic cytotoxicity studies conducted *in vitro* (Song et al. 2010; Auffan et al. 2009b; Buerki-Thurnherr et al. 2012) as well as an *in vivo* intratracheal instillation study (Cho et al. 2011).

6.3 In vitro toxicity

As with other ENMs, numerous *in vitro* cytotoxicity experiments using various different cell types[103] or experiments investigating the mechanistic detail of various aspects of the inflammatory response of ZnO NPs have been published (e.g. Cho et al. 2012a, 2012b; Deng et al. 2009; Feltis et al. 2012; Gerloff et al. 2009; Hanley et al. 2009; Heng et al. 2010, 2011a, 2011b; Hsiao and Huang 2011; Kao et al. 2012b; Kocbek et al. 2010; Lenz et al. 2009; Osman et al. 2010; Otero-Gonzalez et al. 2012; Prach et al. 2013; Pujalte et al. 2011; Zhang et al. 2011c; Song et al. 2010; Sun et al. 2011a; Xie et al. 2012). While noting these studies are important for unravelling the molecular mechanisms of ENM induced toxicity at this time they are of limited value for identifying and managing ENM risks in the workplace. Such investigations are therefore not described in detail in this review.

[103] For example, neural stem cells, immune cells, bronchial epithelial cells, foetal lung fibroblasts, cancer cells, skin cells, kidney cells, and cardiac microvascular endothelial cells.

Interesting findings from these *in vitro* experiments include:

- Hsiao and Huang (2011) found that at a fixed size and surface area, nanorod ZnO particles were more cytotoxic to human lung epithelial cells than the corresponding spherical NPs. This suggests the shape of ZnO NPs may influence their cytotoxicity.
- Song et al. (2010) also concluded that particle shape may impact the cytotoxicity of ZnO NPs in mouse macrophages.
- Lenz et al. (2009) developed and validated a novel air-liquid interface cell exposure system (ALICE) using three NMs (Au, ZnO, and carbon black NPs) and solutes (such as NaCl). ALICE generates a dense cloud of droplets with a vibrating membrane nebuliser and uses combined cloud settling and single particle sedimentation for fast (~10 min; entire exposure) and efficient delivery of NPs or dissolved substances to the liquid interface of cultured cells. The authors found that exposures of human alveolar epithelial cells to 1 µg/cm^2 ZnO NPs did not exert cellular responses, which they suggest indicates ZnO NPs are not cytotoxic at occupationally allowed exposure levels[104].
- Some studies have shown that ZnO NP induced cytotoxicity is dependent on size, charge, and solubility factors (Prach et al. 2013, Auffan et al. 2009b, Cho et al. 2012b). In Prach et al. (2012), ZnO NPs (70 nm) were more toxic to a human monocytic cell line than the bulk form (<44 µm mesh).
- Distinct patterns of oxidative stress were observed in alveolar type II epithelial cells exposed to aerosolised ZnO NPs and NPs in suspension at the same cellular doses (Xie et al. 2012). This could suggest different mechanisms of toxicity may exist in ZnO NPs at the air liquid interface than in submersed cultures (i.e. the mode of action is different from that of dissolved Zn^{2+} inducing toxicity).
- *In vitro* cytotoxicity assays with ZnO NPs employing three different cell systems, four different exposure doses, two different time points, and two biomarkers/endpoints, all deliberately similar to those used in *in vivo* intratracheal and acute inhalation studies did not accurately predict the effects observed *in vivo* (Warheit et al. 2009a). This supports the conclusion that *in vitro* cytotoxicity assays do not exhibit predictive concordance with effects observed *in vivo* (Section 3.6).

[104] To put the experimental dose into perspective, Lenz et al. (2009) provided the following context. The US Occupational Safety and Health Administration (OSHA) permissible exposure limit (PEL) for ZnO fumes is 5 mg/m^3 (this is the same as in Australia) averaged over an 8-hour work shift. Assuming an accumulated breathing volume of 3 m^3 in 8 hours, a lung surface area of 140 m^2, an alveolar deposition efficiency of 10-50% (depending on particle size) and a 70% long-term clearance from the alveolar regime the OSHA standard corresponds to an average (long-term) daily alveolar surface dose of 0.32-1.6 ng/cm^2. Hence the maximum lifetime dose accumulated by a worker is 3.6-18µg/cm^2 (5 workdays per week, 50 weeks, 45 years).

6.4 In vivo toxicity

6.4.1 Acute inhalation

Warheit et al. (2009a) compared the inhalational toxicity of nanoscale ZnO with that of fine ZnO in rats. Although different sizes were claimed by the supplier, the two particle types were very similar in size (~100 nm when dry, 170-370 nm when wet), albeit the particle size of the ZnO NPs were still smaller than for fine ZnO. Rats were exposed by inhalation to aerosols of 25 or 50 mg/m^3 for 1 or 3 hours. These concentrations are 5-10 times higher than the current Australian workplace exposure standard for zinc oxide fume[105]. Lung inflammation, cytotoxicity, and histopathological endpoints were assessed at several time points post exposure (24, 72 and 192 hours). Inhalation of both nano- and fine- ZnO produced 'metal fume fever' type responses at both doses, characterised by transient, short term lung inflammatory or cytotoxic responses. The severity of the response was concentration-related. No significant differences were observed in the potency or time course between responses to nano- or fine- ZnO. Full recovery was observed 8 days post-exposure.

6.4.2 Acute oral

No mortalities, clinical abnormalities, or gross pathological changes were observed in mice administered a single dose of 5 g/kg body weight ZnO NPs (50 nm) or ZnO microparticles (1.2 µm) via gavage for the duration of the observation period (14 days) (Li et al. 2012a).

Pasupuleti et al. (2012) compared the acute oral toxicity of nano-sized ZnO (60 nm; 225 nm in suspension) with its micron-sized counterpart by administering doses of 5, 50, 300, 1000 and 2000 mg/kg bw of these particle suspensions[106] once via oral gavage. Biochemical and haematological parameters were analysed 14 days after administration, and organs collected for histopathology. Liver toxicity as judged by serum ALT and AST was evident with the nano-ZnO but not with micro-ZnO. In addition the toxicity was inversely correlated with dose of the nanoparticles. This is contrary to what would be expected and suggests something unusual with the study, it is noted the number of animals per group was low (n=5).

In a recent review, Schilling et al. (2010) concluded that the acute oral toxicity of ZnO NPs is very low.

6.4.3 Repeat inhalation

Repeat inhalation toxicity studies have not been conducted with ZnO NPs to date.

6.4.4 Repeat oral

Two repeat oral toxicity studies were located in the literature specifically investigating renal toxicity.

[105] The current workplace exposure standard for zinc oxide fume is 5 mg/m^3 (Safe Work Australia 2013b).

[106] Particle suspensions were prepared in distilled water (Pasupuleti et al. 2012).

The first is a study investigating the ameliorative effects of quercetin and/or arginine on nephrotoxicity caused by ZnO NPs. The NPs[107] were administered by gavage at two doses (either 600 mg or 1 g/kg body weight/day for 5 consecutive days) to rats (Faddah et al. 2012). Biochemical indicators of nephrotoxicity were monitored and histopathological examination of kidney tissue was performed at the conclusion of the study period (24 hours after last dose). Nano ZnO-induced nephrotoxicity was confirmed by the elevation of several inflammatory markers[108], a significant increase in serum urea and creatinine levels, and a significant decrease in the non-enzymatic antioxidant reduced glutathione (GSH) in kidney tissue coupled with an increase in serum glucose levels. These biochemical findings were supported by histopathological findings in kidneys in the high dose group, which showed numerous kidney glomeruli underwent atrophy and fragmentation. Epithelial desquamation, degeneration and necrosis were also observed in the renal tubules, with some tubules showing casts in their lumina. Severe congestion was also observed in the renal interstitium. The effects were dose dependent (Faddah et al. 2012). Neither the concentrations of zinc in the kidney nor presence of nano-ZnO was determined.

The second repeat dose study which investigated nephrotoxicity is Yan et al. (2012). They applied a metabonomic approach[109], coupled with haematology, clinical biochemistry and histopathology methods after dosing rats daily with 100, 300 or 1000 mg/kg ZnO NPs (50 nm) by gavage for 14 days. Over 7 days, hypourocrinia and drowsiness were observed in the 300 and 1000 mg/kg groups. Additionally, the animals at these doses showed a dramatic weight and food consumption loss. No obvious abnormalities were observed in the 100 mg/kg dose group. Most of the rats in the 1000 mg/kg group and one in the 300 mg/kg group showed overt tubular epithelial cell necrosis, whereas no overt pathologic abnormalities were found in the kidneys of the 100 mg/kg dose group. The animals at the top two doses also had increases in serum biomarkers for renal damage, creatinine and blood urea nitrogen, whereas the low dose group did not. Marked changes in urinary metabolites were identified in ZnO NP-treated rats, including glucose, choline, phosphocholine, lactate, taurine, and the Krebs cycle products. Because these metabolites are involved in cell membrane constitution and energy metabolism, the authors suggested ZnO NPs can cause renal mitochondria and membrane damage. Neither the concentrations of zinc in the kidney nor presence of nano-ZnO was determined.

[107] The size of the NPs reported by the supplier was 50 nm; the authors did not independently characterise the NP physical properties (Faddah et al. 2012).

[108] The elevated inflammatory markers were tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and C-reactive protein (CRP). Moreover, immunoglobulin (Igg), vascular endothelium growth factor (VEGF) and nitric oxide (NO) were significantly increased in rat serum compared to controls.

[109] Yan et al. (2012) used ^{1}H NMR spectroscopic techniques to analyse metabolites in urine and the kidneys of rats.

Sharma et al. (2011) also investigated the oral toxicity of ZnO NPs (30 or 270 nm)[110] by administering doses of 50 or 300 mg/kg/d for 14 consecutive days to mice. In the 300 mg/kg dose group, they found significant accumulation of zinc in the liver leading to cellular injury, evident by elevated ALT and ALP serum levels and pathological lesions in the liver. Oxidative DNA damage in the liver and kidney cells of mice was evaluated by the fpg-modified comet assay[111] after the exposure period. A statistically significant increase in oxidative DNA damage was observed in the liver of mice exposed to 300 mg/kg ZnO NPs; no significant DNA damage was observed in mice given the lower dose (50 mg/kg). It is stated by the authors "*Our results conclusively demonstrate that sub-acute oral exposure to ZnO nanoparticles in mice leads to an accumulation of nanoparticles in the liver causing oxidative stress mediated DNA damage and apoptosis*". Unfortunately, only an increase in zinc ions was demonstrated in the study, not an increase in ZnO nanoparticles. As discussed elsewhere in this report nano-metal oxides will be solubilised in the gastrointestinal tract.

6.5 Genotoxicity

Results obtained in standard genotoxicity assays with ZnO NPs have been conflicting. ZnO NPs (50 nm) tested negative in both the micronucleus assay and the Ames test (Li et al. 2012a). Landsiedel et al. (2010b) also found no genotoxicity of ZnO NPs (30-200 nm) in a standard genotoxicity battery which included *in vitro* and *in vivo* tests[112]. On the other hand, ZnO NPs (primary particle size of 20 nm, forming 50 nm aggregates) induced significant DNA damage in intestinal Caco-2 cells, a human cancer cell line (Gerloff et al. 2009). DNA damage was also observed in mouse liver after 14 days gavage administration of 300 mg/kg/d (Sharma et al. 2011). In another study, ZnO NPs[113] also increased DNA and cytogenetic damage in a concentration- and time-dependent manner in *in vitro* comet and cytokinesis-blocked micronucleus assays (Osman et al. 2010).

In a recent review on the human safety of TiO_2 and ZnO NPs in sunscreens, Schilling et al. (2010) concluded that overall both substances were non-genotoxic and non-photo-genotoxic, and no major

[110] Sharma et al. (2011) characterised the size of the ZnO NPs using dynamic light scattering, and found the mean hydrodynamic diameter to be 272 nm. However, the average size reported by the commercial supplier of the material (Sigma-Aldrich) using the Brunauer-Emmett-Teller (BET) method followed by transmission electron microscopy (TEM) was 30 nm. Sharma et al. (2011) commented that the difference in size could be attributed to the different principles involved in each measurement technique and the agglomeration of NPs in the aqueous medium used for dose administration.

[111] The fpg-modified comet assay is more sensitive than the unmodified comet assay at detecting DNA damage.

[112] This standard genotoxicity test battery included the *in vitro* Ames' Salmonella gene mutation test and V79 micronucleus chromosome mutation test, and the *in vivo* mouse bone marrow micronucleus test and comet DNA damage assay in lung cells from rats exposed to ZnO NPs via inhalation (Landsiedel et al. 2010b).

[113] The ZnO NPs used by Osman et al. (2010) varied in particle size based on the concentration used (ranging from 17 nm in 10 µg/mL to 420 nm in 50 µg/mL solution). This means as the concentration in cell medium increased the particles tended to form aggregates.

differences were observed in the hazard profiles for micro- and nano- structured particles in terms their potential mutagenic/genotoxic hazards.

6.6 Workplace implications

6.6.1 Exposure

Information on the likelihood and expected concentrations of workplace exposure to zinc oxide NPs during the production phase was not found in the literature. As for other nanomaterials, the inhalation and dermal routes are probably the most likely routes of exposure. Inhalation is, however, probably more important, since ZnO NPs are unlikely to penetrate into or through viable layers of the skin (Section 3.5.1) Osmond and McCall (2010) discussed the potential for human exposure and the health hazards of ZnO NPs in modern sunscreens at each stage of their manufacturing and use. In their review, they highlighted the lack of information on inhalation exposures for workers and other personnel during the manufacturing phase.

When comparing the lung inflammation induced by ZnO particles (35 and 250 nm) after a single inhalation exposure to a low, moderate or high dose in rats[114] Ho et al. (2011) concluded the mass concentration and surface area, but not the number of particles, were significantly correlated with the percentage of neutrophils, number of neutrophils and total cells.

6.6.2 Limiting workplace exposure

Specific WES for nano-forms of ZnO have not been established.

6.6.3 Risk assessment

There are no studies investigating the reproductive toxicity of ZnO NPs, nor are there suitable repeat inhalation or oral studies that could be used to develop acceptable exposures. There is clearly a dearth of information on the chronic toxicity of ZnO NPs that can be reasonably assigned to the nano-form of the substance, this precludes health based risk assessment for inhalation exposures to these particles in the workplace. Nevertheless based on the information to date ENMs of nano-ZnO appear to be of low toxicity.

6.7 Summary and conclusions

The toxicological database of nano-ZnO is not as extensive as some other nano-metal oxides (nMeOs). As with other nMeOs, nano-ZnO in *in vitro* cell culture systems is able to cause cytotoxicity and indirect DNA damage via oxidative stress. It appears to do this by releasing zinc ions within the cell after the nano-form has been translocated from the media into the cell.

[114] In Ho et al. (2011), the exposure concentrations were 2.4 mg/m^3 and 7.2 mg/m^3 (low), 3.7 and 11.5 mg/m^3 (moderate), or 12.1 and 45.2 mg/m^3 (high) for 35 and 250 nm particles, respectively.

Use of acute inhalation information for workplace assessment is compromised by the very high concentrations that have been used. This results in the expected lung inflammation and cytotoxic responses. While the available acute and sub-chronic studies with nano-ZnO show increased metal concentration in various tissues, plus or minus indications of tissue damage, after high gavage doses they are compromised by lack of evidence that the nano-ZnO has actually been absorbed from the gastrointestinal tract. In this milieu nano-ZnO is likely to be extensively solubilised.

The use of nano-ZnO in sunscreens has prompted sophisticated human and murine studies to be conducted in Australia whereby very sensitive stable isotope techniques have been able to show small fractions (<0.001%) of the applied zinc is absorbed into the blood. Furthermore the absorption continues for some days after repeat application stops and the skin has been washed. It cannot be determined whether the increased blood zinc is the result of zinc ion or nano-zinc absorption. Relative to normal circulating levels of zinc, the amount absorbed from nano-ZnO sunscreen is tiny and does not affect the homeostatic balance of zinc in tissues. No adverse effects are expected. The weight of literature evidence indicates nano-ZnO does not penetrate the skin. Others may not have detected the very small absorption of metal because their analytical techniques were not sensitive enough.

7. Cerium oxide nanoparticles

Cerium oxide NPs (CeO_2 NPs) exhibit anti-inflammatory properties, and are being studied for a variety of potential therapeutic applications, including cancer treatment (Asati et al. 2010). They are currently used in coatings, electronics, energy and as fuel additives (Cassee et al. 2011b).

7.1 Distribution

The tissue distribution, accumulation and elimination of three different sizes (nominally < 5000, 40, 5–10 nm) of cerium oxide obtained from Sigma-Aldrich (United Kingdom), Umicore (Belgium), and Antaria (Australia), respectively were studied in rats after inhalation exposure. The solubility of the particles was extremely poor in water at neutral pH (Geraets et al. 2012). Although the actual primary particle sizes were different[115], powder aerosolisation resulted in comparable aggregate/agglomerate mass median aerodynamic diameters of 1.4, 1.17 and 1.02 µm. The mean mass concentration in the inhalation studies were 55.00, 19.95, and 10.79 mg/m^3 respectively. It is noted these concentrations are likely to result in pulmonary overload and therefore pulmonary biokinetics will likely be different from that of lower exposures. The number of daily exposures were 1, 11 or 20 at 6 hr/d with tissue evaluation performed 1, 48 or 72hr after the last exposure.

[115] The primary particle sizes were 615.3 $\pm$ 430.5, 28.4 $\pm$ 10.4 and 44.9 $\pm$ 14.6 nm for Sigma-Aldrich, Umicore and Antaria particles, respectively (Geraets et al. 2012).

- After a single exposure, approximately 10% of the total inhaled dose of cerium was measured in lung tissue. No consistent pulmonary deposition differences between the micro- and nano-sized particles were observed.
- After a single 6-hour exposure, cerium from each CeO_2 sample was also distributed to other tissues, such as the liver, kidney, spleen, brain, testis and epididymis. The highest concentrations were found in the lung, whereas <0.2% of the total inhaled dose of cerium was detected in the extrapulmonary tissues. Again, no clear particle size-dependent effect on extrapulmonary tissue distribution was observed. There was no confirmation of nano-CeO_2 in the tissues, but because the ENMs were poorly soluble it may be most of the tissue cerium was associated with nanoparticles.
- Repeated exposure to CeO_2 resulted in significant accumulation of cerium in the extrapulmonary tissue. Tissue clearance was slow, and overall, insignificant amounts of cerium were eliminated from the body at 48- to 72-hours post-exposure. Therefore, an elimination half-life could not be reliably determined.
- Cerium was not detected in blood, indicating fast distribution from blood to tissues.

