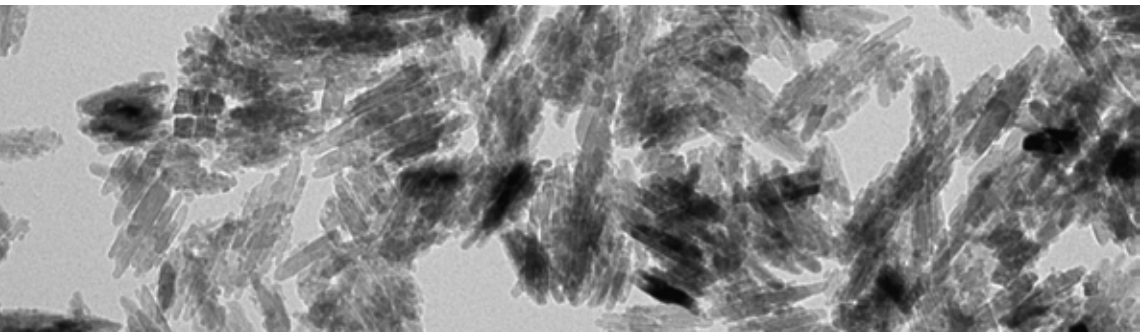
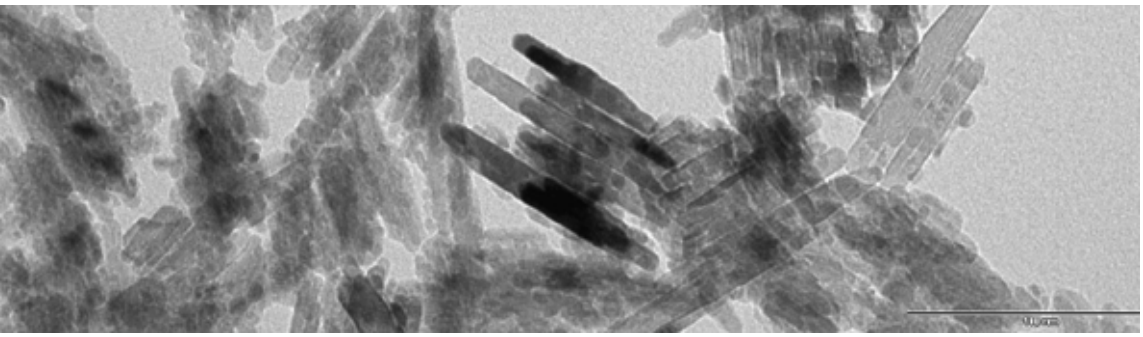
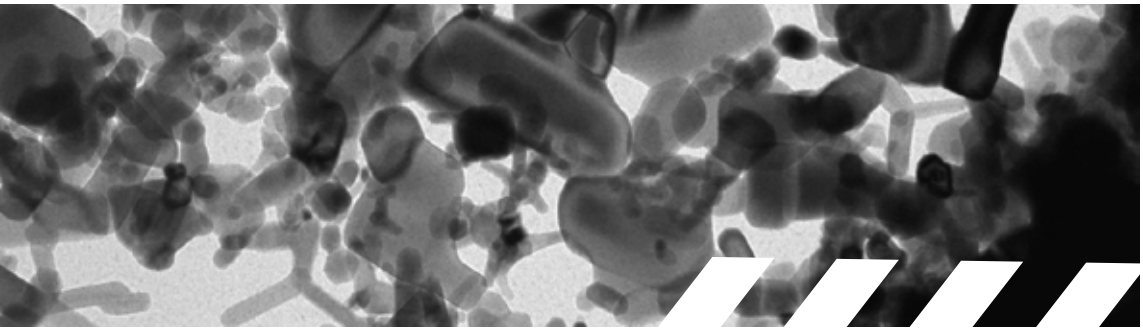
ENGINEERED NANOMATERIALS: A REVIEW OF THE TOXICOLOGY AND HEALTH HAZARDS







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Engineered nanomaterials: A review of the toxicology and health hazards

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Toxikos Pty Ltd is a consulting company specialising in toxicology and health based risk assessment of chemicals in the human environment. Its charter is to assist industry and government make science based decisions regarding potential effects and management of environmental and occupational chemicals. For more than 20 years staff have provided toxicology and health risk assessment advice to clients in a wide range of industries and government in Australia, New Zealand and South Africa.

The report was independently reviewed by staff of SAFENANO at the United Kingdom's Institute of Occupational Medicine (IOM) during drafting. IOM has international renowned research programs investigating the toxicology of engineered nanomaterials. SAFENANO is an initiative that provides strategic, independent and impartial advice to stakeholders concerning the potential risks to human health and the environment from nanomaterials.

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Executive summary

This review reports the current understanding of the toxicology and health hazards associated with engineered nanomaterials, and the implications in regard to health hazards in occupational settings (i.e. during manufacture, handling, and use). It updates a previous review by the Australian Safety and Compensation Council. The information in this review is based on scientific literature published from 2006 to 2008, however, during the editorial phase some important publications from the first half of 2009 have been incorporated.

There are two primary streams of health/toxicology research involving nanoparticles (NPs) currently being carried out internationally. The first involves public and occupational health impacts of fine, and ultrafine, particulate air pollution. This type of human exposure to NPs has been in existence for millennia but more so since the industrial revolution. By virtue of the historically longer exposure times, and hence longer investigation effort, it is arguably the most advanced of the two research areas. Much of the concern about NPs is rooted in epidemiological evidence showing ambient or occupational air pollution by fine particulates may induce/exacerbate airways disease and increase mortality incidence in susceptible individuals, most notably in those with compromised pulmonary and cardiovascular function. In the occupational arena much is known about the illnesses induced by quartz and asbestos, and how they come about.

The second NP research stream is focussed on the toxicology of engineered nanoparticles (ENPs). This is a rapidly expanding field. While there are theoretical concerns regarding public health impact, the major thrust of the research is in relation to identifying potential hazards for assessment of occupational safety since working with ENPs is likely to be where most exposure occurs. In contrast to ambient particulate air pollution, where health effects have been observed and research has been aimed at discovering the causative agents and mechanisms, the reverse is true for ENPs. The challenge is to find out what health impacts might occur should there be exposure, and what level of exposure is necessary for those effects. Naturally, efforts to identify hazards associated with ENPs are influenced by experiences with the other particulates and with chemicals in general. We know that particle size and surface chemistry respectively dictate where a material ends up in the body and what toxicity could occur, especially in the lungs.

Approaches to the hazard identification of ENPs have been informed, indeed driven, by the considerable toxicological and health knowledge base that exists for fine particles polluting ambient air, and occupational exposures to crystalline silica (quartz) and asbestos. Understanding the toxicological paradigms underpinning current awareness of the pulmonary responses to particles and fibres is fundamental for interpretation of many of the *in vitro* and *in vivo* toxicological experiments that have been conducted with ENPs. These paradigms are described in the body of this review. Biopersistence in the lung is a pivotal feature of particulates, including ENPs that cause pulmonary toxicity.

Mechanistic understanding of the association between fine particle air pollution and adverse cardiovascular outcomes is developing and is informative regarding possible hazards of ENPs. However the occupational experiences with respirable quartz dust, asbestos and man-made mineral fibres dictate that the lung is the primary target organ for airborne fine particles in the workplace; consequently, and correctly, potential

pulmonary effects of ENPs have been the main focus of the nanotoxicology literature over the last few years. There have been only few attempts to evaluate the systemic toxicity of ENPs following inhalation exposure, titanium dioxide (TiO_2) is perhaps the exception. Because ENP's may be used in consumer products other routes of exposure, in addition to inhalation are also being investigated.

The question of whether the very small size of ENPs could confer unique toxicity to materials, i.e. toxicity not previously known or identified for the same material of larger particle size, has been a speculative concern in much of the scientific and lay literature over the last five years or so. In the literature examined for this review persuasive evidence for such a 'unique toxicity' phenomenon was not found, however it is acknowledged that sufficient tests (acute and chronic) in relevant test models that look at a wide variety of endpoints have not yet been conducted for most ENPs. There is much evidence to suggest that nanoparticles tend to be more bioreactive/toxic than larger particles of the same material, and so, in the context of this review, while producing the same spectrum of *in vivo* toxicities associated with larger particles they do so at somewhat smaller mass doses, i.e. the nanomaterials are more potent. Our current understanding indicates that by and large the pulmonary hazards of nanoparticles may be predicted from a combination of contemporary knowledge of the hazards of fine particulates and fibres *per se* and targeted *in vivo* and toxicokinetic testing of ENPs. This however is tempered by the possibility that nanoparticles with different surface coatings and low biopersistence will have different potency to cause the lung effects than nanoparticles that are highly reactive and/or biopersistent.

There are also questions regarding whether traditional protocols used for safety testing chemicals are appropriate for ENPs. The evidence would suggest that some of those tests may need to be modified if they are to yield data useful for occupational risk assessment of ENPs. It is also noted that some ENPs can be transported to parts of the body, and be retained, which is not observed for their larger counterpart. However investigations have not yet been conducted to determine whether there are adverse *in vivo* effects associated with these disposition patterns. So the question of the whether the unique physical properties of ENPs can produce a different (i.e. unique) spectrum of toxicity compared with larger particles of the same material awaits the results of long term *in vivo* tests that have examined more end points than just lung pathology. There have been numerous *in vitro* (test tube) experiments performed with a wide range ENPs that have shown a number of effects. However, on their own, these results are not sufficient for assessing health risks in the workplace. They do however indicate the need for more investigation into the potential health impacts of ENPs.

Many scientific and agency reviews of nanotechnology and the nanotoxicology literature have been published since 2006. For various aspects of nanotoxicology these reviews provide an introduction and insight to the earlier literature. However due to lack of definitive studies many of the reviews speculate, or build hypotheses regarding potential toxicity and health effects of ENPs. When reading the reviews care is required in separating opinions founded on empirical information from those based on assumption.

With the exception of titanium dioxide (TiO_2), long term exposure inhalation studies in experimental animals for ENPs are not referenced in these reviews and none were located in the literature search conducted for this review. Patently this is a significant data gap. Hazard identification and occupational risk assessment of most ENPs are therefore currently constrained to extrapolation of rodent single dose studies that have incorporated pulmonary response evaluation at varying post treatment times. However data from sub-chronic repeat inhalation investigations were recently presented at a

toxicology scientific society meeting in the United States. Although this information awaits peer review and publication in scientific journals it is included in this review at the appropriate places to provide an indication of the type of data that will be forthcoming over the next few years.

Experimental conditions

There are important aspects of the experimental procedures used to assess the toxicity of ENPs that must be considered when evaluating the data and drawing possible implications for worker health impact.

Intratracheal administration

For most ENPs it is difficult to generate aerosols suitable for inhalation experiments, consequently instillation of a suspension of ENPs directly into the upper tracheobronchial region of rats has been the favoured experimental technique for delivering ENPs into the lungs. However rats are particularly prone to pulmonary particle overload by intratracheal instillation. The pulmonary reactions to large doses of poorly soluble, persistent respirable particles are currently well known and qualitatively predictable. Because these reactions are a result of the particulate overload and not reflective of the intrinsic toxicity of the material, data derived from such high exposure experiments should not be solely relied upon for hazard identification or human risk assessment for ENPs. The problem in interpreting these studies is exacerbated because many have employed only one or two doses and have not attempted to identify doses that are without effects.

Intratracheal mass particulate doses greater than about 200 – 300 µg/rat have the potential to overwhelm pulmonary clearance mechanisms. It has been recommended that intratracheal instillation studies be limited to particulate doses of less than 100 µg/rat. Many *in vivo* ENP toxicity studies have employed intratracheal doses much greater than this and are essentially studying the pulmonary overload phenomenon and not the intrinsic toxicity of the ENP.

Depending on the equations used to extrapolate the dose to humans, 100 µg/rat is equivalent to an instant deposition of the mass inhaled by a human during continuous 8 hour exposure to an airborne ENP concentration of between 2.5 – 5 mg/m^3.

More recently pharyngeal aspiration has been used in mice to investigate the lung responses to ENPs. This technique is less invasive, is unlikely to cause particle overload, and is amenable to repeat exposures. It is however not ideal as it potentially allows aspiration of saliva and bacteria/endotoxin into the lung at the same time the ENPs are being introduced. If this occurs pulmonary reactions to the administered agent may be modified.

Dispersal medium

In intratracheal experimental protocols, exposure of the lower reaches of the lungs requires the material to diffuse away from the site of instillation. The ability of the administered material to move from the bronchioles and reach the target alveolar area is critically dependent on the choice of dispersion agent. While many studies have characterised ENPs as they have been synthesised it is only recently that physical characterisation has been undertaken in the media used to deliver the material to the

experimental system. There are significant differences in the pulmonary distribution and toxicity of agglomerated versus dispersed ENPs.

An array of dispersal media have been used for delivering nanomaterials to animals or cell cultures; unless protein and/or surfactant are included in the media, ENPs can markedly agglomerate and precipitate. While the best dispersal media seems to be one based on the constituents of bronchiolar-alveolar fluid an agreed dispersion protocol for either *in vivo* or *in vitro* experiments has not been established. This is probably not critical providing that for any given experiment it has been shown agglomeration is minimised and there is effective dispersion in the media being used.

If pulmonary response data from animals is used for hazard identification or risk assessment there are tacit assumptions that the physical state of the ENP during experimental exposure, and ENP behaviour in the lung of animals, is the same as that for humans. It should be feasible to determine the agglomeration state of ENPs in workplace air. However, while in experimental situations it may be possible to determine if the administered ENP agglomerates or de-agglomerates in the lung, it is unlikely that similar *in situ* information will be available for humans.

Characterisation

In toxicological investigations, it is essential that ENPs be thoroughly characterised in the medium in which they are delivered to the test system. Many *in vivo* and *in vitro* studies have failed to adequately define and describe the ENPs being investigated; this severely limits the utility of the data generated by these experiments.

Characterisation of the biological responses is often undertaken by comparing the results with very fine or nano-size particulates of material for which the toxicity and health effects are reasonably well known (e.g. quartz, carbon black or asbestos). Standard ENPs which can be used for similar purpose are not yet available.

In vitro systems

The literature over the last few years contains a huge number of *in vitro* experiments conducted with all sorts of different nanomaterials. A large and bewildering range of cellular endpoints have been measured in a vast array of cultured cell types. Since current mechanistic knowledge indicates excessive oxidative stress and consequential prolonged inflammation are pivotal events in toxicological responses to particulates and fibres, many *in vitro* investigations measure various genomic and biochemical steps in the oxidative stress/inflammatory cascade and/or the cellular consequences of undue redox imbalance (e.g. cytotoxicity and other end points of cell viability).

In isolation, the data emerging from these experiments are of limited value in appraising ENP safety or in assessing risk to workers. The studies are potentially useful if they are part of a program testing a mechanistic hypothesis or comparing effects of a novel ENP with a standard material. The most useful application of such *in vitro* tests lies in investigating the influence of ENP purification, or different functional coatings on biocompatibility, and in developing nanomaterial which comparatively has lower adverse cellular effects than others within its family. Ultimately however the ENP selected for commercialisation will need to be tested in whole animals in order for its biological fate and hazards to be properly investigated.

Carbon nanotubes (CNTs)

In the main text of this report the recent toxicological data for single walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) are presented separately. Although the toxicity of SWCNTs has not been as extensively studied as MWCNTs, they both produce pulmonary inflammation, oxidative stress, interstitial fibrosis and granulomas. Hence, with respect to their cytotoxicity and pulmonary toxicity associated with inflammation, the information for these substances are summarised here as carbon nanotubes (CNTs). Both types of CNTs appear to be able to act as adjuvants to enhance the immunological response of inhaled allergens. The information relating to possible fibre-like carcinogenic activity of MWCNTs is presented separately as similar studies have not yet been conducted for SWCNTs.

As with other NPs the surface chemistry of CNTs is critical to their *in vivo* behaviour. Non-functionalised, i.e. pristine, CNTs have hydrophobic surfaces and are non-specific in binding proteins on their surfaces. This binding may change the shape of the protein. A variety of organic compounds are used to alter the surface chemistry of CNTs to make them more biocompatible; polyethylene glycol (PEG) is often used to covalently coat CNTs to form stable, water dispersible materials intended for biomedical application.

Chronic inhalation toxicity studies published in the scientific literature for CNTs were not located. This is not surprising given the practical difficulties in consistently generating respirable aerosols of CNTs with defined characteristics for experimental purposes. However such methods have been recently been developed and at a recent scientific meeting preliminary data for repeat inhalation exposures were presented. The information by and large qualitatively corroborates the findings from intratracheal instillation and aspiration experiments.

Physiochemical properties of non-functionalised CNTs are different to functionalised CNTs, the latter tending to be more water soluble and to have lower biopersistence. Generally, increasing the functionalisation of CNTs decreases their *in vitro* cytotoxicity. Because many functionalised CNTs are being investigated for potential clinical applications in diagnosis and treatment of diseases, the primary route of human exposure in these situations is not considered to be inhalation but rather parenteral (injection or infusion). Consequently most *in vivo* data for functionalised CNT has been generated by intravenous administration to experimental animals. When pristine CNTs are intravenously injected into mice the plasma half life of the material is short, of the order of minutes. They are widely distributed in the body and being avidly taken up by the reticuloendothelial system, the liver, spleen and lungs have the highest concentrations. Lack of suitable assay methods for CNTs in biological matrices hamper measurements of low concentrations in tissues, but after high intravenous doses of 2 mg/mouse (i.e. approximately 80 mg/kg) SWCNTs can be found in nearly all tissues, including the brain. Non-functionalised CNTs appear not to be excreted in urine and tissue concentrations remain high for very long periods. In contrast CNTs whose surface has been functionalised have longer plasma half lives due to lower/slower uptake by the reticuloendothelial system, and while still widely distributed in the body have shorter tissue retention. Studies investigating the systemic distribution of CNTs after inhalation were not found for this review.

Recent toxicological studies examining the systemic toxicity of CNTs are limited to observations made up to three months after single intravenous doses. Nonetheless, as variously judged by lack of clinical signs, maintenance of body weight, normal blood

chemistry and tissue histological evaluation (albeit limited), neither pristine nor functionalised CNTs appear to exert acute systemic toxicity after intravenous administration. While this is reassuring, the long tissue retention of CNTs (both pristine and functionalised) highlights the need for thorough evaluation of a range of toxicological endpoints after chronic repeat dose studies, using all relevant human exposure routes, using a few 'model' CNTs which have been physically and chemically well characterised, and which have different toxicities. Such studies could act as comparative benchmarks for other CNTs.

Most *in vivo* toxicology studies of non-functionalised CNTs investigating potential pulmonary toxicity employ non-physiological exposures such as single dose intratracheal instillation to rats, or single exposures of mice by pharyngeal aspiration. Animals are then evaluated at various times for up to 3 months post exposure. At high doses non-functionalised CNTs (whether single walled or multi walled) are able to elicit inflammation, fibrosis and granuloma formation in the lungs of both species. Similar effects are observed when they are administered in vehicles that minimise aggregation (i.e. allow more efficient dispersion within the lung) and at doses that do not significantly compromise normal lung clearance mechanisms. The pulmonary reaction of CNTs is therefore not conditional on the rat particle overload phenomenon.

The World Health Organisation (WHO) has defined pathogenic fibres as those which are longer than 5 μm, have a diameter less than 3 μm, and have an aspect ratio (length to width ratio) greater than 3:1. Experimental protocols developed for screening materials for potential fibrogenic activity involve injecting high amounts into the abdominal cavity (i.e. the peritoneum) of mice and evaluating the peritoneal mesothelial tissues at various times afterwards. Recent experiments with thin, long straight non-functionalised MWCNTs of fibre length greater than 20 μm, i.e. those meeting the WHO pathogenic fibre definition, have shown a sustained inflammatory reaction, granuloma formation, and tumours of the mesothelial tissue lining the peritoneal cavity similar to pathogenic asbestos fibres. This did not occur with shorter or long tangled MWCNTs, or shorter asbestos fibres, or with the tested fullerenes.

In vitro experiments support the hypothesis that pathogenic fibre-like CNTs are able to initiate genomic responses in mesothelial cells that may lead to uncontrolled cell growth and mesothelioma formation. In addition recent preliminary, unpublished data indicates MWCNTs, when inhaled or administered by pharangeal aspiration to mice can reach the mesothelial lining of the pleural cavity and initiate inflammatory reaction.

Although SWCNTs of pathogenic fibre dimensions have not been tested in the rodent 'peritoneal fibre' screening assay it would be prudent, in the absence of data, to assume such SWCNTs, if they were biopersisitant, would produce similar effects as MWCNTs of pathogenic fibre dimensions if sufficient numbers reached the mesothelial target tissue after inhalation.

Summary

Non-functionalised CNT are able to elicit toxicological responses similar to both fine particulates and respirable pathogenic fibres. While impurities, particularly metals, can modulate the responses, recent experiments have shown they are not responsible for the primary effects. The effects are intrinsic to the non-functionalised CNT *per se*.

The experimental data broadly indicate some of the toxicological effects of non-functionalised CNT are dose related but there is insufficient information available for

reliable identification of doses which do not cause adverse effects. While no observed adverse effect levels (NOAELs) are assumed to exist, experimental designs to date have not allowed good dose response analysis and determination of NOAELs, or their equivalent.

At this time there is information to support an initial view that functionalised CNTs may present different (lower) health hazards than non-functionalised CNTs if the former are less biopersistent. It is stressed however that this is a preliminary opinion since equivalent *in vivo* experiments delivering functionalised CNTs to the lung have not been conducted, and functionalised CNTs of pathogenic fibre dimensions have not been screened in the 'peritoneal fibre' assay.

Titanium dioxide

Compared to other ENPs the database for nano-titanium dioxide is relatively rich as it has been used as a test material for developing and trialling toxicity test protocols for nano-particulates, and it has been in commercial use for a relatively long time. Titanium dioxide (TiO_2) is poorly soluble and generally regarded as a low toxicity substance. Nonetheless a number of intratracheal and inhalation studies have shown both fine (about 0.1 - 10 µm diameter) and ultra-fine (i.e. nano-sized, <0.1 µm) TiO_2 can induce pulmonary inflammation, fibrosis and lung cancer if sufficient material can reach the lungs to exceed the threshold load necessary for prolonged inflammation. Relative to quartz the acute inflammatory response to low doses of nano-TiO_2 is transient and without clinical significance.

The International Agency for Research on Cancer has recently evaluated the data for TiO_2 and classified the material as possibly carcinogenic to humans (i.e. Group 2B in the IARC classification scheme). The basis for the classification is rat studies with high exposures leading to particulate overload and cancer. IARC considered the biological mechanisms operable in the rat relevant to workers in dusty jobs, i.e. situations of high exposures. Nonetheless there is consensus that the critical mode of action, sustained inflammation, has a threshold and hence there will be exposures without significant harm to humans.

Data was not located for this review that enabled identification of no observed effect levels associated with long term repeat inhalation studies. However the existing rat cancer bioassay studies potentially lend themselves to detailed dose response modelling that should enable safe levels for human exposure to be determined.

There is sufficient data indicating nano-TiO_2 is more efficient at inducing pulmonary inflammation than fine particulate TiO_2, and that anatase nano-TiO_2 is potentially more toxic than the equivalent sized rutile nano-material. However all forms and sizes of TiO_2 are able to induce a pulmonary inflammatory response if the exposure is high enough.

Reviews of dermal absorption studies for nanoscale TiO_2 consistently conclude that TiO_2 is not absorbed through intact skin. However it is noted dermal penetration has not been studied in damaged or compromised skin.

High dose oral studies show nano-TiO_2 can be absorbed from the gastrointestinal tract and be distributed throughout the body. Neither intravenous or oral studies indicate nano-TiO_2 retention within blood cells, plasma, brain or lymph nodes but there is significant retention by the liver, spleen, kidney and lung for observation periods of

either fourteen or twenty eight days. Distribution studies after repeated low oral doses or inhalation exposure were not found for this review. Repeat subcutaneous administration, giving a very high total dose of anatase-TiO_2 to pregnant mice causes the material to be distributed to the tissues of off-spring. Although a number of biological changes are observed in the off-spring, investigations of adverse functional effects have not yet been conducted. In addition the data is difficult to inteprete with respect to human health due to deficiences in experimental design and reporting.

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.

Application of a variety of traditional hazard/safety tests to nano-TiO_2 has shown the material to be of low acute toxicity. There are probably also low chronic risks to human health if exposures are kept below the threshold for initiating sustained pulmonary inflammation.

General comments

- Interpreting nanotoxicology publications requires special attention to the experimental details and physical characterisation of the ENPs in the dosing medium in order to adequately gauge the relevance of the information.

- Agglomeration of ENPs can significantly alter their *in vivo* and *in vitro* toxicity.

- Chronic or repeat dose inhalation toxicity studies for the majority of ENPs have not yet been conducted. This is most likely because it is very difficult and resource intensive to generate, and demonstrate, consistent aerosol concentrations of ENPs.

- Studies predating the period of this review have shown direct absorption of inhaled ENPs into the systemic circulation may occur through the lungs. More recent investigations were not located.

- Previous reviews have indicated NPs cause more intense pulmonary reactions than the equivalent mass of coarser (fine) material. Particle surface area is consequently considered a better predictor of the toxicity and pathologic response of inhaled ENPs than is particle mass dose.

- Once in the systemic circulation ENPs may be distributed throughout the body, including the foetus. Transport into various tissues is dependent upon the relative size of the ENPs and the pore holes of the capillaries serving the tissue. ENPs are rapidly taken up from the blood by the reticuloendothelial system and sequestered primarily by the liver, spleen and lungs where they may remain for long time.

- Overall there is a consistent theme in the recent literature on the kinetics and disposition of CNTs, quantum dots (QDs) and fullerenes:

    - they are quickly taken up by the reticuloendothelial system, but nonetheless distributed widely in the body.

    - there is strong tissue retention once the material is taken up.

- functionalised CNTs and QDs are less avidly taken up by the reticuloendothelial system and so have longer plasma half lives.

- the major route of elimination from the body appears to be biliary excretion with excretion in urine being absent or minimal. Functionalisation that increases solubility increases urinary excretion.

- Intravenous injection of a limited number of ENPs into rodents has not shown significant toxicity for up to 3 months observation after the injection, suggesting low acute systemic toxicity. It is noted however only limited toxicological endpoints have been evaluated in these acute tests. Similarly application of traditional acute toxicity test protocols to nano-TiO_2 has shown them to have low acute toxicity.

- However, since chronic repeat dose toxicological studies have not been conducted for most ENPs, the long term consequences of the avid accumulation of ENPs by the reticuloendothelial system are unknown.

- Similarly, adequate evaluation of the potential for reproductive toxicity of ENPs using relevant exposure routes and different dose levels has not been conducted to date. However, for the few ENPs so far examined, there are indications that multiple injections of very high doses to pregnant mice can cause biochemical and physiological changes in some organ systems in the off-spring. These studies suffer from experimental design and reporting deficiencies, consequently other than signalling caution when women of child bearing age are working with ENPs, the broader relevance of these findings for hazard and risk to humans is not clear.

- In a number of different *in vitro* test systems, ENPs have been shown to have deleterious dose and time dependent effects on cell function and viability. These are observed mostly at high applied concentrations. On their own *in vitro* data are of limited assistance in risk assessment but may be useful for:

    - identifying ENPs requiring further toxicological characterisation,

    - choosing an ENP from within a group that may have lower adverse biological effects for commercialization, or,

    - occupational hazard identification when appropriate criteria are developed,

    - investigating possible mechanisms of toxicity, and

    - adding to a ‘weight of evidence’ evaluation in risk assessment.

- Unless the ENP has been functionalised leaving a reactive ‘organic’ surface, or contains impurities, no information has been recently published that provides compelling evidence that poorly soluble ENPs behave differently to other well characterised insoluble fine particulates with respect to causing DNA damage. That is, the secondary genotoxicity pathway mediated by reactive oxygen species (ROS) will be the dominant, if not the only, *in vivo* mechanism causing DNA damage.

- Biologically persistent ENPs (e.g. metal oxides and certain size CNTs) can cause typical particulate and fibre responses in the lungs after high doses via unphysiological exposure routes. Although these studies, if well conducted, serve to identify potential occupational hazards they do not provide information to assess potential risk. Understanding the dose response relationships of long term inhalation exposures to the types of ENPs in workplace air is required for establishing risk.

- Concern is often expressed regarding the potential for adverse cardiovascular effects arising from inhalation exposure to ENPs. From ambient particulate pollution and the limited number of studies published in the last few years there is sufficient data available to legitimise the concern for susceptible individuals. However there is insufficient information to be able to judge whether, at levels of airborne ENPS which workers are likely to encounter, this concern is significant for worker health.

- Where meaningfully investigated, recent studies have shown low concordance between *in vitro* and *in vivo* toxicity. The predictive power of *in vitro* tests for an *in vivo* outcome is therefore questionable. Overall, *in vitro* cellular systems need to be further developed, standardized, and validated (relative to *in vivo* effects) in order to provide useful screening data on the potential effects of inhaled ENPs of unknown toxicity.

- Based on the information retrieved for this review fullerenes appear not to be very efficient in generating free radicals, they don't alter macrophage function and are not intrinsically cytotoxic towards macrophages. These properties may explain why a recent 10 day (3 hr/d) inhalation study with dry nano-fullerenes did not reveal acute pulmonary toxicity. *In vivo,* they are also not directly toxic to skin, do not alter DNA synthesis in skin and are not cancer promoters in the skin cancer bioassay. Similarly in a modified 'intraperitoneal' fibre screening assay in which the observation period was extended to 25 weeks they were negative in causing mesothelioma. Whether these general comments can be applied to all fullerenes is not known at this time since their toxicity is likely to be highly dependent on whether they are functionalised, and on the type of functional groups they may contain. Some studies have shown *in vitro* effects on cell function. It is noted that increasing water solubility with fullerene surface coatings decreases the *in vitro* effects.

- Occupational exposure of the eye to nanomaterials is most likely to occur from airborne exposures, and perhaps also by hand-eye transfer. Studies where material has been injected into the eye do not provide relevant information for occupational risk assessment other than an idea of what might happen if substantial amounts were to penetrate the eye. The available hazard data, albeit limited, indicates most ENPs are unlikely to produce adverse eye effects with workplace exposures.

Conclusions and Issues for Further Consideration

These issues are not presented in any particular order; the order of presentation should not be taken as signifying perceived importance of one over another.

1. International criteria for conducting meaningful nanotoxicology experiments could be collated, endorsed and summarised for the benefit of Australian investigators.

2. One of the reasons why repeat inhalation toxicity studies have not been widely done for many ENPs is the fact that ENPs in air readily agglomerate and it is difficult to generate aerosols of individual ENPs and sufficient material for such studies is often not available. Agglomeration also occurs in aqueous suspensions and while addition of lipoprotein, albumin, serum and/or surfactant lessens agglomeration there is uncertainty whether the nanomaterial being evaluated in intratracheal and/or aspiration toxicity studies reflects the physical state of the nanomaterial to which workers may be exposed. To promote the generation and use of experimental dose-effect information in occupational risk assessments of ENPs, there is an urgent need to:

    - characterise in detail the nature of airborne ENPs in workplace air that is potentially breathed by workers, and

    - determine the dissolution behaviour of such ENPs in lung fluid.

3. Biopersistence is a critical ENP property for induction of particulate- and/or fibre-like responses in the lung. The absence of standard/validated tests and criterion for characterising pulmonary persistence of NPs needs to be addressed.

4. The recent nanotoxicology literature is vast and expanding quickly. A wide variety of *in vitro* and *in vivo* experimental protocols have been used to assess biological responses to NPs, some of these yield more useful data for occupational risk assessment than others. Some are potentially misleading. The creation of a set(s) of conditions/criterion for collective evaluation of nanotoxicology publications would assist weight of evidence assessment of the literature. This would necessitate multidisciplinary input and would include such aspects as description of the ENP, its physical characterisation in the experimental exposure milieu, dose and dose metric aspects, relevance of various experimental models, appropriateness of end point measurements for key experimental models, etc.

5. It is clear non-functionalised biopersistant 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.

    - published information was not located for this review to suggest the pulmonary responses to SWCNT that met the physical requirements (i.e. biopersistence and dimensions) of fibrogenicity would be/are substantially different from those of physically similar MWCNT. Consequently, for the purposes of managing workplace exposure and

minimising possible health effects, no distinction should be made at this time between pathogenic fibre size SWCNTs and MWCNTs as to their potential hazards and risks. Further data is however required on the comparative potential of SWCNTs and MWCNTs for induction of pathogenic fibre-like responses.

- while functionalised CNTs are likely to have lower toxicity than non-functionalised CNTs, this has not yet been verified using equivalent *in vivo* assays to deliver the material to the lungs. Consequently no distinction between their potential pulmonary toxicity should be made at this time. Movement from this default for individual CNTs should be based on appropriate data.

6. Evidence leads to a conclusion that as a precautionary default:

    - all biopersistent CNTs, or aggregates of CNTs, of pathogenic fibre dimensions could be considered as presenting a potential fibrogenic and mesothelioma hazard unless demonstrated otherwise by appropriate tests (note CNTs or structures of CNTs, such as ropes, can have such dimensions even if the smallest dimension is greater than 100nm, so while they don't meet the accepted definition of nanoparticle they may still be a pathogenic fibre hazard), and

    - data criteria are required to facilitate advancement away from this default.

    - consideration be given to establishing an occupational exposure standard for CNTs. Since current optical microscopy methods for counting asbestos fibres are inadequate for CNTs, a method of counting CNTs would also need to be considered.

7. The toxicological findings of this report indicate that generating guidelines for hazard classification, provision of material safety data sheet (MSDS) information and workplace labelling of ENPs in Australia should be considered.

8. Consideration should be given to establishing an occupational exposure standard for non-functionalised nano-TiO_2.

9. Recent data suggests carbon based NPs may augment immunological responses to airborne allergens, and other ENPs may suppress immunologic activity. A weight of evidence evaluation of the entire literature was beyond the scope of this update review. Nevertheless it represents an important area for consideration in the workplace and needs to be examined further for ENPs.

10. Validated protocols for conducting *in vitro* genotoxicity assays for ENPs currently do not exist. International progress in this area may be reviewed to identify opportunities for contributing research from Australia.

11. There is a significant need for chronic, or at least, repeat exposure inhalation studies of ENPs.

12. Exposure metrics describing the exposure-response relationships of ENPs are important and should be expressed in several ways to facilitate comparison across studies.

With consideration of the capacity of Australian researchers, some of the above issues could be addressed in Australia to facilitate scientific evaluation of nanomaterial hazards and the safe handling of nanomaterials in the workplace. However due to complexity, cost and other factors, a number of the experimental studies could best be performed under the auspices of organisations in, for example, Europe and North America, such as the Institute of Occupational Medicine (IOM) and NIOSH, that have the expertise, equipment, raw material, management systems and finances to generate significant data under Good Laboratory Practice (GLP) conditions. Australian researchers have started to work collaboratively with these organisations on such projects and if appropriate funding was made available this work could continue.

Contents

1. Scope

This review reports the current understanding of the toxicology and health hazards associated with engineered nanomaterials, and the implications in regard to health hazards in occupational settings (during manufacture, handling, use and disposal). It updates the health hazard information provided in the Australian Safety and Compensation Council's (ASCC) previous review report entitled "A Review of the Potential Occupational Health & Safety Implications from Nanotechnology" (ASCC 2006).

This report provides:

- A review of recent research
- A current understanding of toxicology and health hazards associated with nanomaterials
- Information on the toxicology of specific engineered nanomaterials
- Implications for the protection of health in the workplace
- Suggestions on focus areas for future research.

The review focuses on published research results from 2006 to approximately August 2008. Additional information that became available in late 2008 and early 2009 has been included during the editorial phase of producing this report; however a systematic search for papers has not been done for this latter period. The literature search was undertaken using standard databases (e.g. Medline, Toxline, PubMed, Access Medicine, Science direct etc). For the period in question more than 1,750 hits were recorded with the search terms "nanoparticle and (toxicity or health)". Approximately 500 articles with relevant titles have been read but not all are cited in this review. The bibliography of this review contains all articles located that were considered relevant to the project's scope; articles that are cited in the report are marked with an asterisk.

The previous ASCC review (2006) provided an introduction to the jargon and nomenclature of the world of nanotechnology and a brief description of nanomaterial manufacture. That review noted that various health and materials science specialists had expressed concern the unique physical properties of nanomaterials, conferred by their size, may have untoward effects on human and environmental health. The 2006 review identified significant data gaps with regard to setting priorities for acquisition of information necessary to set workplace exposure standards. In the three years since that review many governmental and academic research initiatives aimed at characterizing and assessing the possible health effects of nanomaterials have been funded. The outcomes of these programs are just now starting to be published in the scientific literature. The concerns expressed publicly regarding the toxicity of engineered nanomaterials were, and still are far ranging. It is not surprising therefore that the data now emerging is only a small fraction of that which will materialize over the next several years, and which is needed to address the expressed concerns. It will be a significant challenge to stay abreast, comprehend and appropriately apply the information. As indicated above this report provides a review and critique of the current scientific information on the hazards of engineered nanomaterials.

The concerns expressed in ASCC (2006) regarding ability to effectively, and economically, measure airborne nanoparticles in the workplace are still valid today. Although assessment of worker exposures was not part of this updated review, it appears that more work is still required to fill the knowledge gap identified in 2006 regarding potential exposures of workers to nanomaterials.

At the time of writing the previous review, the occupational health and safety aspects of engineered nanoparticles were mostly unknown and there was little information available upon which to base hazard and/or risk informed decisions. There is now much more information. Much of it has to do with sorting out what toxicological hazard data is required and how to go about obtaining it so it can be unambiguously interpreted. The lack of knowledge that existed at the time of the previous review is being rapidly addressed. Although published data from long term repeat inhalation exposures are not yet available, these studies are underway. There is however a need for developmental, reproduction and neurobehavioral investigations on ENPs to be undertaken. In addition there are numerous publications of *in vitro* and *in vivo* studies that do not meaningfully contribute to understanding occupational health hazards and risks of engineered nanomaterials. The breadth of *in vitro* and *in vivo* experimental protocols that have been recently applied to a myriad of different nanomaterials to investigate their potential biological interactions is extensive. Some of this 'collateral information' is the result of investigators asking the question, 'what does this ENP do in my test system?' rather than trying to discover the innate toxicological properties of the ENP.

It is evident that no single or few investigations provide definitive hazard data for any particular nanomaterial. Indeed such studies could potentially lead a person to invalid conclusions if they are quoted in isolation. This updated review advocates a 'weight of evidence' evaluation for illuminating the hazards of a given nanomaterial, and that experimental protocols be especially scrutinised during the evaluation. While many experimental approaches have been devised, it currently remains there are no regulatory validated techniques specific for hazard identification of nanomaterials. Nevertheless there is now sufficient robust information to support the initial notions that for some nanomaterials it is prudent and appropriately precautionary to minimise worker exposure; especially during handling activities that may inherently allow the material to become airborne. Application of a precautionary approach should be consistent with current hierarchical approaches for restricting workplace exposure to any material that is known, or suspected to be hazardous.

The ASCC (2006) review remarked that the knowledge gaps would be best addressed at a multidisciplinary level. Indeed it has transpired that the best data is emerging from academic and governmental research centres that have embraced this concept.

2. Glossary

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

- Nanotechnology Now:
  - http://www.nanotech-now.com/nanotechnology-glossary-A-C.htm
- Institute of Nanotechnology, UK:
  - http://www.nano.org.uk/nano/glossary.htm
- Northwestern University:
  - http://www.discovernano.northwestern.edu/whatis/Glossary
- U.S. National Cancer Institute:
  - http://nano.cancer.gov/resource_center/nanotech_glossary.asp
- Nanoforum (European Nanotechnology Gateway):
  - http://www.nanoforum.org/nf06~struktur~0~modul~loadin~folder~143~.html
- NanoSAFE:
  - http://www.nanosafe.org/glossary
- Nanotech BC, Canada:
  - http://www.nanotechbc.ca/main/resources_/1083/

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

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.

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.

blood-brain barrier: A CNS epithelial cell barrier that is impermeable 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 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.

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

engineered nanoparticle(s): A nanoparticle with at least one dimensions between approximately 1 nm and 100 nm and manufactured to have specific properties or composition. Abbreviation ENP(s).

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.

equivalent diameter: Diameter of a sphere which behaves like the observed particle relative to or deduced from a chosen property.

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

fullerene: A new allotrope of carbon characterized 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). Abbreviation ip.

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.

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

micelle: A submicroscopic aggregation of molecules, as a droplet in a colloidal system.

mobility diameter: Diameter of spherical particle with the same mobility as the particle under consideration.

MSDS: Material Safety Data Sheet

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

MWCNTs: Abbreviation for multi-walled carbon nanotubes.

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

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.

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: Contain only a few thousand or tens of thousands of atoms, rather than the millions or billions of atoms in particles of their bulk counterparts.

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: NP(s).

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.

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: Nanometre sized objects. 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.

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

nanotubes: Nanometre-sized tubes 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.

necropsy: The procedure of post-mortem 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.

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

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) guideline/standard for maximum workplace exposure over an 8-hour time weighted average (TWA) exposure. Equivalent to Australian National Exposure Standard (or WES [workplace exposure standard]).

Per os: Latin expression meaning 'by mouth'. Abbreviation po.

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

proteasome: A complex barrel-shaped multi-protein structure inside the cell which functions to digest other proteins into short polypeptides and amino acids (either self or non-self). The proteasome system is essential for many cellular processes including cell cycle, signal transduction and regulation of gene expression.

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.

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.

SWCNTs: Abbreviation for single-walled carbon nanotubes.

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.

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

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

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.

workplace exposure standard (WES): Australian National Exposure Standard for maximum workplace exposure over an 8-hour time weighted average (TWA) exposure. Equivalent to US PEL (Permissible Exposure Limit).

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

3. General Reviews

3.1 Regulatory

Between 2006 and 2008 a number of government and non government organisations have undertaken reviews on various aspects of ENPs. The scope and objectives vary from report to report, however the data used are frequently common and conclusions reached are generally very similar.

Information from the following are broadly summarised below.

- Ostinguy, C., Lapointe, G., Menard, L., Cloutier, Y., Trottier, M., Boutin, M., Antoun, M., and Normand, C. (2006). Nanoparticles - Actual knowledge about occupational health and safety risks and prevention measures, Institut de recherche Robert-Sauvé en santé et en sécurité du travail du Québec (IRSST), Montreal, Quebec.

- GR (2006). Health significance of nanotechnologies, Health Council of the Netherlands, The Hague, The Netherlands.

- SCENIHR (2006). The appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies, Scientific Committee on emerging and newly identified health risks.

- DEFRA (2007). Characterising the potential risks posed by engineered nanoparticles: A second UK government research report, Department of Environment, Food and Rural Affairs, London, UK.

- SCCP (2007). Opinion on safety of nanomaterials in cosmetic products, Scientific Committee of Consumer Products.

- US EPA (2007b). Nanotechnology white paper, United States Environmental Protection Agency, Washington, D.C.

- UK Royal Commission (2008). 27th Report: Novel Materials in the Environment: The case of nanotechnology. Royal Commission on Environmental Pollution, London.

Overall the regulatory reviews articulate the concerns regarding the health hazard and risks. In the main the concerns relate to lack of hazard information for specific engineered nanoparticles (ENPs), the concerns arise because there is a dearth of inhalational and long term toxicological studies. Consequently some of the concerns are hypothetical rather than being based on empirical data. The summary of scientific reviews in the next section is intended to provide the reader with a basic understanding of the general state of knowledge.

Recurrent themes within the agency reviews include[1]:

- Identification of data gaps and uncertainties in understanding of health impacts
- Toxicological effects are poorly understood
- Concern that NPs are more toxic than their bulk counterpart due to the greater surface area and expected higher surface chemistry
- Recognition that altering the surface of NPs can change their *in vitro* cell effects
- Concern that NPs may readily enter the systemic circulation and be translocated to tissues
- The need for thorough characterisation of ENPs and techniques to do so
- Determining which descriptions of dose, or exposure, will best describe the dose response relationships of ENPs (e.g. mass, number of particles/m^3, or particle surface area relative to body weight, lung weight or lung surface area)
- The need for validation of *in vitro* methods
- Development of classes of nanoparticles and ENPs that can be extensively tested and used as reference materials
- Prioritisation to hazard characterisation of ENPs with potential high occupational exposure
- Broad descriptions of government programs relating to nanotechnology
- Identification of current research initiatives and ongoing research needs
- How traditional data (both on chemical and physical properties and toxicology) can be used to inform ENP health risk assessments.

3.2 Reviews from the general scientific literature

In recent years there have been several reviews of the toxicology literature of engineered nanoparticles (ENPs). These are summarised in **Table 3.1** and provide pertinent overviews of the some of the issues associated with toxicology research of ENPs. There have also been recent publications of 'nanotoxicology' and 'particle toxicology' books (Donaldson and Borm 2007, Monteiro-Riviere and Tran 2007); these books give general background information as well as synopsis of specific topics of interest to researchers. Much of the literature information prior to 2006 can be found in the reviews and books.

The large number of scientific reviews (32 in **Table 3.1**) indicates the extent of nanotoxicology research over the last few years.

[1] These are not listed in any order of priority.

Excellent recent reviews are available on quantum dots (QDs) (Hardman 2006), carbon nanotubes (CNTs)(Lam et al. 2006; Helland et al. 2007), fibres and CNTs (Sanchez et al. 2009), and nanomaterials in general (Maysinger et al. 2007; Nel et al. 2006).

Maynard and Aitken (2007) examine current abilities for measuring airborne nanomaterials in the workplace and discuss future requirements. The need for accurate, portable and cost effective measurement techniques is echoed by Tsuji et al. (2006).

Hoet and Boczkowski (2008) provide brief summaries of what they consider to be the new influential information. This includes new knowledge and ideas on the movement of nanomaterials through biological membranes; with CNTs they report studies of DNA damage *in vitro*, and *in vivo* pulmonary and systemic effects. They noted that many studies failed to follow recent recommendations concerning good practice for nanotoxicological research[2]. This has also been noted in the material reviewed for this report and has contributed to the formulation of recommendation 4 in the **Executive Summary**.

Schins and Knaapen (2007) in a review of the genotoxicity of poorly soluble particles conclude this effect is via secondary genotoxic mechanisms.

The review by Maynard et al. (2006) focuses on the safe handling of nanotechnology and recommends research strategies that will support the sustainability of nanotechnologies through minimizing environmental and health risks. The review is written by an international panel of scientists and present five grand challenges selected to stimulate research and to bring focus to a range of complex multidisciplinary issues.

These are:

- Develop instruments to assess exposure to engineered nanomaterials in air and water, within the next 3–10 years
- Develop and validate methods to evaluate the toxicity of engineered nanomaterials, within the next 5–15 years
- Develop models for predicting the potential impact of engineered nanomaterials on the environment and human health, within the next 10 years
- Develop robust systems for evaluating the health and environmental impact of engineered nanomaterials over their entire life, within the next 5 years
- Develop strategic programmes that enable relevant risk-focused research, within the next 12 months.

The long time frames of these research challenges reflect the extent of work required to develop experimental protocols for studying the toxicology of ENPs and gather the necessary data for meaningful risk assessments. The current relative dearth of data

[2] See Oberdörster et al. (2005a). Principles for characterising the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Particle and Fibre Toxicity2:8 doi:10.1186/1743-8977-2-8. Also Balbus et al. (2007) for biochemical pulmonary evaluation techniques.

dictates that precaution is required when handling ENPs. This is no different from any other material for which there is minimum information on health effects.

Table 3.1: Summary of scientific reviews of the toxicology of engineered nanoparticles.

Author	Title	Review comments and conclusions
Borm et al. (2006a)	Research strategies for safety evaluation of nanomaterials, Part V: Role of dissolution in biological fate and effects of nanoscale particles.	Dissolution is a critical step for some ENPs in determining fate and toxicity. The review is intended to provide a useful basis for developing relevant dissolution assay(s) for NPs.
Hardman (2006)	A toxicological review of Quantum Dots: Toxicity depends on physicochemical and environmental factors.	The reviewed literature suggests several key points. Engineered QDs cannot be considered a uniform group of substances. QD absorption, distribution, metabolism, excretion, and toxicity depend on multiple physicochemical properties and environmental conditions; QD size, charge, concentration, outer coating bioactivity, and oxidative, photolytic, and mechanical stability have each been implicated as determining factors in QD toxicity. QDs may pose risks to human health under certain conditions.
Hurt et al. (2006)	Toxicology of carbon nanomaterials: Status, trends, and perspectives on the special issue.	Minimum required characterization includes complete chemical composition (specifically metals and heteroatom content >0.1%), surface area, detailed morphology description by electron microscopy. There is little support for either extreme viewpoint—that ENPs pose no health risks, or that nanotechnology presents extreme risks that warrant cessation of development activities.
Lam et al. (2006)	A review of carbon nanotube toxicity and assessment of potential occupational and environmental health risks.	Rodent intratracheal and pharyngeal aspiration studies collectively show that regardless of the process by which CNTs were synthesized and the types and amounts of metals they contained, CNTs were capable of producing pulmonary inflammation, epithelioid granulomas, and fibrosis. The possible mechanisms of CNT lung pathogenesis are discussed.
Maynard et al. (2006)	Safe handling of nanotechnology.	Five challenges to stimulate research on the toxicity of ENPs are proposed (see text in this Section) which are supported by the general discussion in the review.
TGA (2006)	A review of the scientific literature on the safety of nanoparticulate titanium dioxide or zinc oxide in sunscreens	There is *in vitro* evidence that ZnO and TiO_2 can induce free radical formation in the presence of light and that this may damage these cells. However this would only be of concern in people using sunscreens if the ZnO and TiO_2 penetrated into viable skin cells. The weight of current evidence is they remain on the outer dead layer of intact skin.
Borm et al. (2006b)	The potential risks of nanomaterials: a review carried out for ECETOC.	Oxidative stress-related inflammatory reactions occur with a number of NPs. Tumour-related effects have only been observed in rats, and might be related to overload conditions. NP translocation into the systemic circulation may occur after inhalation but there is conflicting evidence on the extent. Despite the existing database on NPs no blanket statements about human toxicity can be given at this time.

Author	Title	Review comments and conclusions
Buzea et al. (2007)	Nanomaterials and nanoparticles: Sources and toxicity.	Humans have been exposed to fine particulates for millennia. Inhaled NPs are less efficiently removed than larger particles by macrophage clearance mechanisms in the lungs, causing lung damage. NPs can translocate through the circulatory, lymphatic, and nervous systems to many tissues and organs. The review discusses human evolution in the presence of NPs and how the body has adapted to defend itself. This provides a good overview of different types of particulates and the body's defence mechanisms.
Fadeel et al. (2007)	There's plenty of room at the forum: Potential risks and safety assessment of engineered nanomaterials.	Report of a symposium on nanotoxicology devoted to definitions and standardization in nanotoxicological research, nano-specific risk assessment and regulatory/legislative issues. Examples of studies with carbon-based nanomaterials using a wide range of *in vitro* and *in vivo* model systems are presented.
Hagens et al. (2007)	What do we (need to) know about the kinetic properties of nanoparticles in the body?	The current knowledge on kinetic properties of NPs is reviewed and knowledge gaps identified.
Helland et al. (2007)	Reviewing the environmental and human health knowledge base of carbon nanotubes.	Absorption, distribution, metabolism, excretion, and toxicity of CNTs depend on functionalisation, coating, length, and agglomeration state. Characterized exposure scenarios could be useful when conducting toxicological studies. In sufficient quantity CNTs produce lung toxicity in a time and dose-dependent manner.
Hansen et al. (2007)	Categorization framework to aid hazard identification of nanomaterials.	A suggested hazard identification framework according to inherent physical/chemical properties associated with observed effects. The scheme is tested with a case study. Nine NP properties are proposed for measurement, and inclusion on MSDS. NPs should be characterized according to location in process and/or exposure cycle.
McAuliffe and Perry (2007)	Are nanoparticles potential male reproductive toxicants? A literature review.	Very few studies available and none for inhalation exposure. NPs given ip, iv or po suggest they can cross the blood testes barrier and deposit in the testes. No toxicity observed. Suggestions for future research strategies are outlined.
Maynard and Aitken (2007)	Assessing exposure to airborne nanomaterials: Current abilities and future requirements.	Available techniques to measure occupational exposure for particle number, surface area and mass concentration are reviewed. It is clear that no single method for monitoring nanoaerosol exposure will suit all nanomaterials. Most techniques are inappropriate for making routine personal exposure measurements on a regular basis. The notion of a universal aerosol monitor measuring all three metrics simultaneously is explored.
Mossman et al. (2007)	Mechanisms of action of inhaled fibres, particles and nanoparticles in lung and cardiovascular diseases.	A number of host susceptibility factors implicated in adverse health effects associated with inhaled pathogenic particulates are elucidated. A detailed discussion of mechanisms of asbestos-induced cell injury is presented.

Author	Title	Review comments and conclusions
Oberdörster et al. (2007c)	Toxicology of nanoparticles: A historical perspective.	Discoveries of NP-specific concepts of toxicology related to their small size and large specific surface area go back to the early parts of the 20th century. The ability of NP to cross cell barriers, enter cells and interact with subcellular structures is well established, as is the induction of oxidative stress as a major mechanism of NP toxicity. Health risks are different for different nanomaterials, ranging from perceived and very low for most, to real and very high for some. There are many questions that remain to be addressed.
Peters et al. (2007)	Translocation and potential neurological effects of fine and ultrafine particles: A critical update.	The mechanisms via which ultrafine particles penetrate pulmonary tissue and enter capillaries and translocate has not been elucidated. Histological neurodegeneration occurs in canine and human brains exposed to high ambient PM levels. The oxidative stress pathway may be involved.
Schins and Knaapen (2007)	Genotoxicity of poorly soluble particles.	Particles have two principle modes of action, primary (in the absence of pulmonary inflammation) and secondary (due to reactive oxygen/nitrogen). Current data indicates tumorigenesis of poorly soluble particulates is via secondary genotoxicity.
Ayres et al. (2008)	Evaluating the Toxicity of Airborne Particulate Matter and Nanoparticles by Measuring Oxidative Stress Potential - A Workshop Report and Consensus Statement.	Due to cost advantages *in vitro* test methods have a role in toxicity screening of NPs. There is a need to compare tests using standardized samples and to establish correlations with *in vivo* data.
Bastús et al. (2008)	Reactivity of engineered inorganic nanoparticles and carbon nanostructures in biological media.	ENPs which incidentally come in contact with living organisms normally do not cause acute toxicity but there may be long term effects. This review focuses on the particular physico-chemical properties of inorganic ENPs to understand interactions with biological material.
Hallock et al. (2008)	Potential risks of nanomaterials and how to safely handle materials of uncertain toxicity.	Cell effects and toxicity depends on the base material of the NP, its size, structure, substituents and coatings. The review discusses best practices used by universities to prevent exposure.
Helland et al. (2008)	Risk Assessment of Engineered Nanomaterials: A Survey of Industrial Approaches.	An overview of industry risk and safety approaches based on a survey of 40 companies in Germany and Switzerland. Only 32.5% performed risk assessments of their nanomaterials. Fate in use and disposal received little attention and the majority of companies did not foresee unintentional release throughout the life cycle. The development of risk and safety decision frameworks in industry seems necessary to ensure potential risks of ENPs are systematically evaluated.
Hirano (2008)	Health effects of nanoparticles and nanomaterials (I): Recent overview of the health effects of nanoparticles.(Japan)	NPs are considered to be to be highly permeable to lung and skin tissues but small enough to evade phagocytosis by the reticuloendothelial system. Dose metrics are critical.
Hoet and Boczkowski (2008)	What's new in nanotoxicology? Brief review of the 2007 literature.	Reviewed is new information on the movement of nanomaterials through biological membranes, with CNTs there are new studies of DNA damage *in vitro* and *in vivo* pulmonary and systemic effects. The authors note many studies failed to follow recent recommendations concerning good practice for nanotoxicological research.

Author	Title	Review comments and conclusions
Hoyt and Mason (2008)	Nanotechnology: Emerging health issues.	Toxicological effects are poorly understood. Safe handling practices include engineering controls such as closed systems or hoods to prevent airborne material from reaching an employee's breathing zone, respiratory protection, preferably supplied air, and use of impervious gloves to prevent absorption through the skin. A rigorous risk control assessment should be performed before handling any nanomaterials.
Lewinski et al. (2008)	Cytotoxicity of nanoparticles.	The review summarises the *in vitro* cytotoxicity data available on carbon, metal and semiconductor nanoparticles. Different data has been published for each due to varying experimental conditions and physiochemical properties.
Li et al. (2008a)	The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles.	This is a review of detailed mechanisms for generation of oxidative stress and impacts of oxidant injury in the respiratory tract. Based on induction of oxidative stress the potential effects of ENPs are discussed. It stresses cell ROS production per se does not automatically lead to adverse effects.
Nohynek et al. (2007)	Grey goo on the skin? Nanotechnology, cosmetic and sunscreen safety.	Most theoretical and experimental evidence suggests insoluble NP do not penetrate into or through human skin. Oral and topical toxicity data suggest TiO_2 and ZnO NP have low systemic toxicity and are well tolerated on the skin. *In vitro* cytotoxicity, genotoxicity, and photogenotoxicity studies on TiO_2 or other insoluble NP that report uptake by cells, oxidative cell damage, or genotoxicity should be interpreted with caution, since such toxicities may be secondary to high concentrations of insoluble particles. There is little evidence supporting the notion smaller particles have greater effects on the skin or other tissues, or produce novel toxicities relative to micro-sized materials. Overall, the current weight of evidence suggests that nano-materials such as nano-sized vesicles, or TiO_2 and ZnO nanoparticles currently used in cosmetic preparations or sunscreens pose no risk to human skin or human health, although other NP may have properties that warrant safety evaluation on a case-by-case basis before human use.
Ray et al. (2009)	Toxicity and environmental risks of nanomaterials: challenges and future needs.	Discusses the fate, behaviour, and toxicity of different class of nanomaterials in the environment. Attempts to address the question - how toxic are nanomaterials at the potential concentrations at which they might be used? Presents many *in vitro* results, particularly for Au NPs, but does not comment on experimental protocols and interpretation for humans but does say it is difficult to compare results from different research groups. Still considerable research challenges.

Author	Title	Review comments and conclusions
Sanchez et al. (2009)	Biopersistence and potential adverse health impacts of fibrous nanomaterials: what have we learned from asbestos?	The mechanisms by which fibres are cleared from the respiratory tract are reviewed (mucociliary escalator, engulfment, and removal by macrophages, or through splitting and chemical modification). The need for biopersistence is emphasised and compared to long asbestos fibers which can cause inflammation, granuloma formation, fibrosis, and cancer. Although the mechanisms of nanomaterial toxicity are not elucidated, recent evidence suggests similarity with asbestos fibers, including generation of reactive oxygen species, oxidative stress, and genotoxicity.
Tsuji et al. (2006)	Research strategies for safety evaluation of nanomaterials, Part IV: Risk assessment of nanoparticles.	Airborne exposures depend on whether NPs are dispersed or aggregated. Accurate, portable, and cost effective measurement techniques are essential for understanding workplace exposures. Under many conditions dermal penetration of NPs is limited. In mice CNTs have greater pulmonary toxicity than graphite, and their metal content affects toxicity. For some substances it is incorrect to assume smaller particles are always more toxic. Recent toxicity and exposure data, combined with therapeutic and other related literature, are beginning to shape risk assessments that will be used to regulate the use of nanomaterials in consumer products.
Yang et al. (2008)	Inhaled nanoparticles - A current review.	This briefly reviews the toxicity of NPs and the factors influencing the fate and disposition of inhaled nanomaterials. The lung is an ideal target organ for non-invasive local and systemic drug delivery, especially for protein and poorly water-soluble drugs. The potential application of pulmonary drug delivery of NPs in the context of published toxicology is reviewed.

4. Experimental considerations

4.1 Intratracheal exposures

When assessing the potential toxicity of airborne substances the inhalation exposure route is preferred since this is the natural exposure mode for humans. However, for various reasons, both technical and financial, it cannot always be used in animal studies. A convenient alternative technique is administration of a solution/dispersion trans-orally into the tracheal lumen using an intubation needle or catheter. This direct intratracheal instillation procedure requires the animal to be lightly anaesthetized and is discussed in detail in **Appendix 1**.

Particle persistence and load is critical for development of adverse effects in the lungs. The biopersistence can be the result of their intrinsic properties (size, shape, stability etc), or consequence to high exposures in experimental situations overwhelming the normal mechanisms for removing particles from the lungs. While the latter has historically been known to occur in situations of excessive occupational particulate exposure, in animal experiments it can easily arise when dispersions are intratracheally administered in amounts significantly disproportionate to expected human exposures. Careful evaluation of experimental design is necessary to determine if the reported pulmonary particle persistence, or pulmonary effect, is an aberration of experimental design. The mass dose, particulate concentration and carrier vehicle can all contribute to particle overload in intratracheal experiments. In these situations the relevance of the intratracheal instillation study to human hazard and risk assessment is often obscure; the relevance is sometimes taken for granted, or conversely dismissed out of hand on grounds the dose route was non-physiological.

Although on face value the list of disadvantages (in **Appendix 1**) appear to outweigh the list of advantages, it is nonetheless possible to obtain useful information from intratracheal experiments. It is also a fact of reality at this time, and for the foreseeable future, that as part of a pragmatic tiered approach to assessing human health risks from ENPs, there will be far more experiments conducted with intratracheal exposures than with inhalation exposure. The data therefore should not be dismissed out of hand, it maybe all that is available for any given ENP. Conversely caution and care are required with interpretation of intratracheal instillation data to ensure only legitimate health concerns are raised.

The pulmonary reactions to large doses of poorly soluble, persistent respirable particles are well known and predictable (see **Section 5**). Rats are particularly prone to particle overload; it is considered that data derived from such experiments are not relevant for human risk assessment (Morrow 1988, 1992; Oberdörster 1995a, 1995b; Valberg and Watson 1996; Morrow et al. 1996; Warheit 1997; Mauderly 1997; ILSI 2000, Mohr et al. 2006).

To address the issue of particle overload the Inhalation Specialty Section of the US Society of Toxicology recommended intratracheal instillation studies be limited to doses of less than 100 µg per rat (Driscoll et al. 2000), the threshold for particulate

overload seems to be about 200 – 300 µg/rat[3] (Bellman et al. 1991, Warheit et al. 2007b). IARC consider impaired lung clearance in rodents exposed to ultrafine particles occurs at much lower mass concentrations than with fine particles[4] (Baan 2007).

Other recommendations for the appropriate conduct of intratracheal experiments made by Driscoll et al. (2000) included validation of the procedure by the laboratory, rational choice of delivery vehicle, the inclusion of appropriate control materials, experimental design to allow dose response evaluation, and post instillation evaluation times. They also recommended the intratracheal instillation procedure should be considered as a method only for single exposure of the lungs. They noted there is little data to support/validate the use of repeat instillation exposures.

The mouse pharyngeal aspiration technique is less invasive than the intratracheal instillation technique and inherently limits the amount of material that can be delivered to the lung. It is consequently more amenable for repeat exposure use and is unlikely to cause particulate overload in the lungs[5]. Even so it can be argued that like intratracheal administration, pharyngeal aspiration delivering a single bolus exposure to the lungs is an artificial exposure where the large single dose may contribute to the pulmonary response. Hence the response may not be relevant to evaluation of the chronic lower dose exposures most likely to occur in occupational settings. Kisin et al. (2009) have examined the relative pulmonary responses to SWCNTs in mice after inhalation or aspiration exposure[6]. They found qualitatively similar pathology was produced by both techniques but effects were produced more easily after inhalation.

It is noted however the aspiration technique has the potential to allow aspiration of saliva, and therefore bacteria and endotoxins, into the lung at the same time as the ENPs and therefore may confound the results. Particularly if only short time frame (a few days) oxidative stress biomarkers are measured. This should not be a significant drawback of the method as careful evaluation of pulmonary response will be able to readily distinguish between bacterial/endotoxin reactions and those associated with persistent NPs.

Particle overload in the lungs of rats invokes indirect, secondary toxicity mechanisms (see **Section 5**) that are independent of the particular ENPs being investigated. Unfortunately many ENP toxicological studies employing high intratracheal doses have examined this secondary toxicity and not the intrinsic toxicity of the specific ENPs. Consequently the data is of little value for determination of explicit health hazards associated with the ENPs.

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[3] For example, Bellman et al. (1991) exposed rats to a toner (not well characterised but size ~ 4 µm) at different inhalation concentrations for different periods up to 24 months. At various times alveolar macrophage clearance of radiolabled tracer particles (^{59}Fe-labeled iron oxide and ^{85}Sr-labeled polystyrene particles respectively 0.39 and 3.5 µm) were determined. At a pulmonary load of 0.1 mg/lung there was no difference in macrophage clearance, >0.2-0.3 mg/lung clearance became impaired, at 1 mg/lung clearance was approximately 50% lower and virtually ceased at 10 mg/lung.

[4] For contextual information ultrafine TiO_2 has a primary particle size of 10-50 nm but fine TiO_2 is 200 – 300 nm (Baan 2007).

[5] In addition, mice are inherently less prone to lung particle overload than are rats (see Appendix 1).