The dust aerosolisation technique employed by Geraets et al. (2012) was intended to mimic occupational exposure to NPs, thus aggregation or agglomeration of CeO_2 NPs likely can also be expected to occur in the workplace. Geraets et al. (2012) made no comment on whether or not any toxicological effects were observed in the rats throughout the experiment.

Similar extrapulmonary and systemic distribution of CeO_2 NPs to the liver, spleen, kidney, testis and brain has also been observed following intratracheal instillation (He et al. 2010) and intravenous administration (Dan et al. 2012, Hardas et al. 2010, Yokel et al. 2009, 2012a).

To better understand the fate of ceria as a model ENM Dan et al. (2012) compared the tissue distribution and rate of clearance from blood of four different sizes of cubic or polyhedral ceria nanoparticles, a mixture of cubes and rods, and the cerium ion after intravenous infusion into rats. The kinetics and distribution of the cerium ion did not predict those of the ceria ENMs. A 5 nm ceria ENM, which was very resistant to agglomeration and settling *in vitro*, was cleared much more slowly from blood than larger ceria ENMs, presumably because it was too large to be cleared by glomerular filtration and perhaps too small or too hydrophilic to be readily recognised by the reticuloendothelial system. Ceria ENMs larger than 5 nm were very rapidly cleared from circulating blood. All the ceria ENMs had a much greater steady state volume of distribution than the cerium ion, consistent with their extensive distribution and prolonged retention throughout the rat.

Hardas et al. (2010) specifically studied the distribution of 5 nm CeO_2 from blood into brain after intravenous infusion of 100 mg/kg. Using TEM nanoparticles of ceria were not seen in microvascular

endothelial or brain cells and produced little oxidative stress effect to the hippocampus and cerebellum. The authors noted this was contrary to what had been found for 30 nm cerium oxide particles and concluded the results were contrary to the hypothesis that a smaller ENM would more readily permeate the blood brain barrier.

Yokel et al. (2012a) intravenously infused over an hour nanoceria into rats at approximately 100 mg/kg. Less than 1% of the nanoceria was excreted in the first 2 weeks, 98% in faeces. Ceria was primarily retained in the spleen, liver, and bone marrow. There was little decrease of ceria in any tissue over the 90 days. Granulomas were observed in the liver. Time-dependent oxidative stress changes were seen in the liver and spleen.

Potential exposure via the oral route following inhalation of particles (e.g. pulmonary clearance by the mucociliary escalator after phagocytosis) may contribute to extrapulmonary tissue deposition. He et al. (2010) showed that after intratracheal instillation of CeO_2 NPs (primary particle size: 6.6 $\pm$ 0.9 nm) to rats approximately 25% of the given dose was cleared via the faeces, pointing toward particle transport from the lung to the gastrointestinal tract. Furthermore, approximately 90% of the faecal elimination occurred on the first day post-exposure, and nearly 100% after 3 days (He et al. 2010). This suggests limited gastrointestinal absorption of CeO_2 NPs. He et al. (2010) calculated an elimination half-life from the lung of 103 days for intratracheally instilled CeO_2 NPs.

The internal cellular distribution of polymer-coated cerium oxide NPs (3-4 nm) is dependent on surface charge (Asati et al. 2010). CeO_2 NPs with positive or neutral charge entered most cell lines studied, whereas negatively charged NPs were internalised only in the lung cancer cell line. Once internalised, neutral CeO_2 NPs remained in the cytoplasm of the cell, and were not cytotoxic as judged by the MTT assay. Positively charged NPs were localised in lysosomes, as were negatively charged NPs (only cancer cells), both resulting in cytotoxicity of those cells into which uptake was observed.

CeO_2 NPs were effectively immobilised by undiluted human cervicovaginal mucus[116], therefore negligible fractions are expected to penetrate to the tracheobronchial epithelium (Jachak et al. 2012).

7.2 Mode of action

It has been proposed other metal oxide NPs may exert their toxicity by a Trojan horse-type mechanism, in which the nanoparticulate size facilitates their uptake into cells, where they are

[116] In Jachak et al. (2012), CeO_2 NPs were purchased from Sigma Aldrich, with a measured average diameter of 495 $\pm$ 66 nm. The exposure concentration used in the experiment was 23 µg/cm^2, calculated using the US workplace exposure limit for CeO_2 of 15 mg/m^3. Assuming an 8 hour workday, a breathing rate of 4.8 m^3/h (healthy male working adults) and a 10% deposition fraction, the authors calculated a mass dose of CeO_2 NPs deposited in the tracheobronchial airways of 58 mg. Using the surface area of the tracheobronchial region (2471 cm^2), the dose per area was calculated.

subsequently dissolved to their metal ion constituents, which are in turn responsible for the observed toxicity (Sections 3.1, 6.2, 8.2). The study by Asati et al. (2010), where cytotoxicity was only observed if CeO_2 NPs were localised within lysosomes of cells (i.e. acidic environment potentially capable of releasing the cerium ions) lends support to a Trojan-horse type mechanism for these NPs. However there is insufficient information to assign a toxicological mode of action to nano CeO_2 particles.

7.3 In vitro toxicity

As with other ENMs, numerous *in vitro* cytotoxicity experiments using various different cell types or experiments investigating the mechanistic detail of various aspects of CeO_2 NP toxicity have been published (e.g. Asati et al. 2010, Auffan et al. 2009a, Cho et al. 2012b, Culcasi et al. 2012, Gojova et al. 2009, Hussain et al. 2012b, Kitchin et al. 2011, Kroll et al. 2011, Lanone et al. 2009, Rothen-Rutishauser et al. 2009, Rotoli et al. 2012, Steiner et al. 2012). While noting these studies are important for unravelling the molecular mechanisms of ENM induced toxicity at this time they are of limited value for identifying and managing ENM risks in the workplace. Such investigations are therefore not described in detail in this review.

Interesting findings/conclusions from these *in vitro* experiments include:

- CeO_2 NPs are able to be reduced in biological media. Generally metallic NPs with strong oxidant or reductive power can be cytotoxic and genotoxic towards biological targets *in vitro* (Auffan et al. 2009b).
- CeO_2 NPs (44 nm primary particle size) caused very little inflammatory response in human aortic endothelial cells after exposure to aerosols generated using flame spray pyrolysis, even at the highest concentration tested (50 µg/mL) (Gojova et al. 2009). The authors concluded the results indicate the material is rather benign compared to Y_2O_3 and ZnO NPs.
- The cytotoxicity of 23 different ENMs was tested using three standard *in vitro* assays and 10 different cell lines (Kroll et al. 2011). The authors found that even slight differences in their surface chemistry played an important role in triggering CeO_2 NP induced oxidative stress. However, none of the five CeO_2 NPs tested produced cell death or a change in the cells' metabolic activity.
- CeO_2 NPs interfered with the DCF fluorescence (oxidative stress), MTT light absorption (metabolic activity), and INT light absorption (cell death) assays at either the higher of two administered doses or both (10 or 50 µg/cm^2) (Kroll et al. 2011).
- Lanone et al. (2009) screened 24 different types of NPs using two cytotoxicity assays and two pulmonary cell lines, and found no correlation between cytotoxicity and equivalent spherical diameter or specific surface area. CeO_2 showed moderate cytotoxicity in the assays, with Cu-based and Zn-based NPs being the most toxic.

- CeO_2 NPs were not cytotoxic to lung macrophages or epithelial cells *in vitro* at any of the doses tested (15-240 µg/cm^2) (Rotoli et al. 2012).

7.4 In vivo toxicity

7.4.1 Acute inhalation or instillation

Srinivas et al. (2011) conducted an acute inhalation toxicity study with CeO_2 NPs[117] in rats. Rats were exposed in an inhalation exposure unit (nose-only exposure chamber) to an average aerosol concentration of 641 mg/m^3 CeO_2 NPs for 4 hours. This is an extraordinarily high concentration. Cytotoxicity, oxidative stress and inflammation were evaluated at 1, 2 or 14 days post-exposure. Cell viability in BALF measured by tryphan blue assay was significantly decreased from controls at all post-exposure periods. The percentage of neutrophils in BALF was significantly increased compared to controls, with the maximum increase of 44% observed 48 hours post-exposure. The concentrations of pro-inflammatory cytokines were significantly elevated in BALF and blood throughout the observation period. Histopathology of the lung on post-exposure day 14 revealed most animals had developed a moderate to marked level of multifocal microgranulomas distributed throughout the pulmonary parenchyma. Other extra-pulmonary organs collected (liver, spleen, kidney, thymus, brain) and examined did not show any CeO_2 NPs related toxic responses or the presence of NPs. The exposure concentration used by Srinivas et al. (2011) is much higher than the threshold for particle overload in the rat (Safe Work Australia 2009a), and considerably higher than expected exposure concentrations in the workplace. Therefore this test is of limited usefulness for informing hazards of CeO_2 NPs in the workplace.

Other authors have used intratracheal instillation as an indication for inhalation exposure to high concentrations.

CeO_2 NPs (20-30 nm)[118] were dose-dependently inflammogenic to the lungs of rats 24 hours and 4 weeks after intratracheal instillation of two high doses (50 or 150 cm^2/rat)[119] (Cho et al. 2010). At 4 weeks post-exposure there was a modest residual neutrophilic/mild cytotoxic inflammation still evident. These doses are approximately equivalent to or above the commencement of particle overload (Safe

[117] Average primary particle size of 55 nm by SEM; the generated aerosol had an average MMAD of 2.28 µm, with an average GSD of 2.94 (Srinivas et al. 2011).

[118] Although primary particle size of CeO_2 NPs was 20-30 nm, the particles formed aggregates of 88.1 $\pm$ 29.3 nm (Cho et al. 2010). The NPs were dispersed in 5% rat serum for administration.

[119] The equivalent mass doses of CeO_2 NPs were 625 µg/rat (150 cm^2) and 208 µg/rat (50 cm^2) (Cho et al. 2010). These doses are approximately equivalent to or above the commencement of particle overload (Safe Work Australia 2009a). Using the formula in Safe Work Australia (2009a, Appendix 2), these concentrations approximately equivalent to 21 and 7 mg/m^3 inhaled by a worker during a shift, respectively.

Work Australia 2009a), consequently the data is of little value for determination of explicit health hazards associated with CeO_2 NPs.

Another study investigated the inflammatory effects in rat after a single intratracheal instillation of 0.15, 0.5, 1, 3.5 or 7 mg CeO_2 NPs/kg body weight[120] (Ma et al. 2011). Exposure at all doses induced significant lung inflammation and cytotoxicity to lung cells[121]. The first three doses administered are below or at the doses resulting in particle overload (0.2 – 3 mg/rat) (Safe Work Australia 2009a), therefore this study may have some relevance for workplace exposure and may be an indicator that high but realistic work shift exposure concentrations of CeO_2 NPs (1-7 mg/m^3) could potentially be associated with inflammatory effects in the lung.

In a study by the same research group, Ma et al. (2012b) exposed rats to the same concentrations of CeO_2 NPs by a single intratracheal instillation. The treated animals were sacrificed at 1-, 3-, 10-, 28-, or 84-days post-exposure. Alveolar macrophages (AM) were isolated by bronchial alveolar lavage (BAL). CeO_2 exposure significantly increased fibrotic cytokine TGF-ß1 and osteopontin production by AM above controls. The collagen degradation enzymes, matrix metalloproteinase (MMP)-2 and -9 and the tissue inhibitor of MMP were markedly increased in the BAL fluid at 1 day and subsequently declined at 28 days post-exposure, but remained higher than in controls. Phospholipids in BAL fluid were elevated and hydroxyproline content in lung tissue was increased in a dose- and time-dependent manner. Morphological analysis revealed increased collagen fibres in the lungs exposed to 3.5 mg/kg CeO_2 and sacrificed at 28 days post-exposure. The authors concluded the results show that CeO_2 induced fibrotic lung injury in rats (Ma et al. 2012b).

The studies discussed above have all investigated the toxicity of CeO_2 NPs to the lung. Systemic toxicity in rats intratracheally instilled with 1, 3.5, or 7 mg/kg CeO_2 NPs[122] has been investigated by Nalabotu et al. (2011). Animals were sacrificed 28 days post exposure. Exposures were associated

[120] CeO_2 NPs (reported diameter of ~20 nm) were obtained from Sigma Aldrich. Nanoparticles were diluted in saline for animal exposures. In saline suspensions, CeO_2 NPs formed agglomerates of 0.5-3.5 µm in size (Ma et al. 2011). The first three doses administered are below or at the doses resulting in particle overload (0.2 – 3 mg/rat) (Safe Work Australia 2009a). The doses are approximately equivalent to 1, 3.4, 6.7, 23.6, and 47.1 mg/m^3 inhaled by a human worker over a work shift (Safe Work Australia 2009a, Appendix B).

[121] These effects were marked by statistically significantly increased numbers of alveolar macrophages, polymorphonuclear leukocyte infiltration, increased lactate dehydrogenase, increased serum albumin (all doses, most post-exposure times), and increased apoptosis of alveolar macrophages 24-hours post-exposure at all doses tested.

[122] The CeO_2 NPs (reported average diameter of 20 nm) were obtained from Sigma Aldrich, and saline was used as a vehicle for instillation (Nalabotu et al. 2011). The diameter of the primary CeO_2 NPs was determined to be 10.14 $\pm$ 0.76 nm by TEM, and agglomerates of 0.5 – 3.5 µm were formed in saline as described by Ma et al. (2011). The concentrations used by Nalabotu et al. (2011) are approximately equivalent to 6.7, 23.6, and 47.1 mg/m^3 inhaled by a human worker over the duration of a workshift (Safe Work Australia 2009a, Appendix B).

with increased liver ceria levels, reductions in liver weight, and evidence of liver damage (e.g. elevations in serum alanine transaminase, reduced albumin, diminished sodium-potassium ratio, decreased serum triglyceride levels, dose dependent hydropic degeneration, hepatocyte enlargement, sinusoidal dilatation and accumulation of granular material). However, the latter observed effects only reached statistical significance in the highest dose group. Only the reduction in the sodium-potassium ratio was statistically significant in the other dose groups. No histopathological alterations were observed in the kidney, spleen and heart. Overall, this study only observed systemic hepatic effects at the highest dose tested, approximately equivalent to a human inhaling 47.1 mg/m^3 of CeO_2 NPs over the duration of a work shift[123]. As such high exposures in controlled workplaces are unlikely (Section 7.6.1), the observed effects have limited applicability to the workplace.

7.4.2 Acute oral

No studies investigating the oral toxicity of CeO_2 NPs could be located.

7.4.3 Repeat inhalation

Cassee et al. (2012) exposed atherosclerosis-prone apolipoprotein E knockout mice by inhalation to diluted exhaust (1.7 mg/m^3 particle concentration, 20, 60 or 180 minutes, 5 days/week for 4 weeks) from an engine using standard diesel fuel (DE) or the same diesel fuel containing 9 ppm CeO NPs (DCeE). Changes in haematological indices, clinical chemistry, atherosclerotic burden, tissue levels of inflammatory cytokines and pathology of the major organs were assessed. Addition of CeO_2 NPs to fuel resulted in a reduction of the number (30%) and surface area (10%) of the particles in the exhaust, whereas the gaseous co-pollutants were increased (6-8%). There was, however, a trend towards an increased size and complexity of the atherosclerotic plaques following DE exposure, which was not evident in the DCeE group. There were no clear signs of altered haematological or pathological changes induced by either treatment. However, levels of proinflammatory cytokines were modulated in the brain region and liver following DCeE exposure. These results imply that addition of CeO_2 NPs to fuel may reduce atherosclerotic burden associated with exposure to standard diesel fuel. However, the authors recommended further testing to ensure there would be no chronic inflammatory response induced by the CeO_2 NPs.

Another set of authors conducted a repeat exposure inhalational study with CeO_2 NPs in mice (Aalapati et al. 2013). Male mice were subjected to nose only inhalation exposure of CeO_2 NPs[124] for 6 hours/day for 0, 7, 14 or 28 days with 14 or 28 days recovery time at an aerosol concentration of 2 mg/m^3. BALF analysis revealed the induction of pulmonary inflammation, as evident by an increase in the influx of neutrophils with a significant secretion of pro-inflammatory cytokines that lead to

[123] This was calculated using Equation A2 in Appendix 2 of Safe Work Australia (2009a).
[124] The CeO_2 NP aerosol had a MMAD (GSD) of 1.4 µm (2.4). Examination of the CeO_2 NP aerosols collected on nitrocellulose filters demonstrated the geometric mean diameter of the particles was 45 nm.

generation of oxidative stress and cytotoxicity, as is evident by induction of lipid peroxidation, depletion of GSH and increased BALF LDH and protein. The histopathological examination revealed that these inhaled CeO_2 NPs were located all over the pulmonary parenchyma, inducing a severe, chronic, active inflammatory response characterised by necrosis, proteinosis, fibrosis and well-formed discrete granulomas in the pulmonary tissue and tubular degeneration leading to coagulative necrosis in kidneys. ICP-OES results showed a significant accumulation of these NPs in the pulmonary and extra-pulmonary tissues, however a significant decline in Ce deposition was noted during the recovery period (by day 28 post-exposure, the concentrations of Ce in liver, kidney, heart, and brain were comparable to those seen at day 7). Together, these findings suggest that repeat inhalation exposure of CeO_2 NPs at concentrations relevant to workplace exposures may induce pulmonary and extra-pulmonary toxicity, especially since clearance of NPs was found to be low.

The very different results obtained in the two repeat inhalation toxicity studies described above may be attributed to the higher exposure concentration of pure CeO_2 NPs used by Aalapati et al. (2013). The CeO_2 NPs used in the study by Cassee et al. (2012) only made up a small fraction of the total particulate exposure of diesel exhaust.

7.4.4 Repeat oral

No studies investigating the oral toxicity of CeO_2 NPs could be located.

7.5 Genotoxicity

Auffan et al. (2009a) investigated the genotoxic potential of CeO_2 NPs by monitoring DNA single-strand breaks and micronuclei in dermal fibroblasts. The particles induced strong DNA lesions and chromosome damage related to oxidative stress even at low concentrations (i.e. 6×10^{-10} g/cell). Although the formation of aggregates was common in the cell culture medium[125] used, agglomeration or aggregation of CeO_2 NPs was not observed in a similar medium[126] by Kato et al. (2010). This discrepancy places uncertainty on the results observed.