[6] Kisin et al. (2009) exposed mice to aerosolised SWCNT (5 hr/d for 4 days) or to varying doses of the same SWCNT by aspiration. Early inflammatory and oxidative stress response culminating in development of multifocal granulomatous pneumonia and interstitial fibrosis was produced by both techniques.

In evaluating the relevance of hazard data generated by intratracheal administration of ENPs it is essential to carefully consider the amounts of material that have been applied to the lungs. We consider the pharyngeal aspiration technique provides better data than intratracheal administration.

4.2 Intraperitoneal fibre experimental model

Intraperitoneal administration of insoluble mineral fibres has been used for many decades to probe the processes of mesothelioma (e.g. Davis 1974, Moalli et al. 1987, Roller et al. 1997) and more recently for investigating the toxicity of carbon nanotubes (CNTs) (Poland et al. 2008, Takagi et al. 2008a, Muller et al. 2009).

Quite naturally one questions the relevance to humans of data that has been produced by injecting a bolus mass of fibre into the abdominal cavity of a rodent; there are concerns about the unphysiological nature of the exposure and possible 'overload' perturbation of normal physiology.

Based on the brief evaluation of the technique in **Appendix 1.2**, we consider the experimental intraperitoneal fibre toxicity model to be a reasonable screening system for identifying fibrogenic hazards of fibres. Nevertheless care needs to be taken in extrapolating, and articulating, the results to humans. The data indicate what might happen if a critical load of persistent fibrogenic fibre was in long term contact with pleural mesothelial tissue. Some relevant questions for risk assessment:

- Are the fibres biopersistent?
- Do they reach the pleural cavity after inhalation in humans?
- Is the form of the fibre inhaled by workers the same as that used in the intraperitoneal hazard screening test?

The above 'intraperitoneal fibre' animal model has been made more sensitive with the use of genetically modified mice that are deficient in their ability to make a protein[7] called p53 (Donehower et al. 1992, Harvey et al. 1993, Marsella et al. 1997, Vaslet et al. 2002, Tazawa et al. 2007, Takagi et al. 2008a)). However as discussed in **Appendix 1.2**, if this model was used as a screening test for identifying fibrogenic potential of ENPs results would need to be carefully interpreted given there is potential for false positive results arising from the increased sensitivity of the test system.

[7] Expression of p53 protein is an early cellular response to DNA damage, the protein induces transcription of genes that activate cell cycle checkpoints and direct a cell with DNA damage to self destruct via apoptosis. The absence of p53 therefore allows the DNA damaged cell to potentially survive and divide thereby fixing the damage in all daughter cells. There are many studies showing mice deficient in p53 are very susceptible to chemical carcinogens and also to cancer induced as the result of chronic inflammation (see also Appendix A1.2).

4.3 Dose considerations

4.3.1 *In vivo*

Intratracheal administration of SWCNTs into a rat at 5 mg/kg (1,250 µg/animal) does not resemble any relevant human *in vivo* exposure (Balbus et al. 2008); indeed about 15% of the rats so treated died because the SWCNT agglomerated and suffocated the animals (Warheit et al. 2004).

Elder et al. (2007) intratracheally dosed rats (200 – 250g body weight) with platinum nanoparticles (Pt-NPs) at 100 µg/rat. Using a predictive lung deposition model developed in the US and Netherlands, the human to rat ratio for total inhaled mass deposited in the alveoli was calculated. A human would have to inhale a NP-containing aerosol at a mass concentration of 4.6 mg/m^3 over an 8 hour period in order to deposit a similar mass in the lung as what was delivered to a rat via an intratracheal dose of 100 µg/rat.

Based on the equations provided in standard text books (Whalan et al. 2006, Valentine and Kennedy 2001) a similar dose conversion method is presented in **Appendix 2**. At an intratracheal dose of 5 mg/kg to a rat the equivalent deposited amount for humans would require an 8 hour exposure to 25.5 mg/m^3. If the equation in **Appendix 2** is applied to the instillation dose used by Elder et al. (2007), i.e. 100 µg/200g rat the equivalent human exposure concentration for an 8 hour exposure for equivalent deposition in the lung is 2.5 mg/m^3, versus 4.6 mg/m^3 calculated by Elder et al. (2007). Assuming Elder et al. (2007), who had the benefit of a sophisticated physiologically based dispositional model, have more accurately converted their administered dose to an inhaled air concentration for humans it would appear the simple equations in **Appendix 2** underestimate the equivalent human exposure to a given intratracheal dose by about 2 fold.

Nevertheless, the equations in **Appendix 2** are useful for quickly converting intratracheal instillation doses into 'equivalent' human air exposures. The conversion of the common 5 mg/kg bw rat installation dose to 34 mg/m^3 (or about 60 mg/m^3 if the method of Elder et al. (2007) is used) demonstrates the experimental intratracheal doses may represent extraordinary, unrealistic concentrations for workplace exposures, and also misrepresent the depositional doses achieved in inhalation experimental studies.

For example:

- Inhalation exposure of rats to TiO_2 ultrafine NP (100 nm) from a fluidised bed powder generator at 10 mg/m^3 for 8 hr gave a measured deposited dose of 38 µg/rat. (Nurkiewicz et al. 2008). Interestingly the measured deposition was 2- 5 times less than that expected from calculations using 200 ml/rat as the minute ventilation and 10% deposition for rats

- Mice exposed to 7 mg/m^3 of aerosolized 5 nm or 21 nm TiO_2 NP for 4 hr gave a deposited mass of approximately 12.5 µg/mouse (Grassian et al. 2007a).

Thus the pulmonary tissue doses that can be achieved following inhalation exposure, even at very high air concentrations, are much lower than those that can be administered intratracheally to rats. This suggests that intratracheal doses of NPs that

give rise to sustained oxidative stress may be the result of secondary toxicity subsequent to pulmonary overload. It is considered the importance/usefulness of the intratracheal data for occupational hazard and risk assessment should be judged by comparison of the delivered intratracheal dose to:

- the benchmark intratracheal dose recommended by Driscoll et al. (2000) i.e. 100 µg/rat, and the approximate dose of 200 – 300 µg/rat that appears to correlate with the commencement of overload associated decreased macrophage clearance of particles (see **Section 4.1** and **Appendix 1.2**), and

- the equivalent air concentration for humans that would deliver the same deposited dose as was delivered by the intratracheal instillation (**Appendix 2**). This may however require assumptions to be made regarding the respired dose fraction for alveolar deposition of the particular ENP under consideration for both the inhalation and intratracheal exposures.

The *in vivo* experimental doses can be further put into perspective by comparison with measurements of nanoparticles in workplace air. **Table 4.1** provides a quick appreciation of likely workplace air concentrations of NPs. Generally concentrations are higher where engineering controls are not in place. It is also interesting that a combination of measurements are reported; mass/volume of air (e.g. mg/m^3), as are the OELs for dusts of low toxicity, or as number of particles/volume of air which is more akin to OELs for fibrogenic materials. Exposure measurement comparisons between workplaces are therefore quite difficult.

Although an exhaustive literature search was not undertaken for this review, it is apparent that at least in the open scientific literature there is a dearth of information regarding workplace exposures to ENPs. This is particularly so for Australian workplaces. It is important that information on workplace exposures be gathered, and made available to the general scientific community so it can be judged whether ENPs as characterised in experimental or safety test settings are comparable to the physical ENPs in workplace air. In Australia there are many instances in which nanomaterials are handled in research laboratories.

While it might expected that ENPs may become airborne when they are weighed, it has also been recently demonstrated for fullerenes (C_{60}) and underivatised and hydroxylated MWCNT that there is an increase in airborne particle concentrations while they are sonicated in water during preparation for mixing into relevant matrices for experimentation (Johnson[8] et al. 2009).

It is suggested that for workplace air measurements of NPs in Australia that mass/volume ($µg/m^3$), particle number/volume (respirable particles/m^3), surface area/mass (cm^2/mg) and agglomeration states be reported. Although this will require additional characterisation of the particles in addition to normal concentration measurement, it will enable comparison with a range of experimental dose metrics and OELs when, if, they are declared.

[8] The Johnson et al. (2009) information comes from a poster presented at the American Society of Toxicology annual scientific meeting. It has not yet been published in a peer reviewed scientific journal.

An example where a relatively low dose of intratracheally administered nanoparticle has not caused an inflammatory response is TiO_2; inflammation was observed with a single dose of 5 mg/kg but not with 1 mg/kg (~1,250 and ~ 250 µg/rat respectively) (Warheit et al. 2007b).

Mercer et al. (2008) used the pharyngeal aspiration technique in mice to administer SWCNTs dispersed in phosphate buffered saline (PBS)[9] to mice. The authors considered the dose of 10 µg/mouse was occupationally relevant by comparing it with peaks of 53 µg/m^3 reported by Maynard et al. (2004) during laboratory handling of SWCNTs. The rationale is as follows; with an assumption of human minute ventilation of 20,000 ml/min during light work and 21% alveolar deposition for that particle size, 106.8 µg of the SWCNTs would be predicted to deposit on the alveolar surface of a worker per day. Normalising to the equivalent alveolar epithelial surface area in human (102 m^2/lung) and mouse (0.05 m^2/lung) a worker would receive a unit alveolar lung burden equivalent to the mouse pharyngeal aspiration of 10 µg in approximately 200 work days. This dose resulted in a highly dispersed interstitial distribution of SWCNTs throughout the lung, accompanied by mild transient parenchymal inflammation that resolved within the first week of dosing. A relatively even connective tissue response of alveolar thickening was observed 7 days and 1 month post dosing.

Matching ENP doses in toxicological investigations to likely human exposure is important for realistic risk assessments and to inform the design of mechanical and management systems for limiting exposure to levels judged to be safe. Many of the toxicological experiments done to date have been with NP exposures not representative of work environments with good occupational hygiene. The publication of Song et al. (2009) illustrates what can potentially happen when there is a gross failure of engineering and occupational hygiene processes, including hygiene training, to protect workers.

Song et al. (2009) report serious lung injury in seven Chinese females (two of which died) working in a print facility where they heat cured polystyrene boards coated with a mixture of polyacrylic ester. The process created smoke/fumes to which the workers were exposed. Occupational hygiene in the workplace was extremely poor. Important aspects are:

- The process room was small; 70m^2 with one door and no windows
- 5 months prior to respiratory symptoms developing, the single ventilation system broke down and was not repaired, and the door was kept closed due to the cold

[9] Dispersed SWCNT were prepared from purified (low iron) SWCNTs suspended in water which was passed through a 200 µm screen and sonicated. This preparation was then dispersed in acetone in an ultrasonic bath at room temperature for 24 hour; compared to SWCNTs dispersed in distilled water there was a 150 fold increase in the volume of the SWCNTs due to dissociation of NTs as the nonionic solvent, acetone, reduced van der Waals forces binding the SWCNTs together. Large dispersed SWCNT were then filtered from the solution using a nylon mesh screen that had a 4 µm hole diameter. The smaller dispersed SWCNTs in the acetone solution were collected on a 0.2 µm filter and washed several times with distilled water to remove residual acetone. The filter was dried overnight in vacuum and weighed to determine the quantity of dispersed SWCNT. Before use, the filter containing the SWCNTs was placed in PBS and SWCNTs released into the vehicle by brief sonication. The distribution of average area equivalent diameter of the dispersed SWCNTs was approximately normally distributed with a mean of 0.69 µm and a variance of 0.49 with a maximum area equivalent diameter of 3.7 µm.

- Cotton face masks were only occasionally used
- The workers had no knowledge of occupational hygiene or that the process fumes may be harmful.

From the above it is clear air circulation in the room was dreadfully low or non existent, hence exposure to process smoke/fumes was most likely very extensive. Fumes coming from the process would contain a mixture of chemicals as well as heat generated particulates, some of which will be in the nano-size range. It is well known that excessive exposure to such fumes can cause varying degrees of respiratory distress and damage (Masami et al. 2006, Rom and Markowitz 2007). In that sense the article does not provide new information. However the detailed characterisation of the pulmonary reaction by Song et al. (2009) adds to the overall knowledge of how humans react to over-exposure to these types of fumes.

The observation of nanoparticles in lung fluid and cells of the affected individuals is interesting but their presence may not be unusual since similiar investigations have not been previously undertaken in cases of fume-induced lung damage. Due to the mixed exposure, which is uncharacterised, the finding does not necessarily implicate the nanoparticles as being the primary causative agents of the condition. The conclusion of the authors that the nanoparticles did cause the respiratory condition should be viewed with caution.

Presumably the manufacturing process was not new to the facility and there had not been previous incidents of severe respiratory symptoms. In this tragic report, the fact that nanoparticles were part of the hazardous milieu to which the workers were exposed is of secondary importance to the collapse of the occupational hygiene processes. The question of whether gross over exposure to nanoparticles could cause serious lung damage in humans is not answered by this article. Nevertheless animal experiments suggest such damage might occur; hence it is prudent to ensure all practical means are taken to minimise worker exposure – the same philosophy as with any hazardous material.

Table 4.1: Concentrations of Nanoparticles in the Workplace[(a)]

Type of nanoparticle	Concentration range	Reference
MWCNT (avg. tube length = 1.5 µm)[(b)]	0.21 – 0.43 mg/m^3 (before control measures) Non-detectable level (after control measures)	Han et al. (2008a)
Ultrafine particles (total)[(c)]	10.6 - 14.8 x 10^4 part/cm^3 (no control measures in place)	Brouwer et al. (2004)
Aerosol containing SWCNT[(d)]	0.7- 53 µg/m^3	Maynard et al. (2004)
Ultrafine particles (total between 0.01 – 1 µm)[(e)]	2 x 10^4 – 3 x 10^5 part/cm3	Thomassen et al. (2006)
Nanoparticles (11-100 nm)	4.5 x 10^5 – 1.3 x 10^7 part/cm^3	Nasterlack et al. (2006)
Ultrafine particles[(f)]	5.0 x 10^5 – 2.5 x 10^6 part/cm^3	BIA (2003)
Carbon black in respirable dust (1-500 nm)	0.09 – 0.96 mg/m^3	Gardiner et al. (1996)
Nanoparticles[(g)]	7.78 x 10^5 part/cm^3 (with ventilation at top of booth) 1.48 x 10^4 part/cm^3 (with modified ventilation system)	Lee et al. (2007)

Notes:

(a) The data in this table does is not a comprehensive depiction of workplace measurements that may be available in the literature. The data is provided to give the reader an appreciation of airborne particulate concentrations in controlled and uncontrolled workplaces and how these relate to experimental exposures. The above concentrations are for a range of job activities (research laboratory, welding stations, grinding stations, working in vicinity of forklift, laser ablation, nanotube production, anode changing in aluminium smelter, applying wax to skis, carbon black manufacture) encountered in industrial sites using nanoparticles. No attempt has been made to separate exposures according to job description, although some jobs would inherently be associated with higher exposures.

(b) The aerodynamic diameter of MWCNTs in this study ranged between 0.5 – 20 µm.

(c) The mid-point aerodynamic diameter of ultrafine particles in this study was 70 nm. After assuming a density of 5,500 kg/m^3, specific surface area of a particle was calculated as 109 m^2/g (using the radius of a single particle as 5 nm).

(d) The aerodynamic diameter of SWCNTs in this study was between 1 – 10 µm. A nominal density of 1,000 kg/m^3 was assumed. However, the article states that it would have been more appropriate to use a density between 100 and 1000 kg/m^3, but a generic density was used in the absence of individual density information.

(e) These consisted of singlet particles, (carbon) agglomerates, and (to a minor extent) small fibres.

(f) No particle size was given for these concentration ranges.

(g) Geometric mean size of particles was 181 nm, with a geometric standard deviation of 1.8. After analysis, particles were found to be composed of hazardous heavy metals with varying morphologies (Mn, Cr, Ni).

4.3.2 *In vitro*

Walker and Bucher (2009), while exploring the application of new predictive *in vitro* approaches to address the human health risks for the thousands of agents that may be of public health concern, questioned whether such approaches would be generally applicable for the broad area of nanotoxicology. They noted the following:

- The specific composition of an *in vitro* and *in vivo* test system will likely play a huge role in how a nanomaterial interacts with a cell, or other biological target
- Agglomeration and aggregation are recognised major issues in both the *in vitro* and *in vivo* evaluations of effects of nanomaterials
- It is essential that the appropriate physicochemical properties of a tested nanomaterial should be characterised within the experimental test systems of both *in vitro* and *in vivo* studies. Agglomeration/aggregation or changing particle size may affect 'the dose of concern' for the nanomaterial
- It will be important to determine the likelihood that an ENP will actually present itself to humans as a nanomaterial, or as the material that has been tested, or as something else.

Hardly any investigations published over the last few years have attempted to provide information, or comment on the relevance of the ENP doses used in their *in vitro* studies. Information is provided in **Appendix 2** regarding dose conversions for *in vitro* experiments.

Morin et al. (2008) states that diesel soot concentrations of 10-100 µg/cm^2 applied to a cell culture monolayer are equivalent to instant dust inhalation of 10-100 g by a human of 70 kg. However the authors do not describe the basis (either qualitative or quantitative) for their extrapolation.

Elder et al. (2007) investigated the *in vitro* toxicity of different shaped platinum nanoparticles (Pt -NP) as part of a correlation study of acellular *in vitro* and *in vivo* techniques for assessing ENPs of unknown toxicity. They used *in vitro* doses of 0 – 500 µg/well corresponding to 0 – 132 µg/cm^2 of culture dish surface area, and the number dose range was 0 – 1 x 10^{12} particles. Without providing the rationale, they considered the highest dose (the only dose providing evidence, albeit equivocal, of cytotoxicity) was extremely high and of doubtful physiological relevance.

Of interpretative importance, but not mentioned by other investigators, is the comment by Elder et al. (2007) that in some cases variation in *in vitro* responses were greater due to cell culture passage number rather to NP dose.

In their correlative investigations of *in vitro* techniques predicting *in vivo* outcome, Sayes et al. (2007b) used five NPs at doses of 0.052 – 520 µg/cm^2 in cultures of rat lung epithelial cells or alveolar macrophages. They cited Faux et al. (2003) who had determined *in vitro* surface area doses of 1–3 cm^2/cm^2 to be an overload dose for low toxicity dusts such as fine-sized TiO_2. Sayes et al. (2007b) converted their doses to cm^2/cm^2 and deduced doses equivalent to 52 and 520 µg/cm^2 were overload doses. It was noted that these were the doses primarily, but not solely, giving positive *in vitro* cytotoxicity responses in their study.

More recently Donaldson et al. (2008b) has investigated the concordance of *in vitro* and *in vivo* dosimetry for the proinflammatory[10] effects of low-toxicity, low-solubility particles. Expressing the dose of barium sulphate or TiO_2 in A549 epithelial cells as surface area of particulate per unit of cultured cells gave a threshold of 1 cm^2/cm^2. The same threshold was obtained when sub-chronic or chronic rat inhalation data for the same substances was expressed as particle surface area per unit of proximal alveolar region of the lung. While this data is not specifically for nanoparticles it elegantly demonstrates thresholds for key events in pulmonary responses to particulates and in the context of this review the importance of surface area dosimetry for comparing *in vitro* and *in vivo* data.

A reliable and validated method for extrapolating ENP concentrations applied to isolated or cultured cells to either an animal or human exposure is not known to these reviewers. *In vitro* investigations are therefore of limited use in risk assessment, but as noted previously may contribute to 'weight of evidence' evaluation for potential hazard providing one is cognisant of experimental nuances, particularly dose and agglomeration state (see below), that may skew the outcome.

4.3.3 Dispersal, aggregation and biological dose

The phenomena of agglomeration/aggregation, together with lack of characterisation of ENP in the experimental media, are arguably the biggest experimental issues in nanotoxicology experiments influencing interpretation of the data. Agglomeration/aggregation changes the dose of nanoparticle at the surface of the cells in culture and is significantly influenced by the dispersal medium used in the test. Agglomeration/aggregation effectively decreases the amount of ENP in nano-form that is bioavailable to the cell. There are many publications in which it is noted the ENP precipitated in culture media or sat as lumps on the surface of the cells. The issue is discussed in detail in **Appendix 1**.

Agglomeration/aggregation adds an extra difficulty over and above the usual issues of extrapolating experimental *in vitro* and *in vivo* chemical data to humans for hazard and risk assessment. Agglomeration, of nanoparticles generally appears to give rise to lesser pulmonary toxicity than dispersions of the original nanoparticle. For any particular NP the relevance of the data for human health will depend upon whether workers are exposed primarily to dispersed nanoparticles or to agglomerates.

The dispersal medium for the ENP is critical (Shvedova et al. 2005a); it not only affects agglomeration but may also alter surface properties of the NP and thence also its toxicity (Murdock et al. 2008). In recent years many researchers have investigated the issue and the general consensus is that phosphate buffered saline (PBS) is unsuitable

[10] As noted in **Sections 5** and **7.1.1** prolonged inflammation is a pivotal event for particle induced lung pathology. The proinflammatory measurement used by Donaldson et al. (2008b) was gene expression of interleukin 8 (IL-8) which is representative of the chemokines mediating inflammation, it is especially important in acute inflammation caused by particles. This *in vitro* data emanated from Monteiller at al. 2007). The *in vivo* data for the same particles (median aerodynamic diameter of 4.1 µm for $BaSO_4$ and 2.1 µm for TiO_2) was published under Tran et al (2000) and established the importance of particle surface area as a dose metric. Polymorphonuclear leukocytes (PMN) numbers in bronchioalveolar lavage (BAL) washes was used as the marker for pulmonary inflammatory response. The proximal alveolar region of the lung is the primary area where respirable particles deposit.

unless it also contains a surfactant and/or protein, and the mixture has been sonicated (see **Appendix 1**).

It is however noted that an agreed dispersion protocol has not been established.

Dissolution is an important mechanism for removal of particles from the lung (see also **Sections 5** and **7.1.1**), it is however difficult to predict dissolution behaviour in complex biological systems. Borm et al. (2006a) have reviewed various aspects of material science and analytical chemistry with the aim providing a basis for developing relevant dissolution assays for nanomaterials.

4.3.4 Exposure metrics

Many reviews comment /discuss what exposure metric is the most appropriate for describing the toxicity of particular NPs. Although there is not consensus it would appear that particle number or surface area may better describe some dose responses than mass concentration of ENP.

Consideration of exposure metric also applies to measurements intended to define worker exposure. Maynard et al. (2007) present a useful discourse on the applicability of different physical exposure metrics for a range of particle class/attributes and combinations. They also reviewed currently available techniques to measure exposure as particle number, surface area and mass concentration. They explore the idea of an inexpensive, easy to use in the field, universal aerosol monitor which would enable personal exposure measurements to be collected simultaneously for all three metrics.

4.3.5 Characterisation of nanoparticles

It has been known for a long time that proper characterisation of ENP physico/chemical characteristics is essential for understanding and extrapolating toxicity data. In reviewing the kinetic properties of ENPs, Hagens et al. (2007) remarked that almost all such studies describe the properties of analysed ENPs in a different way. Minimum requirements have recently been suggested (Hurt et al. 2006, Hansen et al. 2007). It is pointed out several times in this review that many recent investigations do not adequately characterise the ENP being investigated. A recurring concern regarding the application of toxicological research data for any given ENP is whether the material tested is the same as, or sufficiently similar, to the material to which workers may be exposed.

Wittmaack (2007) explored which dose metrics were most relevant for quantifying the lung inflammatory response to NP exposure. It was concluded the physical characterisation of NPs have to be improved significantly before the appropriate dose metric for lung inflammation, which also needed quantisation refinements, could be safely identified. Powers et al. (2006) describe the issues relating to measurement of NP size, shape, and dispersion when evaluating the toxicity of NPs. However they conclude size, size distribution, shape, particle number and surface area are not enough for complete characterisation. The authors suggest some general protocols to address the common issues faced with NP characterisation.

In proposing a categorization framework to aid hazard identification of ENPs, Hansen et al. (2007) emphasise the need for physical characterisation of nanomaterials according to where the material is located in the manufacturing and/or exposure cycle.

They nominate nine relevant physical and chemical properties identified from an extensive literature search as potentially determining the inherent hazards of nanomaterials. Furthermore they advocate these properties be included on material safety data sheets (MSDS) and made available for regulatory registration of nanomaterials. The parameters are:

- Chemical composition
- Size
- Shape
- Crystal structure
- Surface area
- Surface chemistry
- Surface charge
- Solubility
- Adhesion.

Warheit (2008) has drawn up a similar list but with slightly different names and has added the need for characterisation in the dry and wet state. Murdock et al. (2008) point out characterisation of NPs in solution before assessing the *in vitro* toxicity is a high priority but also lament regarding the lack of well-defined techniques for characterisation of wet nanomaterials in aqueous or biological solutions. They explore the issue using dynamic light scattering and other techniques for a wide range of nanomaterials. It was found that addition of serum to cell culture media can, in some cases, have a significant effect on particle toxicity possibly due to changes in agglomeration or surface chemistry.

4.3.6 Reference materials

The incorporation of positive controls is an essential feature for robustness of experimental design. Experiments without the ability to benchmark an ENP of unknown toxicity with a reference NP whose toxicity has been characterised should be given lower evaluative weight. However there is an issue of what constitutes an appropriate reference material.

The REFNANO project was commissioned by the UK Government to provide a priority list of candidates for inclusion in a set of reference materials to support measurement, toxicology and risk assessment of ENPs in the UK. Aitken et al. (2008) report the outcome of a workshop of leading UK experts in toxicology, metrology and risk assessment from academia, government and industry. A consensus was reached on:

- A rationale for the selection and development of primary reference/test materials
- A priority list of materials

- The quantities required and the matrix in which they are provided
- A set of characteristics to be determined for the reference materials.

4.4 Summary

1. *The techniques and methods employed in nanotoxicology research have a profound effect on the outcome of the experiments.*
2. *There have been many suggestions in the literature regarding conditions and criteria that can inform and guide the conduct of experimental investigations for ENPs. It would be very useful for this information to be gathered, evaluated and a set of recommended experimental criteria created, and disseminated, for the benefit of Australian researchers.*
3. *Intratracheal instillation of particulates at loading of more than about 100 – 200 µg per rat lung has the potential to cause effects associated with particulate overload of the lung.*
4. *Exposing mice by nasopharyngeal aspiration provides better particle dispersion in the lower lung and is not as susceptible to lung overload issues as is intratracheal instillation.*
5. *The correct vehicle for administering ENPs to the experimental system is essential for avoiding excessive agglomeration of ENPs.*
6. *Agglomeration of ENPs can significantly alter the in vivo and in vitro toxicity.*
7. *Exposure metrics describing the exposure-response relationships are important and should be expressed in several ways to facilitate comparison across studies.*
8. *High in vitro concentrations of ENPs which have no relevance to the real world exposures, together with other in vitro ENP nuances, curtail the application of in vitro data.*
9. *It is essential to physically characterise ENPs in the experimental delivery media using several parameters.*
10. *Information on Australian exposures to ENPs in the workplace need to be gathered, and made available to the general scientific community. This will enable comparison of ENPs used in experimental, or safety tests to be compared to the physical characteristics of ENPs in workplace air.*

5. Mode of action for fine insoluble particulates

5.1 General concepts

In the previous ASCC report essential background information was presented that dealt with barriers of entry for nanoparticles into the body, and defensive mechanisms that may be mobilised should these barriers be compromised and nanoparticles enter the systemic circulation. In that report brief mention was made of the role of reactive oxygen species and macrophage ingestion of particulates.

Over the last decade much has been learned about the possible mode(s) of action for the toxicological effects of low-toxicity particulates of fine size. This has given rise to the 'particulate mode of action paradigm' and in fact has driven much of the research investigating NP toxicology. Understanding the paradigm facilitates interpretation of the experimental data.

Particles may cause direct or indirect toxicity. Direct toxicity is associated with their chemistry or size/shape and is the result of substance interaction with cellular macromolecules or structures inducing a change in cell and tissue function. The toxicity is manifested when function cannot be restored by adaptive or repair mechanisms. For nanoparticles there is a question of whether their size and physicochemical properties can result in cellular interactions that are not only different from the corresponding material of larger size, but which may be unique in the sense that such interactions may give rise to toxicity not previously known for the larger size material. There is also concern as to whether the adaptive responses of the body will be able to overcome injury mediated by apparently unique toxicological mechanisms (if they occur). It is pointed out however nanoparticles of various kinds occur naturally and ubiquitously in the environment (GR 2006, Buzea et al. 2007).

Indirect toxicity can occur as the result of excessive, or dysregulated, adaptive responses which lead to prolonged inflammation in the lung. Although the inflammation is initiated by the presence of particles, it is not associated with the properties of the particle *per se*. Most particles are readily removed from the lungs but the clearance mechanisms can be overwhelmed if tissue load is excessive. In addition, the minute size of individual nanoparticles enables them to bypass or perhaps damage the lungs' clearing mechanisms. Any particles, including nanoparticles or their aggregates, which are not readily dissolved or broken down tend to accumulate. The accumulation over time can give rise to inflammation mediated lung pathology.

Pulmonary inflammation is an acute response to viruses, bacteria and particulates, which coupled with the fact that prolonged inflammation is an underlying cause of chronic particulate associated occupational diseases, provides the rationale for many of the end points investigated in ENP *in vitro* and *in vivo* experiments. Wilson and Wynn (2009) have reviewed the pathogenesis, regulation and aetiology of pulmonary fibrosis. A three phase model of wound repair is presented that includes; injury/insult, inflammation and repair. In most pulmonary fibrotic conditions dysregulation at one or more of these phases has been reported. Chronic inflammation can lead to an imbalance in the production of chemokines, cytokines, growth factors, and disrupt cellular recruitment. These changes coupled with excessive pro-fibrotic IL-13 and/or TGFβ1 production can turn a well controlled healing response into a pathogenic fibrotic response.

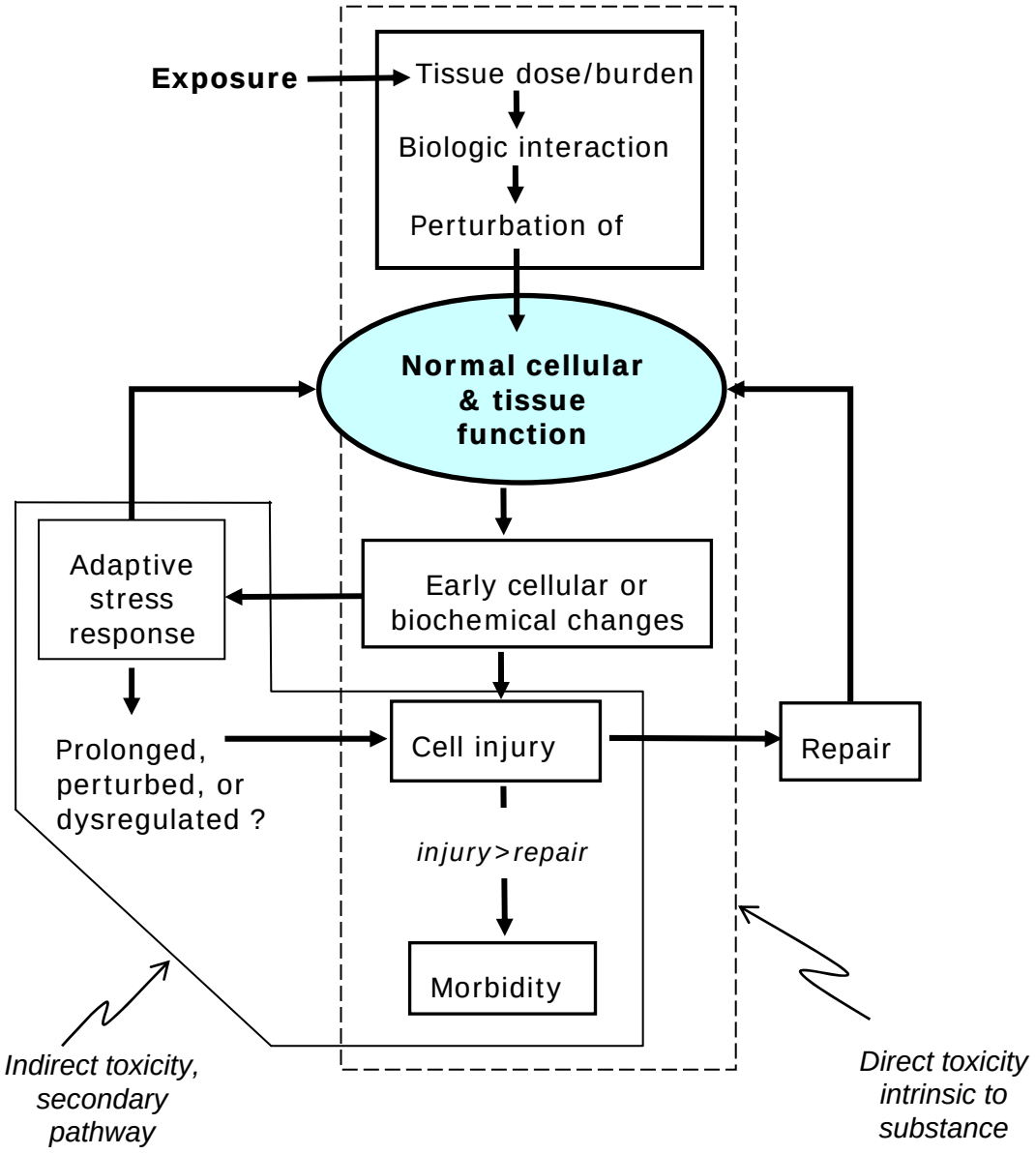
An overview of the general concepts of toxicological cell and tissue damage are summarised in **Figure 5.1**. The figure reflects one of the basic tenets in toxicology; that is the well known axiom - 'the dose makes the poison'.