7.6 Workplace implications

7.6.1 Exposure

Two publications have reviewed or predicted the potential human exposure as a result of using CeO_2 NPs as a fuel additive (Cassee et al. 2011b, Johnson and Park 2012), but only one was found in relation to potential occupational exposure (Leppaenen et al. 2012).

[125] The NPs had a primary particle size of approximately 7 nm, but easily formed aggregates ranging from 300 – 3000 nm in Dulbeco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal calf serum, glutamine, sodium pyruvate, and gentamicin (Auffan et al. 2009a).

[126] Kato et al. (2010) used Dulbeco's Modified Eagle's Medium (DMEM), supplemented with 10% heat-inactivated foetal bovine serum, penicillin, streptomycin and amphotericin B.

In this study, exposure levels of CeO_2 NPs (20-40 nm primary particle size) were measured during enclosed flame spray processes used for coating and surface modification of materials (Leppaenen et al. 2012). The average particle number concentration varied from 4.7 x 10^3 to 2.1 x 10^5/cm^3 inside the enclosure, and from 4.6 x 10^3 to 1.4 x 10^4/cm^3 outside the enclosure. These concentrations are 0.2 to 10.5 times (inside enclosure) and 0.2 to 0.7 times (outside enclosure) the German IFA (2012) benchmark of 20,000 particles/cm^3 for metal oxide NPs (Appendix C). This suggests that in some instances, current occupational exposures to CeO_2 NPs may not be adequately controlled. However, it should be noted that the latter benchmark is not substantiated toxicologically, and therefore it is unknown if such exposures would be associated with health effects. The average mass concentrations of the particles in the study were 320 and 66 µg/m^3 inside and outside of the enclosure, respectively (Leppaenen et al. 2012). The particles were present mainly as chain-like aggregates (~500-1000 nm), which supports the notion that workers' inhalational exposure to ENMs is potentially more likely to be to aggregates, rather than primary particles.

7.6.2 Hazards

Overall, the results from biodistribution and toxicological studies have shown inhaled CeO_2 NPs can distribute systemically from the lungs to other tissues and organs in the body (e.g. liver, kidney, spleen, testis, brain). Being highly insoluble, CeO_2 NPs are slowly eliminated, and concentrations may bioaccumulate in the body.

No chronic toxicological information was found for CeO_2 NP exposure, and no toxicity information was found relating to oral exposure. However, a biodistribution study by He et al. (2010) suggests absorption of CeO_2 NPs from the GI tract is likely to be low.

The usefulness of data generated on the toxicity of CeO_2 NPs via inhalation or intratracheal instillation is hampered by the use of unrealistic exposure concentrations. However there are a few studies which have used reasonable exposure concentrations. From these studies, the following can be tentatively concluded with respect to workplace hazards of CeO_2 NPs:

- Based on a study using intratracheal instillation in rats (Ma et al. 2011), high but realistic work shift exposure concentrations of CeO_2 NPs in the order of 1-7 mg/m^3 may potentially be associated with inflammatory effects in the lung.
- Inhalation exposure to low concentrations of CeO_2 NPs, such as when used as additives in diesel exhaust is unlikely to result in adverse effects in the short term. Effects from long term exposures have not been investigated.
- Exposures during enclosed flame spray processes used for coating and surface modification of materials were on average 320 and 66 µg/m^3 inside and outside of the enclosure, respectively (Leppaenen et al. 2012). Presumably, flame spraying is likely to reflect an occupation associated with high-end exposure to CeO_2 NPs.

- Repeat inhalation exposure of CeO_2 NPs at concentrations potentially relevant to high-end workplace exposures (i.e. 2 mg/m^3) may induce pulmonary and extra-pulmonary toxicity, especially since clearance of NPs was found to be low.

7.7 Summary and conclusions

CeO_2 nanoparticles have low solubility and are potentially retained in the lungs. High inhalation exposures have resulted in typical particle pathology changes in the lung. Biokinetic studies have been peformed by measuring the fate of cerium, rather than the nanoparticle *per se*. However because they are poorly soluble and stable it is presumed tissue cerium concentrations are reasonably associated with particulates. Soon after inhalation of moderate amounts of nanoceria approximately 25% is excreted in faeces, of this more than 90% in the first 24 hours. This indicates the initial clearance from the lung is via the mucociliary ladder and gastrointestinal absorption is limited. Once in the systemic circulation CeO_2 nanoparticles may be widely distributed with the highest tissue concentrations found in the reticuloendothelial system. Once there they are retained for a long time.

A mode of toxicological action has not yet been assigned to nanoceria. Nevertheless *in vitro* and high exposure *in vivo* inhalation and intratracheal investigations indicate a typical oxidative stress and inflammatory response associated with biopersistent particulates; including formation of pulmonary granulomas. Not unexpectantly for a particle that produces oxidative stress after entering cells, nanoceria can cause DNA strand breaks in *in vitro* systems.

8. Silver nanoparticles

Johnston et al. (2010b) have reviewed the *in vivo* and *in vitro* toxicity of silver and gold particulates with respect to their attributes and biological mechanisms responsible for the observed toxicity. These authors contend that of all the metal ENMs, the quantity of available hazard information is arguably greatest for silver particulates (Ag-NPs). This is due to their widespread inclusion in a number of diverse products (including clothes and wound dressings) that take advantage of their antibacterial behaviour.

8.1 Distribution

The intracellular distribution of Ag-NPs is primarily to lysosomes. However it is not possible to state that the silver content of any cell distribution is due to silver nanoparticles or to silver ions, since the analytical methods measuring silver in tissues do not discriminate between them (Johnston et al. 2010b).

If intravenously administered, the liver, spleen and kidneys are the primary sites of accumulation of Ag-NPs. But increased concentrations in other organs are also noted (Johnston et al. 2010b, Tiwari et al. 2011). Presumably if Ag-NPs are absorbed across the lung a similar tissue distribution will occur.

Blood kinetics and tissue distribution of 20, 80 and 110 nm Ag-NPs were investigated in rats after intravenous administration once daily for 5 consecutive days. Following both single and repeated injection, Ag-NPs disappeared rapidly from the blood and distributed to all organs evaluated (liver, lungs, spleen, brain, heart, kidneys and testes) regardless of size. The 20 nm particles distributed mainly to liver, followed by kidneys and spleen, whereas the larger particles distributed mainly to spleen followed by liver and lung. In the other organs evaluated, no major differences between the sizes were observed (Lankveld et al. 2010). The authors developed a physiologically based pharmacokinetic (PBPK) model describing the kinetics.

Leavans et al. (2012) used isolated perfused porcine skin flaps to determine that Ag-citrate NP and Ag-silica NP distribution was greater than the extravascular space and it was most likely Ag^+ that was distributing from the vascular space into the interstitial fluid[127].

After three intravenous injections of Ag-NPs[128] to pregnant mice on gestation days 7, 8 and 9. At gestation day 10, Ag-NPs distributed to most maternal organs, extra-embryonic tissues, and to a small extent embryos, but did not accumulate significantly in embryos. Thus silver accumulation was significantly higher in liver, spleen, lung, visceral yolk sac, and endometrium compared with other organs from silver NP-treated mice. A similar distribution was observed with $AgNO_3$ injections. For Ag-NPs very little silver reached the developing embryos (less than 0.01% of total injected silver). The percentage of total injected silver measured in embryos was about 25-fold less than that seen in the placenta or visceral yolk sac, suggesting that these extra-embryonic tissues did not transfer large quantities of silver from mother to embryo. (Austin et al. 2012).

It is noted that all studies have the liver as the organ with the highest amount of accumulated Ag-NPs from the systemic circulation. Thus for identification of hazards associated with inhaled Ag-NPs in the workplace particular attention should be given to liver effects (as well as respiratory) in animal inhalation experiments.

8.2 Mode of action

There is a growing body of evidence indicating the toxicological effects of Ag-NPs may be influenced more by silver ions than their nano-form. For example:

[127] The interstitial fluid is the solution that bathes and surrounds the cells of tissues.

[128] The intravenous doses (in citrate buffer) used by Austin et al. (2012) into pregnant CD-1 mice on gestation days 7, 8, and 9 at dose levels of 0, 35, or 66 µg Ag/mouse. The average agglomerate particle diameter was determined to be approximately 50 nm by TEM. Note evaluation was one day after the last injection.

- Ag^+ is released from Ag-NPs by surface oxidation (Liu and Hurt 2010).
- Kittler et al. (2010) showed the toxicity of Ag-NPs increases during storage due to the release of Ag^+.
- The initial Ag^+ concentration in Ag-NP suspensions contributes greatly to the toxicity of Ag NPs *in vitro* (Beer et al. 2011, 2012). The size of the Ag-NPs may still be important as it would be expected more release of Ag^+ from smaller particles.
- Studies suggest that the toxicity of Ag-NPs is due to a so-called Trojan-horse mechanism. Ag-NPs are taken up by the cells, partition to lysosomes where the lower pH facilitates subsequent intracellular release of Ag^+. This leads to excess ROS and cytotoxicity mediated oxidative stress pathways being activated to initiate cell death (via apoptosis) and genotoxic damage (Miura and Shinohara 2009, Park 2010a, b; Karlsson et al. 2009, Liu et al. 2010a, Midander et al. 2009, Chairuangkitti et al. 2012, Foldbjerg et al. 2009, Cronholm et al. 2013).
- The cytotoxic effects of citrate-coated Ag-NPs on cultured rat brain endothelial cells was explained by the intrinsic toxicity of the Ag^+ released from the particles (Grosse et al. 2013).
- In some studies the cytotoxicity of $AgNO_3$ is greater than Ag-NPs[129] (e.g. Miura and Shinohara 2009).
- Many studies have shown the genotoxicity of Ag-NPs is likely due to Ag^+ and associated oxidative stress (Section 8.5).
- The uptake of 'solid' particles and ensuing release of metal ions occurs with other metal-NPs, e.g. CuO-NP, cobalt oxide (Co_3O_4)-NP and manganese oxide (Mn_3O_4)-NPs (Cronholm et al. 2013).

Selected recent studies supporting the notion that Ag^+ is the toxic component of Ag-NPs are briefly described below.

Cronholm et al. (2013) used human lung cell lines and cytotoxic and DNA damage to investigate the role of a Trojan horse type mechanism for the toxicity of Ag-NPs and copper oxide nanoparticles (CuO-NPs). They found high uptake of CuO-NP and Ag-NPs compared to no uptake of $CuCl_2$, or low uptake of $AgNO_3$. CuO-NP induced both cell death and DNA damage whereas $CuCl_2$ induced no toxicity. The opposite was observed for silver, where Ag-NPs did not cause any toxicity, but $AgNO_3$ induced a high level of cell death. Cytotoxicity and DNA damage was evaluated after 4 hours exposure of cells to the NPs. At 4 hours the intracellular aggregates of CuO-NP were totally removed but those of Ag-NPs were still intact, it was not until 24 hours that aggregates of Ag-NPs were

[129] In Miura and Shinohara (2009) the higher cytotoxicity of Ag^+ cultured HeLa cells may partially be due to agglomeration of the Ag-NPs in the culture medium. In ultra pure water the Ag-NPs were well dispersed with uniform diam 2 – 5 nm whereas in culture medium there were agglomerates >50nm diam. It is also noted the Ag-NPs were purchased from an industrial engineering company and included a protectant (patented material) to suppress ionising of Ag. Other studies with Ag-NPs purchased from commercial suppliers do not mention the inclusion of stabilisers, one wonders if this is a common practice and how it may influence toxicity studies.

observed to decrease in size. The authors concluded CuO-NP toxicity is predominantly mediated by intracellular uptake and subsequent rapid release of copper ions. No toxicity was observed for Ag-NPs due to only low release of silver ions within the short time period used for assessing biological response. The results are consistent with the Trojan horse type mechanism.

Liu et al. (2010a) using Ag-NPs of ~ 5, 20 and 50 nm demonstrated that smaller Ag-NPs are more easily taken up by cells[130] and have greater effects than larger Ag-NPs. At equal concentrations (1 µg/mL) in the culture media the intracellular silver concentration after 6 hour incubation with Ag-NPs_{5nm} was approximately the same as with $AgNO_3$ but 3 – 5 times higher than for the other Ag-NPs. Furthermore Ag^+ at the same incubation concentrations as Ag-NPs_{5nm} produced the same spectrum of effects to a similar extent as Ag-NPs_{5nm}. Park et al. (2011b) also found small Ag-NPs had greater toxicity in a range of tests (Section 8.3) than larger ones. In addition, depending on cell type, the potency of small Ag-NPs on metabolic activity and cell membrane integrity was about the same as, or slightly more potent, than for Ag^+.

Notwithstanding the above, Powers et al. (2011) in an unusual cultured cell system[131] presents evidence that Ag-NPs work through a combination of Ag^+ release and mechanisms that may be a reflection of the nanoform in combination with the chemistry of silver.

8.3 In vitro toxicity

While there are many studies investigating *in vitro* cellular toxicity and genotoxicity (Sections 8.2 & 8.5), there is a lack of *in vivo* toxicity information that allows correlations between *in vitro* and *in vivo* findings to be made. This is exacerbated by the excessively high particle concentrations frequently used in *in vitro* experiments.

Park et al. (2011b) studied the effects of silver nanoparticles of different sizes (20, 80, 113 nm) in *in vitro* assays for cytotoxicity, inflammation, genotoxicity and developmental toxicity[132]. In all toxicity

[130] Liu et al. (2010a) utilised four human cell models (A549, SGC-7901, HepG2 and MCF-7). Endpoints included cell morphology, cell viability, cellular membrane integrity, oxidative stress and cell cycle progression. 'Intracellular' concentrations of Ag were determined after washing cells three times, however as pointed out by others membrane-bound Ag-NPs or Ag^+ may not be completely excluded.

[131] Powers et al. (2011) used PC12 cells which are derived from a rat pheochromocytoma (adrenal medulla cancer). The cells differentiate when treated with nerve growth factor and are frequently used as a model for differentiation of sympathetic neurons (Das et al. 2004, Joen et al. 2010).

endpoints studied, silver nanoparticles of 20 nm were more toxic than the larger nanoparticles. They concluded:

- The effects of silver nanoparticles on different toxic endpoints may be the consequence of their ability to inflict cell damage by decreasing metabolic activity. Interestingly the generation of ROS in macrophages occurred only at concentrations above those decreasing metabolic activity, the authors surmised generation of ROS was a secondary effect rather than causing the onset of cytotoxicity.
- The potency of silver in the form of nanoparticles to induce cell damage compared to silver ions is cell type and size-dependent.

A similar technique to that used in the genotoxic screening assay of Duffin et al. (2013) (Section 8.5) has been applied to determine the relative potency of different Ag-NPs to generate intracellular oxidative stress (Prasad et al. 2013). Three key stress-responsive pathways were evaluated in a high throughput battery of stable luciferase-reporter HepG2 cell lines. All pathways were activated by the Ag-NPs but with different potency. Citrate-coated and PVP-coated Ag-NPs had higher intracellular silver concentrations compared with $AgNO_3$ (ionic). The smaller (10-nm) were more potent than the larger (75-nm) regardless of whether they were coated or not[133]. As judged by the determined intracellular concentrations of silver, $AgNO_3$ was markedly more potent than any of the Ag-NPs. Consequently, and because the cellular stress response profiles after Ag-NP exposure were similar to that of $AgNO_3$, the authors concluded the oxidative stress and inflammatory effects of Ag-NPs are likely due to the cytotoxicity of silver ions.

It is apparent, and perhaps not surprising that the *in vitro* cytotoxic effect of Ag-NPs is dependent on dose, exposure time and on the cell line tested. Differences exist between the sensitivity of cell lines which can potentially be understood in terms of their natural antioxidant levels (Mukherjee et al. 2012).

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[132] Park et al. (2011b) sourced the Ag-NPs from a commercial fabricator of nanoparticles and characterised in phosphate buffer rather than cell incubation media. Cytotoxicity was investigated in L929 murine fibroblasts and RAW 264.7 murine macrophages, and effects of Ag-NPs compared to Ag^+. Measured were generation of ROS and parameters of inflammation. Developmental toxicity was investigated *in vitro* in mouse embryonic stem cells (the end point was inhibition of embryoid body differentiation measured as number of spontaneously contracting myocardial foci). Effects of Ag-NPs on embryonic stem cell differentiation occurred only at concentrations similar to or above those decreasing metabolic activity, indicating the effects on stem cell differentiation were a direct result of cytotoxicity, rather than a specific effect of the nanoparticles on differentiation of the embryonic stem cells. Genotoxicity (mutation frequency in the *lacZ* gene) was assessed in embryonic fibroblasts, in this system significant DNA damage was not detected.

[133] In Prasad et al. (2013) the hydrodynamic diameters for the aggregated Ag NP in culture medium ranged from 66.2-77.2 nm for the 10-nm Ag NP and 119-170 nm for the 75-nm Ag NP.

As discussed with other ENMs in Section 3.2, protein adsorption has a significant effect on the biological response to clusters of silver nanoparticles (Ag-NCs)[134] in cultured cells (Shang et al. 2012). These are ultra-small ENMs that are very efficiently taken up by cells. The amount of Ag-NCs internalised by cells is substantially reduced in the presence of human serum albumin and correspondingly decreases the cytotoxicity.

8.4 In vivo toxicity

In an experiment reminiscent of the *ex-vivo* isolated organ bath systems of classical pharmacology, Gonzalez et al. (2011) studied the contraction of rat tracheal rings before and after exposing them to Ag-NPs[135]. Ag-NPs by themselves did not modify the basal smooth muscle tone of rat trachea, but after treatment with acetylcholine the Ag-NPs induced contraction. Ag-NPs modified the contractile action of acetylcholine on the trachea through nitrogen oxide production. The effects were not observed with bulk $AgNO_3$. The authors speculated Ag-NP exposure could possibly induce hyper-reactivity of tracheal smooth muscle but this hypothesis has not yet been investigated in whole animal studies.

Jun et al. (2011) has shown Ag-NPs can increase platelet aggregation and pro-coagulant activation in freshly harvested human or rat platelets. The effect occurred in a concentration dependent manner starting from 100 µg/ml. Silver metal microparticles (5–8 mm) induced aggregation was substantially weaker. Platelets obtained from rats 6 hours after intratracheal administration of Ag-NPs (~4 mg/rat) aggregated faster when stimulated with thrombin. Since platelet aggregation and procoagulant activation are key contributors to thrombotic diseases, on face value this experiment appears relevant for human risk assessment. However the concentrations of Ag-NPs *in vitro* to cause platelet aggregation and the *in vivo* intratracheal dose are very high. It seems highly unlikely these blood concentrations, or airborne concentrations matching the intratracheal dose, would be achieved in workplace exposure scenarios.

8.4.1 Acute inhalation

Sung et al. (2011b) followed OECD Test Guideline 403 under GLP[136] to investigate the acute inhalation toxicity (4 hour exposure) of Ag-NPs to rats[137] and observed them for 14 days. No

[134] Average diameter of Ag-NCs was 1.3 nm ± 0.3 nm, hydrodynamic diameter of dihydrolipoic acid-capped Ag-NCs (DHLA–Ag-NCs) in PBS was 2.1 nm ± 0.4 nm (Shang et al. 2012).

[135] The Ag-NPs in Gonzalez et al. (2011) were purchased from Sigma chemical company with nominal diameter of 45 nm, they were dispersed in Krebs – Henseleit physiologic solution in which diameter was 4 to 90 nm.

[136] GLP = Good Laboratory Practice. OECD Guideline is primarily conducted to determine the LC_{50} (Lethal Concentration to 50% of animals) which is used for hazard classification purposes.

significant body weight changes or clinical effects were observed, and there were no significant differences from controls in lung function tests at exposures up to the top concentration of 750 µg/m^3. The authors note that due to technical difficulties in generating aerosols of Ag-NPs a short term exposure lethal dose to rats may not be achievable. They nominated the LC_{50} as > 750 µg/m^3 (the concentration of the top dose).

8.4.2 Repeat inhalation exposure

- *10 day:* In contrast to *in vitro* studies, when mice were exposed (whole body) sub-acutely by inhalation to Ag-NPs (3.3 mg/m^3, 4 hours/day, 10 days, 5 ± 2 nm primary size)[138] there were minimal inflammatory or toxicological effects on the lung as judged by traditional measurements in bronchoalveolar lavage fluid or by histopathology (Stebounova et al. 2011). The median retained dose of silver in the lungs was 31 µg/g lung (dry weight, dw) immediately after the final exposure, 10 µg/g following a 3-wk rest period. Silver concentrations in the heart, liver, and brain were all below the detection limit (1.8 µg/g dw). The authors calculated 803 µg Ag/g lung (dw) was delivered to the lungs and the amount of Ag detected immediately after the final exposure corresponded to a hypothetical lung burden accumulated by a 70 kg person exposed to 1.0 mg/m^3 for 16.6 hours. The authors considered their observations with Ag-NPs are in agreement with other studies, which indicate that much higher exposure doses of Ag-NPs than other metals (e.g. Cu NPs, TiO_2 NPs) are needed to induce significant inflammation.
- *28 day:* Hyun et al. (2008) evaluated upper respiratory tract effects in rats after 6 h per day, 5 times a week for 28 days at 0.5 µg/m^3, 3.5 µg/m^3 and 61 µg/m^3. The authors concluded that while the Ag-NPs influenced the pattern of neutral mucins in the respiratory mucosa, the results did not suggest any toxicological significance in this model.

  Ji et al. (2007) exposed Sprague-Dawley rats to Ag-NPs (1.98–64.9 nm), at 0.48 µg/m^3 , 3.48 µg/m^3 , and 61 µg/m^3 for 6 h/day, 5 days/week for 28 days following OECD test guideline 412. The authors concluded that the exposures did not appear to have any significant health effects in the rats.
- *90 day:* Daily 6 hour (5d/week) whole body exposure of rats for 90 days caused histological demonstrable alveoli inflammation and alterations in lung function at the highest exposure

[137] Seven-week-old rats, weighing approximately 218 g (males) and 153 g (females), were exposed to Ag-NPs (average diameter 18-20 nm) for 4 hours in a whole-body inhalation chamber. Fresh-air control, low-dose (0.94x10^6 particle/cm^3, 76 µg/m^3), middle-dose (1.64 x10^6 particle/cm^3, 135 µg/m^3), and high-dose (3.08x10^6 particle/cm^3, 750 µg/m^3) (n=5/gp).

[138] In the exposure chamber of the study by Stebounova et al. (2011) there were agglomerates of mean particle size 79 ± 1.5 nm. Only one exposure was used.

tested[139]. There were clear dose related increases in blood levels of silver with accumulation evident in the liver, olfactory bulb, brain, and kidneys. There were no significant dose-related differences in the haematology and no significant dose-related differences in the blood biochemistry. Toxicity was observed in the liver as minimal bile-duct hyperplasia at the top dose but serum liver enzymes were unchanged (Sung et al. 2009). Because these were whole body exposures it is uncertain whether the liver observations were the result of translocation of silver from the lung or due to ingestion from grooming. Particularly when it is considered that oral Ag-NPs produce systemic toxicity (Johnston et al. 2010b). A similar exposure regime was negative for the bone marrow micronucleus test (Kim et al. 2011b). However this may be because silver did not reach the bone marrow, silver content of bone marrow was not measured.

Concentrations used in the Sung et al. (2009) study were based on the ACGIH silver dust threshold limit value (TLV) of 100 µg/m^3; therefore in terms of mass dose, the low, middle, and high doses were approximately 0.5, 1, and 5 times the ACGIH silver dust TLV. The NOAEC in Sung et al. (2009) is 133 µg/m^3 and the ACGIH TLV for soluble silver (i.e. Ag^+) is 10 µg/m^3.

It is not inconceivable that the lack of effect in Stebounova et al. (2011) at high concentrations (3,300 µg/m^3) is consistent with the NOAEC of 133 µg/m^3 from Sung et al. (2009). The difference in concentration could be due to lower exposure times (4 hr/d x 10d vs 6hr/d x 90d) and different species (mouse vs. rat).

8.4.3 Repeat oral exposure

Ag-NPs induce subchronic oral toxicity in 28- and 90-day oral studies.

- The NOAEL and LOAEL in a 28 day study were 30 and 300 mg/kg, respectively. This was based on significant dose-dependent changes in blood alkaline phosphatase and cholesterol suggesting slight liver damage. There were no statistically significant differences in the micronucleated polychromatic erythrocytes suggesting that Ag-NPs did not induce genetic toxicity in the bone marrow. The tissue distribution of silver showed a dose-dependent accumulation in all tissues examined. There was twice as much silver in the kidneys of female rats compared to males (Kim et al. 2008).

- In a 90-day oral study the authors suggested a NOAEL of 30 mg/kg and LOAEL of 125 mg/kg (Kim et al. 2010b). The difference in the LOAEL from the 28 day oral study reflects different

[139] The exposures in Sung et al. (2009) were 49 µg/m^3 (equivalent to 0.6 × 10^6 particles/cm^3), 133 µg/m^3 (1.4x10^6 particle/cm^3), and 515 µg/m^3 (3.0x10^6 particle/cm^3). The experimental procedures followed OECD test guideline 413 and GLP.

dose spacing in the two studies[140]. However all treated groups had a higher incidence of bile duct hyperplasia and liver necrosis, albeit with no dose response, than the control group in which the incidence was 0% for both lesions. Without further clarification it would be prudent to consider that this study did not identify a clear NOAEL. Tissue concentrations were higher in females than males.

8.4.4 Safety tests

Kim et al. (2012a) evaluated the genotoxicity, acute oral and dermal toxicity, eye irritation, dermal irritation and corrosion and skin sensitisation of commercially manufactured Ag-NPs[141] according to the OECD test guidelines and GLP. The Ag-NPs were not found to induce genotoxicity in a bacterial reverse mutation test and chromosomal aberration test, although some cytotoxicity was observed. This is different from the positive micronuclei results of Jiang et al. (2013, Section 8.5) using the same test system. It is noted the Ag-NPs in Jiang et al. (2013) were coated with BSA but in Kim et al. (2012a) the Ag-NPs were pristine and dispersed in citric acid for adding to the test systems. In acute oral and dermal toxicity tests using rats, none of the rats showed any abnormal signs or mortality at a dose level of ~ 2,000 mg/kg. Similarly, acute eye and dermal irritation and corrosion tests using rabbits revealed no significant clinical signs or mortality and no acute irritation or corrosion reaction for the eyes and skin. In a skin sensitisation test (guinea pig maximisation test), one animal (1/20) showed discrete or patchy erythema, thus Ag-NPs were classified as weak skin sensitisers. Maneewattanapinyo et al. (2011) assessed the acute oral toxicity of Ag-NPs and found no significant effects[142] with doses up to 5,000 mg/kg. All toxicity tests in this study suggest that colloidal Ag-NPs could be relatively safe when administered orally, or to the eye or skin for short periods of time. It is known that long-term exposure to colloidal silver or silver salts affects human skin and eyes, resulting in irreversible pigmentation of the skin and pigmentation of the eyes (citations in Kim et al. 2012a).

[140] Dose spacing in the Kim et al. (2012a) 28 day oral study was 30, 300 and 1,000 mg/kg/d but in 90 day study of Kim et al. (2010b) it was 30, 125 and 500 mg/kg/d.

[141] Kim et al. (2012a) conducted safety tests on Ag-NPs obtained from ABC Nanotech Co., Ltd. (Daejeon, Korea) and dispersed in 1% citric acid. Their physico-chemical properties were received from the manufacturer. Further characterisation was not undertaken.

[142] Maneewattanapinyo et al. (2011) used Ag-NPs with a spherical configuration and primary particle diameter of 10–20 nm dispersed in distilled water, Ag^+ was very low (less than 0.04%). The haematological and blood chemistry analysis showed no significant differences from control mice. There were no gross or histopathological lesions in various organs. The LD_{50} was greater than 5,000 mg/kg body weight. In the acute eye irritation and corrosion study, no mortality and toxic signs were observed during 72 hr observation period after various doses were instilled into guinea pig eyes. However, the instillation of Ag-NPs at 5,000 ppm produced transient eye irritation at the 24 hr observation time. No gross abnormality was noted in the skins of the guinea pigs exposed to various doses of colloidal Ag-NPs and no significant Ag-NP exposure relating to dermal tissue changes was observed microscopically.

8.5 Genotoxicity

Jiang et al. (2013) using multiplatform genotoxicity analyses in CHO cells[143] showed Ag-NPs[144], but also silver ions (Ag^+), induced bulky-DNA adducts, 8-oxodG[145] and micronuclei formation in a concentration-dependent manner. However there were quantitative and qualitative differences between the particulate and ionic form of silver, Ag^+ being more potent than Ag-NPs. Specifically, Ag^+ induced the formation of bulky DNA adducts and micronuclei approximately 2-fold more than Ag-NPs. The intracellular IC_{50} for the concentration dependent inhibition of mitochrondrial activity indicated Ag^+ were more toxic than the Ag-NPs. In addition Ag^+ induced more ROS than Ag-NPs at the same silver concentration. ROS has been considered the major source of spontaneous damage to DNA (Foldbjerg et al. 2011; Kim et al. 2011c). Paradoxically the amount of 8- oxodG was 44 % higher in cells exposed to Ag-NPs compared to cells exposed to Ag^+. The Ag-NPs were located in endosomes and/or lysosomes, not in mitochondria or the nucleus. Absence of Ag-NPs in the nucleus suggests the genotoxicity may be indirect rather than direct. Eom and Choi (2010) reached this conclusion when investigating oxidative stress endpoints in cultured Jurkat T cells; both Ag-NPs and Ag^+ induced similar levels of cellular reactive oxygen species during the initial exposure period.

De Lima et al. (2012) have reviewed the *in vitro* and *in vivo* cytotoxicity and genotoxicity of Ag-NPs in a variety of organisms and concluded human cells have a greater resistance to the toxic effects of Ag-NPs in comparison with other organisms. It is however obscure how this conclusion was derived as the review does not contain a quantitative analysis of dose (or concentration) effect responses for the different cells.

As noted previously the bone marrow micronucleus test was negative after 90 days exposure to concentrations that had been shown to cause lung and liver toxicity (Sung et al. 2009, Kim et al. 2011b).

8.6 Workplace implications

8.6.1 Occupational exposures

Lee et al. (2012c) estimated the potential exposure of workers; personal sampling, area monitoring, and real time monitoring were conducted over 3 days using a scanning mobility particle sizer and dust

[143] The Chinese hamster ovary cell line CHO-K1 was chosen by Jiang et al. (2013) because it is recommended by the OECD for use in the micronucleus genotoxicity assay.

[144] Jiang et al. (2013) used BSA coated Ag NPs (mean particle size = 15.9 ± 7.6 nm, n = 490) that had been previously characterised (Foldbjerg et al. 2012).

[145] The DNA oxidative adduct 8- oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG) is characteristic of DNA damage by ROS.

monitor at a workplace where the workers handle nanomaterials. The area sampling concentrations[146] obtained from the injection room showed the highest concentration, ranging from 0.00501 to 0.28873 mg/m^3. (Note the precision of these numbers is not commensurate with the accuracy of the measurements). None of the area samplings obtained from other locations showed a concentration higher than 0.0013 mg/m^3. The personal sampling concentrations ranged from 0.00004 to 0.00243 mg/m^3 over the 3 days of sampling, these are comparable with previous measurements (Lee et al. 2011, 2012a). The authors stress the importance of obtaining robust background measurements of airborne nanoparticles as being essential in being able to interpret measurements taken when nanoparticle manufacturing processes are operational.

8.6.2 Hazards

Overall, toxicity of Ag-NPs in animal experiements is low. An acute inhalation toxicity study found no significant clinical effects on rats at any of the exposure concentrations (up to 750 µg/m^3) (Sung et al. 2011b). The concentrations at which no adverse effects (NOAECs) were observed in repeat inhalation studies with Ag-NPs are summarised in Table 8.1.

[146] In the study of Lee et al. (2012c) the particle number concentrations at the silver nanoparticle manufacturing workplace were 911,170 (1st day), 1,631,230 (2nd day), and 1,265,024 (3rd day) particles/ cm^3 with a size range of 15–710.5 nm during the operation of the reactor, while the concentration decreased to 877,364.9 (1st day), 492,732 (2nd day), and 344,343 (3rd day) particles/cm^3 when the reactor was stopped.

Table 8.1: Repeat inhalation studies with Ag-NPS

Exposure duration	Critical effect	NOAEC (µg/m^3)	Reference
10-day	None (NOAEC = highest dose tested)	3,300 [a]	Stebounova et al. 2011
28-day	None (NOAEC = highest dose tested)	61	Hyun et al. 2008 Ji et al. 2007
90-day	Alveoli inflammation and alterations in lung function	133	Sung et al. 2009

[a] The lack of effect in Stebounova et al. (2011) at high concentrations (3,300 µg/m^3) compared with the NOAECs from the other studies could be due to lower exposure times and different species (mouse vs. rat).

With respect to oral hazards, a 28-day oral toxicity study identified a NOAEL of 30 mg/kg for liver damage in rats administered with Ag-NPs (Kim et al. 2008), and no clear NOAEL can be delineated from a 90-day oral study (Kim et al. 2010b).

8.6.3 Workplace exposure limits

Based on differences in toxic concentrations between nanoparticles of different sizes in *in vitro* test systems Park et al. (2011b) suggest that derivation of workplace exposure limits based on mass concentrations (i.e. mg Ag-NPs/m^3) is inappropriate. They suggest derivation of safe exposure limits (WESs) for Ag-NPs should be done on a case-by-case approach. 'Case-by-case' derivation of WESs implies there will be data available for each Ag-NP, clearly this will not be the case. In addition, such an approach does not easily meld with existing regulatory systems, or benchmarks used by industry for control of exposure to workplace substances.

Lee et al. (2011, 2012c) use the WES set by the ACGIH of 0.1 mg/m^3 for silver dust and 0.01 mg/m^3 for silver soluble compounds as the benchmarks for hygiene interpretation of their measurements. While it is debatable whether these are ideally suitable for Ag-NPs, it is noted the weight of available hazard information points to silver ions as being a significant contributor to the toxicity observed with Ag-NPs.

It is acknowledged it is a complex question whether there is a higher risk associated with exposure to Ag-NPs compared to similar airborne concentrations of silver ions. There are a number of factors that impart uncertainty on such considerations. For example, organ bioavailability of Ag-NPs vs Ag^+, relative distribution pattern of both forms of Ag, the Ag-NP size and likely surface chemistry which affect Ag^+ release or intracellular generation once inside the cell. Nevertheless the WES for soluble silver compounds[147] might be a pragmatic starting point for establishing an interim exposure limit for Ag-NPs. On the assumption that the potency of small Ag-NPs (Liu et al. 2010a, Park et al. 2011b), in some test systems, may be greater than Ag^+ *per se* a precautionary uncertainty factor of 5 or 10 could

[147] The reader should note that the basis of the ACGIH TLVs for silver dust and soluble silver has not been assessed for this review. It would be essential for any person wishing to apply, or modify these limits for exposure to Ag-NPs to thoroughly understand the empirical basis upon which they were set.

be applied to the 0.01 mg/m^3 exposure limit for soluble silver compounds. However, there is some evidence indicating silver ions are toxicologically more potent than Ag-NPs. This suggests the existing WES for soluble silver compounds (without application of an uncertainty factor) may be suitable for Ag-NPs.

It would also be possible to use the 90 day NOAEC of 133 µg/m^3 from Sung et al. (2009), make adjustments for the exposure time, comparative regional distribution of small particulates in rats and humans with uncertainty factors to account for less than chronic exposure to derive a WES for Ag-NPs.

8.6.4 Risk assessments

Park et al. (2011b) note the concentrations inducing cytotoxicity were much higher than those reported to have antimicrobial activity. They suggest there may be a margin of safety between exposure for antimicrobial application of Ag-NPs and adverse effects observed in human cells. The challenge for assessing risk from biocide use of Ag-NPs in various products is to quantitate the range of 'margin of safety(s)'.

Quadros and Marr (2011) have evaluated the exposure to silver from consumer products that claim to contain silver nanoparticles or ions. Products emitted 0.24-56 ng of silver in aerosols (1 – 25 µm) per spray action. The size of the emitted aerosol was largely independent of the silver size distributions in the liquid phase. Exposure modelling suggested up to 70 ng of silver may deposit in the respiratory tract during product use.

Using information in the open literature, and following the classical regulatory risk assessment approach, Christensen et al. (2010) has undertaken a human health risk assessment for Ag-NPs that includes consideration of occupational exposures. They found that gaps in the available data set, both in relation to exposures and hazard do not allow definite conclusions to be made that could be used for regulatory decision making. However they concluded the available information indicates that repeated inhalation of Ag-NPs in the workplace, and possibly inhalation by consumers, has associated risks of health effects.

8.7 Summary and conclusions

Intravenous studies with Ag-NPs show silver accumulating in the liver, spleen and kidneys but increased concentrations in other organs are also noted.