Thus the toxicity paradigm for particulates inherently calls up a threshold exposure, or lung burden, which needs to be exceeded for either acute or chronic adverse pulmonary effects to be manifested. That is, if the dose of particulate is large enough, such that adaptive responses to induced perturbations of cellular or lung function cannot cope to return the tissue to normal function, then injury may occur. Not all adaptive responses to cellular stress are necessarily beneficial. In particular, if pulmonary inflammatory responses are prolonged or become dysregulated they can paradoxically cause tissue injury in their own right. Thus if the injury is severe enough, frequently repeated, or occurs in situations of compromised defence mechanisms such that repair processes cannot cope, or they malfunction, then tissue damage, leading to morbidity, may occur (Gregus 2008, Wilson and Wynn 2009).

Important aspects of this scheme are:

- There are cellular and tissue mechanisms to deal with the direct toxicity of small doses of materials
- Morbidity occurs when injury is greater than repair or when repair processes are compromised
- Prolonged or inappropriate adaptive responses to cellular responses can lead to tissue injury.

Figure 5.1: Overview of the direct and indirect toxicological pathways leading to particle adverse health effects.



Notes:

Because the possibility of overexposure may be important for hazard identification both the direct and indirect toxicity pathways may contribute to considerations of acute or chronic hazard identification. However for assessing risk, it is usually the direct toxicity that is more relevant because this is manifested at exposures closer to that of humans and lower than the exposures causing indirect toxicity. The issue of what dose data are relevant for hazard and risk assessment for ENPs is analogous to the debate that has taken place for experimental carcinogens; i.e. should a carcinogenic responses that is only observed in excess of the maximum tolerated dose be used for cancer hazard and risk assessment.

5.2 Particle paradigm

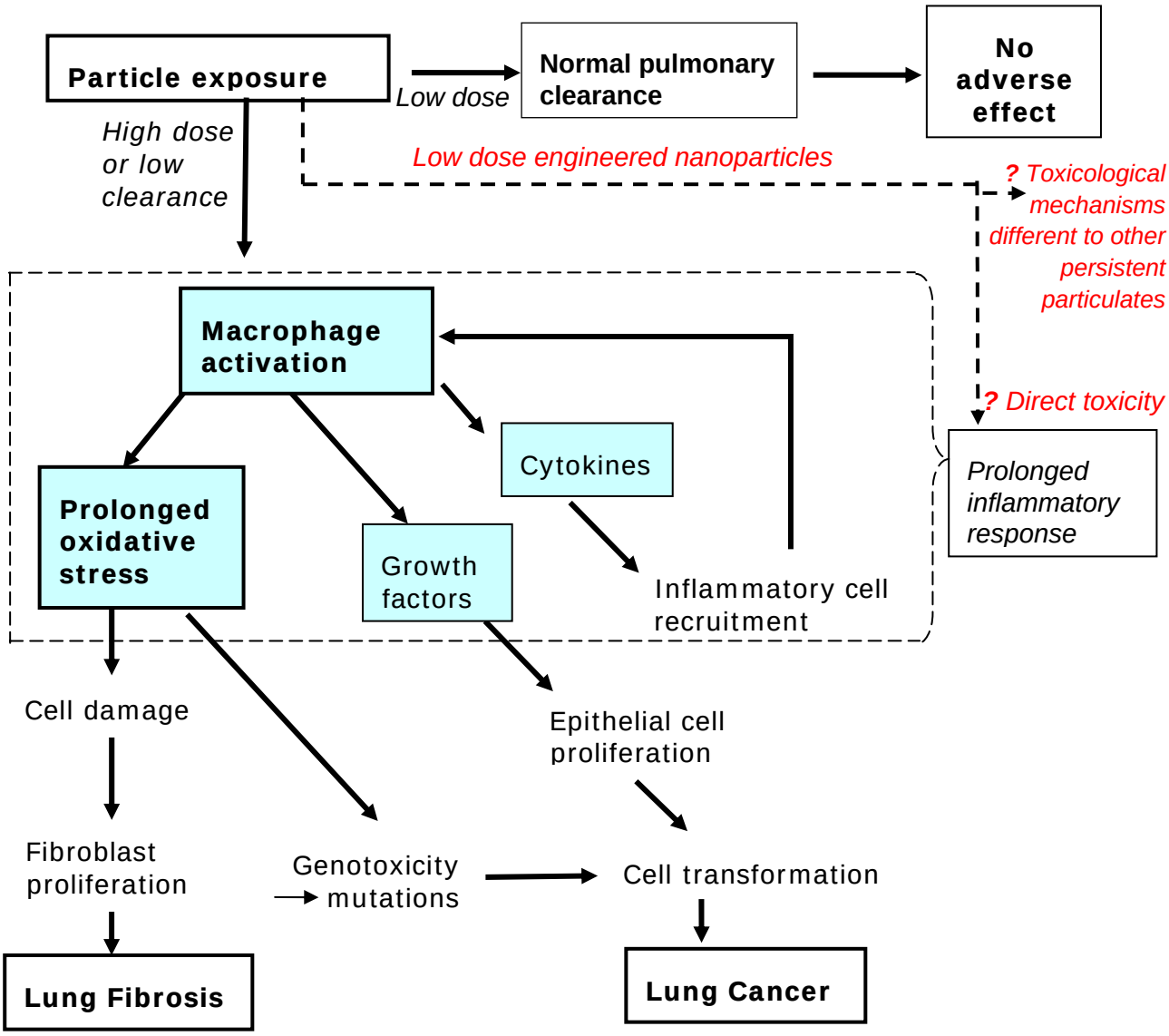
It is well known that if the lung is not overloaded with dust, dust-laden macrophages on the alveolar surface will migrate upward and be carried by the mucociliary escalator system up the trachea, and cleared into the oesophagus.

Particle transport by macrophages from the alveolar region toward the larynx is however rather slow in humans and it eliminates only a fraction of the deposited particles in the deep peripheral lung. The remainder may accumulate unless the particles are biodegradable or cleared by simple chemical dissolution in lung fluids. Thus macrophages laden with particulates may remain in the lower lungs for some time, the continued interaction between the cells and the trapped dust will give rise to a prolonged production of oxidative stress and an exacerbated inflammatory response (Lam et al. 2006, Borm et al. 2006a, Li et al. 2008a). The situation can be brought about by a few high exposures, or a number of much lower exposures that over time gradually allow the lung burden of particulate to increase. Patently, for equivalent exposures higher burdens will be attained sooner for biopersistent particles compared to those that are easily removed from the lung.

A more detailed summary of the toxicological mode of action for inhaled fine particulates is in **Figure 5.2**. This paradigm has been particularly informed by the toxicity of crystalline silica and other poorly soluble fine particles where chronic excessive occupational exposure has given rise to lung burdens causing prolonged inflammation, clinical fibrosis and cancer. It is such real life experiences that have driven concern regarding what the chronic health effects of occupational exposure to ENPs might be. Note the toxicological mode of action of particulates is similar to, but slightly different to that of biopersistent fibres described in **Section 7.1.1**.

The particulate paradigm highlights what the long term effects might be if over exposure occurs and an excessive lung burden of particulates accumulate. The scheme does not preclude particulates having acute direct toxic effects. However to ensure an effect is observed, most *in vivo* ENP experiments have employed acute exposures that have placed high burdens of particulate into the lung which have overwhelmed clearance mechanisms and indeed given rise to relatively long lasting inflammatory responses. The usual rationale for such high single doses is they may mimic the ultimate pulmonary load accumulated over many years if the particle has very slow intrinsic clearance. These experiments have not investigated the intrinsic *in vivo* toxicity of ENPs per se, i.e. toxicity directly associated with ENP physics (optical, electric, and magnetic properties), chemical reactivity or size. There have been very few low dose *in vivo* experiments and no chronic inhalation studies, performed to date, that seek to reveal if ENPs possess unique toxicological effects outside of those mediated by the predictable oxidative stress and inflammation associated with doses that exceed pulmonary particulate clearance mechanisms.

Figure 5.2: Summary of the particle toxicity paradigm for chronic effects



Notes:

For clarity the adaptive and repair pathways in Figure 5.1 have not been included in this figure, nonetheless they are still operative and there is a requirement for sustained adaptive response and injury to be greater than repair. Note the pivotal roles of (i) high dose and/or low pulmonary clearance and (ii) prolonged inflammatory response with associated oxidative stress. Key parameters used for investigating the pulmonary toxicity of engineered nanoparticles are therefore persistence in the lungs (equates to low clearance) and measures of oxidative stress. It should also be noted that as a consequence of the postulated common mode of action (oxidative stress) for lung fibrosis and lung cancer there may be incidence, or temporal concordance between them. However they are separate disease entities; fibrosis does not progress to cancer. Also, due to the much shorter times required, all experimental protocols for ENPs to date have measured pre-inflammatory or early fibrosis parameters. Tests for genotoxicity are limited to *in vitro* investigations, the relevance of which for workplace exposures is obscure at this time. No published chronic repeat exposure animal experiments were located for the ENPs. There are questions whether ENPs because of their unique chemical properties could cause direct toxicity not associated with oxidative stress or inflammation, whether early inflammatory responses associated with low (non-overload) doses are transient or associated with later development of adverse effects, and also whether lung burdens required to set off the chronic inflammation cascade are lower for ENPs than for fine particulates studied to date.

However it is important to keep in mind that in the general population adverse effects of particulates (smoke, smog, automobile exhaust, etc.) are not limited to chronic exposure. Epidemiological studies have correlated, with up to a few days lag time, increased visits to hospital emergency departments, increased bronchodilator use in asthmatics, and increased mortality from exacerbation of respiratory and cardiovascular diseases with ambient pollution events of particulates of size up to 10 μm (PM10)(EC 1997, RIVM 2002, US EPA 2003, Mills et al. 2007).

Although biological persistence is a key feature of ENPs that could lead to toxicity, no information apart from that for fibres (see **Section 7.1**) was located regarding experimental criteria that would define pulmonary persistence. This is considered to be an important area for clarification.

5.3 Summary

1. *The toxicity paradigm for particulates has and continues to provide the experimental framework for investigating potential health effects of ENPs, particularly chronic health effects. The paradigm is significantly informed by data for crystalline silica.*

2. *The paradigm inherently incorporates the concept of a threshold dose for pulmonary effects. Unfortunately research over the past few years with ENPs has not been conducted with the intent of identifying threshold exposures.*

3. *As a consequence of the particulate paradigm most experiments with ENPs have concentrated on oxidative stress and pro-inflammatory toxicological end points.*

6 Effects of engineered nanoparticles

6.1 *In vitro* test systems

6.1.1 Inflammation and oxidative stress

Since prolonged inflammation and associated oxidative stress is considered to be one of the most important mechanisms leading to particulate induced toxicity it is not surprising that the vast majority of ENP investigations have concentrated on these parameters. Unfried et al. (2007) and Li et al. (2008a) have reviewed the detailed mechanisms for the production of oxidative stress and the importance of cellular antioxidant and detoxification pathways in protecting against particle induced lung damage. The authors highlight that induction of reactive oxygen species (ROS) *per se* does not automatically lead to adverse biological reactions. Thus measurement of ROS in *in vitro* systems is in of itself not a good indication of particle hazard potential. In terms of characterising the toxicological effects of ENPs, it is widely agreed that no one test is likely to be fully applicable, but that in fact a suite of tests may be needed and that ROS production may form a useful part of that suite.

Required for toxicity is failure of the normal cellar responses to provide adequate protection, under these circumstances further increase in ROS can result in proinflammatory effects followed by cytotoxicity. Li et al. (2008a) define oxidative stress as a state of redox disequilibrium characterised as a decrease in the cellular glutathione (GSH)/glutathione disulphide (GSSG) ratio, but functionally should be seen as a cellular stress response that activates a number of the redox-sensitive signalling pathways. This is a useful working paradigm for assessing and interpreting *in vitro* data on ENPs. Besides the cell membrane, mitochondria and cell nucleus are considered as major cell compartments relevant for possible NP-induced toxicity.

Assessment of oxidative stress is relatively easy to evaluate in cell cultures; reactive oxygen species (ROS), production of pro-inflammatory cytokines, and changes in the antioxidant defence mechanisms can be measured (e.g. reduced sulphydryl status and changes in linked enzyme systems, tocopherol levels, superoxide dismutase activity, heme oxygenase activity, etc.) plus gene activity associated with the enzyme systems and cytokine production. Induction of oxidative stress is repeatedly reported (Hoet and Boczkowski 2008); a bewildering number of cell types[11] have been utilised in these studies with a wide array of concentrations and endpoints.

Donaldson et al. (2009) point out that three different conventional particle types (PM_{10}, asbestos and quartz) cause diverse pathological effects[12] (**Table 6.1**). Nonetheless all

[11] For example: Human alveolar epithelial cells (Park et al. 2007), rat lung epithelial cells (Sharma et al. 2007), BJ foreskin cells (Sarker et al. 2007), human A549 cells (Limbach et al. 2007, Monteiller et al. 2007), J774 cells (Wilson et al. 2007), BEAS-2B cells (Veranth et al. 2007).

[12] All these particles cause pulmonary inflammation and fibrosis, and lung cancer but only PM_{10} is also associated with cardiovascular effects. Both quartz and asbestos are associated with autoimmunity and

these particles cause very similar oxidative stress events in cells in culture. Consequently demonstration of oxidative stress in *in vitro* cell systems does not inform on possible *in vivo* effects. Oxidative stress is a central mechanistic tenet for the toxicity of a large number of chemicals and metals at the cellular level. Donaldson et al. (2009) consider such data on its own is of highly questionable value for predicting toxicity of ENPs and needs to be interpreted with great care. They state "it is a self fulfilling prophecy that adding particles to cells will cause oxidative stress if the dose is high enough".

Table 6.1: Particle-specific adverse health effects of conventional particles

Particle	Adverse Effects	
	Pulmonary	**Extra-pulmonary**
Asbestos	Interstitial fibrosis, bronchogenic carcinoma, pleural mesothelioma, pleural fibrosis, pleural plagues.	Peritoneal mesothelioma, autoimmune disease.
Quartz	Nodular fibrosis, small airways disease, bronchogenic carcinoma, pleural fibrosis.	Autoimmune disease
PM_{10}	Increased lung cancer risk Exacerbations of COPD[(a)] Development of COPD Exacerbations of asthma	Deaths and hospitalisations for cardiovascular disease.

Notes:

(a) COPD = Chronic Obstructive Pulmonary Disease

Source: Donaldson et al. (2009)

As with any *in vitro* cell technique it is enormously difficult to extrapolate the results to the whole animal and relate the concentrations applied in cell cultures to an equivalent human or animal exposure. Collectively the *in vitro* oxidative stress and pro-inflammatory data indicates that at some concentration a variety of, and maybe most, ENPs may be able to induce oxidative stress in cultured cells. Even if the potency of an ENP for oxidative stress is compared with a reference compound, e.g. nano-sized crystalline silica, the relevance for occupational risk remains obscure. Donaldson et al. (2009) consider the only way forward to put *in vitro* studies on a relevant and plausible footing with regard to dose and exposure in use, what target cells to study, and ultimately the usefulness of *in vitro* data in hazard identification is to undertake toxicokinetic studies of the ENP. The issues of *in vitro* to *in vivo* dose considerations are discussed in **Sections 4.3.1 – 4.3.4**, and a brief discussion of the concordance of *in vitro* with *in vivo* data in **Section 6.1.4**.

The utility of the *in vitro* techniques lies in monitoring the change in biological activity of a given type of ENP as it is produced and customised, for example:

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pleural fibrosis (PM_{10} is not) but only asbestos causes pleural plagues, pleural mesothelioma and peritoneal mesothelioma.

- according to the type of impurities it contains
- as it is purified
- as its surface characteristics are modified
- as its size distribution is changed
- as it aggregates or disperses, etc.

The above applications of *in vitro* techniques rely on the specific ENP under evaluation being modified from a 'base' state. The techniques are therefore useful in developing an ENP with given technical characteristics but which has minimal biological activity within the particular ENP family.

6.1.2 Cytotoxicity

In vitro cytotoxicity is by far the most common cellular endpoint measured in toxicological studies of ENPs (Hansen et al. 2007). It can be measured using different methods: tetrazolium dye assays (e.g., MTT, XTT, WST-1), viability markers (e.g. trypan blue exclusion, neutral red, thymidine uptake), apoptotic and necrotic markers with immunocytochemistry and flow cytometry staining, and cell growth (Cunningham 2007).

Wörle-Knirsch et al. (2006) have demonstrated fake cytotoxic effects with SWCNTs when cell viability is measured with MTT but the same cell treatment evaluated with WST-1, LDH, FACS assisted mitochondrial membrane potential determination, and Annexin-V/PI staining do not reveal cytotoxicity. SWCNTs appear to interact with tetrazolium salts such as MTT but not with others (such as WST-1). The authors strongly suggest verifying cytotoxicity data with at least two or more independent test systems for nanomaterials. Monteiro-Riviere and Inman (2006) reached the same conclusions when investigating *in vitro* the effects of carbon black (CB) on human keratinocyte cytotoxicity; CB interfered with determination of some end points indicating it was not appropriate as a standard for cytotoxicity tests.

Thus there are significant interpretational and methodological issues that curtail the usefulness of some of the data (See **Section 4** and **Appendix 1**). Some results of individual cytotoxicity assays are provided in the sections dealing with specific ENPs.

Lanone et al. (2009), as part of the NanoSAFE 2 European project, evaluated the toxic effect of 24 NPs of similar spherical diameter and various elemental composition on two human pulmonary cell lines[13]. One of the aims of the study was to elaborate a generic experimental set-up that would allow the rapid screening of the cytotoxic effect of NPs. Two cytotoxicity assays, based on metabolic activity and membrane permeability (MTT and neutral red respectively), were compared and analysed at two time points (3 and 24 hours) for each cell type and nanomaterial. Each nanomaterial was analysed by two independent laboratories, out of the three different laboratories, participating in the study. A549 cells showed less sensitivity than THP-1 cells and in

[13] A549 (representative of alveolar type II cells) and TPH-1 cells (Phorbol Myristate Acetate (PMA) activated/differentiated monocytes to macrophages.

most cases MTT was more sensitive than the neutral red assay. For highly cytotoxic NPs and those that were not toxic there was good concordance between laboratories, however for NPs of intermediate cytotoxicity the reproducibility between laboratories was poor. The authors concluded the study clearly highlighted the difference in sensitivity between cell types and cytotoxicity assays that has to be carefully taken into account when assessing the data.

6.1.3 DNA damage

Determining whether an agent is able to directly interact with and alter the functionality of DNA is an important consideration in the overall hazard profile of a substance and in assessing its health risks. DNA damage may also occur as a result of secondary mechanisms that do not require the agent to be directly genotoxic. These largely occur with high doses and include excessive production of ROS and cytotoxicity (Vallyathan and Shi 1997, MacNee and Donaldson 2003, Knaapen et al. 2004, Kisin et al. 2007, Schins and Knaappen 2007).

Gonzalez et al. (2008) have recently evaluated the *in vitro* and *in vivo* genotoxicity data available for ENPs. Due to the limited number of data, incomplete physico-chemical characterization of ENPs examined and shortcomings in experimental approaches the authors could not draw a definitive conclusion concerning the genotoxic activity of ENPs. The evaluation revealed gaps in the genotoxicity studies of ENPs in that focus was mainly on ROS as mode of action. The authors concluded there is a need to develop adequate positive controls for genotoxicity assays when conducted with nanomaterials and have proposed minimal criteria for conducting nano-genotoxicity assays.

According to a recent review by the European Commission, “if the genotoxic effects of nanoparticles are related to inflammation, simple *in vitro* assays may not be adequate in showing the genotoxic potential” (SCCP 2007). Interpretation of such studies is particularly confounded when they are carried out in the absence of reference compounds (i.e. positive controls).

In relation to NPs the European review (SCCP 2007) also states:

There are presently no validated standard methods for assessing genotoxic effects in the expected target tissues in vivo, but techniques such as the comet assay, micronucleus test, and gene mutation analysis in transgenic animals could probably be applied.

To date no OECD test guidelines have been developed to address the genotoxicity of nanoparticles. Further research into the differentiation between the inflammatory response and genotoxicity, as well as validation of *in vitro* and *in vivo* genotoxicity tests for NPs, is required for an informed discussion of the genotoxicity and its health implications (NSTC 2008).

Appendix 3 contains details of the effects of ENPs on DNA and **Table 6.2** summarises some recent genotoxicity studies undertaken with ENPs. There have been surprisingly few studies published for the period covered by this review. This may be a reflection of the fact that standard *in vitro* genotoxicity testing protocols are not readily adapted for use with ENPs without investment of major workup. Generally the data from large multidisciplinary investigative groups is of better quality. The ENPs are better characterised, multiple genotoxic endpoints are used, appropriate positive controls are

incorporated into the experimental design and care has been taken not to introduce artefacts. While such studies are data rich, the down side is the information is difficult to assimilate and contextualise.

Overall:

- *Unless the ENP has been functionalised leaving a reactive 'organic' surface, or contains impurities, no information has been recently published that provides compelling evidence for poorly soluble ENPs behaving differently to other well characterised insoluble fine particulates with respect to causing DNA damage, i.e. the secondary genotoxicity pathway mediated by ROS will be the dominant, if not the only, in vivo mechanism causing DNA damage.*
- *As far as this review has been able to ascertain, validated genotoxicity tests for nanoparticles have not yet been established. International progress in this area could be reviewed to identify opportunities for contributing research from Australia.*
- *As with other areas of nanotoxicology research, preparation and characterisation of the ENP is critical to obtaining meaningful information.*

6.1.4 *In vitro* versus *in vivo* data

Notwithstanding the above discussions regarding the potential usefulness of *in vitro* data, in the opinion of the authors at this time, on its own *in vitro* oxidative stress, pro-inflammatory, cytotoxicity or genotoxicity data for ENPs is of limited usefulness for hazard identification, regulatory hazard classification, or for occupational risk assessment purposes. *In vitro* data may however contribute to a weight of evidence evaluation (WoE) for hazard and potential risk, and elucidating toxicological mechanisms.

There is broad qualitative concordance between *in vitro* and *in vivo* data in that at some experimental level of exposure markers of inflammation and oxidative stress responses can be observed in both types of experiments. The issues of course are whether the *in vitro* experiments which bypass *in vivo* kinetic considerations are physiologically similar enough to the *in vivo* situation to allow valid comparison of descriptive responses, and whether cellular and tissue dose metrics line up (see **Section 4**).

Appendix 4 describes recently published studies that compare *in vitro* ENP data with the *in vivo* information. Comparisons of *in vivo* and *in vitro* measurements for fullerenes demonstrated little correlation (Sayes 2007a, b). Whereas that for platinum nanoparticles was reasonable in that nothing of significance was found in any of the tests (Elder et al. 2007). On the other hand, Sayes et al. (2007b) found when investigating five different particle types, the comparisons of *in vivo* and *in vitro* measurements demonstrated little correlation, particularly when considering many of the variables assessed in the study (such as cell types, culture conditions and time course of exposure, measured end points). The authors concluded it is clear that *in vitro* cellular systems need to be further developed, standardized, and validated (relative to *in vivo* effects) in order to provide useful screening data on the relative toxicity of inhaled particle types. We suggest that this applies to all types of *in vitro* test

systems that might be considered as forming part of a tiered testing approach for ENPs, not just those used for elucidating cytotoxicity and genotoxicity.

Table 6.2: Summary of *in vitro* genotoxicity tests [(a)]

ENP	Cell system	ENP characterisation	Concentration	Genotoxicity	Comments	Reference
UF - TiO_2	WIL2-NS	None, used as purchased	26 µg/ml 65 and 130 µg/ml	↑ Apoptosis ↑ Cytotoxicity (MTT) + Micronucleated binucleated cells + HGPRT + Comet assay	WIL2-NS are Human B-cell lymphoblastoid cells (transformed with Epstein-Barr virus) with a p53 mutation. Sensitive to ROS. Liquid styrene oxide was positive control.	Wang et al. (2007a)
Iron oxide (Fe_2O_3 - DMSA coated)	Cultured fresh fibroblasts from infant foreskin	Size range Structure by X-ray absorption	1,000 µg/ml	± Weak cytotoxicity (WST-1 test for mitochondrial activity) - Comet assay.	Negative genotoxicity attributed to coating. Particle internalisation by endocytosis after adhesion to cell identified by TEM.	Auffan et al. (2006)
Colloidal C_{60} fullerene	Freshly isolated human lymphocyte	Colloid concentration. Size and charge. TEM imaging.	2 – 4 µg/ml	+ Comet assay	Two C_{60} colloid preps, made by EtOH to H_20 or extended H_20 mixing. Hypothesised genotoxicity mechanisms: ROS or fullerene e^- transfer mediated.	Dhawan et al. (2006)
SWCNTs	Chinese hamster V79 lung fibroblasts	Acid washed to remove metals.	0, 24, 48, 96 µg/cm^2 (83% dispersed by sonication in culture medium)	↑ Cytotoxicity with TP exclusion at two highest doses. - Micronucleus. + Comet assay at two highest doses. - Salmonella up to 240 µg/plate.	Purity of SWCNT assessed by several techniques. Diameter: 0.4 – 1.2 nm. Length: 1 – 3 µm SA:1,040 m^2/g	Kisin et al. (2007)

ENP	Cell system	ENP characterisation	Concentration	Genotoxicity	Comments	Reference
Capped water soluble FePt	Bacterial reverse mutation. Salmonella (TA98, 100, 1535, 1537) E.Coli	Laboratory made & characterised Done by several methods	Up to 5,000 µg/plate	Pre-incubation ± S9 ± Weakly +ve in only TA100 –S9. - In all other strains - E.Coli	NPs capped with tetramethyl ammonium hydroxide. Mean diameter: 9 nm., 11% face centred cubic crystal structure.	Maenosono et al. (2007)

Notes:

(a) + = positive, - = negative, ± = ambiguous result, ↑ = increase

6.2. Kinetics and translocation

The reader is referred to Peters et al. (2006) and Unfried et al. (2007) for recent reviews of the detailed mechanisms by which ENPs may cross cell membranes, the review by Buzea et al. (2007) also contains good information relating to translocation of ENPs in the body.

Although investigations of the systemic distribution of ENPs after inhalation exposure were not found for this review studies predating the period of this review have shown direct absorption of ENPs into the systemic circulation may occur through the lungs (e.g. Nemmar et al. 2002, Kreyling et al. 2002, Mills et al. 2006, Oberdörster et al. 2002, 2007a).

Although not often considered by experimenters there are particle kinetic issues associated with testing NPs in *in vitro* cell systems. The problem of dispersion and agglomeration has already been discussed (**Section 4.3.3** and **Appendix 1**). Teeguarden et al. (2007) review dosimetry conundrums of *in vitro* experiments and point out that while equal mass concentrations may imply equal doses for dissimilar materials the corresponding particle number, or particle surface area per area or number of treated cells may differ by orders of magnitude. They advocate that incorporating particokinetics and principles of dosimetry in *in vitro* experiments would significantly improve the basis for NP toxicity assessment.

Peters et al. (2006), citing examples of oxidative stress and neuro-degeneration, makes a case that the brain is a target organ for fine particulates in polluted ambient urban air but does not extend the argument to ENPs. Hagens et al. (2007) has reviewed the absorption of different NPs via the traditional exposure routes and also point out there is evidence for uptake of NPs into the brain of rats from the olfactory mucosa via the olfactory nerve. This absorption route had been described in 1940 for nanosized polio virus. No additional information on this exposure route for NPs was located during this review. It is not known how important it may be for humans and more work is required.

Hagens et al. (2007) undertook a review of the toxicokinetic properties of NPs and concluded it is unclear to what extent the different NP characteristics contribute to their kinetics since these authors could not extract and correlate common kinetic and physicochemical properties from the available data. NPs present experimental challenges in studies of absorption, distribution, and excretion because many of the techniques used to characterise/measure NPs after synthesis or in dosing media are not suitable for biological matrices. They considered the development of validated detection and characterisation methods for NPs in biological fluids and tissues is urgently needed (see also **Section 4.3.5**). Major studies published over the last few years contributing meaningful information on the fate and toxicity of NPs have tended to use advanced electron microscope techniques, Raman spectroscopic signatures, and NPs incorporating stable isotopes to characterise and/or measure amounts of NP in tissues (e.g. Liu et al. 2008).

Factors affecting the fate of nanomaterials in the body are reviewed by Yang et al. (2008) and Bastús et al. (2008). Translocation of ENPs in the body occurs via blood vessels and the lymphatic system. Healthy capillary vessels are permeable to NPs but have a pore size cut-offs depending on the location and type of capillary blood vessel (**Table 6.3**).

Table 6.3: Pore size cut-offs of blood vessels and intracellular structures

Blood vessels	**Pore size (nm)**
Tight junction capillary (e.g. CNS blood brain barrier, testis)	<1
Continuous capillaries in most tissues (e.g. muscle, lung, skin)	~6
Fenestrated capillaries (kidneys, intestine, some exocrine & endocrine glands)	50 – 60
Sinusoid capillaries (liver, spleen, bone marrow)	100 – 1000
Tumour blood vessels	400 – 600
Intracellular membranes	**Pore size (nm)**
Nuclear membrane pores	8 – 9
Cytoplasm cytoskeleton mesh	~ 20

Source: Bastús et al. (2008).

Patently size, shape, and surface charge play a role in the uptake and translocation of NPs.

Surface coating with protein can significantly enhance uptake of NPs by cells; it is noted in *in vitro* investigations and in NP experiment dispersion techniques that protein avidly adheres to many nanomaterials. Concern has been raised regarding the physical and biological stability of coated NPs, should the coating be metabolised or deteriorate on storage then the core NP with different biological reactivity may become exposed (Bastús et al. 2008).

The fate of inhaled NPs in the lungs depends on the regional deposition within the lung and the complex kinetics of absorption and non-absorptive clearance mechanisms. Bastús et al. (2008) points out that when airborne NPs pass through a filter or a specific channel (e.g. the respiratory tract) the smaller particles impact more often with the channel walls and get retained more efficiently because Brownian motion increases as particles become smaller. The balance between Brownian and translation motions has been modelled under conditions of human nose breathing to predict the fractional deposition of NPs in different regions of the respiratory tract. **Table 6.4** summarises the predicted deposition fractions for different size NPs, see also **Appendix 1** for more information.

Table 6.4: Fractional deposition of NPs in human lung when nose breathing

Size (nm)	**Fractional deposition (%) per region**		
	Nasopharyngeal	**Tracheobronchial**	**Alveolar**
1	90	10	0
5	~33	~33	~33
20			50

Source: Bastús et al. (2008).

Human alveolar macrophages measure between 14 and 21 µm, while rat alveolar macrophages measure between 10 and 13 µm. Macrophages can engulf particles of a size comparable to their own dimensions, but are significantly less effective with particles that are much larger[14] or smaller (Buzea et al. 2007). Particles less than 50 – 100 nm are taken up by macrophages less efficiently than their larger counter parts[15] (Chono et al. 2006, Buzea et al. 2007). At high concentrations, nanoparticles tend to cluster, forming agglomerates more often larger than 100 nm.

NPs are predominantly cleared from the lungs via the mucociliary escalator if phagocytosed, or by absorption into the lymphatic and circulatory systems. From the latter they can be distributed throughout the body. Although lymphatic drainage can remove particles up to a size of about 500 nm, according to Yang et al. (2008) there is uncertainty regarding the real contribution of this clearance pathway to systemic appearance of NPs after inhalation.

Pristine ^{13}C labelled[16] SWCNTs administered intravenously to mice were cleared quickly from the blood and distributed throughout the entire body; major accumulations occurred in mononuclear phagocytes of the reticuloendothelial system of the lungs, liver, and spleen and were maintained over the 28 day observation period. SWCNTs were clearly detectable in the brain. Clearance from organs was generally slow, with an estimated average total clearance rate of less than 1 µg of SWCNTs per day. It was stated that animals did not show clinical signs of acute toxicity up to intravenous doses of 2 mg/mouse (80 mg/kg), although not explicit in the paper we presume the animals were only monitored for standard 'in life' signs of toxicity such as grooming and other behaviour, fur staining, food consumption and body weight (Yang et al. 2007b).

The distribution of pristine SWCNTs is different from the chemically modified/functionalized SWCNTs. The latter are retained in plasma much longer, less avidly taken up by the reticuloendothelial system, and removed from tissues more quickly than are their non-functionalised counter parts, albeit still slowly (Li et al. 2008a). Some functionalized SWCNTs are quickly excreted in urine (Singh et al. 2006) whereas pristine nanotubes could hardly be detected in urine and faeces (Yang et al. 2007b). Although this data may suggest pristine SWCNTs may have different systemic toxicities to functionalized SWCNTs no acute toxicity, as judged by clinical signs and limited pathology investigation, following intravenous administration has been demonstrated for either (Li et al. 2008a).

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[14] The sizes of the macrophages probably determines the length of fibres (>20µm) considered to cause frustrated phagocytosis and ensuing pathogenic responses of fibrosis and mesothelioma (see **Section 7**).

[15] According to Buzea et al. (2007) there are contradictory *in vitro* vs *in vivo* reports regarding the phagocytosis of nanoparticles smaller than 100 nm. *In vitro* studies show that nanoparticles activate and are phagocytized by alveolar macrophages. However, macrophage lavage recovery studies show that nanoparticles smaller than 100 nm are not efficiently phagocytized in comparison with particles between 1 and 3 µm.

[16] SWNT were bundles of 10-30 nm in diameter and 2 – 3 µm long administered via tail vein injection (200 µg/mouse) in an aqueous suspension with 1% Tween 80. Tissue distribution at 1, 7 and 28 days was measured using isotopic abundance ($^{13}C/^{12}C$ ratio) determined by isotope ratio mass spectroscopy. The detection limit for the $^{13}C/^{12}C$ ratio is about 1 ppm.

The distribution of quantum dots 705 (QD705) was determined in mice after single intravenous injection[17] by Yang et al. (2007a). The introduction of this research paper contains a brief review of the distribution, kinetics and toxicity of a variety of quantum dots (QDs). Essentially the coating of the QDs determines the target tissue for accumulation, QDs are quickly cleared from the blood, but accumulate in the liver, kidneys, spleen and lungs and have long tissue half lives but according to Yang et al. (2007a) are without apparent toxic effects. Notwithstanding the absence of *in vivo* effects, a large number of *in vitro* cytotoxicity studies have been conducted, some of which depending on QD coating, show adverse effects. Embryogenesis in Xenopus frogs was not affected by injection of QD.

Yang et al. (2007a) produced distribution data for QD705 that is consistent with the limited existing literature for QDs. The plasma half-life of QD705 in mice was short (18.5 hr), but analyses revealed QD705 persisted and continued to increase in the spleen, liver, and kidney 28 days after the intravenous dose. Time-dependent redistribution from body mass to liver and kidney was apparent. Faecal and urinary excretion of QD705 was not appreciable. It was concluded QD705 has a very long half-life, potentially weeks or even months, in the body. This signals a need for chronic toxicity testing[18].

Clift et al. (2008) investigated in J774.A1 murine 'macrophage-like' cells, the uptake of different surface coated QDs. COOH QDs were clearly taken up by the cells but uptake of NH_2 (PEG) QDs was not detectable by live cell imaging, but was observed following 3D reconstruction of fixed cells, as well as by flow cytometry.

Overall there is a consistent theme in the recent literature on the kinetics and disposition of CNTs, QDs and fullerenes (see Section 7.7):

- *They are quickly taken up by the reticuloendothelial system,*
- *but nonetheless distributed widely in the body.*
- *There is strong tissue retention once the material is taken up.*
- *Functionalised CNT and coated QD are less avidly taken up by the reticuloendothelial system and so have longer plasma half lives.*
- *Based on a relatively small number of studies available, the major route of elimination from the body appears to be biliary excretion with excretion in urine being absent or minimal for nonfunctionalised material. Functionalisation or surface coatings that increases water solubility increases excretion in urine.*

[17] The intravenous dose of QD705 was 40 pmol and animals were assessed for up to 28 days. QD705 has a CdTe core with a ZnS shell. They respectively had 46, 11, and 1% Cd, Se, and Te. These nanocrystals were surface modified with methoxy-polyethylene glycol (PEG-5000), an inert biologically compatible polymer.

[18] In Yang et al. (2007a) at 28 days pathological examination revealed sinusoidal congestion and increased presence of giant cells in the vascular regions of the spleen. Liver and kidneys showed no remarkable abnormalities either grossly or microscopically.

6.3. Cardiovascular effects

A number of epidemiological studies have demonstrated an association between exposure to fine particulates in ambient air and adverse cardiovascular effects (US EPA 2003, Pope et al. 2006, Buzea et al. 2007). Hence there are questions regarding similar effects occurring with ENPs.

NPs can enter the alveolar interstitium and can migrate into pulmonary capillaries and thence be translocated to other sites (see **Section 6.2**). The heart is the first organ to receive blood from the lungs and therefore, second to the lung, potentially the highest concentration of NPs. There is the theoretical possibility of direct vascular effects, or oxidative stress in the lung causing the release of mediators that could impair endothelial and blood cell homeostasis and affect blood clotting (Simeonova et al. 2007a). Part of this concern lies in the recognised association between particulate air pollution and adverse cardiovascular outcomes in people with compromised cardiovascular function (Pope and Dockery 2006).

There is evidence in an atherosclerotic mouse model that SWCNTs can cause oxidative stress in cardiac and aortic tissue and enhance plaque formation (Shvedova et al. 2007). *In vitro*, metal impurities in SWCNTs can induce lipoprotein oxidation by vascular cells and therefore possibly contribute to arterial plaque formation (Cai and Harrison 2000). However metal impurities are likely not to be critical as purified SWCNTs can induce cardiovascular-oxidative stress in ApoE-/- transgenic mice (a widely used model of human atherosclerosis) after high doses (10 – 40 µg/mouse) by pharyngeal aspiration (Simeonova et al. 2007a).

Fine particulates and NPs may produce cardiovascular effects by several theoretical mechanisms (Hoet et al. 2007). While inflammation is considered to be a key event, exactly where in the cardiopulmonary system inhaled particles elicit the effect is unclear. Possibilities are a direct effect on cardiac or post pulmonary vascular tissue after NPs have translocated into the systemic circulation, or the effect may be mediated by lung derived mediators released in response to particulates in the lung. Totlandsdal et al. (2008) have produced *in vitro* data that supports the latter hypothesis. Cardiac cell release of the inflammatory cytokine IL-6 was strongly induced by exposure to cell culture media from rat epithelial lung cells exposed to ultrafine carbon black (12 -17 nm), much more so than with direct particle exposure. It was demonstrated IL-1 from the lung cells was necessary but not sufficient for the cardiac cell response.

Hoet et al. (2007) have reviewed the mechanisms and techniques for investigating haemostatic and thrombotic effects of particulate exposure. They note that the differences in thrombogenesis between animal and human must be taken into account. They stress that collection of high quality blood samples is essential as poor sampling will lead to incorrect conclusions, and advise that such studies be done in close collaboration with an expert in the field of coagulation and vascular biology.

In summary:

- *There is concern regarding the potential for adverse cardiovascular effects arising from inhalation exposure to ENPs.*
- *From ambient particulate pollution and the limited number of studies published in the last few years there is sufficient available data to legitimise the concern but there is insufficient information to be able to judge the relevance for worker health.*

6.4. Eye effects

There is limited information regarding *in vivo* toxicity of nanoparticles to the eye.

Miyawaki et al. (2008) conducted traditional eye irritation tests on single walled carbon nanohorns (SWCNHs), albeit with instillations 5 times less than that prescribed by the regulatory approved protocol, and found no irritation regardless whether the eyes were washed or not (see also **Section 7.4**).

The majority of eye studies with ENPs have been concerned with efficient drug or gene therapy delivery to the eye and have involved injection into various intraocular regions (Prow and Lutty 2007). A variety of NPs have been evaluated for this purpose in recent years:

- Nanosized complexes of phosphorothionate oligonucleotides in poly(lactide-co-glycolide) microspheres (Gomes dos Santos et al. 2006)
- DNA tethered to gold NP, semiconductor nanocrystals or magnetic NPs (Prow et al. 2006a)
- Compacted DNA NPs (Farjo et al. 2006)
- Chitosan, PCEP, or magnetic NP (Lutty et al. 2006).

Most of the 'ocular therapy delivery' *in vitro* studies and some *in vivo* studies have shown low or no toxicity of nanoparticles to the eye. For example, Prow et al. (2006a) showed that there was no detectable increase in oxidative stress generated by iron nanoparticles *in vitro*. Studies with condensed DNA nanoparticles (Farjo et al. 2006) and poly(lactide-co-glyolide) (PLGA) nanoparticles (Bejjani et al. 2005; Aukunuru et al. 2003) have also shown no toxicity *in vitro*. An *in vivo* study where poly-lactic acid nanospheres were applied topically to rabbit corneas also showed these nanoparticles to be non-toxic (Giannavola et al. 2003). Similarly, histological analysis following intravitreal injection of polylactide nanoparticles in rat eyes revealed no toxic effects other than an inflammatory response between 18 and 24 hours after injection that eventually subsided within 48 hours (Bourges et al. 2003). In another study, no toxicity or inflammatory reaction was observed following subconjunctival injection of microspheres in rabbits (Gomes dos Santos et al. 2006). In a modified Draize test Huczko and Lange (2001) observed no eye abnormalities 72 hours after instillation of carbon nanotube-containing soot into rabbit eyes. Even though *in vitro* studies of

chitosan nanoparticles showed them to be non-toxic (De Campos et al. 2004), Prow et al. (2007a) observed inflammation after intravitreal injection *in vivo*. In a related study, Lutty et al. (2006) found chitosan nanoparticles caused large amounts of inflammation and hazy vitreous in 38% of rabbit eyes injected intravitreally. Although magnetic NPs did not cause inflammation or retinal dysfunction in intravitreally injected eyes when rabbits were sub-retinally injected, varying toxicities and levels of retinal dysfunction were observed.

Occupational exposure of the eye to nanomaterials is most likely to occur from airborne exposures, and perhaps also by hand eye transfer. Studies where material has been injected into the eye do not provide relevant information for occupational risk assessment other than an idea of what might happen if substantial amounts were to penetrate the eye.

Nonetheless the available data suggests most ENPs do not produce adverse eye effects.

6.5. Skin effects

CNT and fullerenes:

Monteiro-Riviere et al. (2007) has reviewed the literature regarding the toxicity and nanomaterial interactions with skin cells (keratinocytes) or with intact skin. They note there are a few reports of dermal irritation in humans such as carbon fibre dermatitis and hyperkeratosis that according to the authors suggest carbon NPs may gain entry into the viable epidermis after topical exposure. Should access to the epidermis occur then, at least theoretically, adverse effects may ensue since CNT and fullerenes can apparently penetrate and produce oxidative stress in cultured keratinocytes and other cell types. Not surprisingly, subcutaneously implanted MWCNT induces a foreign body response and granuloma at the implantation site.

At the time of the Monteiro-Riviere et al. (2007) review, information regarding the biodistribution and metabolism of fullerenes in skin was limited, probably due to the fact that C_{60} is insoluble in aqueous solutions plus the fact there was a paucity of sensitive analytical techniques available for measuring the material in biological matrices. Topical administration of fullerenes to mouse skin resulted in no effect on either DNA synthesis or ornithine decarboxylase activity. Fullerenes did not act as promoters in the two stage mouse skin carcinogenetic test. Water-soluble functional groups on the surface of fullerenes can dramatically decrease the cytotoxicity of pristine C_{60} in cultured keratinocytes. The least derivatised and most aggregated form of C_{60} was more toxic than the highly soluble derivatives. A fullerene C_{60} peptide with a fluorescent marker topically applied to porcine skin in flow-through diffusion cells resulted in penetration of the fluorescent label through all epidermal layers, this was significantly enhanced by surfactants.

There is a direct correlation between particle penetration and skin flexing (Rouse et al. 2007, Monteiro-Riviere et al. 2007). Hagens et al. (2007) also describes a few studies where penetration of microsized particles (500 – 1000 nm) and C_{60} fullerenes through the epidermis occurred after flexing motion.

Quantum dots:

Ryman-Rasmussen et al. (2006) topically applied commercially available QD 565 and 655 with three different surface coatings (PEG, PEG amines, or carboxylic acids) to porcine skin in flow-through diffusion cells and found penetration of the intact stratum corneum to the viable layers of the epidermis (and, in some cases, the dermis) within 8 to 24 hours.

Metal oxides:

Based on the studies available at the time, Monteiro-Riviere et al. (2007) concluded that nano-sized TiO_2 does not penetrate the viable layers of skin in healthy adults.

The Australian Therapeutic Goods Administration has reviewed the scientific literature on the safety of nano-TiO_2 or nano-zinc oxide (ZnO) in sunscreens (TGA 2006). They noted nano-TiO_2 or nano-ZnO can produce free radicals and cause adverse effects in *in vitro* cell preparations. Since the TGA did not have access to some of the pertinent studies they quoted the European evaluation which stated "micronised material (ZnO) has been found to be clastogenic, possibly aneugenic and inducing DNA damage in cultured mammalian cells in vitro under the influence of UV light". No evidence of photo-toxicity on intact skin of human volunteers was found. An *in vitro* assay with human skin stripped of the stratum corneum indicated transfer of 0.34% of applied ZnO into the receptor fluid. Furthermore 7 of 8 dermal penetration studies showed an inability of nano-TiO_2 or nano-ZnO to reach viable cells. The TGA (2006) concluded the weight of evidence indicated nano-TiO_2 or nano-ZnO remain on the surface of the skin and in the outer layer of the skin.

Nohynek et al. (2007) concluded most available theoretical and experimental evidence suggests that insoluble NPs do not penetrate into or through normal as well as compromised human skin. They indicate oral and topical toxicity data suggest TiO_2 and ZnO NPs have low systemic toxicity and are well tolerated on the skin. *In vitro* cytotoxicity, genotoxicity, and photogenotoxicity studies on nano-TiO_2 or other insoluble NPs reporting uptake by cells, oxidative cell damage, or genotoxicity should be interpreted with caution, since such toxicities may be secondary to phagocytosis of mammalian cells exposed to high concentrations of insoluble particles. The overall assessment of Nohynek et al. (2007) was there is "little evidence supporting the principle that smaller particles have greater effects on the skin or other tissues or produce novel toxicities relative to micro-sized materials. Overall, the current weight of evidence suggests that nano-materials such as nano-sized vesicles or TiO_2 and ZnO nanoparticles currently used in cosmetic preparations or sunscreens pose no risk to human skin or human health, although other NPs may have properties that warrant safety evaluation on a case-by-case basis before human use". See also **Section 8.3**.

To date the available data indicates:

- *That a range of ENPs do not have direct adverse effects on the skin.*
- *Some ENPs may penetrate into the skin, especially in areas where there is mechanical flexing. However no studies were located suggesting the skin was an important route of systemic exposure to ENPs.*

6.6 Immunological effects

Dobrovolskaia and McNeil (2007) have reviewed the immunological effects of ENPs intended for parenteral administration in medical applications. The immunostimulatory properties of ENPs include their antigenicity, adjuvant properties, inflammatory responses and the mechanisms through which they are recognized by the immune system. While ENPs destined for medical applications can be altered and tailored to provide the desired immunological properties, ENPs made for industrial use may not have been evaluated for such effects.

SWCNTs, MWCNTs and C_{60} fullerenes were examined by Hamilton et al. (2007) for their effects on macrophage function and airways responsiveness of mice[19]. *In vitro* alterations of normal antigen-presenting function of a macrophage/T cell mixture response to ovalbumin protein antigen were found to occur with SWCNTs and MWCNTs (both ≥50 µg/ml) but not with the fullerenes. Similar results were obtained for alveolar and bone marrow macrophages. The authors considered the changes were due to the carbon nanoparticles (CNPs) accumulated in the cell plasma membrane which disrupted the membrane lipid rafts. Airways response to nebulised methacholine 24 hours after intranasal administration of 150µg CNP/mouse was enhanced by the three CNPs.

The use of only one high dose of CNPs in this study significantly hampers the interpretation of the data with respect to hazard and risk assessment. The authors noted the dose of CNPs was high, and may not reflect human occupational or environmental exposure. In addition the bolus doses used in the experiment would not accurately model the effects of cumulative exposures over time. Since methacholine causes bronchoconstriction by stimulation of the parasympathetic nervous system the enhanced responsiveness in this model would appear to have little to do with immunological mechanisms.

Nygaard et al. (2009) examined whether SWCNTs, MWCNTs and ultrafine carbon black (CB) have the capacity to promote allergic responses in mice. The NPs were mixed with allergen ovalbumin and animals either exposed by a series of subcutaneous foot pad injections or intranasally on three consecutive days. It was demonstrated that both SWCNTs and MWCNTs strongly increased the immunological response to ovalbumin, particularly IgE, however the response profile of SWCNTs was different from that of MWCNTs and ultrafine CB. The authors concluded CNTs promote allergic responses in mice.

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[19] SWCNTs average diameter 1.2 – 1.4nm and length 2 – 5 µm; MWCNTs average diam 1.2 - 20 nm and length 100 – 200 nm and 5 – 20 layers. C_{60} were 99.5% pure and 10 – 20 nm in diameter. Primary metal contaminants were nickel and yttrium at 0.3% each. The CNPs were prepared in heat-inactivated foetal BSA, suspensions dispersed with Dounce homogeniser and sonicated. Suspensions were frozen at -20^{0}C until used and resonicated when thawed. Intranasal administration of CNPs (150 µg/mouse) was done in ketamine immobilised animals supported in an upright position while 30 µl of suspension was slowly pipetted into the nasal passage. 24 hr later airways responsiveness to nebulised methacholine was measured with barometric whole body plethymography. Methacholine is a non-selective muscarinic receptor agonist in the parasympathetic nervous system that constricts the bronchi.

The adjuvant activity of MWCNTs has also been investigated after repeat intratracheal exposures in mice[20] (Inoue et al. 2009). Serum ovalbumin specific allergen IgG_1 and IgE were significantly enhanced by MWCNTs administration. Also lung responses to the ovalbumin allergen were amplified by the MWCNTs. The authors concluded the study showed MWCNTs can exacerbate allergic airway inflammation. The enhancing effects were concomitant with the increased lung expression of Th cytokines and chemokines related to inflammatory leukocyte recruitment.

Dutta et al. (2008) derivatised the amine terminal of poly (propyleneimine) (PPI) dendrimers with a number of chemicals to target specific cells for drug delivery. After intravenous administration to rats, underivatised PPI caused changes in blood cell profile indicative of haemolysis plus increased serum enzyme changes characteristic of liver toxicity which was confirmed histologically. None of the derivatised PPI caused toxicity. Also investigated was immunogenicity in Balb/C mice after intramuscular injection of 2.5, 25 or 250 mg/kg. Neither PPI nor any of its engineered derivatives showed any signs of immunogenicity as judged by sensitive analysis of IgG antibody titre. One of the derivatised PPI contained tuftsin, a tetra-peptide which is a natural macrophage activator that elicits an immunogenic response.

Xiang et al. (2006) have reviewed the use of NPs for the delivery of antigens for vaccination and discuss the cellular uptake mechanisms for pathogens and particles of different sizes. Since particle size and composition can influence the immune response, inducing humoral and/or cellular immunity, activating CD8 T cells and/or CD4 T cells of T helper 1 (Th1) and/or T helper 2 (Th2) type, particle characteristics can have a major impact on vaccine efficiency. For example when carboxyl-modified polystyrene beads were conjugated with ovalbumin antigen and injected into the hind foot pad of mice it was found the size of the bead influenced whether the antigenic response to the antigen was typical of induction of type 1 or type 2 T-cells (Mottram et al. 2007). This data is useful in the development of effective vaccines against common human pathogens, and suggests perhaps the presence of NPs might modify immune responses to environmental agents.

Li et al. (2008a), in reviewing the role of oxidative stress in lung diseases caused by particulates, consider there is increasing evidence that ambient particulate pollutants act as an adjuvant for allergic sensitisation to common allergens. Several studies seem to indicate oxidative stress is capable of shifting the immune response from Th1 to Th2 dominance. However they acknowledge the topic is controversial.

Inoue et al. (2007b) showed inhalation by mice of diesel engine-derived nanoparticles [21] (mean diameter 20 – 30 nm) exacerbated, in a dose dependent manner, the pulmonary inflammatory response to bacterial endotoxin lipopolysaccharide (LPS). Previous experiments with diesel exhaust particulates of different origin and size

[20] Mice were divided into 4 groups; vehicle control (phosphate buffered saline with 0.05% Tween 80), ovalbumin (1 µg/animal every two weeks for 6 weeks), MWCNTs group (25 or 50 µg/animal every week for 6 weeks), and MWCNTs + ovalbumin. The authors relied on a previous characterization in 2005 of the MWCNTs for this study.

[21] The particles were 20 – 30 nm average diameter and exposure was 15, 36, or 169 µg/m^3 for 5 hours. The bacterial endotoxin lipopolysaccharide (LPS) was given intratracheally 12 hours before inhalation exposure of mice to the diesel exhaust nanoparticles.

(mean diameter >100 nm) did not show exacerbation of lung inflammatory responses to LPS but instead attenuation (Inoue et al. 2006).

These researchers have further demonstrated intratracheally administered CB nanoparticles (14 and 56 nm diameter) can enhance lung hyperresponsiveness to ovalbumin antigen (Inoue et al. 2007a), and exacerbate lung inflammation related to bacterial endotoxin (LPS) and subsequent blood coagulatory disturbance (Inoue et al. 2006). The effect is greater for the smaller nanoparticles and appears to be mediated through the increased local expression of IL-1β and chemokines (Inoue et al. 2006). The 14 nm nanoparticles also enhance allergic airway inflammation, including lung expression of cytokines and chemokines related to ovalbumin antigen and immunoglobulin production (antigen specific IgG and IgE) compared with 56 nm nanoparticles (Inoue et al. 2005a).

In summary;

- *There is limited data suggesting carbon nanoparticles may contribute to pulmonary morbidity by acting as an adjuvant for air borne allergens. These effects have long been suspected for fine combustion particles in ambient air pollution, and it is difficult to judge the importance of such interactions for the workplace at this time. A weight of evidence evaluation of the entire literature was beyond the scope of this update review. Nevertheless this is an important area for consideration in the workplace and needs to be examined further.*

- *ENPs that have been functionalised or coated with an antigenic protein may elicit an immunogenic response, the nature of which may be modulated by the size of the NP.*

- *The preliminary data to date suggests non-functionalised ENPs are not antigenic.*

6.7 Summary and conclusions

Smaller particles have a higher toxicity than larger particles of the same composition and crystalline structure, and they generate a consistently higher inflammatory reaction in the lungs. Inflammation is the normal response of the body to injury. NPs have been shown to generate more free radicals and reactive oxygen species than larger particles, likely due to their higher surface area. Smaller NPs are correlated with adverse reactions such as impaired macrophage clearance, inflammation, accumulation of particles, and epithelial cell proliferation, followed by fibrosis, emphysema, and the appearance of tumours (Buzea et al. 2007).

1. ENP persistence is recognised as a key physical feature that may lead to critical particulate lung burdens being attained. However while the physicochemical attributes contributing to persistence are known, criteria have not yet been articulated that set the bounds for defining ‘biological persistence’.

2. There have been a plethora of *in vitro* studies with a variety of ENPs showing them capable of eliciting an oxidative stress response. The utility of this

information for occupational hazard identification and risk assessment remains obscure.

3. Several recent publications demonstrate ENPs can potentially interfere with endpoint evaluations of *in vitro* tests and give misleading results. This may arise in several ways, for example the ENP binds a reagent used in the evaluation, or precipitated agglomerates distort spectrophotometric quantisation.

4. It remains that current standard genotoxicity protocols require adaptation and validation before they can be reliably used for hazard identification of intrinsic genotoxicity.

5. Where meaningfully investigated, recent studies have shown low concordance between *in vitro* and *in vivo* toxicity. The predictive power of *in vitro* tests for an *in vivo* outcome is therefore questionable.

6. Overall, *in vitro* cellular systems need to be further developed, standardized, and validated (relative to *in vivo* effects) in order to provide useful screening data on the relative toxicity of inhaled ENPs of unknown toxicity.

7. ENPs, once in the systemic circulation are rapidly taken up from the blood by the reticuloendothelial system and sequestered primarily in the liver, spleen and lungs where they may remain for long time.

8. Since chronic repeat dose toxicological studies have not been conducted for most ENPs, the long term consequences of the avid accumulation of ENPs by the reticuloendothelial system are unknown.

9. It appears from the available studies that ENPs exert minimal effects on the eye and skin. Skin sensitisation is not expected unless the ENP can penetrate into the epidermal layer and also has an antigen functional group on its surface.

10. The investigational effort on the toxicology and potential health effects of ENPs has exponentially exploded in the last few years. There is much complex, confusing and conflicting data, only a relatively small proportion of which is likely to meaningfully inform on workplace hazards and assessment of risk. For these reasons reliance should not be placed on the results of a single or singular series of experiments. Weight of evidence assessments will be needed to appropriately glean useful information. It is suggested that set(s) of conditions/criterion for collective evaluation of nanotoxicology publications be drawn up to assist weight of evidence assessment of the literature.

11. Given the complexity of the emerging data on the toxicology of ENPs and factors that affect it, guidelines should be considered to enable hazard classification, provision of MSDS information and workplace labelling of ENPs in Australia.

7. Carbon nanoparticles

7.1 General information

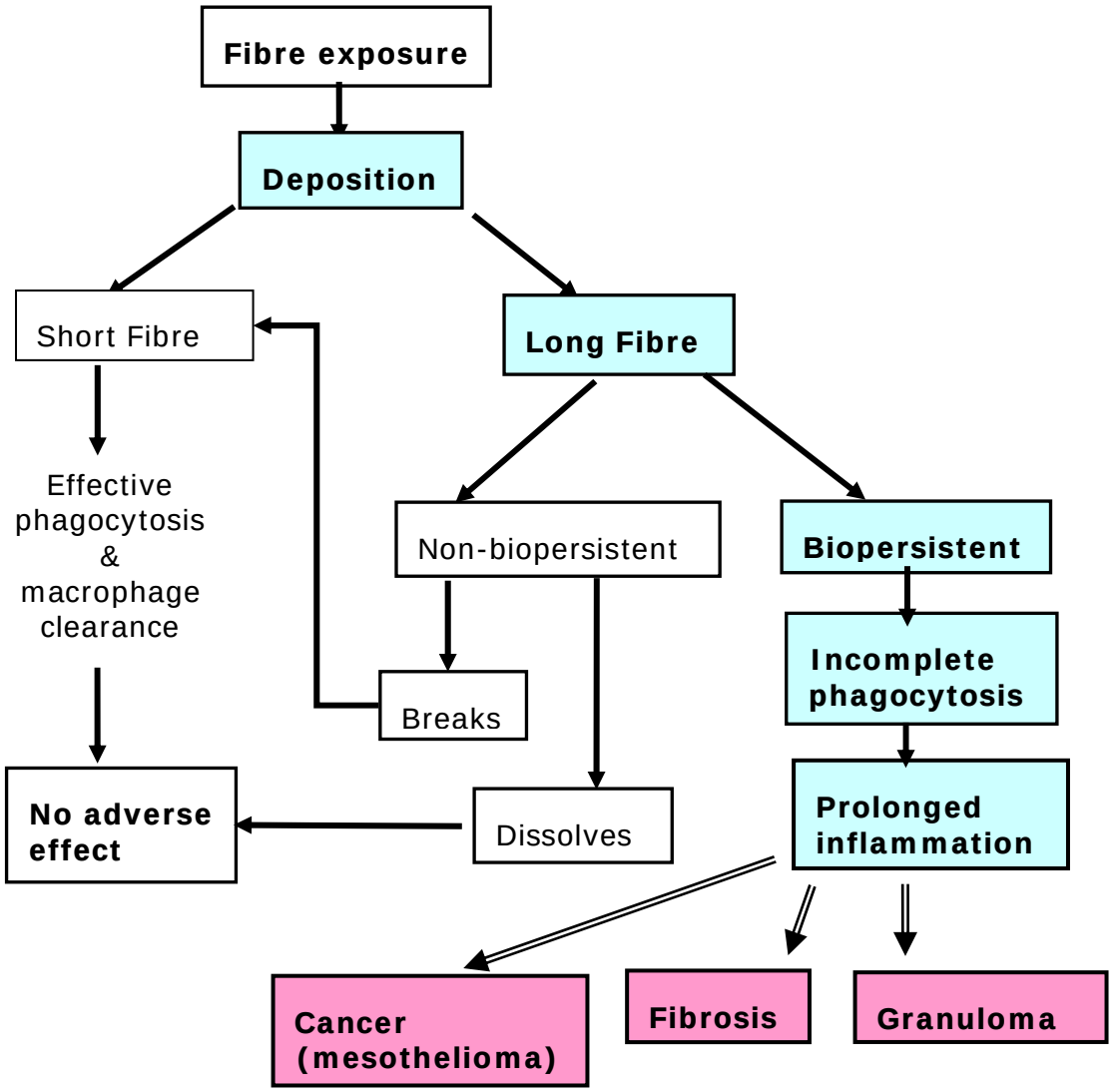
7.1.1 Fibre toxicity paradigm and persistence

CNTs can agglomerate into relatively stable larger forms of similar size to mineral fibres that historically have been associated with occupational disease. Not surprisingly therefore toxicological investigations of the hazards and potential health effects of CNTs have been informed by prior investigations on mineral and synthetic vitreous fibres. The toxicological mode of action for the pulmonary effects of fibres is similar but different to that for fine particulates (e.g. Hesterberg and Hart 2001, Hei et al. 2006, Nguea et al. 2008). **Figure 7.1** summarises the salient features. As for poorly soluble particulates the essential requirements for causing lung effects are biopersistence leading to prolonged inflammation and oxidative stress. Whereas for particles the protracted inflammation is considered to be primarily the result of excessive exposures achieving a lung burden that overwhelms particle clearance mechanisms and pulmonary defence systems, for biopersistent fibres it is size and dimension characteristics that primarily determine whether the fibre will, or will not, be efficiently cleared from the lung. The inflammatory response is an essential component of host defence and is normally transient. However, excessive or persistent inflammation can result in irreversible damage to the tissues. Inhaled fibres that are too long to be phagocytosed by macrophages and translocated out of the lung and too durable to be broken-down, chronically irritate and inflame the lung tissues. A prolonged inflammatory and fibrotic reaction can then potentially occur.

A pivotal aspect of lung fibre pathology is that fibres need to be long and thin so that when encountered by macrophages, phagocytosis can begin but not be completed. The length of the fibre determines if the macrophage is able to completely engulf the fibre and thence transport it from the alveolar. In situations of frustrated phagocytosis, electron or light microscopy (depending on the fibre) typically reveals fibres protruding from macrophages. The ‘long thin’ requirement for fibre pathogenicity has been determined from many toxicological and epidemiological investigations of different types of mineral and synthetic vitreous fibres, and has lead authorities to be able to identify potential pathogenicity based on the relative dimensions of the fibre (WHO 1985, **Section 7.1.2**).

The pathological response to such fibres includes granuloma formation as well as fibrosis and cancer. Granulomas are not cancers and do not progress to cancer. Many people develop granulomas during their lifetime that are without clinical effects and no treatment is necessary. While biopersistent, long thin fibrogenic fibres can cause lung tumours, the signature cancer for these materials is mesothelioma; a tumour of the membranes that cover the outer surface of the lung. These membranes are in the pleural cavity, the space between the lung and the rib cage.

Figure 7.1: Summary of the fibre toxicity paradigm



Notes:

Adapted from Donaldson et al. (2006b), others have also produced similar toxicity models that include detail on biochemical and cell signalling events (e.g. Hei et al. 2006, Jain et al. 2007). The overarching mode of action themes of biopersistence and prolonged inflammation/oxidative stress of the fibre paradigm are the same as for particulate toxicity mode of action (**Figures 5.1** and **5.2**). However where the particulate paradigm invokes lung overload and macrophage clearance mechanisms being unable to cope, the fibre paradigm has the central notion of fibre size being critical to incomplete phagocytosis leading to the prolonged inflammatory response. An additional difference lies in the granuloma response; if the fibre is persistent and too large to be engulfed by a macrophage these and other cells congregate to coat and isolate the fibre. Fibrotic responses occur at the same time resulting in the formation of a granuloma. Granulomas are not cancers and do not progress to cancer, they can form in any tissue where an 'indigestible' foreign body may lodge or injury has occurred, and more often than not do not cause clinical signs or symptoms.