There is a growing body of evidence indicating the toxicological effects of Ag-NPs may be influenced more by silver ions than their nano-form. While there are many studies investigating *in vitro* cellular toxicity and genotoxicity there is a lack of *in vivo* toxicity information that allows correlations between *in vitro* and *in vivo* findings to be made. This is exacerbated by the excessively high particle

concentrations frequently used in *in vitro* experiments. The *in vitro* work points to intracellular oxidative stress as being the principal, although perhaps not the sole mode of action as a number of esoteric *in vivo* effects have been observed that do not rely on oxidative stress to occur.

For Ag-NP there are a number of short term (10, 28 and 90 day) repeat exposure inhalation studies. Some of these have been conducted according to OECD inhalation guidelines designed to generate safety data for chemicals. While there are clear dose related increases in blood and tissue silver concentrations it appears significant effects (alveoli inflammation and alterations in lung function) only occur in the lungs, and then at high exposure concentrations. The bone marrow micronucleus test was negative after 90 days exposure to concentrations that had been shown to cause lung and liver toxicity. Silver ions and Ag-NP can form DNA adducts and micronuclei in a concentration-dependent manner, with silver ions being more potent.

Limited monitoring of workplace air at facilities making or handling Ag-NPs shows very small mass concentrations of silver in the air that are orders of magnitude lower than employed in toxicological studies. While it is acknowledged that it is a complex question whether there is a higher risk associated with exposure to Ag-NPs compared to similar airborne concentrations of silver ions the available toxicological data point to silver ions as being the ultimate toxic entity of Ag-NPs. Furthermore there is some evidence indicating silver ions are toxicologically more potent than Ag-NPs. This suggests that perhaps the existing WES for soluble silver compounds may be suitable for Ag-NPs.

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Appendix A: Literature search strategy

This review is not a comprehensive review of health hazards, but has been conducted to determine key advances in target areas of knowledge since 2009, especially information relevant to health hazards evaluation to the worker.

In order not to limit the literature search due to non-descriptive search terms, literature dealing with the toxicology of engineered nanoparticles, published within the time span of the year 2009 to early 2013 was shortlisted. Although the comprehensive literature search was completed in January 2013, some articles of high relevance were found after January 2013 which may also have been incorporated in the review.

From the previous review, it was found that for the database literature search, the most useful combination of search terms, which generated the largest number of relevant hits, was determined as: “nanoparticle* and (toxicity or health).”

In total, we searched nine databases and three journals (which we believed to be relevant, but were not a part of any database’s search scheme). We restricted our search to articles published between 2009 and early 2013. Abstracts for this literature were screened to determine whether or not full text articles would be required. An endnote library for this literature was constructed.
Subject matters of interest for shortlisting included the following:

- carbon nanotube toxicity
- dermal absorption
- health
- eye effects
- *in vitro* studies
- *in vivo* studies
- inhalation studies
- intratracheal instillation studies
- whole animal studies
- kinetics
- mode of action
- reviews
- toxicity studies
- workplace concentrations.

The databases and journals that were searched are the following:

- Toxline (includes PubMed)
- Medline
- ScienceDirect
- American Chemical Society Publications (ACS)
- Informaworld
- Blackwell Synergy
- Knovel Library
- Wiley Interscience
- Canadian Centre of Occupational Health and Safety (CCOHS)
- Nanotoxicology
- Nature Nano
- Particle and Fibre Toxicology

It is our experience that open scientific literature searching even when multiple databases are employed only yields a certain percentage of the overall material useful for a targeted toxicology review. Therefore, the bibliographies of relevant literature for which full text articles were acquired were also screened for relevant articles and author's names. Critical citations, which may contain key information older than 2009 were also acquired in this manner. Our endnote library consists of a total of 1,210 shortlisted references. Out of these, approximately 200 were reviews.

A number of national and international agency websites were consulted in order to determine if any relevant reviews or exposure limits for nanomaterials had been published which had not been previously identified in the 2009 review. The agencies were the following:

- Safe Work Australia
- National Health and Medical Research Council (NH&MRC)
- National Industrial Chemicals Assessment and Notification Scheme (NICNAS)
- Therapeutic Goods Administration (TGA)
- Australian Pesticides and Veterinary Medicines Authority (APVMA)
- German Federal Institute for Occupational Safety and Health (BAuA)
- German Federal Environment Agency (Umweltbundesamt)
- US Occupational Safety and Health Administration (OSHA)
- US National Institute for Occupational Safety and Health (NIOSH)
- UK Health and Safety Executive (UK HSE)
- Canadian Institut de recherche Robert-Sauve en santé et en securite du travail (IRSST)
- Dutch Health Council of the Netherlands (Gezondheidsraad)

- US Department of Health and Human Services (US DHHS)
- Nordic Council of Ministers
- Royal Commission on Environmental Pollution
- UK NanoSafety Partnership Group (UKNSPG)
- Swedish KEMI
- European Chemicals Information System (ESIS)
- International Programme on Chemical Safety (IPCS Inchem)
- World Health Organisation (WHO)
- US Agency for Toxic Substances and Disease Registry (ATSDR)
- International Agency for Research on Cancer (IARC)
- Dutch National Institute for Public Health (RIVM)
- Health Canada
- Environment Canada
- United States Environmental Protection Agency (US EPA)
- California Office of Environmental Health and Hazard Assessment (OEHHA)
- Organisation for Economic Co-operation and Development (OECD)
- US National Toxicology Program (NTP)

In total, over 90 relevant reports/communications/reviews were identified from agency websites and sourced for the review. This is in addition to the short-listed references discussed above.

Appendix B: Summary of agency reviews

This appendix contains a small number of pre-2009 agency reviews, which were not identified or referenced in the 2009 review.

Agency (Reference)	Title	Brief overview
Institute of Occupational Medicine, UK (Aitken et al. 2009)	EMERGNANO: A review of completed and near completed environment, health and safety research on nanomaterials and nanotechnology.	- Identified & assessed worldwide progress in relation to nanotech risk issues - Mapped 260 projects against research objectives set in UK - Brief summaries of individual projects of most relevance are provided - The specific objectives of Task Force Area 1 (Metrology, characterisation, standardisation and reference materials) are sparsely addressed. - In Task Force Area 2 (Exposures, sources, pathways and technologies), there is a lack of information regarding engineering controls in practical settings. - In TFA3 (Human health hazard & risk assessment), there is a lack of studies describing accumulation of particles in organs after inhalation, as well as a scarcity of dermal uptake studies.
Australian Pesticides and Veterinary Medicines Authority (APVMA 2009)	The APVMA and nanotechnology.	- Brief 2 page overview. - In 2009, APVMA had not identified any existing agvet chemicals & products containing ENMs. However, they had received a small number of applications for such products. - APVMA has put in place a strategy for progressively addressing potential gaps in existing regulatory framework in relation to NMs.

Agency (Reference)	Title	Brief overview
German Federal Institute for Occupational Safety and Health (BAuA) and the German Chemical Industry Association (VCI). (BAuA and VCI 2007)	Guidance for handling and use of nanomaterials at the workplace.	- Provides orientation regarding measures in the production & use of NMs at workplace. - In most products currently manufactured commercially, NPs do not come as individual particles but as aggregates and agglomerates. The latter are not nanoparticles *per se*; they are nanostructured materials whose NPs are linked with each other. Without major energy input a release of NPs from these aggregates or agglomerates is often not possible. - In some instances, NPs are processed into granules, formulations, dispersions or compositions. A release of isolated NPs in subsequent uses is largely no longer to be expected. - Worker exposure in production is possible at filling, sampling, cleaning & maintenance work, or in disruptions of normal operations. - The document provides some specific practical guidance on minimising exposure (pg. 5-6) - Particle mass seems to be of less importance for dose measurement, thus standard gravimetric measuring methods can be used only as accompanying steps when determining exposure[148]. Stationary equipment is used to determine particle number concentration & surface concentrations of NPs in air. - Existing exposure measurement methods include the Condensation Particle Counter (CPC), Scanning Mobility Particle Sizer (SMPS), aerosol mass spectroscopy, energy dispersive X-ray fluorescence analysis, and nano-aerosol sampler (NAS).

[148] This is because the total mass of nanoparticles remains comparatively low at high particle concentrations. In addition, existing exposure measurement methods (at the time of publication of this review, 2007) are not fully standardised yet, so comparison between measurements is often not possible.

Agency (Reference)	Title	Brief overview
German Federal Institute for Occupational Safety and Health (BAuA) and the German Chemical Industry Association (VCI). (BAuA and VCI 2012).	Guidance for handling and use of nanomaterials at the workplace (updated, in German).	- At the workplace, inhalation and dermal exposure to NMs are most important. Based on current knowledge, the greatest health risks are for inhalable, insoluble or poorly soluble, NMs. - Report provides practical recommendations for management of workplace exposures to NMs. - Discussion of: - substitution possibilities - use of closed systems where possible. If not, use of extraction techniques is recommended (i.e. air cannot be recirculated, and machinery would need - to be equipped with HEPA or ULPA filters of at least Class H11-14). - discussions with workers on potential hazards & routine medical examinations at the workplace. To date, the available information does not allow for a standardised method for undertaking the medical examinations. - Biomonitoring of NMs *per se* is probably not appropriate, taking into consideration that mass concentrations are unlikely to reflect particle counts. But biomonitoring may be appropriate for measuring exposure if the NP is associated with a chemical substance which can be biomonitored. - PPE & respirator use - new exposure measurement instruments include: personal particle counters (NanoTracer – 10 to 300 nm, minidisc – 10 to 400 nm, NanoCheck – 25 to 300 nm), Nanoparticle Surface Area Monitor (NSAM – - 10 to 500 nm), EDX.
German Federal Institute of Occupational Safety and Health (BAuA) (BAuA 2010).	Workplace emissions from printers and copiers (in German).	- Brief document entailing a risk assessment for exposure to toner emissions from photocopiers. - Animal studies have shown toner dust to have carcinogenic potential. - Agency used acceptable risk limits together with specific workplace exposure risk estimate extrapolated from the intratracheal animal studies with toner particles to derive 'acceptable' mass concentrations of toner particles in workplace air. - Concluded that generally current exposures to toner emissions in office places lies within acceptable ranges, whereas additional exposure controls may be necessary for personnel servicing printers and workers exposed to emissions from recycled cartridges.

Agency (Reference)	Title	Brief overview
German Federal Institute of Occupational Safety and Health (BAuA) (BAuA 2011a).	Dispersion and retention of dusts consisting of ultrafine primary particles in lungs. Written by Schaudien D, Knebel JW, Mangelsdorf I, Voss J-U, Koch W, and Creutzenberg O	- Investigated dispersion and retention of nanoscaled TiO_2, carbon black, constantan, and zinc oxide *in vitro* (human respiratory tract cell lines) and *in vivo* (Wistar rats) after IT instillation or inhalation of aerosols. - In addition, fate of Eu_2O_3 particles was traced following inhalative deposition in lungs. Only small amounts were detected in remote organs. - In BAL fluid after instillation, TiO_2 P25 (hydrophilic) increased in agglomerate size whereas TiO_2 T805 (hydrophobic) did not show a change compared to the stock suspension. - Authors concluded a tendency of nanoscaled particles to form larger size agglomerates following deposition and interaction with cells (*in vitro*) or the respiratory tract (*in vivo*) is predominant.
German Federal Institute of Occupational Safety and Health (BAuA). (BAuA 2011b).	Genotoxic mode of action of fine and ultrafine dusts in lungs. Written by Ziemann Ch, Rittinghausen S, Ernst H, Kolling A, Mangelsdorf I, and Creutzenberg O.	- Local genotoxicity was assessed by applying immunohistochemical detection and quantification of different markers for DNA damage in rat lung tissue samples from a study previously conducted at Fraunhofer ITEM. In the previously conducted study, rats were exposed intratracheally for 3 months to crystalline silica (1300 nm), amorphous silica (14 nm), or carbon black (14 nm). - A carcinogenicity study with IT instillation of the same particles (at different doses) was also available. - 3-month data concerning BAL & histology allowed correlation of genotoxicity marker expression with the outcome of the carcinogenicity study and alterations of the lung. - For crystalline silica, all biomarkers gave statistically significant positive results, indicating genotoxic stress, occurrence of DSB, and oxidative DNA damage with subsequent repair activity. The response was less pronounced with carbon black, and least with amorphous silica. - Findings correlated well with *in vitro* and *in vivo* literature data
German Federal Institute of Occupational Safety and Health (BAuA) (BAuA 2011c).	Relevance of in vitro methods for the evaluation of chronic toxicity and carcinogenicity of nanomaterials, fine dust and fibers (in German). Written by M. Roller	- Survey of literature on epidemiologic studies, experimental *in vivo* & *in vitro* studies concentrating on validating results of *in vitro* genotox tests with inhalable fibrous and granular inorganic dusts. - No clear correlation of the probability of a positive *in vitro* test with *in vivo* potency was seen across all dusts and studies. - *In vivo* carcinogenicity studies with rats have shown induction of lung tumours. Relative to diesel exhaust, the induction was just as potent, if not more potent with NMs. - The majority of *in vitro* studies (~60%) have also shown a positive effect. - This could mean that the sensitivity of *in vitro* genotox studies is limited to approx. 60% where their specificity is not known, or *in vivo* studies are not necessarily predictive for humans due to the dust overload phenomenon.

Agency (Reference)	Title	Brief overview
German Federal Institute of Occupational Safety and Health (BAuA). (BAuA 2011d).	Research on the carcinogenicity of nanoparticles and other dusts (in German). Written by M. Roller	- Investigated differences between carcinogenicity of granular dusts in rat lung after IT instillation. 19 dusts were chosen. Three of these were quartz, amorphous silica and a coated hydrophobic TiO_2, which had not been previously tested. - The other 16 were defined as *respirable granular bio-durable particles without known specific toxicity* (GBP): 4 were ultrafine dusts (0.01-0.03 μm), the other 12 fine dusts (0.09-4 μm). - All 16 produced higher than expected incidence of lung tumours - Additional cancer risk after exposure of rats to fine GBP was calculated as 1-3% for the General Threshold Limit Value for respirable dust of 3 mg/m^3.
German Research Commission, Deutsche Forschungsgemeinschaft (DFG) (DFG 2011)	New threshold values for 'fine dust' at the workplace. Press Release No. 37.	- This is a press release detailing the update of the general dust MAK (Maximale Arbeitzplatz Konzentration – Maximum Workplace Concentration) value. - As a result of the study conducted by BAuA (2011d), the DFG determined a new MAK value for dust of 0.3 mg/m^3 (respirable), and classified these so-called biopersistent granular dusts (GBS) into Carcinogen Category 4. This identified substances with carcinogenic potential that do not increase cancer risk in humans, provided the corresponding MAK value is observed.
Australian Department of Innovation, Industry, Science and Research (DoIISR 2011)	Nanotechnology Australian capability report, fourth edition	- Snapshot of nanotech related activities in research, industry and government, currently occurring in Australia.
European Commission (EC 2009a)	Annex II: Final version of classification, labelling and packaging of nanomaterials in REACH and CLP.	- Provides guidance on how the existing classification rules in the European CLP/GHS apply to nanomaterials. - If substances are produced both at nanoscale and bulk, a separate classification & labelling may be required if available data indicate a difference in hazard - Hazard characterisation to be carried out on a case-by-case basis
European Commission (EC 2009b)	Engineered nanoparticles: review of health and environmental safety (ENRHES).	- Comprehensive review of health & environmental safety of 4 nanomaterial classes: fullerenes, carbon nanotubes, metals and metal oxides. - Also provide a basic risk assessment inspired by REACH guidance, which showed that for most exposure scenarios the risks from ENMs do not appear to be controlled in the workplace. - Commented on the significant lack of measured & modelled exposure data, and need for further testing strategies
European Commission (EC 2011)	Review of environmental legislation for the regulatory control of nanomaterials.	- Review assessed whether key EU environmental legislation (waste, water, and others) adequately address nanomaterials. - Purpose was to identify gaps in legislation; focus of the report was on possible releases of ENMs into the environment - Not relevant in context of this review

Agency (Reference)	Title	Brief overview
European Commission (EC 2012a)	Nano support project. Scientific technical support on assessment of nanomaterials in REACH registration dossiers and adequacy of available information.	- Reviewed 25 REACH registration dossiers for ENM - These were divided into 3 categories (nanoform, nano-only, and nano-tail) - It was concluded that the methods used were in several cases not appropriate for measurement of particle size distributions for nanomaterials, and did not distinguish between primary particles, aggregates and agglomerates. - It was found the descriptions related to test data provided for physico-chemical & human health endpoints were insufficient.
European Commission (EC 2012b)	Communication regarding Second Regulatory Review of Nanomaterials.	- Communication regarding major EU conclusions regarding nanomaterials. - Generally, agencies found their existing risk assessment paradigms can be used for evaluation of NMs, but are limited to the information available. - A case-by-case approach should be used. - Just as for chemicals, not all NMs are necessarily toxic (e.g TiO_2, carbon black) - Various agencies have developed guidance documents on submitting registration applications for products containing nanomaterials.
European Commission (EC 2012c)	Commission staff working paper. Types and uses of nanomaterials, including safety aspects accompanying the Communication from the Commission to the European Parliament… on the second regulatory review on nanomaterials.	- Presents information on types and uses of NMs, as well as safety aspects, & discusses options for a harmonised database for NMs. - Concludes that toxicological knowledge on NMs is improving continuously. Despite several open questions, available information indicates many NMs are non-hazardous at moderate doses while others are hazardous. - Highlight case-by-case basis risk assessment. - In absence of information, apply precautionary considerations to reduce exposure in the workplace. - Risk characterisation remains at a very preliminary and qualitative level
European Agency for Safety and Health at Work. (EASHW 2009)	European risk observatory literature review: Workplace exposure to nanoparticles.	- Review of workplace exposure & health hazards of nanoparticles. - Current principles of risk assessment seem to be appropriate, however validation of *in vitro* methods & development of testing strategy remain future tasks. - Provides information on risk management options in the workplace (e.g. control banding methods, appropriateness of PPE).
Risk Assessment of Engineered NanoParticles (ENPRA 2011)	Environmental, health and safety impacts of nanoparticles (EXCERPT).	- Report compiles articles' presentations and discussions developed during the 4th meeting of the European Observatory on NanoSafety (EONS) held in 2011.
Environment Canada (Environment Canada 2009)	Guide for the safe handling of nanotechnology-based products	- Provides information on potential risks associated with handling ENMs in research laboratories, and contains practical GLP guidance for ensuring safety of personnel. - Manipulation of solid (dust) NMs should be avoided, liquid formulations are much safer to handle.