Ngeau et al. (2008) have reviewed the features of synthetic vitreous fibres (SVFs) and their lung toxicity. This information is pertinent to how certain CNTs may be evaluated.

- Biopersistence and biodurability are very important. It is pointed out that the extracellular environment of the lung is approximately pH 7.4 but within the alveolar macrophage the pH is 4.5 – 5. A number of citations are provided within the review for measurement of biopersistence and acellular durability.

- There is a difference in the clearance of short and long SVFs, short fibres being cleared quicker than long ones.

- In rat models there is correlation between biopersistence of long SVFs (>20 µm) and occurrence of lung fibrosis and thoracic tumours.

- Inflammation and fibrosis occur before lung cancer.

- Coating of inhaled SVFs by rat pulmonary surfactant enhances phagocytosis by rat macrophages.

- Human alveolar macrophages are approximately twice the size of rat macrophages and can successfully engulf SVFs up to 20 µm but there is incomplete phagocytosis by rat macrophages at lengths of 17 µm.

7.1.2 Classification of fibres

The World Health Organization (WHO 1985) defines pathogenic fibres as fibres which have:

- A length greater than 5 µm

- A width less than 3 µm, and

- An aspect ratio of > 3:1(the fibre is at least three times longer than it is wide).

Fibres have also been examined based upon other characteristics, including biopersistence, retention and clearance rates, and biodurability. Dose, dimension, and durability have been termed the three Ds, all of which are important in determining the carcinogenicity of fibres (NTP 2009). Only fibres of an aspect ratio >3:1, of a length greater than 5 µm, and which are biopersistent are able to cause the pathological responses previously described.

According to the background document on the carcinogenicity of glass wool fibres from the US National Toxicology Program (NTP 2009), biodurability describes the rate of removal through dissolution or disintegration; biopersistence includes biodurability plus physiological clearance and refers to the capacity of a fibre to persist and to conserve its chemical and physical features over time in the lung. Biodurability, because it relates to the chemical and physical properties of the substance is expected to be similar in rats and humans, but biopersistence may be substantially different due to differences in the physiological clearance mechanisms. In general, biodurability of various fibres in the lung has been ranked as follows: glass fibres < refractory ceramic

fibres < chrysotile asbestos < amphibole asbestos. Highly durable fibres, such as asbestos, are resistant to dissolution and transverse breakage.

Hesterberg and Hart (2001) discuss *in vitro* methods for measuring the durability of fibres (dissolution rates) and also a rat biopersistence inhalation model. According to these authors, in Europe a synthetic vitreous fibre is considered to be biopersistent if in an inhalation test the weighted lung clearance half life (WT½) for fibres longer than 20 µm is >10 days or in an intratracheal test WT½ is >40 days (EC 2001). Biopersistent or biodurability data for CNTs was not encountered when reading papers for this review. Guidelines for biopersistence of fibres have been produced by the European Union; for inhalation exposure the EU guideline is ECB/TM/26 rev.7 and after intratracheal instillation the EU guideline is ECB/TM/27 rev.7.

In the European Union the carcinogenic classification of synthetic vitreous fibres may be upgraded from Category 3 (possible carcinogen) to Category 0 (Not classified as a carcinogen) if it passes one of four tests; the inhalation biopersistent test, the intratracheal biopersistence test, an appropriate intraperitoneal injection test[22] that has shown no evidence of excess carcinogenicity, or absence of relevant pathogenicity or neoplastic change in a suitable long term inhalation test (Hesterberg and Hart 2001, EC 2001).

7.1.3 Experimental notes

The experimental techniques used to investigate potential hazards of CNT are essentially the same as employed for NPs. That is, a variety of *in vitro* cell systems are incubated with a variety of CNTs and various inflammatory/oxidative stress biomarkers are measured. The same experimental and interpretation issues surrounding data usefulness for hazard identification and risk assessment of *in vitro* NP data apply to CNTs. For CNTs however some of the issues are exacerbated, for example Herzog et al. (2007) noted in their *in vitro* experiments that the SWCNT[23] settled to the bottom of the culture vessels so the majority of particles did not get into contact with the cells.

As with NPs, 'artificial' biopersistence can be introduced into *in vivo* test systems by excessive loads being delivered to the lung, there are issues associated with CNT physical changes occurring *in situ* after dosing, and whether the material being tested is the same as that in workplace exposures, or indeed the same as that has been physically characterised prior to testing.

One of the experimental difficulties associated with attempting inhalation studies with CNTs is being able to efficiently and consistently aerosolize the material in sufficient quantity. Baron et al. (2008) described a complicated apparatus for aerosolisation of SWCNT that gave an output of up to 25 mg/m^3 of particles with approximately 1 – 10 µm aerodynamic diameter. The authors noted their SWCNTs had a strong propensity to form very open tangled structures (see Section 8.5.2 for toxicological

[22] Such as EU Guideline ECB/TM/18(97) rev1 for Carcinogenicity of Synthethic Mineral Fibres after Intra-peritoneal Injection in Rats and ECB/TM/17(97) rev 2 for Chronic Inhalation Toxicity of Synthetic Mineral Fibres in Rats.

[23] The SWCNTs were HiPCO derived and purchased from a commercial supplier. Dispersion media was not defined in the paper and historic characterisation of the SWCNTs preparation was relied upon by the authors, this indicated the SWCNTs remained bundled after sonication.

implications) and the tendency to spontaneously self-assemble into nanoropes was exacerbated if pressure was applied to the material. They considered the mass concentration range generated was sufficient for animal exposure studies. At the time of writing this review no studies were located that had exposed animals via inhalation to CNTs using this apparatus. Li et al. (2007b) have exposed mice to aerosolised MWCNTs however as discussed in **Section 4** consistent concentrations were not obtained throughout exposure periods.

Mesothelial tissue is not confined to the pleural cavity; it is also the membrane covering abdominal organs and the peritoneal side of the diaphragm. Animal models for testing fibres for fibrogenic potential and mesothelial tissue reactions have therefore been developed that involve injecting the fibre into the abdominal cavity of rodents. Because this is a non-physiological exposure route, which may influence a person's interpretation and application of the data, some features of the technique are described **Section 4.2** and **Appendix 1**.

7.2 Reviews

Donaldson et al. (2006b) have reviewed the toxicology literature of CNTs up to the end of 2005 in relation to workplace safety. They concluded that CNTs seem to have a special ability to stimulate mesenchymal cell growth and to cause granuloma formation and fibrosis. They consider CNTs induce some of their effects through oxidative stress and inflammation. These fibrogenic aspects have been further corroborated in studies described below that have investigated the intrinsic toxicity of CNTs rather than the secondary phenomenon of particle overload.

Donaldson et al. (2006b) point out there was no definitive inhalation study available that would avoid potential artifact effects due to large mats and aggregates forming during instillation exposure procedures.

Lam et al. (2006) reviewed much the same literature as Donaldson et al. (2006b) but have been more forthright in their conclusions[24]. They state:

"*The results of the rodent studies collectively showed that regardless of the process by which CNTs were synthesized and the types and amounts of metals they contained, CNTs were capable of producing inflammation, epithelioid granulomas (microscopic nodules), fibrosis, and biochemical/toxicological changes in the lungs. Comparative toxicity studies in which mice were given equal weights of test materials showed that SWCNTs were more toxic than quartz, which is considered a serious occupational health hazard if it is chronically inhaled; ultrafine carbon black was shown to produce minimal lung responses*".

Furthermore Lam et al. (2006) concluded SWCNTs (administered to the lungs of mice) can produce respiratory function impairments, retard bacterial clearance after bacterial inoculation, damage the mitochondrial DNA in aorta, increase the percent of aortic plaque, and induce atherosclerotic lesions in arteries of the heart.

[24] Lam et al. (2006) was identified in the previous ASCC as an important paper but not reviewed in detail. It was considered that, in the context of this current review, the opinions of Lam et al. (2006) should be presented in greater detail.

Lam et al. (2006) point out that MWCNTs and other carbonaceous nanoparticles in fine (<2.5 μm) particulate matter (PM) have recently been found in combustion streams of methane, propane, and natural-gas flames of typical stoves. Also fine PM samples from indoor and outdoor air samples were reported to contain significant fractions of MWCNTs. Given the experimental data for CNTs, they deduced combustion generated MWCNTs in fine PM in ambient/indoor air may play a significant role in air pollution related cardiopulmonary diseases.

The hazard component of the occupational risk assessment undertaken by Lam et al. (2006) clearly indicates that if CNTs reach the lung in sufficient quantity they have the capacity to produce a toxic response (inflammation, granulomas, fibrosis and from recent research discussed below, possibly mesothelioma). The effects are dose and time dependent. Lam et al. (2006) cite a 2003 NIOSH report[25] in support of the notion that SWCNTs are light-weight and can easily become airborne thereby presenting an occupational inhalation risk. It is pointed out however that because CNTs tend to stick together, forming clumps, it is difficult to separate substantial numbers of particles of respirable size from bulk amounts of CNTs. Nevertheless according to NIOSH scientists, "*generation rates of fine particles from the nanotube material were found to be approximately two orders of magnitude below that for fumed alumina for similar volumes of material.*" It was pointed out that if, as anticipated, very big volumes of CNTs are produced by many manufacturers the numbers of workers potentially exposed during manufacture and application could also be very large. The risk characterization of Lam et al. (2006) concluded inhalation studies are needed to substantiate the pathological findings that were observed in animals exposed by unrealistic exposure routes. However, based on the limited *in vivo* toxicity data available and extrapolation of doses causing lung toxicity in animals, it was recommended by Lam et al. (2006) the occupational exposure limit for respirable CNT dust be set at a value no greater than 0.1 mg/m^3. This is the OSHA permissible exposure limit (PEL) for quartz.

In relation to the ability of CNTs to become airborne, an abstract of a poster presentation at a recent scientific meeting confirmed fullerenes, and underivatised and hydroxylated MWCNTs could become airborne when they are being weighed in the laboratory and transferred to beakers filled with water (Johnson et al. 2009). The preparation of suspensions of dispersed CNTs often necessitates sonication. It was also shown, after accounting for background particle number, there were increases in airborne particle concentrations during the sonication process. It is presumed the particle increase was due to aerosolized CNTs generated during sonication however the abstract did not provide information on this aspect. Although this information has yet to be published in a peer reviewed scientific journal, it indicates care is needed at all stages of handling ENPs during experimental investigations in order to minimize exposures of laboratory personnel. At this time in Australia, it is laboratory personnel who are arguably the population sector most likely to be exposed to CNTs.

Recent data extending the information in the above reviews is discussed below.

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[25] Baron, P.A., Maynard, A.D., and Foley, M. (2003). Evaluation of Aerosol Release during the Handling of Unrefined Single Walled Carbon Nanotube Material. National Institute of Occupational Safety and Health, Cincinnati, OH, April, Report No. NIOSH DART-02-191.

7.3 SWCNTs

7.3.1 *In vitro* data

Shvedova et al. (2005b) had previously demonstrated that pharyngeal aspiration of SWCNTs to mice caused a robust inflammatory response, early onset fibrogenesis and granuloma formation[26]. The group has since conducted follow up genotoxicity studies (Kisin et al. 2007). Their SWCNTs were not genotoxic to two strains of Salmonella typhimurium but in Chinese hamster V79 lung fibroblasts there was a concentration-dependent increase in the frequency of DNA damage, as measured by the Comet assay, but no micronucleus induction. Complicating interpretation was a significant time and concentration dependent loss of cell viability. The authors suggested the SWCNT induced loss of cells might interfere with accurate evaluation of genotoxicity responses to SWCNTs in remaining viable cells.

Non-functionalized purified SWCNTs are not particularly well recognized and phagocytosed by macrophages (Shvedova et al. 2005b, Mercer et al. 2008). SWCNTs are however able to cross the cell membrane and accumulate in cytoplasm of other cell types, e.g. A549 lung epithelial cells (Worle-Knirsch et al., 2006) and Pulskamp et al. (2007) have shown aggregates of SWCNTs are taken up by rat NR8383 macrophages. The differences are probably related to deficiencies in functionality between the cell types[27] and it is unknown how this information relates to the *in vivo* situation.

Kagan et al. (2006) showed well dispersed iron rich (26%) and iron poor (0.23%) SWCNTs were not able to generate intracellular superoxide radicals or nitric oxide in RAW 264.7 macrophages. However, if the cells were stimulated with zymosin in the presence of SWCNTs, the non-purified SWCNTs much more effectively converted extracellular generated superoxide radicals into hydroxyl radicals. Concomitant with the notion that iron rich SWCNTs could enhance cellular oxidative stress induced by other agents there was a larger decrease in intracellular glutathione and increased lipid hydroperoxides. The authors concluded the presence of iron in SWCNT may be important in determining redox-dependent responses of macrophages.

Commercial SWCNTs and MWCNTs[28] were incubated for 24 hours at concentrations up to 100 µg/ml with rat NR8383 or human A549 macrophages by Pulskamp et al. (2007) who used carbon black (CB) and quartz as reference material. No acute toxicity

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[26] Doses were 0, 10, 20 & 40 µg/mouse given in 50 µl PBS. The fibrosis exhibited two distinct morphologies (i) granulomas mainly associated with hypertrophied epithelial cells surrounding aggregates of SWCNTs and (ii) diffuse interstitial fibrosis and alveolar wall thickening likely associated with dispersed SWCNTs.

[27] Shvedova et al. (2005b) used RAW 264.7 cultured cells that are not fully functional at 0.1 mg SWCNTs/ml. RAW 264.7 cells are a mouse leukaemic monocyte macrophage cell line that are mannose receptor (MR) negative. On the other hand rat NR8383 macrophage cells are fully expressive for MR. [MR functions as a first-line host defense receptor in the binding and internalization of a wide range of pathogens by macrophages and it participates in the resolution phase of inflammation by clearing potentially harmful hydrolases and peroxidases from the extracellular space. Expression of the MR is tightly linked to the functional state of the macrophage (Lane et al. 1998)].

[28] The CNTs consisted of large agglomerates forming bundles or ropes with single nanotubes clearly visible. The agglomerates were tightly bound together even after suspensions were sonicated.

on cell viability was observed, and no acute inflammatory mediators were detected with the CNTs. However a dose and time dependent increase in intracellular ROS and a decrease in mitochondrial membrane potential occurred with the commercial CNTs but not when they were purified; the impurities were mainly Co (2.8%) and Mo (4.2%), both of which were significantly higher than manufacturer declared values.

Consistent with the notion that highly purified NPs have low *in vitro* cytotoxicity Fiorito et al. (2006a) have demonstrated purified SWCNTs and C_{60} fullerenes up to a top concentration of 60 µg/ml do not stimulate the release of nitric oxide (NO) by murine macrophage in culture, their uptake by human blood monocyte derived macrophages was very low, and there was very low toxicity against the human macrophages.

In contrast Stoker et al. (2008), using a co-culture of normal human bronchial epithelial cells and normal human fibroblasts as a three dimensional model for the human airway, recently observed NO production was dramatically increased and cell viability[29] was decreased following exposure of different concentrations of SWCNTs (2, 4 and 8 µg/ml). The authors consider their test system[30] to be a viable alternative to *in vivo* tests for evaluating the toxicity of SWCNTs. It is noted however much additional work is required to validate the system.

The Fiorito et al. (2006a), Kagan et al. (2007) and Pulskamp et al. (2007) data support a developing general tenant that impurities, especially metals, can significantly influence cellular responses to SWCNTs. Nevertheless highly purified MWCNTs had direct membrane effects on a cultured mouse macrophage cell line (Hirano et al. 2008). These authors point out that catalytic iron is entrapped within the CNT structure and does not leach out under neutral pH conditions. Thus analytical measurements of total metal impurities may not reflect the bioavailable metal.

As the degree of side wall functionalisation of water dispersible SWCNTs increases the cytotoxicity to cultured human dermal fibroblasts decreases (Sayes et al. 2006a). In this study the SWCNTs in aqueous suspension precipitated and selectively deposited on the cell membrane. It was noted in **Section 4.2.3** and **Appendix 1** that knowing the extent of dispersion of SWCNTs is critical for interpretation of *in vitro* cytotoxicity data. Zhang et al. (2007a) found surfactant dispersed aggregates of functionalised SWCNTs in cell medium results in less cytotoxicity.

Adding to the complexity of data interpretation is the finding of Kaiser et al. (2008) that the effects of purified SWCNTs on cell behaviour[31] were dependent on cell type and

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[29] In the Stoker et al. (2008) study cell viability was measured with the MTT assay. Data provided by the commercial manufacturer was used for characterising the SWCNTs; residual metal content was 3% to 12% by weight, and individual nanotubes were 0.8 to 1.2 nm in diameter and 100 to 1000 nm in length. Dispersion in the application media (water containing Triton X-100) was evaluated using an atomic force microscope, the average length and diameter of nanotube rope were about 500 nm and less than 10 nm, respectively and it is claimed the suspension of well dispersed SWCNTs was stable for 2 months.

[30] Although data was not provided the Stocker et al. (2008) coculture model was stated to show expression of three important phenotypic markers such as mucin (mucous production), F-actin (tight junction), and tubulin (cilia). It had an air interface for epithelial cells and

[31] Cell types were human lung mesothelioma cells (MSTO-211H) and human lung epithelial cells (A549). Cell behaviour end points were cell proliferation, cell activity, cytoskeleton organisation, apoptosis and cell adhesion. Commercial (purified and non-purified) SWCNT bundles (size not given) dispersed with aid of Tween 80 were incubated with cells at 3.75, 7 and 15 µg/ml cell medium. Cell proliferation of mesothelioma cells was decreased to greater extent than with lung epithelial cells, this was speculated

test end point as well as the quality (i.e. purity) and the concentration of the SWCNTs. Yacobi et al. (2007) found SWCNTs altered cellular transport pathways[32] in broadly the same manner as other NPs but the specific pattern was dependent upon the NP composition, shape and/or surface charge.

7.3.2 *In vivo* data

The pre-2007 literature dealing with *in vivo* pulmonary inflammatory responses to SWCNTs and the interaction of SWCNTs with macrophages has been reviewed by Kagan et al. (2007). Although the toxicity has not been extensively studied they produce inflammation, oxidative stress, interstitial fibrosis and granulomas.

Observations suggesting macrophages stimulated by an allergenic substance are more responsive to CNTs (Kagen et al. 2006) have suggestive, but at this time largely theoretical implications for CNTs modifying toxicological responses of other agents.

Because both particulates and ozone are known to influence pulmonary inflammation and injury Han et al. (2008b) postulated there may be synergistic pulmonary effects between CNTs and ozone. Mice were exposed to 20 µg per animal MWCNTs by pharyngeal aspiration and 12 hours later subjected to 3 hours inhalation exposure to 0.5 ppm ozone. The ozone by itself produced a minimal cytotoxicity/inflammation response but the response to MWCNTs was pronounced. Contrary to expectations there was neither an additive nor synergistic response in mice that received both MWCNTs and ozone. In fact, some CNT-induced cytotoxic/inflammatory responses were reduced. There are other circumstances in which the acute pulmonary toxicity of ENPs may be ameliorated; for example attenuation of acute toxicity to Teflon NPs was observed by Johnston et al. (2000) by a series of short non-toxic exposures.

The data from Mercer et al. (2008) are relevant due to low doses of well dispersed SWCNTs delivered to the lungs of mice. Using pharyngeal aspiration, a single 10 µg/mouse dose of SWCNTs[33] resulted in a highly dispersed interstitial distribution of CNTs throughout the lung. This was shown by BAL analysis and histology to cause a response consistent with a mild, transient parenchymal inflammation that resolved within the first week of dosing. The initial inflammation was similar to that seen with large agglomerates of SWCNTs (diameter 15.2 ± 3.2 µm) but instead of granulomatous lesions a relatively even connective tissue response of alveolar thickening was observed 7 days and 1 month post dosing. Since the animals were only observed for one month it is not known whether the alveolar response would resolve or continue, or be exacerbated by additional low exposures to well dispersed SWCNTs. The larger aggregates of SWCNTs deposited in the proximal alveolar region, where the

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to be due to greater uptake of the SWCNTs by mesothelioma cells, however uptake of SWCNTs was not undertaken for either cell type.

[32] Primary cultures of rat alveolar epithelial cells. Measurements were transmonolayer resistance, fluxes of radiolabelled mannitol or inulin, and release of cellular lactate dehydrogenase. The NPs investigated were ultrafine ambient particulate suspensions, polystyrene NP (positively and negatively charged), quantum dots (positively and negatively charged) and SWCNT (nominal diam 0.8 – 1.2 nm and length 100 – 1,000 nm).

[33] See footnotes in **Section 4.3.1** for a description of the preparation and characterisation of the SWCNTs used by Mercer et al. (2008).

granulomas formed; interestingly this is the same injury area associated with ozone exposure.

More recently Kisin[34] et al. (2009) exposed mice to aerosolised SWCNTs (5 hr/d for 4 days) or to varying doses (5 – 20 µg/mouse) of the same SWCNTs by aspiration. Similar pathology of early inflammatory and oxidative stress response culminating in development of multifocal granulomatous pneumonia, collagen deposition and interstitial fibrosis was produced by both modes of exposure, but quantitatively inhalation had a greater effect than aspiration.

Since SWCNTs have potential medical applications[35] for cancer treatment Schipper et al. (2008) injected nude mice with high doses[36] of SWCNTs either loosely or covalently coated with polyethylene glycol (PEG). No unusual behaviour[37] was noted during the 3 month observation period and at termination no untoward histological effects were observed despite there being accumulation of SWCNTs in sinusoidal liver cells[38]. At termination all treated mice had small amounts of a distinctive, fine granular brown-black pigment throughout the liver tissue which was localised in liver macrophages.

7.4 Single walled carbon nano-horns

Single walled carbon nanohorn (SWCNH) aggregates are composed of thousands of graphite tubules of diameter 2-4 nm similar in structure to SWCNTs. The SWCNHs have an overall spherical structure with a diameter of 50 – 100 nm. Based on their morphology SWCNHs are classified into dahlia, bud and seed types. Because SWCNHs are produced by laser ablation of a pure graphite target they do not contain metal catalysts.

Miyawaki et al. (2008) evaluated the toxicity of 'as grown' (i.e. not purified in any way) SWCNHs using traditional toxicity safety tests (**Table 7.1**). The genotoxicity tests, skin and eye irritation tests were negative. A guinea pig skin sensitisation test using adjuvant, irritation (abrasion and sodium lauryl sulphate) and patch induction was also negative. In a regulatory setting the interpretations of the tests are however compromised due to significant precipitation of SWCNHs at relatively low concentrations in the genotoxicity tests, and amounts less than those prescribed in regulatory protocols being applied in the irritation and sensitisation tests. Although a single intratracheal instillation of a large amount of SWCNHs (2,250 µg/rat) resulted in some animals showing black pigmentation on the lungs (anthracosis) there was no

[34] The Kisin et al. (2009) data was a poster presentation at the 2009 American Society of Toxicology annual scientific meeting. Details of the study are not available and it has yet to be published in a peer reviewed scientific journal. Nonetheless, the data indicate SWCNTs produce the same initial biochemical and flow on consequences as do MWCNTs.

[35] Potential biomedical applications include *in vivo* delivery of drugs, proteins, peptides and nucleic acids (for gene transfer or gene silencing), *in vivo* tumour imaging and tumour targeting for anti-neoplastic treatment.

[36] Four mice received a total of 151 mg SWCNTs non-covalently coated with PEG, or 47 mg SWCNTs with covalently coated PEG in two doses at day 0 and day 7. The dose was determined by the solubility limit of the constructs and the maximum volume that can be injected without causing potential cardiovascular effects. Blood pressure was measured before and after each injection, 12 weeks after the first injection animals were killed for histological examination.

[37] Including vocalizations, laboured breathing, difficulties moving, hunching or unusual interactions with cage mates. No ECG or significant blood pressure changes were noted during or following injection.

[38] The sinusoidal cell types were not defined by the author.

corresponding pathology at 7 or 90 days. The authors concluded the results strongly suggested ‘as-grown’ SWCNHs have low acute toxicities; however, caution is required when using the data. The tests are not clear cut in that the SWCNH material as administered to animals was not characterised (it may have well been presented in very large aggregates), broncho-alveolar lavage (BAL) evaluation of early inflammatory responses was not undertaken, and the number of treated animals was small (only 4 or 5). The authors do however point out that additional chronic tests are required.

Table 7.1: Toxicological evaluation of SWCNHs[(a)]

Test		Dose	Findings
Reverse mutation (Ames test)	S.tyhimurium E. Coli	78 – 1,250 µg/plate(b)	No growth inhibition Negative for mutations ±S9.
Chromosomal aberration	CH lung fibroblast cell line	0.01 – 2.5 mg/ml	Negative ±S9.
Primary skin irritation	Rabbit	0.015 g/site(d)	Negative for unabraded and abraded sites. No clinical signs.
Eye irritation	Rabbit	0.02 g/eye(e)	Negative for non-washed and washed eyes.
Skin sensitisation	Guinea pig	0.02 g (f)	Negative.
Acute oral	Rat	2,000 mg/kg (g)	Negative. No deaths, no abnormal clinical signs, no gross pathology, normal body weight gain.
Intratracheal installation(h)	Rat	2,250 µg/rat (17.3 mg/kg)	Black spots on lungs (3 of 5). Foamy macrophage in intra-alveolar spaces (1 of 5). No inflammation. No fibrotic reaction.

Notes:

(a) SWCNHs were used in the tests 'as grown' and not were not characterised prior to testing.

(b) Black precipitation of SWCNTs at doses >78 µg/plate ±S9.

(c) Precipitation of SWCNTs at concentrations >0.039 mg/ml ±S9.

(d) Dose applied was 0.015 g/site but the specified dose for this test 0.5 g/site (i.e. ~33 times less used); the dose used was the maximum that did not overflow from site when patched with cotton lint and occluded (n = 6).

(e) Amount instilled into eye was 0.02 g/eye but the specified dose for this test 0.1 g/eye (i.e. 5 times less applied); the dose used was the maximum that did not overflow from eye (n = 3 for washed and unwashed instillation).

(f) Induction (after intradermal injection with Freud's complete adjuvant and needle abrasion of site) with 3 x 24 hr occluded patches of SWCNHs (0.02 g in 0.1% DCNB/ethanol solution). On day 7, 10% sodium lauryl sulphate applied for 24 hour followed by SWNCHs. Challenge on naïve area at day 22 with 0.01 g SWCNHs in 0.1% DCNB/ethanol for 24 hr. Draize criteria evaluated 24 and 48 hour later. Prescribed induction dose for the test is 0.1 g (i.e. 5 times less was used for induction). (n = 5 for each of test group and -ve & + ve controls).

(g) Single gavage dose of 2,000 mg/kg in DMSO/water (3:7 v/v) at 20 ml/kg with 14 day observation (n = 5).

(h) Single dose of SWCNHs and positive control material administered in 0.025% Tween 80/saline. Positive controls were finely ground quartz (Min-U-Sil 5, 97% <5 µm) and SWCNTs (nominal size 1 ± 0.2 nm diameter and several hundred nm to several µm long as bundled aggregates). Evaluation of gross and histopathology (heart, lungs, kidneys, liver, spleen, brain plus those showing gross pathology) performed 7 and 90 days post instillation. No BAL done. (n = 5/group).

Quartz control showed inflammation, fibrosis and granulomas. For SWCNTs, 1 of 4 had inflammatory cell infiltration and foamy macrophage in the intra-alveolar spaces at day 7, and lungs of 1 of 5 at day 90 had many black particles surround by granuloma.

Source: Miyawaki et al. (2008)

7.5 MWCNTs

7.5.1 *In vitro* data

MWCNTs avidly associate with macrophages via a cell surface receptor MARCO, this is a scavenger receptor that plays a pivotal role in phagocytosis and subsequent clearance of environmental particles and bacteria from the lung. The binding of MWCNTs to MARCO causes macrophage necrosis by injuring the plasma membrane (Hirano et al. 2008).

Using proteomic analysis Witzmann and Monteiro-Riviere (2006) showed MWCNTs are capable of altering protein expression in cultured human neonatal epidermal keratinocytes[39].

Consistent with the findings for SWCNTs, MWCNTs of low Fe content (0.27%) generated only negligible amounts of reactive oxygen species in cultured normal or malignant mesothelial cells (Pacurari et al. 2008). Nevertheless there was dose dependent significant cytotoxicity, DNA damage and apoptosis in both cell types that was similar to that observed with the crocidolite positive control[40]. The effect was greater in malignant mesothelial cells. It was also demonstrated that a number of transcription factors important in carcinogenesis were activated earlier by MWCNTs in normal mesothelial cells compared with malignant cells. The findings demonstrate that MWCNTs are potent activators in mesothelial cells of molecular events associated with mesothelioma development which may be independent of the formation of ROS and inflammatory responses associated with frustrated macrophage phagocytosis.

7.5.2 *In vivo* data

MWCNTs and SWCNTs have the ability to act as adjuvants and enhance immunological responses to allergens (Inoue et al. 2009, Nygaard et al. 2009) (**Section 6.6**).

Li et al. (2007b) demonstrated the alveolar response to exposures of MWCNTs was significantly more severe after intratracheal instillation (50 µg/mouse) than with 5 – 15 day (6 hr/d) inhalation to 32.6 mg/m^3 (discussed in detail in **Section 6.1**).

Two important *in vivo* investigations on the fibrogenic and carcinogenic potential of MWCNTs have been recently published. Poland et al. (2008) injected a single dose of MWCNTs (50 µg/animal) into the intraperitoneal cavity of mice which caused an early inflammatory response and granuloma formation. Takagi et al. (2008) injected a single

[39] MWCNTs were laboratory made and characterisation was described in previous publications from the authors; 2 ml of 5 mg/ml MWCNTs stock solution in culture media + 1% foetal bovine serum was added to 2 ml cell culture containing approximately 96,000 cells.

[40] The MWCNTs were laboratory prepared with a diameter of 81±5 nm, length 8.19±1.7 µm and surface area 26 m^2/g. A stock solution of MWCNTs was suspended in culture medium with 1% foetal bovine serum (FBS) by ultra- and direct probe sonication. TEM of the MWCNTs in 0.1% FBS showed a large number of discrete nanotubes and few agglomerates. *In vitro* concentrations were 5, 12.5, 25, 50, 100 or 125 µg/cm^2 for cell viability and cytotoxicity assays. Apoptosis, DNA damage by Comet assay and transcription induction experiments were conducted with 25 and/or 50 µg/m^2.

high dose of MWCNTs (3,000 µg/animal) into the intraperitoneal cavity of $p53^{+/-}$ mice and let them live long enough to observe mesothelioma development. Although both studies only used a single dose of MWCNTs, an appropriate positive control substance (asbestos) was incorporated into the experimental design to judge whether the test system responded appropriately in the investigators' laboratories. The animal model has been developed for its uniqueness of mesothelial tissue response to fibres; it does not evaluate other toxicological properties of ENPs that may be associated with their particulate nature. For information on the nuances of the animal model see **Section 4.2** and **Appendix 1**.

The Poland et al. (2008) study was undertaken to test the hypotheses that:

- MWCNTs could produce similar mesothelial tissue reactions as known fibrogenic substances as per the fibre toxicity paradigm (see **Figure 7.1**), and

- mesothelial tissue reactions are related to the shape and length of the MWCNTs.

A variety of different shaped fibres were used by Poland et al. (2008), the results summarised in **Table 7.2** clearly showed the inflammatory and granuloma response of the mesothelial tissue to be associated with long straight thin fibres of high aspect ratio (ratio of length and width consistent with WHO description of pathogenic fibres) as predicted by the fibre toxicity paradigm in **Figure 7.1**. Short amosite asbestos fibres and curled/tangled MWCNTs of low aspect ratio were negative, however long straight thin MWCNTs and long amosite fibres gave an unambiguous positive result.

Table 7.2: Summary of the experimental design and results from Poland et al. (2008)

Animal Treatment[(a)] (single intraperitoneal dose of 50 µg/animal)		Inflammatory response at 24 hr[(f)]	Granuloma response[(e)] at 7 d
Amosite (brown asbestos)	Short fibre (4.5%>15 µm, 1%>20 µm)	-	-
	Long fibre (50%>15 µm, 35%>20 µm)	✓	✓
MWCNT – long straight	Commercial[(b)] (24%>15 µm)	✓	✓
	Laboratory made[(c)] (84%>20 µm)	✓	✓[(d)]
MWCNT – curled/tangled	Short fibres forming tightly packed spherical agglomerates, <5 µm diameter	-	-
	Long fibres protruding from central tangled agglomerates many <5 µm diameter	-	-
Controls	0.5% BSA	-	-
	Non-particulate carbon black	-	-

Notes:

(a) Fibres were suspended in 0.5% BSA saline solution by 2 hours ultra-sonication. Fibres were counted in keeping with WHO guidelines, i.e. particles with length to width ratio >3:1 and longer than 5µm. Groups of 3 – 6 animals. The authors note greater confidence in the results would be obtained with larger groups of animals.