Agency (Reference)	Title	Brief overview
Food and Drug Administration (FDA 2012a)	Guidance for Industry: Safety of nanomaterials in cosmetic products (DRAFT)	- Outlines FDA's current thinking on safety assessment of NMs in cosmetics - FDA does not judge all products containing NMs as intrinsically benign or harmful. - If existing toxicity test methods cannot be adequately adapted to cater for NMs, FDA recommends developing new methods. (e.g. Ames test may not be suitable for poorly soluble NPs, as the bacterial cell wall may create a barrier) - Risk assessment based on mass metrics may be adequate for soluble and/or biodegradable NPs (e.g. liposomes and nanoemulsions), but insoluble NPs (e.g. TiO_2, fullerenes, quantum dots) may require other metrics. - Recommend validation of *in vitro* methods for safety testing
Food and Drug Administration (FDA 2012b)	Guidance for industry. Assessing the effects of significant manufacturing process changes, including emerging technologies, on the safety and regulatory status of food ingredients and food contact substances, including food ingredients that are color additives. (DRAFT)	- Guidance to industry and end users regarding nanomaterials for food use. - At the time of the publication, FDA was not aware of any food ingredient or food contact substance intentionally engineered on the nanometre scale for which there are available safety data sufficient to determine if it is GRAS.
Centre for Biosafety, Norway (GenOk 2011)	Nano and the Environment: Potential risks, real uncertainties & urgent issues.	- Outlines central issues facing environmental governance of nanoscale sciences and technologies. - Discusses definitional difficulties. - Recommend to move away from generalised discussion to recognition of case specific differences.
Institute of Occupational Medicine, UK (Hankin et al. 2008)	Cell PEN: A study to identify the physicochemical factors controlling the capacity of nanoparticles to penetrate cells	- Research agenda towards elucidating importance of translocation in NP toxicology. The project focussed on the pulmonary interstitium, other lung cells, blood, blood vessel wall, placenta/foetus and brain. - The research plan was compiled by examining existing literature information & identifying data gaps.

Agency (Reference)	Title	Brief overview
International Agency for Research on Cancer. (IARC 2010)	IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 93: Carbon black, titanium dioxide, and talc.	- IARC reviews the available evidence for the carcinogenicity of carbon black, titanium dioxide, and talc. However, it is pointed out in the front matter the volume does not review ultrafine and engineered nano-forms of these particles, because there are few pertinent studies. - It is also stated that the physical properties and mechanistic studies of ultrafine and nano-particles that are reviewed suggest these particles, due to their greater surface area per unit mass, may be more effective in inducing toxic effects. - Bulk carbon black and TiO_2 are classified as possibly carcinogenic to humans (Group 2B), inhaled talc (not containing asbestos or asbestiform fibres) is not classifiable as to its carcinogenicity (Group 3).
Institut fuer Arbeitsschutz der Deutschen Gesetzlichen Unfallversicherung, Germany (IFA 2012)	Criteria for assessment of the effectiveness of protective measures	- Introduces 'benchmark levels', also called 'nano reference values' for different types of nanoparticles, which are based on a combination of particle number counts in air, density of the nanoparticle, shape and biopersistence. They range from 10,000 to 40,000 particles/cm^3 of workplace air (above background) for different types of ENMs. - These 'nano reference values' are not health-based, but rather
Institute of Energy and Environmental Technology (IUTA), German Federal Institute for Occupational Safety and Health (BAuA), German Social Accident Insurance Institution for the Raw Materials and Chemical Industry (BG RCI), German Chemical Industry Association (VCI), Institute for Occupational Safety and Health of the DGUV (IFA), Technical University of Dresden (IUTA et al. 2011)	Tiered approach to an exposure measurement and assessment of nanoscale aerosols released from engineered nanomaterials in workplace operations.	- Developed harmonised tiered approach towards exposure measurement and assessment of nanoscale aerosols released from ENM in the workplace. - Tier 1: Information gathering according to established industrial hygiene practices - Tier 2: Conduct basic exposure assessment using limited set of easy-to-use equipment. - Tier 3: Employ latest state-of-the-art measurement technology to assess potential for workplace exposure if required. - Existing legally binding WESs must be complied with (e.g. carbon black, amorphous silica). If no such health-based WES exists, the tiered approach is using three criteria for assessment of data: - 1: Interference values exceeded for nanoscale aerosols released from ENMs - 2: Significant increase over aerosol background level in workplace air - 3: Evidence is available for the chemical identity of filter samples indicating source is ENM (i.e. chemical identity of nano-objects and nanoscale aggregates & agglomerates). - If the above three criteria are met, exposure mitigation measures must be taken & their efficiency proven.
Swedish Chemicals Agency (KEMI) (KEMI 2008)	Nanotechnology - high risks with small particles?	- Review of available knowledge concerning risks for health & environment from NT and proposals on how to fill data gaps - Calls for precautionary measures. - Calls for need to develop standardised international methods

Agency (Reference)	Title	Brief overview
Swedish Chemicals Agency (KEMI) (KEMI 2009)	KEMI PM 2/09: Nanomaterial - activities to identify and estimate risks.	- Summary of Swedish involvement in advancing research on the health and environmental effects of nanomaterials. - Discusses high occurrence of NMs in consumer products - Knowledge reg. human health & environmental harm by NMs remains deficient.
Swedish Chemicals Agency (KEMI) (KEMI 2010)	KEMI PM 1/10: Safe use of nanomaterials - need for regulation and other measures.	- Expresses the need to enhance existing legislative rules under REACH and the CLP so they can deal effectively with any risks associated with NMs. - Still indicate there is a lack of an internationally agreed definition for NMs. - Recommends supplementing rules on biocides with requirements that NMs in additives must be identified by applicant for authorisation.
Health Effects Institute, UK (Kennedy et al. 2009)	Uptake and inflammatory effects of nanoparticles in a human vascular endothelial cell line.	- Describes a study in which NPs of Fe, Zn, Y and Ce oxides were generated. - NPs were incubated *in vitro* with human aortic endothelial cells (HAECs) for 4 hours & were evaluated for induction of ROS, markers of oxidative stress & inflammation - ZnO was associated with greatest number of effects, YO with a few effects, the others with none - Particles were taken up by HAECs
Federal Environment Agency, Germany (Umweltbundesamt). (Kuhlbusch et al. 2012)	Fate and behaviour of TiO2 nanomaterials in the environment, influenced by their shape, size and surface area.	- Investigates the applicability of OECD guidelines, specifically those related to soil & sewage plant tests, to nanomaterials. - Concluded the OECD guidelines 303A and 312 are applicable to NMs, specifically TiO_2. OECD guideline 106 was found not to be useful for NM testing due to lack of possibilities for differentiating adsorbed from non adsorbed (agglomerated) TiO_2 NM.
National Institute of Environmental Health Sciences, US Department of Health and Human Services (Kulinowski and Lippy 2011)	Training workers on risks of nanotechnology.	- Discusses implications of NT for worker training. - Suggests a possible training program for NMs, geared towards introductory, advanced or professional levels of expertise and knowledge. - Briefly discusses various workplace control options, e.g. WESs, exposure controls, etc.
Monash University, prepared for the Department of Innovation, Industry, Science and Research (Monash Uni 2009)	The social and economic impacts of nanotechnologies: a literature review.	- Analysis of literature on social & economic impacts of NT - 'Social' is characterised by pronouncements of possible adverse impacts of NT - 'Economic' is characterised by more positive statements about possibilities associated with NT.

Agency (Reference)	Title	Brief overview
The Japanese Research Institute for Safety and Sustainability (RISS) (Nakanishi 2011)	Risk assessment of manufactured nanomaterials - "Approaches" - Overview of approaches and results.	- Provide the methodology for basic risk assessments carried out for TiO_2, fullerene (C_{60}) and CNT. - As part of the project, IT instillation & inhalation studies were conducted. It was considered cancers are unlikely to develop at the concentration less than NOAEL estimated in the study. - Derives period limited WES (WES [PL]) of exposure of a period of ½ to 1/3 of 30-45 years, based on the premise that the results will be reviewed within 10 years using new test results.
The Japanese Research Institute for Safety and Sustainability (RISS) (Hanai et al. 2009)	Risk assessment of manufactured nanomaterials - Titanium dioxide (TiO2)-.	- Interim report on status of developing risk assessment for nanoscale TiO_2. - Proposes method to establish an acceptable exposure concentration in situations with limited inhalation exposure studies. - Proposes a provisional value of an acceptable exposure concentration in the workplace for TiO_2 using this method - Proposed standards are to prevent inflammation in the lung, considered the 'threshold' endpoint, which if prevented, will also prevent cancer.
The Japanese Research Institute for Safety and Sustainability (RISS) (Ogura et al. 2011)	Risk assessment of manufactured nanomaterials - Titanium dioxide (TiO2) -.	- Final report described above, with changes to the proposed WES when compared to the interim report.
The Japanese Research Institute for Safety and Sustainability (RISS) (Shinohara et al. 2011)	Risk assessment of manufactured nanomaterials - Fullerene (C60)	- Final report detailing derivation of proposed provisional period-limited (to 15 yr exposure) WES of 0.39 mg/m^3 for C_{60} fullerenes. - The WES was based on extrapolation of the results from an intratracheal study, supported by inhalation study results. - A particle-size specific WES for fullerenes may be determined from the equations in the publication.
The Japanese Research Institute for Safety and Sustainability (RISS) (Nakanishi et al. 2011)	Risk assessment of manufactured nanomaterials - Carbon nanotubes (CNT) -	- Final report detailing derivation of proposed provisional period-limited WES of 0.03 mg/m^3 for SWCNT (1000 m^2/g) as a WES for CNTs in general, and recommended that if this value is applied to MWCNT with substantially lower specific surface areas, this would be considered safe. - The WES was based on a NOAEL of 0.13 mg/m^3 for SWCNT for 'lung inflammation' from a 4-week inhalational study (3 month observation) and an UF of 2 for conversion into sub-chronic NOAEL of 0.065 mg/m^3. The adjusted NOAEL was then converted to a human equivalent NOAEL of 0.09 mg/m^3. An additional UF of 3 (interspecies toxicokinetic difference) was applied to derive the provisional WES.

Agency (Reference)	Title	Brief overview
National Institute of Environmental Health Sciences (Nazarenko et al. 2012)	Potential for inhalation exposure to engineered nanoparticles from nanotechnology-based cosmetic powders.	- Investigated the potential for human contact and inhalation exposure to NMs when using NT-based cosmetic powders. - Products were characterised using TEM and laser diffraction. TEM showed high agglomeration of NPs in the products. Researchers simulated use of powders by applying them to face of mannequin. - They found a user would be exposed to NM primarily as NP-containing agglomerates > 1-100 nm. This means exposure will primarily be in the trachea-bronchial and head airways, not the alveolar region.
National Industrial Chemicals Notification and Assessment Scheme (NICNAS 2009a)	Proposal for regulatory reform of industrial nanomaterials. Public discussion paper - November 2009.	- Proposal to stakeholders on a reform initiative to introduce new approaches to regulate industrial nanomaterials. - Uses existing NICNAS framework, with some adjustments to address uncertainties in potential health risks of NMs.
Report prepared by Professor Brian G. Priestly for the Department of Health and Ageing, NICNAS. (NICNAS 2009b)	Review of 2007-09 literature on toxicological and health-effects relating to six nanomaterials.	- Reviews literature from 2007-2009 (September) on 6 industrial NMs (fullerenes, CNT, ZnO, TiO_2, CeO and Ag nanoforms). - Report does not address the plethora of *in vitro* studies with the NMs. - Biopersistence is an important factor in determining whether NPs penetrate cells & are retained long enough to lead to pathological changes. - Modification of surface activity modified toxicity potential of NPs, but there is as yet no clear pattern of structure activity relationships to permit predictions of tox. - Lack of useful chronic exposure studies.
National Industrial Chemicals Notification and Assessment Scheme (NICNAS 2011)	Guidance on new chemical requirements for notification of industrial nanomaterials.	- Final documents of NICNAS (2009a) proposal to changes in NM regulation. - Outlines new industrial nanomaterial regulation under NICNAS - From 1st January 2011, nanoforms of new chemicals are not permitted to be introduced under low volume or low concentration exemption categories. - All permit categories remain available, declaration added to forms. - Self-assessment certificate category will not be available for introducers. - Additional data for industrial NMs will also be required (e.g. particle size distribution). - Outlines test methods which may be used to determine NM properties.
National Industrial Chemicals Notification and Assessment Scheme (NICNAS 2013a, b, c)	Nano titanium dioxide fact sheet, technical information sheet, and Appendix: nano titanium dioxide toxicology information and references.	- Summary of health hazard information to July 2012 for TiO_2 NPs. - TiO_2 NPs do not cause adverse effects if accidentally ingested. - TiO_2 NPs do not penetrate through outer layers of skin, and do not cause irritation or allergy. - TiO_2 NPs of small sizes show low toxicity after inhalation. Larger TiO_2 NPs are deposited in lung of rats with small fraction transported to lymph nodes. Breathing in large quantities of TiO_2 NPs over a long period of time may impair normal lung clearance mechanisms. - Mild eye irritation. - Reported effects on reproductive organs are limited and inconclusive.

Agency (Reference)	Title	Brief overview
National Industrial Chemicals Notification and Assessment Scheme (NICNAS 2013d, e, f)	Nano silver fact sheet, technical information sheet, and Appendix: nano silver toxicology information and references.	- Summary of health hazard information to July 2012 for Ag NPs. - Ag NPs have low oral toxicity in rats, mice, and guinea pigs. - Ag NPs are not easily absorbed through the skin, and do not cause irritation but may cause mild allergy (guinea pigs). - The single rat inhalational study was inconclusive due to use of very low doses of Ag NPs. - Studies in rabbits & guinea pigs using small- to medium- sized NPs elicited no eye irritation. - No reproductive or developmental toxicity data are available. - No relevant studies on cancer were reported.
National Institute for Occupational Safety and Health, Department of Health and Human Services, Center for Disease Control and Prevention. (NIOSH 2009a)	Approaches to safe nanotechnology: managing the health and safety concerns associated with engineered nanomaterials.	- Provides overview of potential hazards of ENMs and measures to minimise workplace exposure. - NMs have greatest potential to enter body through respiratory system is they are airborne. - In animal studies, equivalent mass doses of insoluble incidental NPs are more potent than large particles of similar composition in causing pulmonary inflammation & lung tumours. - Studies in workers exposed to aerosols of some manufactured or incidental microscopic (fine) and nanoscale (ultrafine) particles have reported adverse lung effects (e.g. lung function decrements). Implications to ENMs is uncertain. - Mass and bulk chemistry may be less important that particle size and shape, surface area and surface chemistry for ENM monitoring in the workplace. - Background nanoscale particle measurements must be taken into account. - Provide specific recommendations for managing exposures to ENMs.
US National Institute for Occupational Safety and Health, Department of Health and Human Services, Center for Disease Control and Prevention. (NIOSH 2009b)	Current intelligence bulletin 60: Interim guidance for medical screening and hazard surveillance for workers potentially exposed to engineered nanoparticles.	- Currently there is insufficient scientific and medical evidence to recommend the specific medical screening of workers potentially exposed to ENMs. But this does not preclude employers to take precautions beyond existing measures. - Recommendations are to take prudent measures to control exposure to ENMs, conduct hazard surveillance as the basis for implementing controls, and continue the use of established medical surveillance approaches (i.e. if a bulk material of the NM has specific health screening recommendations, then these also apply to its corresponding nanomaterial).

Agency (Reference)	Title	Brief overview
US National Institute for Occupational Safety and Health, Department of Health and Human Services, Center for Disease Control and Prevention. (NIOSH 2010, 2013)	Occupational exposure to carbon nanotubes and nanofibers. November 2010 DRAFT and April 2013 FINAL	- To date NIOSH is not aware of any reports of adverse health effects in workers using or producing CNT or CNF. - NIOSH systematically reviewed 54 laboratory animal studies, many of which indicated CNT/CNF could cause adverse pulmonary effects including inflammation, granulomas, and pulmonary fibrosis. - Estimated the risk of developing early-stage (slight or mild) lung effects over a working lifetime if exposed to CNT at the analytical limit of quantification of 1 µg/m^3 (8-hour TWA) is approx. 0.5%-16%. - Therefore, NIOSH recommended exposures to CNT and CNF be kept below the recommended exposure limit (REL) of 1 µg/m^3 respirable elemental carbon as 8-hour TWA. - The draft NIOSH (2010) report indicated the analytical LoQ was 7µg/m^3 but since then, methodology improvements have lowered this. - Also provide updated screening and medical surveillance guidance for CNTs.
US National Institute for Occupational Safety and Health, Department of Health and Human Services, Center for Disease Control and Prevention. (NIOSH 2011)	Current intelligence bulletin 63: Occupational exposure to titanium dioxide.	- NIOSH concluded TiO_2 is not a direct-acting carcinogen, but acts through a secondary genotoxicity mechanism that is not specific to TiO_2 but related to particle size and surface area. - Exposure to ultrafine (<100 nm) TiO_2 should be considered a potential occupational carcinogen. - NIOSH recommends WESs of 2.4 mg/m^3 for fine TiO_2 and 0.3 mg/m^3 for UF (including ENM) TiO_2 as TWAs for up to 10 hrs/day. These are levels that over a working lifetime are estimated to reduce risks of lung cancer to below 1 in 1,000.
US National Institute for Occupational Safety and Health, Department of Health and Human Services, Center for Disease Control and Prevention. (NIOSH 2012)	General safe practices for working with engineered nanomaterials in research laboratories.	- Guidance document for engineering controls & safe work practices to be followed when working with ENMs in research laboratories. - Recommend the use of a risk management program. - Discusses options for exposure controls and health surveillance in workers exposed to ENMs.
National Science and Technology Council, Committee on Technology. (NNI 2011)	National Nanotechnology Initiative Environmental, Health, and Safety Research Strategy.	- Provides a strategy on research needs that is meant to serve as guidance to the US Federal agencies that produce and use scientific information to develop NT risk assessments that inform risk management and regulatory decisions. - Highlights the need for multicomponent work which overlaps several scientific disciplines.