(b) Ave diameter 85 nm, mean length 13 µm, percentage of long fibre >15 µm = 24%

(c) Ave diameter 165 nm, maximum length 56 µm, percentage of long fibre >20 µm = 84%

(d) Suggestion of dose response in that intensity of inflammatory response (# PMN cells) and area lesion were greater with the MWCNTs with the higher % of long fibres.

(e) Granulomas comprised aggregates of cells containing fibres, considered most likely to be macrophages, and associated collagen deposition.

(f) The cellular inflammatory response, evaluated as number of cells in cavity wash fluid, was essentially over at day 7.

Although Poland et al. (2008) only used a single dose in their investigation, they point out a threshold number of fibres at the mesothelium must be reached to cause chronic activation of inflammatory cells, genotoxicity, fibrosis and cancer in the target tissue (Mossman et al. 1998, Kane et al. 1996).

Takagi et al. (2008) used p53 heterozygous deficient mice to increase the sensitivity of the intraperitoneal fibre animal model (see **Appendix 1**). Groups of 19 animals were injected with either MWCNTs, crocidolite or C_{60} fullerenes at a single dose of 3,000 µg/animal[41] and cumulative deaths due to mesothelioma were monitored for 28 weeks

[41] MWCNTs for injection were suspended at 3 mg/ml 0.5% methyl cellulose & 1% Tween-80 by ultra-sonication. The dose of MWCNTs was 1 x 10^9 particles and crocidolite dose 1 x 10^{10} particles each corresponding to 3,000 µg/head. For size characterisation MWCNT were suspended by sonication in an aqueous suspension of surfactant (0.5% Triton X-100) this has the effect of restricting agglomeration and keeping individual CNTs from aggregating – in this medium 100% of the MWCNTs were <20 µm with approximately 70% <5 µm. The intraperitoneal injection however was in a methyl cellulose/Tween-80

post injection. The MWCNTs caused the highest number of deaths (90%) over the 180 day experiment with 50% deaths in the crocidolite group. It is also noted that although the mass dose was the same there were approximately 8 times more crocidolite particles administered than MWCNTs, but the number of fibres per weight of crocidolite relies upon a count done many years earlier for the material (Moalli et al. 1987). Both MWCNTs and crocidolite showed early mild fibrous adhesions in the peritoneal cavity at day 10. The large mesothelial tumours examined when animals were moribund were invasive to several tissues but no metastases were noted.

Conclusions regarding the relative potency of MWCNTs and asbestos in the Takagi et al. (2008) study cannot be readily made. Although more animals treated with MWCNTs died with mesothelioma than did those that received asbestos, information on the number of tumours per animal is not provided. However, the macroscopic pathology examples of the peritoneal cavity show many more mesothelioma nodules with asbestos than for MWCNTs.

Although on face value the size of the MWCNTs administered by Takagi et al. (2008) appeared to be <20 µm long and therefore within the 'short' fibre dimensions as defined/used by Poland et al. (2008), no conclusions regarding the size requirements for a response can be made from the Takagi et al. (2008) experiment because:

- The MWCNTs were size characterised in a different medium to that which was used for injection. There is therefore uncertainty whether the material injected had the same dimensions as that which was characterised

- The dose was very large, consequently even a small percentage of long fibres (i.e. >20 µm) in the administered preparation of MWCNTs may be sufficient to cause a 'long' fibre response in the sensitive $p53^{+/-}$ mouse model.

The Takagi et al. (2008) study has also been critiqued in the scientific literature (Donaldson et al. 2008a). Concerns were raised regarding the fact the clumps of MWCNTs that were injected into mice would never have reached the part of the lung where macrophage phagocytosis would have occurred if they were inhaled. Also that the dose administered (3,000 µg/mouse) was extremely large. Donaldson et al. (2008a) point out their experience is that a carbon nanotube dose of just 0.1 µg can initiate inflammation and argue that the dose used by Takagi et al. (2008) is markedly higher than any reasonable maximum tolerated dose for irritation. Finally Donaldson et al. (2008a) raise the prospect that the p53 deficient mouse model may be too sensitive and has not been validated regarding its response to irritants. It is implied the response may have been one akin to 'solid state' carcinogenesis and not reflective of a possible fibre carcinogenic response.

In response, Takagi et al. (2008b) maintain there was considerable proportion of dispersed fibres in the suspension of MWCNTs that was administered, that the study was conducted for hazard identification and therefore the question of whether the agglomerates reached the lower lungs when inhaled was beyond the scope of the

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mixture, the extent of agglomeration and hence size distribution in this medium was not characterised other than with light microscope where many fibre/rod-shaped particles >10µm were seen with aggregates that were 50 – 200µm in size. That is, the administered preparation was as clusters of aggregated MWCNTs that may or may not have contained specimens of fibre size tubules.

study. Similarly the hazard identification purpose justified the large dose. In their response to the criticism Takagi et al. (2008) presented preliminary information on a repeat study in which single doses of 3, 30 and 300 µg of MWCNTs were injected per mouse (i.e. respectively 1000, 100 and 10 times less than in the original paper), over the course of the ensuing year mesotheliomas have been found in the peritoneum of all mice that died from each dose group[42]. Regarding the type of carcinogenic response elicited, Takagi et al. (2008b) argue that because the tumour was qualitatively the same as that observed with asbestos it was a fibre response, other agents such as quartz initiate a different type of tumour (see also **Appendix 1.1**).

The lack of dose response data in Poland et al. (2008) and Takagi et al. (2008), together with the different asbestos fibre types in each experiment and the lack of quantitative pathology in each study preclude making conclusions regarding fibrogenic potency of long straight MWCNTs relative to asbestos.

However the work of Poland et al. (2008) and Takagi et al. (2008) provide clear evidence that if sufficient MWCNTs of fibrogenic dimensions are in contact with mesothelial tissue for a prolonged period then the tissue will likely mount a fibrogenic and carcinogenic response which includes inflammation, granuloma formation and induction of mesotheliomas.

To complicate further the extrapolation or generalisation of the work of Poland et al. (2008) and Takagi et al. (2008) is the finding by Muller et al. (2009) that injecting MWCNTs into the peritoneal cavity of rats did not induce mesothelioma, or any other form of cancer after a two year observation period. The administered MWCNTs had previously been shown to be persistent, to induce lung inflammation, granulomas and fibrotic reactions after intratracheal instillation to rats (Muller et al. 2005), and to cause mutations in epithelial cells *in vitro* and *in vivo* (Muller et al. 2008a). These inflammatory and genotoxic activities were related to the presence of defects in the structure of the nanotubes (Fenoglio et al. 2008, Muller et al. 2008b). A single dose of 2 or 20 mg/rat was administered. The failure to induce mesothelioma was not due to the test system since crocidolite (2 mg/rat) gave a mesothelioma incidence of 34.6% after two years.

It is unlikely there would be a significant species difference (rat vs mouse) that would account for the different results between the experiments of Poland et al. (2008) and Takagi et al. (2008) with Muller et al. (2009). The biggest difference is that the MWCNTs used by Muller et al. (2009) were short fibres (about 0.7 µm long and 11 nm diameter) that didn't cause sustained production of free radicals and consequential inflammation. The Muller et al. (2009) MWCNTs were injected as aggregates, similar in nature to the tangled MWCNTs of Poland et al. (2008) that were also negative in the mouse peritoneal assay. In Muller et al. (2009), several months after dosing, an inflammatory reaction was almost absent and limited by a fibrotic encapsulation of the MWCNTs.

Despite the differences in fibre size of the MWCNTs between these studies, it is intriguing that genotoxic MWCNTs shown to be an inducer of pulmonary inflammation, fibrosis and granuloma were negative for tumourigenicity in the peritoneal

[42]The response in each dose group may reflect the sensitivity of the test system.

mesothelioma screening assay. This may be due to the assay being specific for fibres (see **Appendix 1.2**) and particles of pathogenic fibre size were not used by Muller et al. (2009). The long term toxicity of these MWCNTs to the lung remains an open question.

Important caveats in the interpretation of the Poland et al. (2008) and Takagi et al. (2008) studies with respect to the health risk implications of people working with these materials are:

- firstly whether the materials become airborne and retain their fibre like characteristics, and if so
- can they penetrate through the lung to the pleural mesothelium after being inhaled?
- will sufficient numbers of fibres reach the mesothelium to cause cancer?

The first of these questions is a workplace exposure issue and is required to be answered when determining risk to workers. The other questions relate to exposure of the target tissue.

In relation to the second question, Bonner[43] et al. (2009) have observed that mice that inhaled an aerosol of MWCNTs (single exposure of 100 mg/m^3 for 6 hours) developed inflammatory foci on the pleural surface of the lung, even though very little inflammation or fibrosis was observed within the lung parenchyma. CNTs were dispersed throughout the lung on day 1 post exposure with some being embedded within the pleural wall. This study shows inhaled MWCNTs can cause pleural lesions and provides evidence of MWCNTs reaching the pleural mesothelium. It is noted the dose used by Bonner et al. (2009) was very high and there is uncertainty whether the observations are an artifact of the dose rather than an innate ability of the MWCNTs to penetrate to the mesothelium.

Hubbs[44] et al. (2009) have also generated data indicative of MWCNTs being able to access lung pleura. In this study mice were exposed to 20 or 80 µg MWCNTs (mean dimensions 4.2 µm x 49 nm, i.e. fibre like) by pharyngeal aspiration. After 7 or 56 days the MWCNTs accumulated in macrophages (with typical signs of incomplete phagocytosis) and caused granulomas inflammation which extended to the pleura. Subpleural lymphatics were affected and in some mice MWCNTs appeared to penetrate the pleura.

[43] The study of Bonner et al. (2009) was a poster presentation at the American Society of Toxicology annual scientific meeting. The information has been gleaned from a small abstract. It has not been published in a peer reviewed scientific journal and therefore considered as preliminary data only. Physical characteristion of the MWCNTs was not provided in the abstract.

[44] The study of Hubbs et al. (2009) was a poster presentation at the American Society of Toxicology annual scientific meeting. The information provided in this review has been gleaned from a small abstract. The data has not been published in a peer reviewed scientific journal and therefore should be considered as preliminary information.

7.5.3 Repeat inhalation exposures – preliminary data

Over the past few years techniques have been developed to allow NP aerosol generation for repeat exposure inhalation studies (e.g. Teague et al. 2009). Some of the results of these studies were recently presented to the American Society of Toxicology and are briefly described below. It should be noted that this information has not been peer reviewed and published; it should be regarded as preliminary. In addition, the abstracts from which the following has been gleaned provide virtually no data on the generation of aerosols of CNTs or their characterisation.

Inhalation exposure of rats for 5 days (2.5, 10 and 30 mg/m^3) or 90 days (0.1, 0.5 and 2.5 mg/m^3) [hours /day not provided] to CNTs [type not specified] resulted in inflammatory responses in the bronchial epithelium and granulocytic infiltration in the lungs with corresponding increase in biochemical and cytological parameters in BAL fluid. The authors consider the study defines a low observed adverse effect concentration (LOAEC) of 0.1 mg/m^3 (Landsiedel et al. 2009).

As judged by BAL parameters, mice inhaling MWCNTs (10 mg/m^3, 5 hr/d for 2, 4 or 8 days) showed dose dependent increases in pulmonary inflammation and damage (Porter et al. 2009). These effects are qualitatively similar to those observed with intratracheal or aspiration exposure.

The alveolar macrophages of mice exposed by inhalation to respirable aggregates of SWCNTs or MWCNTs at 0.3 or 1 mg/m^3 [size and hr/d not specified] for 14 days contained significant amounts of black particles but there was no or minimal inflammation or pulmonary tissue damage. There was however immunosuppression as judged by reduced T-cell dependent antibody response to sheep erythrocytes (McDonald et al. 2009).

Nose only inhalation by rats of raw or purified SWCNTs (0.3 or 1 mg/m^3 for 6 hours on 1 day) showed transient increase in BAL markers for cytotoxicity 7 days post exposure but all effects were resolved by 28 days. According to the authors, whether the changes observed are suggestive of precursor events to pathological changes or lung remodelling that might develop under more severe or prolonged exposure conditions requires further research (Madl et al. 2009).

7.6 Summary and conclusions for CNTs

7.6.1 Carcinogenic hazard

Studies prior to the period covered by this review have shown relatively high doses of SWCNTs administered by intratracheal instillation or pharyngeal aspiration in rats and mice cause accumulation of agglomerates in the lung that is accompanied by inflammation and development of granulomas. More dispersed CNTs cause interstitial fibrosis. More recently MWCNTs have been shown to interact with mesothelial cells *in vitro* or tissue *in vivo* in mice to induce reactions characteristic of fibrogenic fibres, i.e. inflammation, fibrosis, granuloma formation and mesothelioma (Mercer et al. 2008, Pacurari et al. 2008, Poland et al 2008, Takagi et al. 2008).

The experimental design of Takagi et al. (2008) was not as robust as that of Poland et al. (2008) in that only two types of carbon based NPs were used (long thin MWCNTs

and C_{60} fullerenes). It is noted the dose of MWCNTs was extremely high and given that $p53^{+/-}$ mice are very responsive to foreign body induced tumours, further validation of the intraperitoneal fibre model in $p53^{+/-}$ mice with a range of ENPs and other materials is necessary before it could be considered as a reliable screening tool for NP fibrogenicity. Nevertheless, the results of Takagi et al. (2008) have not been produced in isolation of other data.

When viewed with the data from Poland et al. (2008) and the supporting *in vitro* information considered in this review it is reasonable to conclude that MWCNTs of fibrogenic dimensions present a mesothelioma hazard if sufficient numbers are in contact with mesothelial tissue. Mercer et al. (2008) showed SWCNTs are able to penetrate deep into the lung after pharyngeal aspiration to mice. The preliminary data of Hubbs et al. (2009) and Bonner et al. (2009) appears to support that, after either pharyngeal or inhalation exposure, MWCNTs can penetrate through to the pleural mesothelial tissue and cause lesions. Overall the weight of evidence indicates long, relatively straight MWCNTs are hazardous in a similar manner as other pathogenic fibres when inhaled, but that shorter and entangled specimens do not posses these hazards. Although comparable pathological *in vivo* studies have not yet been reported for SWCNTs, information evaluated for this review does not indicate the carcinogenic hazards associated with inhalation exposure to biopersistent SWCNTs of pathogenic fibre dimensions are likely to be significantly different from those of equivalent sized MWCNTs.

Thus in assessing the potential carcinogenic hazard[45] of pathogenic fibre like MWCNTs the following appears evident:

- *In vitro* they can stimulate mesenchymal cell growth (Donaldson et al. 2006b)
- In cultured mesothelial cells MWCNTs are potent activators of molecular events associated with mesothelioma development (Pacurari et al. 2008)
- In the mouse intraperitoneal fibrogenic screening assay, long (>20 μm) straight thin MWCNTs, which are of pathogenic fibre-like dimensions according to the WHO definition, caused an inflammatory and granuloma response typical of pathogenic fibres that induce mesothelioma in this animal model. Fibres <20μm or which where tangled did not cause the response (Poland et al. 2008)
- In a modification of the mouse intraperitoneal fibrogenic screening assay to make it more sensitive, and letting the animals to live long enough to allow development of tumours, pathogenic fibre-like MWCNTs resulted in deaths due to mesothelioma (Takagi et al. 2008)
- MWCNTs not meeting the WHO size definition for pathogenic fibres did not induce tumours in a two year rat peritoneal fibrogenic screening assay (Muller et al. 2009)

[45] In a carcinogenic hazard assessment, considerations of exposure (i.e. dose) are generally not included since they are more relevant to risk assessment. In a carcinogenic hazard assessment a basic question is asked; is there evidence that the substance knows how to cause cancer?

- In mice pharyngeal aspiration of long thin MWCNTs resulted in sub-pleural lesions and penetration of the pleura by MWCNTs (Hubbs et al. 2009)

- Inhalation exposure of mice to aerosols of MWCNTs resulted in inflammatory foci on the pleural surface and in some MWCNTs becoming embedded within the pleural wall (Bonner et al. 2009).

Each of the above pieces of information individually have experimental uncertainty associated with them, not the least of which is that some of the data has yet to be published in a peer reviewed scientific journal. Nevertheless they collectively form a weight of evidence that suggests long thin MWCNTs (i.e. those of pathogenic fibre dimensions) present a mesothelioma hazard to workers if they are inhaled. Data to date indicates other 'non-pathogenic fibre like' MWCNTs do not have this hazard.

7.6.2 Respiratory hazard

The concept that the type of pulmonary toxicity potentially induced by poorly soluble CNT is dependent on the physical form of the CNT has been strengthened by research over the last few years. While it is unlikely tangled agglomerates CNTs are able to cause mesothelioma they may have other fibrogenic hazards such as diffuse, interstitial fibrosis and granuloma formation typical of high dose insoluble particles deposited in the lungs. On the other hand long CNTs may exist as individual CNTs or agglomerates with dimensions that span those of fibres. The hazards that these ENPs possess appear to be a combination of insoluble particles and persistent fibres. Should exposure be sufficient a mixed particle/fibre toxicological response is envisaged.

While it is important to know the shape of CNT to which a worker may be exposed in order to predict whether fibre type injury or particle type injury is possible, it is somewhat a mute point if the CNT can cause either or both.

Lacking is detailed dose response information and long term, repeat dose studies that are conducted at, or include, reasonably anticipated occupational exposures.

7.6.3 Recommendations

Pending the outcome of a formal weight of evidence evaluation, the following precautionary default positions are suggested for consideration.

1. All poorly soluble CNTs be regarded as being biodurable and biopersistent and potentially fibrogenic unless demonstrated otherwise.

2. All poorly soluble CNTs of pathogenic fibre dimensions[46], or which form agglomerates of pathogenic fibre dimensions, be considered as potential fibrogenic and mesothelioma agents unless demonstrated otherwise.

[46] Pathogenic fibres were defined (WHO definition) in section 7.1.2. Deliberations by the World Health Organization and others on man-made mineral fibres will also inform on suitable criteria.

3. Documentation based on weight of evidence be drawn up to justify the above proposed default position(s).

4. Guidance be created for evidence required to move away from these default positions.

5. Consideration be given to establishing an occupational exposure standard for CNTs. This will require appropriate quantisation methodology (e.g. transmission or scanning electron microscopy) since phase contrast microscopy won't allow counting of CNTs.

6. While functionalised CNTs are likely to have lower toxicity than non-functionalised CNTs, this has not yet been verified using equivalent *in vivo* assays to deliver the material to the lungs. Consequently no distinction between their potential pulmonary toxicity should be made. Movement from this default for individual types of CNTs should be based on appropriate data.

7.7 Fullerenes

Baker et al. (2008) examined the toxicity and lung toxicokinetics of C_{60} fullerene NPs (55nm) and microparticles (930 nm) in rats after 10 days of inhalation[47] exposure. Nasal and eye discharge were observed during exposure but resolved between exposures. At necropsy, no gross or microscopic lesions were observed in either group of C_{60} fullerene exposed rats. There were minor changes in blood parameters but these were different for each group and it is difficult to assign biological significance to them. BAL fluid macrophages from both contained brown pigments consistent with the presence of fullerenes. Although lung particle burdens were greater in nanoparticle-exposed rats and the calculated lung deposition fraction 50% greater, lung half-lives for C_{60} fullerene nanoparticles and microparticles were similar at 26 and 29 days, respectively.

The Baker et al. (2008) study is important in that animals were exposed by a route of exposure relevant to humans, the fullerenes were dry and there was extensive histological evaluation of relevant tissues. The study showed minimal changes in the parameters evaluated, suggesting low inhalational toxicity from acute exposures. The authors note longer duration inhalation exposure studies are needed.

[47] In Baker et al (2008) the C_{60} nanoparticles were 55 nm and at 2.22 mg/m^3, the C_{60} microparticles were 0.93 µm and at 2.35 mg/m^3. Exposure of male rats to both sets of particles was nose only for 3 hr/d for 10 consecutive days. C_{60} aerosols were generated by special designed apparatus described in Gupta et al. (2007). HPLC, XRD, and scanning laser Raman spectroscopy indicated no chemical modification of the C_{60} fullerenes occurred during the aerosol generation. Blood was collected from the retro-orbital sinus, bronchiolar lavage and necropsy done 2 hr after last exposure together with tissue burden analysis and calculation of body burden. Brain, eyes, liver, kidneys, spleen, heart, lungs, large and small intestines, testes, epididymides, urinary bladder, lymph nodes (bronchial, mandibular, mediastinal, mesenteric), larynx (three levels), and nose (three levels) were subject to histological examination.

Stable aqueous suspensions of colloidal C_{60} fullerenes prepared by two methods[48] of solvent exchange were shown to be genotoxic (Comet assay) to freshly prepared human lymphocytes down to concentrations of 2 – 4 µg/L in the assay (Dhawan et al. 2006a). It is noted that there was no statistical difference between the two incubation times used and although cell viability was assessed the results were not reported. The authors claimed this was the first demonstration of genotoxicity by C_{60} and was different from that in *Drosophila melanogaster* by Russian investigators. The authors hypothesized the genotoxicity may be due to production of oxygen radicals and leaky membranes, C_{60} partitioning to DNA, and DNA damage due to oxygen radicals.

The mechanistic conclusions of Dhawan et al. (2006) are also apparently at odds with that of Zhao et al. (2008a) who demonstrated that four different water soluble fullerene preparations could efficiently photogenerate reactive oxygen species. Monomeric but not aggregate C_{60} was cytotoxic to cultured keratinocytes, yet uptake of C_{60} aggregates was higher than for monomeric C_{60}. The authors determined the toxicity was different because the reactive oxygen was sequestered inside the C_{60} aggregates explaining why these preparations were not phototoxic toward human keratinocyte cells. Zhao et al. (2008a) point out that previous studies had shown different preparations of C_{60} in water may induce different toxic effects. For example, aqueous suspensions of C_{60} prepared by THF solvent exchange are reported to be very toxic, while C_{60} aggregates prepared by mechanical milling show no acute or subacute toxicity but powerful antioxidant effects *in vivo*. Neither of the C_{60} in Dhawan et al. (2006) or Zhao et al. (2008a) was prepared by ball milling.

Highly purified fullerenes do not stimulate the release of NO by murine macrophage cells in culture, their uptake by human macrophage cells is very low, and they possess a very low toxicity against human macrophage cells Fiorito et al. (2006a).

Monterio-Riviere et al. (2007) have reviewed some of the studies on fullerenes available up to that time. Topical application to mouse skin had no affect on DNA synthesis and they were not a promoter in the two stage mouse skin carcinogenesis assay. Generally, incorporation of water soluble groups on the surface increased biocompatibility. The least derivatised C_{60} and most aggregated form was more cytotoxic to cultured keratinocytes.

While CNTs altered the *in vitro* antigen presenting function of a mixed culture of macrophage and T-cells, fullerenes did not (Hamilton et al. 2007). It is also noted that fullerenes have been incorporated into experimental designs investigating fibrogenic potential of ENPs as negative controls (Takagi et al. (2008). However, early signs of inflammation were not evaluated in this test.

[48] *n*C_{60} are clusters of C_{60} in aqueous suspension of colloidal size. They are hydrophilic, charged and possibly hydrated, implying fullerene hydrosols are stable and likely to persist in the aqueous environment. The fullerenes used by Dhawan et al. (2006) were prepared by ethanol to water solvent exchange (EtOH/ nC_{60} suspensions) and extended mixing in water (aq/ nC_{60} suspensions). The extended mixing method resulted in the formation of larger (~ 178 nm) and less negatively charged (~ -13.5 mV) nC_{60} colloids than those prepared by ethanol to water solvent exchange (~122 nm, ~-31.6 mV). The C_{60} were characterized (TEM and dynamic light scattering) in the assay medium (PBS), lymphocytes were incubated for 3 or 6 hr in 3 replicate assays, a positive control was used and cell viability assessed by Trypan blue exclusion.

7.7.1 Summary

Overall, from the information reviewed herein, fullerenes appear to be less hazardous than other carbon based NPs because:

- *They are less efficient in producing reactive oxygen and nitrogen species.*
- *Pulmonary toxicity in rats after sub-acute inhalation exposure of moderate concentrations was low.*
- *They do not exhibit direct toxicity to skin.*
- *They do not alter the immunological function of macrophages.*
- *Increasing water solubility decreases the in vitro cytotoxicity.*

7.8 Other carbon nanoparticles

To investigate the potential effects of NP exposure on male reproduction, Yoshida et al. (2008) undertook a repeat dose intratracheal instillation study in mice for carbon black (CB) NPs of three different sizes[49] (14, 56 and 96 nm). Each mouse was instilled with 100 µg for 10 times per week. The serum testosterone levels were elevated significantly in the 14- and 56-nm CB groups. Histological examination showed random partial vacuolation of the seminiferous tubules that was more frequent in the CB treated mice than controls. It was found the effects depended on particle mass rather than particle number.

The authors concluded that carbon nanoparticle-exposure has an adverse effect on the mouse male reproductive function. However this publication is poorly reported and has a number of issues that significantly detract from the authors conclusions; the CB was administered in saline with a surfactant but the extent of agglomeration was not determined, the number of weeks of instillation is not reported, 100 mg/mouse is a high total intratracheal dose, the clinical state of the animals is not reported and organs other than the testis were not examined either grossly or histologically. The review by McAuliffe and Perry (2007) indicated NPs given ip, iv or po could cross the blood testes barrier and deposit in the testes but in those experiments no toxicity was observed.

[49] The surface areas of 14 nm, 56 nm, and 95 nm CB were 300, 45 and 20 m^2/g respectively. The CBs were autoclaved at 250^0C for 2 hr before use. CB NPs were suspended in a normal saline containing 0.05% Tween 80.

8. Titanium dioxide

8.1 Overview

Nano-TiO_2 has been subjected to a range of standard and special toxicity/safety evaluation tests. Safety tests conducted and accessible over the period covered by this review are described below, however it is worth mentioning that the European Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP 2000) have evaluated a large propriety database of toxicological studies[50], most conducted to Good Laboratory Practice (GLP) standards, for a range of commercial microcrystallised TiO_2 which were coated or uncoated[51] and had size of 15 – 60 nm. The assessment was undertaken with skin applications in mind. The studies showed the various nano-TiO_2 preparations were:

- of low acute oral and dermal toxicity
- non-mutagenic in a range of assays
- non-irritant to skin
- some were slightly irritant to the mucous membranes of the eye
- not skin sensitisers
- negative in photo-toxicity tests
- negative for photo-irritation
- negative for photo-mutagenicity
- negative in oral carcinogenicity assays
- some nano-TiO_2 preparations were photocatalytic (able to produce ROS) under UV light but this was much less with coated material.

The SCCNFP (2000) concluded "*the toxicological profile of this material does not give rise to concern in human use, since the substance is not absorbed through the skin*".

The above evaluation of good quality data not available in the public arena is reassuring and provides information useful for hazard identification and risk assessment for workers exposed via skin and potentially by hand-to-mouth transfer. We note however that inhalation studies are conspicuous by their absence in the

[50] The reader is referred to SCCNFP (2000) for a description of the toxicological studies and the findings. The studies included acute (oral and dermal) toxicity, sub-chronic oral (13 weeks), irritation (skin and mucous membranes), sensitisation, toxicokinetics (dermal), mutagenicity, photo-mutagenicity, photo-toxicity, photo-irritation, photo-sensitisation.

[51] Coating materials included alumina, silicon dioxide, aluminium stearate, stearic acid, simethicone, dimethicone.

database examined by SCNFFP. This is the exposure route of most concern for workers and the main focus of this review.

Particularly in relation to pulmonary toxicity, increased surface reactivity predicts that nanoparticles may exhibit greater toxicity per given mass compared with larger particles because the ratio of surface to total atoms or molecules increases exponentially with decreasing particle size (Oberdörster 2005b). This relationship between surface area and toxicity was reported in 2006 (ASCC 2006, Oberdörster 2005b). Beyond understanding the toxicity at the site of contact at high concentrations representative of overload conditions, concern was expressed in ASCC (2006) about the ability of nanoparticles to enter cells and transit across epithelial and endothelial cells into the blood and lymph circulation to reach potentially sensitive target sites such as bone marrow, lymph nodes, spleen, and heart (Oberdörster 2005b, ASCC 2006).

Between 2006 and 2008 additional primary research has extended the understanding of some critical factors (surface treatments, surface activity, and agglomeration) influencing the pulmonary toxicity of TiO_2. Many of these studies were conducted by intratracheal instillation of nanoscale TiO_2 in various particle sizes, crystalline phases, with and without surface treatments. Some compared the responses from intratracheal instillation and acute inhalation exposure.

Research using novel techniques on the role of macrophages in nanoscale TiO_2 clearance and translocation of TiO_2 from the lungs is continuing (Geiser et al. 2005, Mühlfeld et al. 2007, Geiser et al. 2008). This research is being conducted at concentrations that do not cause pulmonary inflammation and the preliminary findings indicate nanoscale TiO_2 may translocate to and cause physiological changes (arteriolar constriction) on the microvasculature (Nurkiewicz et al. 2008).

8.2 Absorption and distribution

A recent critical review of the scientific literature on the ability of nano-scale TiO_2 (diameter is between 10 and 100 nm) to penetrate skin has concluded that there is no evidence that insoluble TiO_2 penetrates into or through intact human skin or may produce human local or system exposure and or adverse effects (Nohynek et al. 2007). These findings are consistent with earlier reviews by the German Federal Institute of Risk Assessment (BAuA et al. 2006), SCCNFP (2000) and the Australian Therapeutics Goods Administration (TGA 2006).

Clearance of inhaled nanoparticles from the lungs depends mainly on particle size, agglomeration surface and other particle characteristics. It was reported following 3 months exposure of rats to ultrafine (20 nm) and fine (200 nm) titanium dioxide (TiO_2) particles by inhalation that the ultrafine particles were cleared significantly more slowly, and showed more translocation to interstitial sites and to regional lymph nodes compared to the fine TiO_2 particles (Oberdörster et al. 1994).

The German National Research Center for Environment and Health, Institute for Inhalation Biology has been conducting research[52] into the potential for nanoscale TiO_2

[52]This research has been conducted using a rat model in which a TiO_2 aerosol (particle size 20 nm, surface area 330 m^2/g) is inhaled by anaesthetised animals via endotracheal tube for 1 hour and

clearance from the lungs and translocation to the vasculature (Geiser et al. 2005, Mühlfeld et al. 2007, Geiser et al. 2008). One hour after aerosol inhalation, 24% of ultrafine TiO_2 particles were located within cells of the main lung tissue compartments but were not membrane bound (Geiser et al. 2005).

Mühlfeld et al. (2007) conducted further analysis of the data of Geiser et al. (2005) by relating the size of a tissue or intracellular compartment to the number of particles located within a particular compartment to create a relative deposition index. Using this novel method for assessment of preferential deposition the authors found that nanoscale TiO_2 particles seem to be transported from airspaces to the connective tissue and then through capillaries into the circulation. Geiser et al. (2008) investigated the role of macrophages in TiO_2 nanoparticle[53] clearance from the lungs. Lung-surface macrophages only took up 0.06 to 0.12% of the particles within 24 hours, indicating that the uptake of TiO_2-nanoparticles by lung-surface macrophages is not a significant clearance mechanism. The authors suggest ineffective macrophage clearance from peripheral lungs prolongs their residence time in the lungs and/or favours translocation into lung tissue and the vasculature.

Tissue distribution studies in male rats following intravenous administration (5 mg/kg bw) did not result in retention within blood cells, plasma, brain or lymph nodes (Fabian et al. 2008). The health and behaviour of the animals was normal throughout the study. Serum clinical chemistry, antigens cytokines were normal indicating there was no detectable inflammatory response or organ toxicity. One hour after injection TiO_2 was not detectable in blood cells, plasma, brain, or lymph nodes. The TiO_2 levels were highest in the liver, followed by spleen, lung and kidney. Levels in the lung and kidney returned to control levels by day 14, only slightly decreased in the spleen at day 28 and not changed in the liver. Similar summary findings have also been reported where subcutaneously injected TiO_2 nanoparticles were not systemically available, but remained at the injection site; intravenously injected TiO_2 nanoparticles were rapidly cleared from the circulation and were found inside macrophages in the liver, spleen and the lungs, suggesting normal clearance by the reticuloendothelial system (Umbreit et al. 2007).

8.3 Toxicological investigations

8.3.1 *In vitro* data

8.3.1.1 ROS and cytotoxicity

There is available a reasonable *in vivo* toxicological database for TiO_2. Consequently the many *in vitro* investigations of TiO_2 NPs have not been reviewed in detail. However the work of Jiang et al. (2008) and Sayes et al. (2006b) are mentioned because they

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observations made at 24 hours post exposure. Observations include conventional transmission electron microscopy (TEM) to screen macrophages for particle agglomerates, and energy-filtering transmission electron microscopy (EFTEM) for elemental microanalysis of individual particles to investigate the nature of particle–cell interaction.