Agency (Reference)	Title	Brief overview
Nordic Council of Ministers (Norden 2012)	Regulatory safety assessment of nanomaterials	- Describes the outcomes of the Nordic NanoNet Workshop and EDC discussion held on the 11-13th October 2011 in Finland. - Major conclusions with respect to NMs from the meeting were: 1) Swift regulatory action should not wait for more scientific information before proceeding with decisions. 2) Need to agree on a NM definition to allow regulatory action to take place. 3) Further measures to ensure safety of NMs under REACH are needed. 4) It would be safer to categorically register NMs as new substances with nano-adapted data requirements. 5) While development of further NM test guidelines is necessary, existing guidelines are an adept starting point for safety and hazard assessment. 6) Grouping NMs remains practically difficult. However, it would be a desirable basis for regulatory guidance. 7) Very few potential new nanospecific endpoints need to be added to existing OECD test guidelines. These new needs are mainly in the area of physico-chemical characterisation.
National Research Council of the National Academies (NRC 2012)	A research strategy for environmental, health, and safety aspects of engineered nanomaterials.	- A strategic approach is presented for developing science & research infrastructure to address uncertainties regarding potential ENM risks. - Little research has been made on the effects of ingested ENMs on human health, on the potential health & environmental effects of complex ENMs, and system integrated approaches are needed that can address
Observatory Nano project (Observatory Nano 2011)	Developments in nanotechnologies regulation & standards - 2011. No. 3. Report prepared by Mantovani E, Porcari A, Morrison M and Geertsma R	- Provides an overview of regulatory developments around the World related to nanotechnology and nanomaterial regulation. - Concludes that so far regulation is still largely based on existing provisions, albeit under revision. - Suggests an appropriate balance between hard and soft regulation still seems the most viable option in the short term.
Organisation for Economic Co-operation and Development (OECD 2009a)	Preliminary analysis of exposure measurement and exposure mitigation in occupational settings: manufactured nanomaterials.	- Currently no consensus standards on measurement techniques for NPs in workplace. However a number of standards & reference materials are under development. - Currently there is no agreement on metrics of exposure to NMs. - Biomonitoring of exposures to NMs is very limited because biomarkers are in early stage of development. However, basic health monitoring has been recommended by a number of organisations. - General occupational health & safety guidelines based on established guidelines for controlling exposures to general aerosols are recommended. More specific guidelines are available for laboratory settings.

Agency (Reference)	Title	Brief overview
Organisation for Economic Co-operation and Development (OECD 2009b)	Series on the safety of manufactured nanomaterial No. 11: Emission assessment for identification of sources and release of airborne manufactured nanomaterials in the workplace: compilation of existing guidance.	- Provides a simple guidance document for semi-quantitative determination of NM release for use by health and safety professionals, e.g. occupational hygienists. - Provides a step by step model for accounting for background particle concentration measurements, identifying potential sources of NP emissions, conducting filter-based area and personal air sampling, surface sampling, quality assurance and control, interpretation of the data for particle size and the limitations of the method. - If concentrations of NMs are 10% higher with the production system on than off, then optional sampling is recommended to determine if worker exposure controls are adequate.
Organisation for Economic Co-operation and Development (OECD 2009c)	Series on the safety of manufactured nanomaterial No. 13: Report of an OECD workshop on exposure assessment and exposure mitigation: manufactured nanomaterials.	- Report of a workshop on exposure assessment & mitigation of ENMs. - Measurement techniques & devices are available in principle and have been tested to measure NPs. But standard processes have to be agreed on that are founded on a reliable basis on reference materials and measurement calibration. - Design of safety measures has to follow the whole life cycle. Many control strategies can be re-designed for NM manufacturing. - Personal, easy-to-use equipment and dose relevant devices are still needed or have to be improved further. Standards for Personal Safety Equipment (PSE) are necessary.
Organisation for Economic Co-operation and Development (OECD 2009d)	Preliminary review of OECD test guidelines for their applicability to manufactured nanomaterials.	- Reviewed the applicability of existing OECD testing guidelines to ENMs. - For guidelines related to physical and chemical properties, 4 of 22 were applicable to ENMs. 2 guidelines are not applicable or provide no useful information. 13 of 22 require further assessment before modification. OECD suggested 17 physical/chemical properties to be a necessary pre-requisite of toxicological assessment. - For guidelines related to health effects testing, in general they are applicable to ENMs with the important proviso that additional consideration needs to be given to the physicochemical characteristics of the material tested, including actual dosing solution. In some cases, there will be a need to further modify the OECD guideline (e.g. inhalation and ADME studies). - Also reviewed ecotoxicity guidelines, but the results are not presented in this overview
Organisation for Economic Co-operation and Development (OECD 2009e)	Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme.	- The objective of the document is to assist sponsors in the development of comprehensive and consistent Dossier Development Plans (DDP), describing the testing programme for a specified ENM. - The organisation of the guidance document follows the intended section organisation of the DDPs. It is expected sponsors will explain and justify the rationale used for making each decision. - The specific sections required include many requiring determination of physical/ chemical properties for the ENM. The guidance document also provides the methods that may be used to determine these (if available).

Agency (Reference)	Title	Brief overview
Organisation for Economic Co-operation and Development (OECD 2010a)	Report of the questionnaire on regulatory regimes for manufactured nanomaterials.	- Identifies applicable (current and proposed) regulatory regimes and how they address information requirements, hazard ID, exposure mitigation, risk assessment & management for ENMs. - None of the respondents have legislation specific to NMs, but rather these products are regulated using existing legislation. - Registration/notification based on trigger quantities may present a problem for jurisdictions wanting to assess ENMs if manufactured below trigger volumes. - A tiered/graduated assessment approach is promising. - Ability to assess substances already in commerce is beneficial. - Ability to distinguish NMs from their larger counterparts with the same composition would assist regulatory agencies with their assessments.
Organisation for Economic Co-operation and Development (OECD 2010b)	Series on the safety of manufactured nanomaterial No. 21: Report of the workshop on risk assessment of manufactured nanomaterials in a regulatory context.	- Discusses the results of a workshop on Risk Assessment of Manufactured NMs in a Regulatory Context, which took place on September 16-18th, 2009. - It was concluded the risk assessment paradigm for chemicals will continue to guide approaches to RA of NMs. However, many of the assumptions & estimations in chemical RA need to be evaluated for NMs. - There does not seem to be a rationale for employing a nano-specific RA uncertainty factor. Application of standard UF should undergo validation. - It is expected empirical results will continue to be reported in terms of mass based units, however RA should include discussion of any limitations this metric may present.
Organisation for Economic Co-operation and Development (OECD 2010c)	Series on the safety of manufactured nanomaterial No. 20: Current development/ activities on the safety of manufactured nanomaterials - tour de table.	- Provides an overview of the status of the work of the Working Party on Manufactured Nanomaterials and compiles information provided by member companies and other delegations on their current developments regarding the safety of ENMs.
Organisation for Economic Co-operation and Development (OECD 2010d)	Guidance manual for the testing of manufactured nanomaterials: OECD's sponsorship programme; first revision.	- Update of OECD (2009e) guidance manual for sponsors on Dossier Development Plans (DDP) for ENMs.

Agency (Reference)	Title	Brief overview
Organisation for Economic Co-operation and Development (OECD 2011)	Nanosafety at the OECD: the first five years 2006-2010.	- Brief overview of what the OECD Working Party on Manufactured Nanomaterials (WPMN) has achieved in 2006-2010. The working party comprises delegates from ministries and agencies responsible for safety of human health and the environment. - Meetings are held every 8 months, in addition to workshops, conferences, etc. - Major outcomes include: 1) OECD database on ENMs to inform research activities. 2) Safety testing of a representative set of ENMs. 3) The role of alternative test methods (e.g. in vitro) in nanotoxicology. 4) Testing guidelines, e.g. guidance note on sample preparation & dosimetry. 5) Co-operation on voluntary schemes and regulatory programmes. 6) Exposure mitigation and measurement documents
Organisation for Economic Co-operation and Development (OECD 2012a)	Six years of OECD work on the safety of manufactured nanomaterials: achievements and future opportunities.	- Nano-brochure providing brief overview of OECD achievements from 2006-2012. - Provides similar information to OECD (2011).
Organisation for Economic Co-operation and Development (OECD 2012b)	Inhalation toxicity testing: expert meeting on potential revisions to OECD test guideline and guidance document.	- Summary of expert meeting on Inhalation Toxicity Testing for NMs on 19-20 October 2011. - While inhalation tox tests with aggregates or agglomerates of nano-sized particles seem adequate for RA for handling of powders, it may be relevant for NPs in production phase to include inhalation tox of aerosols consisting of single NPs as much as possible. Care should be taken that at high mass concentrations size distributions shift towards larger particles due to agglomerations as a function of time & particle number. - Recommended deletion of lower cut-off of size range and minimal mass concentrations in current guidelines. Most agreed to incorporate application of biokinetics into guideline and detailed pathology of brain and other parts of the CNS, while BAL fluid analysis should be a mandatory requirement.
Organisation for Economic Co-operation and Development (OECD 2012c)	Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials.	- Specific guidance document, updating the provisional guidance published in 2010, on sample preparation and dosimetry for ENMs. - Focus is on the kinds of tests that address the endpoints and types of NMs being tested under the OECD sponsorship programme. - Guidance is specific for water insoluble ENMs, as soluble ENMs are unlikely to need different sample preparation techniques than other chemicals, apart from specific reactivity considerations.
Organisation for Economic Co-operation and Development (OECD 2012d)	Important issues on risk assessment of manufactured nanomaterials.	- Discusses the current practices & challenges on RA of ENMs, and strategies for RA where data are limited. - Concludes there is a need for direct research towards specific RA issues. - Many of the conclusions are the same as OECD (2010b). - A margin of exposure may be an alternative approach to understanding risk of ENMs.

Agency (Reference)	Title	Brief overview
Occupational Safety and Health Administration, USA (OSHA 2013)	OSHA fact sheet: working safely with nanomaterials.	- Fact sheet provides basic information to workers & employers on current understanding of potential hazards of ENMs in workplace. - Employers should minimise worker exposure by using hazard control measures and best practices.
Institut de Recherche Robert-Sauve en sante et en securite du travail (IRSST) (Ostiguy et al. 2008)	Health effects of nanoparticles. Second edition.	- This report updates the literature review published in 2006 by the same team (Ostiguy et al. 2006). The latter report was summarised in the previous Safe Work Australia (2009a) review. This 2nd edition incorporates scientific knowledge up to mid-2007. - Insoluble or low solubility NPs are greatest cause for concern. - At equal mass, NPs are more toxic than products of the same chemical composition but of greater size. - Each product and even each synthesised NP batch can have its own toxicity. - Strict prevention methods are recommended.
Institut de Recherche Robert-Sauve en sante et en securite du travail (IRSST) (Ostiguy et al. 2009)	Best practices guide to synthetic nanoparticle risk management.	- Guide assembles then current scientific knowledge on identification of dangers, risk assessment & management of ENMs. - Risk management is an iterative process, allowing continuous improvement. - Authors favour a preventive approach aimed at minimising occupation exposure when a risk assessment cannot be conducted.
Dutch National Institute for Public Health and the Environment (RIVM 2009)	Nanomaterials under REACH - Nanosilver as a case study.	- Proposes an adapted set of minimum information requirements, to be applied to all NMs to be registered under REACH, independent of volume of production & import. - RIVM conducted the hypothetical registration of nanosilver to investigate if REACH was suitable for assessing the safe use of NMs. - They found no definition of a NM is present, and a relevant measure of expressing harmfulness and exposure is not yet known. The standard information requirements were found to be insufficient to assess hazard & exposure.
Dutch National Institute for Public Health and the Environment (RIVM 2010)	Provisional nano-reference values (in Dutch).	- Concluded current scientific knowledge is inadequate to enable health-based WESs for NMs to be derived. As an alternative, provisional nano-reference values (non-health based) can be used as pragmatic benchmark levels to reduce exposure in the workplace. - RIVM evaluated the usefulness of two published methods to derive nano-reference values (BSI, UK and IFA, Germany), and used one of these methods (IFA) for 23 most commonly applied NMs: • 20,000 particles/cm^3 for nano- Ag, Fe, Au, Pb, La, TiO_2, CeO_2, ZnO, SiO_2, Al_2O_3, Fe_xO_y, SnO_2, CoO and nanoclay. • 40,000 particles/cm^3 for C_{60}, carbon black, TiN, Sb_2O_5, polymers, polystyrene, dendrimers and carbon nanotubes for which effects comparable to the effects of asbestos can be excluded. • 0.01 fibres/cm^3 for carbon nanotubes for which effects comparable to the effects of asbestos cannot be excluded.

Agency (Reference)	Title	Brief overview
Dutch National Institute for Public Health and the Environment (RIVM 2012)	Interpretation and implications of the European Commission recommendation on the definition of nanomaterial.	- Univocal definition of NM is essential in EU legislation & regulation. - RIVM considers the definition published by the European Commission in October 2011 to be a good basis for further discussion focusing on two aspects: 1) Proposed size limits for NPs (1 to 100 nm). 2) Requirement that at least 50% of the number of particles should be in this size range. - Materials not covered by definition should not automatically be considered safe.
Safe Work Australia (Safe Work Australia 2009a)	Engineered nanomaterials: a review of the toxicology and health hazards.	- Literature review on the toxicology and health hazards of engineered nanomaterials. References to conclusions made in this review have been made in this report.
Safe Work Australia (Safe Work Australia 2009b)	Engineered nanomaterials: evidence on the effectiveness of workplace controls to prevent exposure.	- Literature review on effectiveness of workplace controls to prevent or minimise exposure to ENMs. - Control & risk management methodologies already known can provide levels of protection for workers from ENMs, but further data are needed to understand the levels of protection & ensure effectiveness. - Precautionary approach should be used. - Examples of workplace controls are elimination, substitution and/or modification, enclosure, extraction, administrative controls, PPE. - Propose use of 'control banding' for risk management in early research stages.
Safe Work Australia (Safe Work Australia 2010a)	Engineered nanomaterials: feasibility of establishing exposure standards and using control banding in Australia.	- Investigated feasibility of using 'control banding' for ENMs in Australia. - While there are some issues associated with the hazard type groups suggested by BSI (2007), they appear to be practical groupings of ENMs.
Safe Work Australia (Safe Work Australia 2010b)	Engineered nanomaterials: investigating substitution and modification options to reduce potential hazards.	- Survey of current substitution/modification practices used in Australian NT-related activities, and a literature review of potential options that may reduce exposure to NMs. - Substitution/modification methodologies are well known & used in Australia, thus there is existing capability that might be applied more broadly to work health & safety purpose. - CNTs can be functionalised and surface-modified to increase solubility & biocompatibility. It is possible to reduce chronic toxicity potential by using short CNTs (<5 μm). - Attaching water solubilising groups to fullerenes will increase solubility & lead to reduced toxicity. - Potential toxicity of TiO_2 product can be controlled by varying crystalline form used.
Safe Work Australia (Safe Work Australia 2011a)	Nanoparticles from printer emissions in workplace environments.	- Investigated exposure of office workers to NPs from laser printer emissions. - It is essential local background particle exposure be accounted for in measurements. - Particle size of emissions was predominantly <300 nm. - Peak and 30-minute printer particle exposure are a better measure than 8-hr TWA. - Provides advice on NP assessment & control strategies.

Agency (Reference)	Title	Brief overview
Safe Work Australia (Safe Work Australia 2011b)	Brief review on health effects of laser printer emissions measured as particles.	- Brief review of potential adverse effects associated with exposure to laser printer emissions measured as particles & consider results in relation to health risk in workplace. - Emissions are primarily aerosol condensates of VOCs or SVOCs. - Assessment of exposure concentrations reported in Safe Work Australia (2011a) against existing guidelines for VOCs/SVOCs indicated the risk of direct toxicity & health effects is negligible, however people responsive to unusual or unexpected odour may detect/react to emissions.
Safe Work Australia (Safe Work Australia 2011c)	Durability of carbon nanotubes and their potential to cause inflammation.	- Determined the durability of CNTs in simulated biological fluid and subsequent fibre pathogenicity, compared with fibre controls. - Results of the study showed that CNTs can be durable, but may also be subject to bio-modification in a sample-specific manner. - Results suggested that if of sufficient length and aspect ratio, pristine CNTs can induce asbestos-like responses in mice, but this may be mitigated if CNTs are of less durable nature.
Safe Work Australia (Safe Work Australia 2012a)	Classification of carbon nanotubes as hazardous chemicals, information sheet.	- NICNAS was commissioned to determine the appropriate hazard classification for CNTs. - Classification as hazardous was recommended for repeated or prolonged inhalation exposure and for carcinogenicity. For all other endpoints, CNTs were not classified as hazardous or could not be classified due to insufficient information.
Safe Work Australia (Safe Work Australia 2012b)	Human health hazard assessment and classification of carbon nanotubes.	- This is the report described briefly in Safe Work Australia (2012a), which describes the basis for the conclusions NICNAS made with respect to the appropriate hazard classification for carbon nanotubes.
Safe Work Australia (Safe Work Australia 2012c)	Safe handling and use of carbon nanotubes.	- Guidance for managing the risks of exposure to CNTs in the workplace. - Two methods are presented: 1) Risk management with detailed hazard analysis & exposure assessment. 2) Risk management by control banding.
Safe Work Australia (Safe Work Australia 2012d)	Safe handling and use of carbon nanotubes in the workplace. Information sheet.	- Short information sheet providing a summary of Safe Work Australia (2012c) described above.
Safe Work Australia (Safe Work Australia 2013a)	Development of an automated high-throughput screening procedure for nanomaterials genotoxicity assessment.	- Researchers at Flinders University describe the development of an automated high-throughput screening procedure for assessing the genotoxicity of ENMs.

Agency (Reference)	Title	Brief overview
Safe Work Australia Safe Work AUS (2013c)	Investigating the emissions of nanomaterials from composites and other solid articles during machining processes. Safe Work Australia.	- This report reviews the particle release from the machining (e.g. wet and dry cutting, drilling, grinding, sanding and abrasion) of composite materials that contain nano-objects (e.g. silica, carbon fibres, CNTs and nanoclay). Matrices include epoxy, polycarbonate, polyurethane, polymethyl methacrylates, and polyamide. - Overall, it was found that the mass of emissions from machining composites containing reinforcing nano-objects is in most instances not significantly different from machining composites not containing nano-objects. High energy processes emit higher numbers of particles than low energy processes. Lower emissions may be achieved (for some processes) using wet instead of dry machining. Nanomaterials are most frequently emitted embedded within the matrix, rather than as free nanomaterials.
Strategic Approach to International Chemicals Management (SAICM 2012)	Progress report on nanotechnologies and manufactured nanomaterials.	- Briefly describes the progress that has been made by the OECD, United Nations, and other international organisation with respect to ENMs. - The progress report primarily highlights discussions among key stakeholders from various governments as being vital, and provides a brief overview of OECD programme outcomes.
Board of Scientific Counselors (Sayler 2010)	Letter concerning the review of the Office of Research and Development (ORD) Nanomaterial case studies workshop: developing a comprehensive environmental assessment research strategy for nanoscale titanium dioxide.	- Review of report of nanomaterial workshop conducted by the US EPA (2009a), focusing on the process to prioritise research needs and to comment on the use of the Nominal Group Technique (NGT) as an overall strategy. - The reviewers found the prioritised list appropriate, however suggested that NGT could be combined with a more formalised decision analysis process in the long run.
Therapeutic Goods Administration, Australia (TGA 2009)	A review of the scientific literature on the safety of nanoparticulate titanium dioxide or zinc oxide in sunscreens	- Potential for TiO_2 and ZnO NPs in sunscreens to cause adverse effects depends on ability of NPs to reach viable skin cells, which the current WoE suggests they do not do. - Isolated *in vitro* experiments have shown that ZnO and TiO_2 may induce radical formation in the presence of light, and this may lead to cell damage (with ZnO). However, recent studies suggest the photo-genotoxicity observed in these studies may be due to a UV-induced experimental artefact rather than the presence of NPs.
Institute of Occupational Medicine, UK (Tran et al. 2008)	An outline scoping study to determine whether high aspect ratio nanoparticles (HARN) should raise the same concerns as do asbestos fibres.	- Scoping study for literature review to determine whether high aspect ratio NPs (HARN) should raise the same concerns as do asbestos fibres. - Review identified many similarities between HRAN and asbestos with respect to physico-chemical properties and toxicological effects and concluded there is sufficient evidence to suggest HARN which have the same characteristics as pathogenic fibres are likely to have similar pathology. - Highlighted lack of key data and suggested research strategy for filling data gaps.

Agency (Reference)	Title	Brief overview
United Kingdom Health and Safety Executive (UK HSE 2009, 2011)	Risk management of carbon nanotubes.	- Information sheet providing occupational health & safety guidance relating to manufacture & manipulation of CNTs. - Since there is uncertainty about the risks of being exposed to CNTs, the regulatory and safe response is to take a precautionary approach. - Provides a number of practical risk management options for workers and employers.
UK NanoSafety Partnership Group (UKNSPG 2012)	Working safely with nanomaterials in research & development.	- Provides guidance on factors relating to establishing a safe workplace and good safety practice when working with ENMs. - Preferred approach, as with other guidance documents, is to prevent potential exposure with rigorous containment rather than using an extensive airborne NM monitoring regime. - Lists examples of current control banding tools developed for NMs, as well as other practical tools for controlling exposure to ENMs.
German Federal Institute for Health and the Environment (i.e. Umweltbundesamt) (Umweltbundesamt 2009)	Nanotechnology for humans and the environment. Promote opportunities and reduce risks.	- Aspects of opportunities (e.g. environmental relief & health protection) & risks (largely unknown) of NT are outlined. - Recommend precautionary principle be followed.
German Federal Institute for Health and the Environment (i.e. Umweltbundesamt) (Umweltbundesamt 2010)	Quantitative biokinetic analysis of radioactively labelled, inhaled titanium dioxide nanoparticles in a rat model.	- Details an experiment to determine the biokinetics of TiO_2 NPs in the whole body of healthy adults rats after inhalation or instillation into the respiratory tract. - Small fractions (~2% of TiO_2-NP deposited in lung) translocate across air-blood-barrier and accumulate in secondary target organs, soft tissue and skeleton. Translocation was accomplished within the first 2-4 hours of inhalation followed by retention in all organs & tissues studies without detectable clearance within 28 days.
United States Environmental Protection Agency (US EPA 2009a)	Nanomaterial research strategy.	- Research strategy devised to guide the US EPA's program in focused NM research. - The key decision-support questions to be answered are: 1) What NMs, in what forms, are most likely to result in environmental exposure? 2) What particular NM properties may raise toxicity concerns? 3) Are NMs with these properties likely to be present in environmental media or biological systems at concentrations of concern? 4) If 'yes', can we change properties or mitigate exposure?
United States Environmental Protection Agency (US EPA 2009b)	Workshop summary for the EPA Board of Scientific Counselors. Nanomaterial case studies workshop: developing a comprehensive environmental assessment research strategy for nanoscale titanium dioxide	- Describes the development of the nano-TiO_2 case studies document (US EPA 2010a), and the results of a workshop which was held to come up with research priorities for the US EPA based on a list of several unanswered questions in the TiO_2 case study document.

Agency (Reference)	Title	Brief overview
United States Environmental Protection Agency (US EPA 2010a)	Nanomaterial case studies: nanoscale titanium dioxide in water treatment and in topical sunscreen.	- Describes case studies of nano-TiO_2 for arsenic removal in drinking water treatment and for topical sunscreens. A product life-cycle perspective was combined with the RA paradigm to provide a holistic examination of a material's potential impacts. - The goal was to provide a foundation for a process to identify and prioritise research directions to support future efforts.
United States Environmental Protection Agency (US EPA 2010b)	Comprehensive environmental assessment: a meta-approach to increase effectiveness of risk management and research planning.	- Describes comprehensive environmental assessment approach to research planning. - The process incorporates a variety of technical and stakeholder viewpoints in a structured process that allows participants to learn from one another yet make their own judgements in prioritising risk trade-offs and/or information gaps. - The process was applied to research planning for NMs (US EPA 2010a).
United States Environmental Protection Agency (US EPA 2010c)	External review draft. Nanomaterial case study: nanoscale silver in disinfectant spray.	- Described case study of nano-Ag use in disinfectant sprays. The intent was to describe what is known/unknown about nano-Ag and to identify and prioritise research needs.
United States Environmental Protection Agency (US EPA 2012)	Approaches for assessing and controlling workplace releases and exposures to new and existing nanomaterials. (DRAFT)	- Guidance document on assessing, monitoring and controlling exposures to ENMs in workplace. The document provides interim practical approaches to: 1) Release and exposure assessment. 2) Inhalation monitoring. 3) Engineering controls. 4) Personal Protective Equipment (PPE).
World Health Organisation (WHO 2012)	Background paper for WHO Guidelines on protecting workers from potential risks of manufactured nanomaterials.	- Background paper that proposes draft critical questions which should be answered in the process of developing guidelines for NT worker safety in low & medium income countries. - Discusses options for occupational risk management of NMs, starting from semi-qualitative (e.g. control banding) to traditional quantitative (WESs). - Raises research questions that need to be considered when developing guidelines.

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Appendix C: Workplace exposure limits summary tables

Table C1: Health-based workplace exposure limits

Nanomaterial	WES	Basis	Reference
Titanium dioxide	0.3 mg/m^3 ultrafine (<100 nm) 40 hours/week, 45 working years	Applied BMD modelling (using various models) to lung cancer risk in rat studies, converted BMDs and BMDLs to human equivalent doses using known species differences in lung surface area, and subsequently estimated working lifetime airborne mass concentrations using human lung dosimetry models. The WES represents the combined model averages (rounded) of BMDL estimated airborne mass concentrations of ultrafine TiO_2 associated with a 1 in 1,000 excess risk of lung cancer in humans after a 45-year working lifetime. No uncertainty factors were applied. A WES for fine TiO_2 of 2.4 mg/m^3 was also derived in the same document.	NIOSH 2011 (USA)
Titanium dioxide	0.6 mg/m^3 Period-limited (15 years)	It was considered prevention of lung inflammation would also prevent cancer. NOAEL (lung inflammation) for TiO_2 was determined to be 2 mg/m^3. This was extrapolated to a human equivalent air concentration of 1.82 mg/m^3. An uncertainty factor of 3 (for selection of dose metric) was applied to derive a WES of 0.61 mg/m^3. This was denoted a 'period-limited' WES with an exposure time of 15 years (to be revised when more knowledge is available).	Ogura et al. 2011 and Nakanishi 2011 (Japan)
Titanium dioxide	(DNEL) 0.017 mg/m^3 8-hour TWA	A NOAEC of 0.5 mg/m^3 from a 13-week inhalation study in rats (6 hrs/d, 5d/week, 21nm particles) was adjusted to 0.25 mg/m^3 for worker exposure (0.5 mg/m^3 x 6h/8h x 6.7m^3/10m^3). An uncertainty factor of 15 (see text) was applied to obtain a DNEL of 0.017 mg/m^3.	EC 2009b (Europe)

Nanomaterial	WES	Basis	Reference
Silver nanoparticles	(DNEL) Inflammatory response: 1) 0.00033 mg/m^3 2) 0.000098 mg/m^3 Systemic liver effects: 0.00067 mg/m^3	Two DNELs were derived using a LOAEC of 0.049 mg/m^3 for lung inflammatory responses in 90-day inhalation study in the rat. This was converted to a worker LOAEC of 0.025 mg/m^3 (6h/8h x 6.7m^3/10m^3 x LOAEC) and extrapolated to a NAEC using factors of 3 (scenario 1) or 10 (scenario 2). A UF of 25 was applied to give a $DNEL_{lung\ scenario1}$ of 0.00033 mg/m^3 and a $DNEL_{lung\ scenario2}$ of 0.000098 mg/m^3. The third DNEL was derived using a NOAEC of 0.133 mg/m^3 for other effects (mainly liver). This was converted to a worker NOAEC of 0.067 mg/m^3 (6h/8h x 6.7m^3/10m^3 x NOAEC). A UF of 100 was applied to give a $DNEL_{liver\ effect}$ of 0.00067 mg/m^3.	EC 2009b (Europe)
Carbon nanotubes/ nanofibres (SWCNT & MWCNT)	0.001 mg/m^3 8-hour TWA, 45 working years	Applied BMD modelling to data for early stage fibrotic and inflammatory lung responses to CNT exposure in short-term and subchronic animal studies. Extrapolated estimated BMDs and BMDLs, as well as NOAELs and LOAELs identified in the experimental studies, to humans by accounting for differences in lung deposition. Subsequently working-lifetime exposure concentrations of CNT were estimated assuming 8-hour TWA exposure during a 40-hour workweek, 50 weeks per year, for 45 years. Working lifetime exposure of 0.2-2 µg/m^3 (8-hour TWA) was estimated to be associated with 10% excess risk of early-stage adverse lung effects (BMDLs). Using NOAELs this was or 4-18 µg/m^3. Uncertainty factors of 20-60 were applied giving zero risk levels of <1 µg/m^3 (8-hour TWA over working lifetime). WES was recommended at current laboratory limit of quantitation of 1 µg/m^3.	NIOSH 2013a (USA)

Nanomaterial	WES	Basis	Reference
Carbon nanotubes (SWCNT & MWCNT)	0.03 mg/m^3 Period-limited (15 years)	Based on 4-week inhalation studies of SWCNT and MWCNT in rats. Lowest NOAEL for lung inflammation of 0.13 mg/m^3 (for SWCNT) used as basis for WES. An uncertainty factor of 2 was applied (for use of subchronic study), prior to conversion into human equivalent concentration of 0.09 mg/m^3. An additional UF of 3 (for interspecies toxicokinetic differences) was applied to give a period-limited (15 years) WES of 0.03 mg/m^3.	Nakanishi et al. 2011 (Japan)
Carbon nanotubes	(DNEL) Short-term exposure: 0.2 mg/m^3 or 0.004 mg/m^3 Long term exposure: 0.034 mg/m^3 or 0.0007 mg/m^3	'Derived No Effect Levels' (DNELs) for worker exposure using 2 scenarios: 1. NOAEC of 5 mg/m^3 for absence of lung inflammation or tissue damage in 14 day mouse inhalation study. NOAEC was converted to worker NAEC (NOAEC x 6h/8h x 6.7 m^3/10 m^3) of 2.5 mg/m^3. UF of 12 (short term exposure) or 75 (long term exposure) (see text) were applied to give $DNEL_{inhalation,\ scenario1,\ short\ term}$ of 0.2 mg/m^3 and a $DNEL_{inhalation,\ scenario1,\ long\ term}$ of 0.034 mg/m^3. 2. LOAEC of 0.3 mg/m^3 for MWCNT for systemic immune effects in the spleen (but not lung) from the same study. Converted to worker LAEC (LOAEC x 6h/8h x 6.7 m^3/10 m^3) of 0.15 mg/m^3. UF of 37.5 (short term exposure) or 225 (long term exposure) (see text) were applied to give $DNEL_{inhalation,\ scenario2,\ short\ term}$ of 0.004 mg/m^3 and a $DNEL_{inhalation,\ scenario2,\ long\ term}$ of 0.0007 mg/m^3.	EC 2009b
Multi-walled carbon nanotubes (Baytubes®)	0.05 mg/m^3 8-hour TWA	NOAEL of 0.1 mg/m^3 for lung and nasal inflammatory responses from 13-week inhalational rat study with Baytubes® adjusted by a factor of 2 for worker exposure time, air intake, deposition, and clearance kinetics to derive a WES of 0.05 mg/m^3.	Pauluhn 2010b (Germany – Bayer, industry)
Multi-walled carbon nanotubes	0.0025 mg/m^3 8-hour TWA	LOAEC of 0.1 mg/m^3 for minimal granulomatous inflammation in the lung from 90-day inhalational rat study. Uncertainty factor of 40 was applied (details not provided) to derive a WES of 0.0025 mg/m^3.	Nanocyl 2009 (Belgium – Nanocyl, industry)

Nanomaterial	WES	Basis	Reference
Multi-walled carbon nanotubes	0.02 mg/m^3 or 0.01 mg/m^3 8-hour TWA	Adjusted NOAEC/LOAEC of 0.1 mg/m^3 for lung and nasal inflammatory responses from MWCNT in two 90-day inhalational rat studies to human equivalent concentration of 0.05 mg/m^3 (0.1 mg/m^3 x 6h/8h x 6.7m^3/10m^3). Then applied an uncertainty factor of 25 or 50 (see text) to give proposed WESs of 0.002 or 0.001 mg/m^3.	Aschberger et al. 2010 (Europe)
Fullerenes	(DNEL) Short term exposure: 0.044 mg/m^3 Long term exposure: 0.0003 mg/m^3	For short term exposure: NOAEC of 2.22 mg/m^3 for absence of lung inflammatory related effects in rats from 10-day inhalation exposure was converted to a worker NAEC of 0.55 mg/m^3 (NOEC x 3h/8h x 6.7 m^3/10 m^3). An assessment factor of 12.5 (see text) was applied to give a DNEL$_{\text{inhalation, acute}}$ of 0.044 mg/m^3. For long term exposure: LOAEC of 0.12 mg/m^3 for up-regulation of genes involved in inflammatory response from subacute inhalation study was converted to worker LAEC of 0.06 mg/m^3 (LOAEC x 6h/8h x 6.7 m^3/10 m^3). An assessment factor of 75 (see text) was applied to give a DNEL$_{\text{inhalation, chronic}}$ of 0.0003 mg/m^3.	EC 2009b (Europe)
C_{60} fullerenes	0.39 mg/m^3 Period-limited (15 years)	Converted NOAEL of 0.7 mg/lung from intratracheal study to an air concentration of 3.1 mg/m^3 using information for clearance, lung retention and physiology of the rat. A human equivalent NOAEL of 3.5 mg/m^3 was calculated. An uncertainty factor of 9 (see text) was applied to derive a provisional period-limited WES of 0.39 mg/m^3. The WES can be adjusted for specific particle size using equations in the publication.	Shinohara et al. 2011 (Japan)
Toner emissions (respirable biopersistent granular particles)	0.6 mg/m^3 ('tolerable' risk) 0.06 mg/m^3 ('acceptable' risk) 0.006 mg/m^3 ('acceptable' risk, as of 2018)	Derivation as per Roller (2006), cited in BAuA 2010. Not described in detail in BAuA 2010. Based on incidence of lung tumours in intratracheal and inhalational animal experiments. [a]	BAuA 2010 (Germany)

WES = workplace exposure standard; BMD = benchmark dose; BMDL = 95% lower confidence limit on benchmark dose; SWCNT = single-walled carbon nanotubes; MWCNT = multi-walled carbon nanotubes; TWA = time-weighted average; DNEL = derived no-effect level; UF = uncertainty factor; WEL = workplace exposure limit; CMAR = Carcinogenic/ mutagenic/asthmagenic/reproductive toxin.

[a] Dose-dependent increases in the incidence of lung tumours have been observed in intratracheal instillation experiments using rats and toner emissions at very high concentrations (Pott and Roller 2005, Roller 2008). Inhalational studies in rats

using toner emissions have resulted in slight or no increases in lung tumour incidence (Muhle et al. 1991, Morimoto et al. 2005). The latter indicates the potential for lung tumours after inhalation exposure to toner emissions.

Table C2: Other workplace exposure limits

Nanomaterial	WES	Basis	Reference
Various	1. 20,000 particles/cm^3 (metals, metal oxides & other biopersistent NMs with a density of >6,000 kg/m^3) 2. 40,000 particles/cm^3 (biopersistent granular NMs with a density <6,000 kg/m^3) 3. 10,000 fibres/m^3 (CNT which do not exhibit properties similar to asbestos)	These benchmarks are to be applied as increases over the aerosol background and as average shift values. They are not substantiated toxicologically. Further details of their derivation are not provided.	IFA 2012 (Germany)
Various	As per IFA 2012	As per IFA 2012 RIVM (2010) considered both the IFA (2012) and BSI (2007) approach (see below), but chose the IFA (2012) approach as it seemed more scientifically sound.	RIVM 2010 (The Netherlands)
Various	WEL for conventional substance (non-nano) x 0.066 for insoluble NMs. WEL x 0.5 for soluble NMs WEL x 0.1 for CMAR NMs OR 20,000 particles/ cm^3 for insoluble NMs. 0.01 fibres/cm^3 for fibrous NMs with aspect ratio greater than 3:1 and length greater than 5,000 nm.	The correction factors of 0.1 for CMAR NMs and 0.5 for soluble NMs are arbitrary and not based on scientific evidence. The correction factor of 0.066 for insoluble NMs is the proportional difference between the NIOSH draft recommended exposure limits of 1.5 mg/m^3 for fine TiO_2 and 0.1 mg/m^3 for ultrafine particles. Note these suggested WESs are from a 2005 NIOSH publication, which has since been updated (NIOSH 2011). The updated recommended provisional WESs are 2.4 and 0.3 mg/m^3 for fine and ultrafine TiO_2, respectively. The proportional difference between these two values is 0.125, approximately double the correction factor originally proposed by BSI (2007).	BSI 2007

WES = workplace exposure standard; BMD = benchmark dose; BMDL = 95% lower confidence limit on benchmark dose; SWCNT = single-walled carbon nanotubes; MWCNT = multi-walled carbon nanotubes; TWA = time-weighted average; DNEL = derived no-effect level; UF = uncertainty factor; WEL = workplace exposure limit; CMAR = Carcinogenic/ mutagenic/asthmagenic/reproductive toxin.

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