[53] The geometric mean size was 20 nm, and mean number concentration 7.2 x 10^6 particles cm^{-3}, resulting in a mass concentration of approximately 0.1 mg/m^3. Rats were anaethetised in airtight plethysmograph boxes and inhaled aerosol for 1 hour via endotracheal tube by negative-pressure ventilation.

illustrate the care that needs to be taken when evaluating studies using *in vitro* systems.

Jiang et al. (2008) measured the ability of different sizes (4-195 nm) and crystal phases of TiO_2 to generate ROS in a cell free system. The highest ROS generating activity per unit area was observed for 30 nm particles, ROS production was constant above this particle size. The ability of different crystal phases to generate ROS was highest for amorphous, followed by anatase, then anatase/rutile mixtures, and lowest for rutile samples. The authors propose that the ROS response is not directly related to surface area but to available surface defect sites. The TiO_2 nanomaterial used in these studies was characterised as the particles were made and collected on filter paper, and not in the chemical medium (PBS) in which the ROS generation occurred. The relevance of the information for either *in vitro* or *in vivo* toxicity is obscure.

Sayes et al. (2006b) compared the *in vitro* cytotoxicity[54] of anatase, anatase/rutile and rutile TiO_2. Cytotoxicity and inflammation was observed only at high concentrations (100 µg/ml) but thereafter in classic dose response and time response fashion. The crystal phase of TiO_2 strongly correlated to cytotoxicity; anatase TiO_2 being 100 times more cytotoxic than rutile. The authors stated no dependency on surface area was found but also noted each nano-TiO_2 sample exhibited similar changes in aggregation in culture medium and stated NP size was not a variable in these studies.

8.3.1.2 Genotoxicity

The literature search for this review did not find *in vivo* genotoxicity studies for TiO_2.

The *in vitro* genotoxicity studies conducted to date that report positive results are unable to differentiate whether the effects are secondary to initiation of the inflammatory response or are potentially an intrinsic property of the material (Wang et al. 2007b, Falck et al. 2007). For example, Wang et al. (2007b) observed increases in micronucleated binucleated cells, increased DNA damage in the Comet assay and increased mutations in the HPRT gene mutation assay. However these effects occurred at concentrations that significantly decreased cell viability and increased apoptosis. Interestingly Falck et al. (2007) found coarse TiO_2 (average 1 µm) to be more effective than nano-TiO_2 in inducing DNA damage measured in cultured human bronchial epithelial cells.

Kang et al. (2008) investigated the mechanism for genotoxic effects they observed in lymphocytes cultured with up to 100 µg/ml commercial[55] TiO_2 NP. They concluded the

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[54] In Sayes et al. (2006b) the TiO_2 particle size was approximately 3-10 nm diameter and surface area 110-155 m^2/g. The cell lines were human dermal fibroblasts and human lung carcinoma cells. Cells were exposed at concentrations of 3 µg/ml to 3 mg/ml for 48 hours and tested for viability (oxidative stress), LDH (membrane integrity), MTT (metabolism) and IL-8 (inflammation). The nano-TiO_2 particles were suspended in deionised water. NP characterisation was done on dry powder but the particles were suspended in ultrapure water. Analysis in culture medium showed increased aggregation. Anatase phase TiO_2 catalytically dissociates water to form hydroxyl radicals under illumination, whereas rutile-phase TiO_2 has comparatively little catalytic activity. Anatase-phase TiO_2 exhibits significantly greater cytotoxicity and release of the inflammatory cytokine, IL-8, than rutile-phase TiO_2 in primary human dermal fibroblasts and lung A549 cells, and cytotoxicity is potentiated in the presence of UV light (Sayes et al. 2006b).

[55] The commercial TiO_2 NP used by Kang et al. (2008) were anatase:rutile (~80:20). The particles were characterised as dry powder with surface area 50 m^2/g and size ~30 nm.

nano-TiO_2 caused the generation of ROS which induced the DNA damage as this was ameliorated by the antioxidant N-acetylcysteine. The ROS induced cell damage also activated p53 leading to apoptosis.

Warheit et al (2007c) reported negative results for ultrafine TiO_2 in the *in vitro* bacterial reverse mutation assay and chromosome aberration with Chinese hamster ovary cells (see also **Section 6.1.3**). The abstract by Falck et al. (2007) used a reference substance to compare the response of nano and fine TiO_2 with an *in vitro* human bronchial epithelial cell genotoxicity assays. DNA damage was assessed using the comet assay and a micronucleus assay. Both the fine and nano-scale TiO_2 were reported to cause DNA damage in the comet assay (but not the micronucleus assay) in a dose dependent manner with the coarse form being "somewhat more effective".

8.3.2 *In vivo* data

8.3.2.1 Intratracheal and inhalation

After intratracheal administration transient inflammatory responses are observed with fine TiO_2 of size about 300 nm, the effects are not sustained when compared to quartz related effects and do not lead to fibrotic type responses (Warheit et al. 2006a, b). The investigators consider inhalation of such TiO_2 particles would have low risk potential for producing adverse pulmonary health effects and have led to these particles being used as negative controls in experiments with ultra-fine TiO_2, i.e. nanoscale particles.

Warheit et al. (2006a) considered the shape and nature of nanoscale TiO_2 by comparing the acute lung toxicity in rats of intratracheally instilled pigment grade TiO_2 (rutile), TiO_2 rods (anatase) and TiO_2 dots[56] (anatase) with a positive control (quartz). A single intratracheal dose of either 1 or 5 mg/kg bw (i.e. 250 or 1,250 µg/rat) was administered in PBS and observations made for up to 3 months post instillation. No significant[57] adverse pulmonary effects were observed in any of the TiO_2 formulations tested; the response to nanoscale TiO_2 was not different from the larger size material. The high dose quartz, but not the low dose, resulted in the expected significant adverse effects that led to fibrosis.

Warheit et al. (2007b) has further evaluated the toxicity of other ultrafine TiO_2 particles[58]. No significant adverse pulmonary effects were observed with fine rutile TiO_2 (used as the negative control), or ultrafine TiO_2 rutile particles. However ultrafine

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[56] In Warheit et al. (2006a) the pigment grade TiO_2 was rutile particles (ca 300 nm, sa 6 m^2/g) TiO_2 rods were anatase (200 nm x 35 nm, sa 26 m^2/g) and TiO_2 dots were anatase (ca 10 nm, sa 169 m^2/g). The nano-TiO_2 rods which were synthesised with surfactant had little aggregation when characterised in water, but the dots synthesised without surfactant exhibited moderate to severe aggregation. They were administered to rats in PBS after dispersement by sonication. Observations on time course and dose response intensity of pulmonary inflammation and cytotoxicity, cell proliferation, and histopathology were made at 24 hr, 1 week, 1 month and 3 months.

[57] Nanoscale TiO_2 rods and dots did produce some minor transient and reversible signs of inflammation but these were considered by the authors to be due to the effects of the instillation procedure. The low dose quartz also produced transient inflammatory responses.

[58] In Warheit et al. (2007b) the ultrafine TiO_2 was approx 130 nm and the fine TiO_2 380nm when characterised in water. In PBS however, the vehicle in which the materials were administered, there was significant aggregation of all TiO_2 samples that resulted in median size aggregates of about 2,500 nm. Doses were 1 and 5 mg/kg with evaluations for inflammatory responses in BAL and via histology at 24 hr, 1 week, 1 month and 3 months.

anatase TiO_2 particles produced pulmonary toxicity which at 3 months post instillation included macrophage aggregates containing TiO_2 and tissue thickening indicative of fibrosis. Interestingly the authors did not entirely attribute the pulmonary toxicity to the crystalline nature of anatase. Unlike the production of ultrafine rutile TiO_2, the production of commercial ultrafine anatase does not include an aqueous or wet surface treatment step to neutralise and remove the acidic chloride ions from the surface of the particles. Thus the authors considered the different response to anatase could be related to crystal structure, the inherent pH of the particles or surface chemical reactivity.

The surface reactivity of nanoscale TiO_2 may change when different coatings are present on the nanoparticles. Previously Warheit et al. (2005) did a comparison study of fine rutile particles in inhalation and instillation experiments and found only NPs with alumina and amorphous silica coatings caused adverse effects. Similarly Oberdörster (2000) reported that ultrafine TiO_2 coated with a silane compound caused markedly less pulmonary inflammation compared with uncoated material.

Either by inhalation or intratracheal administration in mice, the early inflammatory response to 3-5 nm or 20 nm TiO_2 particles was the same. There was not a greater response with the smaller, larger surface area NPs. With the smaller sized particle no pulmonary toxicity was seen with intratracheal 0.4 mg/kg, moderate toxicity at 4 mg/kg (approximately 100 µg/mouse), and lung overload at 40 mg/kg (Li et al. 2007a). Grassian et al. (2007b) exposed mice either by inhalation[59] or by nasopharyngeal aspiration. The results showed the larger TiO_2 NPs (anatase/rutile) to be moderately, but significantly more toxic than the smaller ones (anatase). Because the different size NP preparations in water had different agglomeration states, Grassian et al. (2007a) concluded that the difference in the nature of the agglomeration may be an equally important factor as the surface and other particle characteristics in determining the toxicity of nanomaterials.

Nurkiewicz et al. (2008) conducted an inhalation study to determine if acute inhalation[60] of ultrafine TiO_2 produces greater microvasculature dysfunction than fine TiO_2. TiO_2 aerosols were produced using a fluidized-bed powder generator which was able to disperse particles and avoid agglomeration. By histological evaluation no inflammatory changes were seen in the lung however particle containing macrophages were observed in intimate association with the alveolar wall. Both fine and ultrafine TiO_2 caused arteriolar constriction in a dose dependent manner. At similar pulmonary burdens the arteriolar constriction for the ultrafine was more intense. The no effect lung burden for ultrafine and fine TiO_2 was 4 µg and 8 µg, respectively (equivalent to 1.5 and 3 mg/m^3). For ultrafine TiO_2, the arteriolar constriction was significant with a lung deposition of 38 µg (achieved by an exposure of 10 mg/m^3 for 8 hours).

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[59] Air concentrations were produced by nebulisation of water suspensions to give TiO_2 concentrations of approximately 0.7 or 7 mg TiO_2/m^3.

[60] In Nurkiewicz et al. (2008) aerosol concentrations were between 1.5 and 20 mg/m^3 with exposure times of 240-720 minutes (2-8 hours). 24 hours post exposure rats were anaesthetised, particle deposition in the lungs was measured (ICP-AES) and the right spinotrapezius muscle was used for *in* vivo microscopy to determine endothelium dependent artiolar by assessing the capacity for Ca^{++} dependent endothelial NO formation in response to intraluminal infusion of the calcium ionophore A23187. The primary mode aerosol particle sizes for the fine and ultrafine aerosols generated by the exposure system were 710 and 100 nm, respectively.

Studies investigating the ability of nanoscale TiO_2 to cause systemic adverse toxicological effects following repeated inhalation exposure were not identified.

8.3.2.2 Cancer

In February 2006, an IARC Monographs Working Group re-evaluated the carcinogenic hazards to humans from TiO_2. Based on evidence from cancer studies with rats TiO_2 was evaluated as possibly carcinogenic to humans (i.e. Group 2B under the IARC classification scheme) (IARC 2006). Following chronic inhalation lung tumours have been observed in rats with fine (<2.5 µm) TiO_2 at 250 mg/m^3 and ultrafine (<0.1 µm) TiO_2 10 mg/m^3), but not in mice or hamsters (Lee et al. 1985a, 1985b, 1986; National Toxicology Program, 1993; Heinrich et al. 1995; Nikula et al. 1995).

Hamsters had relatively effective lung clearance and also little overload or adverse response compared to mice or rats, whereas rats had more severe lung responses, including inflammation and tissue injury, compared with mice or hamsters (Bermudez et al. 2002, 2004; Elder et al. 2005).

No detectable excess cancer risks were observed in epidemiological studies with workers exposed during production of titanium dioxide. Levels of exposure to respirable dust in these occupations ranged between <1 and 5 mg/m^3 (geometric mean) but have declined over time (Baan 2006).

The IARC Working Group noted that prolonged exposure to inhaled particles at sufficiently high concentrations in experimental animals may lead to impairment of normal clearance mechanisms in the alveolar region of the lung, resulting in a continued build-up of particles that eventually leads to excessive lung burdens accompanied by chronic alveolar inflammation. Because exposure to ultrafine particles was considered to cause lung overload at much lower mass concentrations than with fine particles (see **Section 4.1** and **Appendix 1.1**) the cascade of events leading to cancer were regarded by IARC as being relevant to humans working for a long time in dusty jobs (IARC 2006, Baan 2006).

The mechanism of tumour induction in rats appears to be a secondary genotoxic mechanism associated with persistent inflammation and the inflammatory response shows evidence of a threshold. The US National Institute for Occupational Safety and Health (NIOSH) are exploring dosimetry and dose response models to obtain risk estimates for humans (Dankovic et al. 2007). Using ICRP particle deposition (see **Appendix 1**), estimated human occupational exposures yielding equivalent inflammatory lung burdens as rats range from approximately 1 – 40 mg/m^3 for fine TiO_2. This work is ongoing but the techniques being investigated offer a promising approach to the risk assessment of ENPs and setting occupational exposure limits. This work is however only possible because of the comparatively large *in vivo* database for fine and ultra-fine TiO_2.

8.3.2.3 Oral and dermal

Warheit et al. (2007c) report the results of testing three[61] different TiO_2 particles in a base set of hazard safety tests that included negative and positive controls. The tests and results are presented in **Table 8.1**. The authors concluded the results of most studies demonstrated a low hazard potential following acute exposures to ultrafine TiO_2 particle types. Wang et al. (2007a) conducted a standard (OECD Test Guideline 420) acute oral toxicity study with fine and ultrafine[62] TiO_2 administered to mice at 5,000 mg/kg bw in a suspension of 0.5% hydroxypropyl methylcellulose. No symptoms of ill health or deaths occurred. However clinical chemistry suggested there had been organ damage with the ultrafine but not the fine TiO_2 particles, but pathology was seen in animals treated with 80 nm TiO_2 and fine (155 nm) TiO_2, but not 20 nm sized TiO_2. Overall the results do not point to significant size dependent difference (i.e. large vs small) in the spectrum of TiO_2 adverse effects.

- Liver damage was indicated by elevated liver enzymes in the serum and spotty necrosis of hepatocytes.
- Nephrotoxicity by elevated BUN, serious swelling of renal glomerulus and proteinic liquids in the renal tubule.
- There was a slight brain lesion manifested by vacuoles in the hippocampus.
- Inflammation cells in the stomach attributed to the particle overload.

The TiO_2 tissue distribution was different for 80 nm particles compared to the 20 nm and fine TiO_2. For the latter, in descending order, the spleen, kidney, lung and brain retain the highest amounts. None is retained by the liver. However with the 80 nm TiO_2 the liver is by far the largest accumulator.

Consistent with Wang et al. (2007a), Warheit et al (2007b) reported an LD_{50} of greater than 5,000 mg/kg in female rats for nano-scale TiO_2 (80% rutile and 20% anatase 140 nm) with no symptoms of ill health or deaths.

8.3.2.4 Developmental effects

Investigations of the developmental toxicity of nano-TiO_2 conducted according to established protocols for such studies were not found for this review.

Takeda et al. (2009) observed TiO_2 in the brain and testis of male offspring of pregnant mice that had been subcutaneously injected with nano-TiO_2 (4 injections approximately 4 days apart starting on day 3 postcoitum, each injection 100 µg/mouse; total approximate dose 10,000 µg/kg). Although the authors report undertaking characterisation of their TiO_2 (25 – 70 nm) by scanning electron microscopy, it is not stated whether this was in the medium used for injection (saline plus 0.05% Tween

[61] The TiO_2 particles tested by Warheit et al. (2007c) were TiO_2 rutile core with 2% alumina surface coating, mean size 136 nm in phosphate buffered water; TiO_2 rutile core with silica (~7%) and alumina (5%) surface coating, 149 nm; and TiO_2 rutile/ anatase (79%/21%), 140 nm.

[62] The grades of TiO_2 assessed by Wang et al. (2007a) were fine TiO_2 155±33 nm, and ultrafine (nano-sized) at 25nm and 80 nm.

80). The testicular observations consisted of aggregates of TiO_2 (100-200 nm) in Leydig cells, Sertoli cells and spermatids of 4 day and 6 week old offspring. In 6 week old mice testicular morphology appeared abnormal in that the seminiferous tubules were disorganized and there were fewer mature sperm. However sperm morphology was normal. No information is provided on the condition of the dams during the treatment and given that the body weight of the male offspring at birth was significantly less than controls there is an open question of whether poor health (e.g. decreased body weight gain) of the dams may have contributed to the observed testicular effects. The authors state the effects were dependent upon dose, however only one dose is reported in the publication. TiO_2 was also observed in the brain of 6 week old males (data for 4 day olds not reported) with biochemical and morphological evidence suggestive of apoptosis; none of this information was subject to quantitative analysis in the publication. The authors considered TiO_2 reached the brain because the blood brain barrier of the *in utero* foetus is not fully established.

Overall the Takeda et al. (2009) investigation demonstrates that TiO_2 repeatedly injected into pregnant mice can be distributed to the testis and brain of the foetus. Since a control of non-nano TiO_2 was not included in the experimental design it is not known if the distribution is a specific feature of the nano-size of the injected TiO_2. The Takeda et al. (2009) data support the need for additional developmental studies of ENPs; however due to reporting deficiencies and the non-physiological route of exposure no conclusions regarding hazard or risk to humans can be drawn from this study.

Shimizu et al. (2009) subcutaneously injected pregnant mice four times with anatase[63] TiO_2 (100µg/mouse, total dose approximately 9,000 µg/kg) and using cDNA microarray analysis measured gene expression in the brain of male off spring on postnatal days 2, 7, 14 and 21. Relative to the control group there were hundreds of genes whose expression (up or down regulated) was changed. While the authors speculated the changes may cause a range of diseases it has not been demonstrated that adverse functional changes arise from such patterns of gene expression. Interpretation of this data is further complicated by the fact that the dose of TiO_2 was very large and given by a non-physiological route of exposure. Furthermore the condition of the dams and whether there were changes in weight gain was not reported.

[63] Particle characterisation was supplied by the manufacturer (25–70 nm; surface area 20–25 m^2/g and was not reascertained in the administrating vehicle of sonicated water containing 0.05% Tween 80).

Table 8.1: Toxicity test results with ultrafine TiO_2

Test	Observations	Conclusion
Pulmonary assay[(b)] (Rat)	80:20 anatase:rutile produced transient pulmonary inflammation, cytotoxicity and adverse lung effects. No effects with other TiO_2 ultrafines.	Low toxicity
Acute oral – up down procedure (Rat) (OECD 425)	No mortality. Grey coloured faeces. No biologically important weight loss No gross lesions at necropsy LD_{50} >5,000 mg/kg	Low toxicity
Skin irritation (Rat) (OECD 404)	No dermal irritation No clinical signs of toxicity No body weight loss	Not skin irritant
Eye irritation (Rabbit) (OECD 405)	Conjuctival redness (score 1 or 2) No corneal injury Eyes normal at 24 or 48 hr No clinical signs	Minor ocular conjunctival redness
Dermal sensitisation, local lymph node assay (OECD 429)	Stimulation indices <3, therefore EC3 value could not be calculated. +ve control worked OK	Not a sensitiser
E. coli & S.typhimurium reverse mutation (OECD 471, plate incorporation)	No positive mutagenic responses. No compound related toxicity.	Negative
Mammalian cell chromosome aberration (OECD 473)	Preliminary assay at 5 mg/mL had precipitation. At >750 µg/mL substantial inhibition mitotic activity. Test conducted at 62.5 – 2,500 µg/mL. No significant induction of chromosomal aberrations.	Negative

Notes:

(a) The pulmonary assay is as conducted by Warheit et al. (2006a, 2007b) with intratracheal administration of 1 mg/kg and 5 mg/kg as described elsewhere in this text. Other tests were conducted according to OECD guidelines.

Source: Warheit et al. (2007c).

8.3.3 Effect of ultraviolet light

Barker and Branch (2008) have recently reported an increasing incidence of unsightly appearance defects upon relatively newly installed prepainted steel roofs and other durable surface coatings. The defects are due to the aggressive photocatalytic activity of nano-particulate, photocatalytic grades of TiO_2 and zinc oxide (ZnO) in sunscreens that have been transferred to the building materials during construction handling. The photocatalytic particles accelerated the weathering of the steel surface by more than 100 fold.

It has been known for some time that photocatalytic grades of nano-size metal oxides in the presence of UV light can potentially generate ROS in *in vitro* cell systems and potentially damage cells and DNA. Some of these studies have been reviewed in Nohyek et al. (2007) and TGA (2006) who have concluded the *in vitro* data is probably not relevant for human risk since the NP containing sunscreens are applied to the stratum corneum which acts as a barrier preventing access to the live cells. The TGA (2006) concluded the "*weight of current evidence is that they remain on the surface of the skin and in the outer dead layer (stratum corneum) of the skin*".

A similar conclusion to the TGA (2006) was reached by SCCNFP (2000). They also indicated coated TiO_2 preparations show much less photo-catalytic activity than the uncoated material. Many of the coating substances applied to TiO_2 were already used as ingredients in cosmetics, and it was considered if they are acceptable in this role they should be acceptable as coatings for titanium dioxide.

Furthermore there are issues with high concentrations used in the in vitro studies and the possibility that the UV irradiation is enhancing the susceptibility of mammalian cells to the nanomaterial rather than the effect being a genuine photo-toxic property (Dufour et al. 2006).

SCCNFP (2003) evaluated the toxicological database of ZnO in relation to its use in sunscreens. The SCCNFP was of the opinion that more information was needed to enable a proper safety evaluation of micronised ZnO for use as a UV filter in cosmetic products. Consequently, an appropriate safety dossier on micronised ZnO itself, including possible pathways of cutaneous penetration and systemic exposure, was required.

The potential for nano- TiO_2 plus UV catalysed biological effects continues to be explored:

- Theogaraj et al. (2007) investigated eight different classes of ultrafine TiO_2 particles for photo-clastogenicity in Chinese hamster ovary (CHO) cells with and without UV light. None of the particles tested induced any increase in chromosomal aberration frequencies either in the absence or presence of UV

- On the other hand, in *in vitro* systems it has been demonstrated the combination of different sized ZnO nanoparticles (Guo et al. 2007) or nano-TiO_2 (Song et al. 2006) and daunorubicin, an anticancer drug, under UV irradiation have interactive cytotoxic effects on leukaemia cancer cells which according to the authors presents opportunities for enhanced cancer treatment in the future

- Arsac and Hidaka (2007) found the concentration of sodium chloride in anionic vesicles did not influence the damage caused by nano- TiO_2 (uncharacterised) to DNA in an acellular system.

Overall, the UV enhanced *in vitro* cytotoxicity and genotoxicity of nano-TiO_2 may or may not be relevant for human safety. Most information points to there not being a significant hazard if the material in sunscreens is applied to intact skin. Data for effects on damaged skin were not located for the publication period covered for this review. It is noted that, as with other ENPs, surface coating can change the photocatalytic activity of nano-metal oxides (SCCNFP 2000, Hidaka et al. 2006) and it is not clear whether the nano-material tested *in vitro* in the scientific literature is the same as that which is used in sunscreens or cosmetics. The weight of evidence from propriety data

(see **Section 8.1**) indicates various coated and uncoated nano-TiO_2 are negative in tests for photo-toxicity, photo-irritation, and negative for photo-mutagenicity.

Finally this brief discussion on the relationships between ENPs and UV light does not constitute an assessment of the safety or otherwise of sunscreens containing potentially photoactive ENPs. The reader is referred to the appropriate authority for such assessment, in Australia it is the Therapeutic Goods Administration (TGA 2006).

8.4 Conclusions for TiO_2

Titanium dioxide (TiO_2) is generally regarded as a poorly soluble low toxicity particle (ASCC 2006, NIOSH 2005).

Prolonged exposure to inhaled particles at sufficiently high concentrations in experimental animals may lead to impairment of normal clearance mechanisms in the alveolar region of the lung, resulting in a continued build-up of particles that eventually leads to excessive lung burdens accompanied by chronic alveolar inflammation.

The International Agency on Cancer (IARC) has recently classified TiO_2 as a Group 2B carcinogen – possibly carcinogenic to humans. The pivotal data were lung tumours in rats after chronic high exposures. Mice and hamsters did not develop tumours. It is acknowledged the rat tumours are the result of particle overload, but since the mechanisms involved in the overload scenario are potentially applicable to humans should lung burdens become high, the rat data was considered relevant for humans. Nevertheless there is consensus that the mechanism involves ROS and chronic inflammation in the lung which has a threshold.

Some intratracheal and acute inhalation studies did not observe the expected increased pulmonary toxicity with increased surface area (Grassian et al. 2007b, Li et al. 2007b).

The media (deionised water and phosphate buffered saline) used to prepare suspensions for intratracheal instillation and aerosols inhalation has resulted in agglomeration and aggregation of TiO_2 particles thus complicating the particle characterisation and interpretation of the results (Foucaud et al. 2007, Grassian et al. 2007b, Ma-Hock et al. 2007, Sager et al. 2007, Warheit et al. 2007a).

Reviews of dermal absorption studies for nanoscale TiO_2 consistently conclude that TiO_2 is not absorbed through skin (BAuA et al., 2006, TGA 2006, Nohynek et al. 2007).

Systemic distribution was investigated following single dose toxicity studies via intravenous and oral routes of administration (Fabian et al. 2008, Umbreit et al. 2007, Wang et al. 2007a). The high dose oral studies show nano-TiO_2 can be absorbed from the gastrointestinal tract and be distributed throughout the body. Neither the intravenous or oral studies indicate nano-TiO_2 retention within blood cells, plasma, brain or lymph nodes but they do show significant retention by the liver, spleen, kidney and lung for observation periods of either 14 or 28 days. Distribution studies after repeated doses, low oral doses or inhalation exposure were not found.

There is sufficient data indicating anatase nano-TiO_2 is potentially more toxic than the equivalent sized rutile nano material. However all forms and sizes are able to induce a

pulmonary inflammatory response if the exposure is high enough, and, as evidenced by the rat inhalation cancer studies, such high doses can lead to long term effects.

Consideration should be given to adoption of the occupational exposure standards for crystalline quartz as a default occupational exposure standard for non-functionalised anatase nano-size TiO_2 until such time that a specific standard can be created, or adopted from overseas.

Application of a variety of traditional hazard/safety tests to nano-TiO_2 has shown the material to be of low acute toxicity. Although these have not examined long term inhalation exposure, there are probably also low chronic risks to human health if exposures are below the threshold for initiating sustained pulmonary inflammation.

The existing long term rat cancer studies provide an opportunity for detailed dose response analysis and identification of safe exposures for workers. This work is currently underway in the US. In the mean time it would be prudent to minimise worker exposure to nano- TiO_2 concentrations as much as possible.

9. Quantum dots

Quantum dots are semiconductor nanocrystals (~ 2–100 nm) that have metal cores and unique optical and electrical properties useful in biomedical imaging and electronics industries.

Hardman (2006) has reviewed the literature pertaining to the toxicology of quantum dots (QDs). As with other nanomaterials understanding the potential toxicity of QDs requires a fundamental grasp of their physicochemical properties. Structurally, QDs consist of a metalloid crystalline core and a “cap” or “shell” that shields the core and renders QDs bioavailable. Cores of QDs consist of a variety of metal complexes such as semiconductors, noble metals, and magnetic transition metals. Newly synthesized QDs are inherently hydrophobic. To render them biologically compatible/active, the QDs are “functionalized,” or given secondary coatings, which improves water solubility, core durability, suspension characteristics in fluids, and assigns a desired bioactivity. Obviously not all QDs are alike. A key factor in the toxicity of QDs is their stability (*in vivo* and during synthesis and storage) and as a consequence the bioavailability of metal in the core.

Much concern has been expressed in the literature regarding leaching of the metals from QDs and possible toxicity. For example Cho et al. (2007) demonstrated the *in vitro* toxicity and cell death of human breast cancer MCF-7 cells by Cd/Se quantum dots was a combination of the direct toxicity of Cd^{++} and the reactive oxygen species (ROS) induced as part of the oxidative stress associated with the dot per se. Stern et al. (2008) also concluded QD cytotoxicity is dependent upon the properties of the particle as a whole and not exclusively the metal core materials. These authors discovered extensive autophagy[64] in porcine renal proximal tubule cells in the absence of oxidative stress and suggest it may represent a common cellular response for nano-materials.

Section 6.2 discusses aspects of the absorption, distribution, metabolism and excretion of ENPs, including QDs. Not surprisingly, these parameters are highly variable due to the wide variation in the physicochemical properties of QDs (Hardman 2006). Metabolic processes and excretory mechanisms are poorly understood and have not been well studied.

Dose dependent cytotoxicity has been demonstrated for some QDs. However the relevance of such *in vitro* toxicity is debatable since some QDs shown to be cytotoxic have no observable toxicity when injected into animals. In many instances QDs are recognized as foreign by the body and are sequestered by the reticuloendothelial

[64] Autophagy, or autophagocytosis, is a catabolic process involving the degradation of a cell's own components through the lysosomal machinery. Autophagy was originally defined as a process of protein recycling and releasing cellular nutrients in times of stress. Autophagic cell death is a caspase-independent process that exhibits an extensive autophagic degradation of the Golgi apparatus, the polyribosomes, and the endoplasmic reticulum, with all these features preceding the destruction of the nucleus. Autophagy differs from apoptosis in that it is a caspase-dependent process characterized by the condensation of cytoplasm and the preservation of organelles, essentially without any autophagic degradation.

system of the major organs but appear not to cause acute toxicity (**Section 6**). Long term studies have not been conducted.

In medical imaging applications the route of exposure to QDs is well defined and likely to be intravenous, hence this is the route most used in distribution studies. However, in the workplace the likely routes of exposure are dermal and inhalation. Under what conditions QDs aerosolize and whether they form aggregates in ambient air are not known.

9.1 Summary

- *Quantum dots (QDs) have metal cores which may be exposed and perhaps initiate toxicity, if the material is not stable either in storage or in the body.*
- *A variety of surface coatings have been applied to QDs to make them biocompatible and confer desired functionality. Consequently their distribution after injection is variable.*
- *In many instances QDs are recognized as foreign by the body and are sequestered by the reticuloendothelial system of the major organs, as are many other ENPs, but appear not to cause acute toxicity after intravenous injection.*
- *Inhalation studies with QDs were not located for this review.*

10. Conclusions

Over the last few years there have been numerous publications reporting a variety of biological and toxicological interactions of ENPs in *in vitro* and *in vivo* experimental systems. A wide range of biochemical and toxicological endpoints within each system have been reported. Most have been directed to proinflammatory and inflammatory markers since existing knowledge on the health effects of ambient fine particulates and ENPs has identified a central role for oxidative stress and inflammation in the toxicological mode of action of NPs.

In isolation *in vitro* data, at least with the current state of knowledge, is unlikely to be able to be used for hazard identification, regulatory classification, or in risk assessments. However the *in vitro* toxicological methods can be used to inform on success of ENP purification processes, batch preparation consistency and the impact that ENP functionalisation or coating may have on their biological effects. In addition, results from *in vitro* experiments could contribute to a weight of evidence evaluation of the safety of ENPs.

Workers are exposed to ENPs primarily by inhalation and any potential effects are the result of a number of integrated physiological processes (e.g. absorption, distribution, elimination, defence mechanisms etc). Since these processes are not present in *in vitro* experiments inhalation data is more appropriate for workplace hazard and risk assessment than is *in vitro* data. Until very recently there had been few inhalation experiments with ENPs, especially with repeat exposures. Chronic inhalation exposure studies for ENPs are not available. This represents a significant knowledge gap primarily responsible for uncertainty regarding the long term occupational safety of ENPs.

Most whole animal toxicological information has been obtained from single intratracheal instillation of ENPs directly into rat lungs. While these studies have shown many ENPs can elicit a burst pulmonary inflammatory response this is a common defence response within the lungs to a variety of agents (infectious and non-infectious). In some circumstances the inflammation is prolonged, and at least within the experimental observational time frame (usually at most 28 days post exposure) pathological changes, typical of high lung burdens of biopersistent particulates, have been observed. In evaluating these studies special care needs to be given to the administered dose as in many instances the defence mechanisms of the rodent lung may have been overwhelmed by particle overload and the study does not investigate the direct intrinsic toxicity of the ENP. During the period covered by this review, data from a few mouse repeat pharyngeal aspiration exposure experiments have been published. While this technique is less invasive than intratracheal instillation it is still not equivalent to inhalation exposure. Nevertheless the results broadly confirm the data obtained by intratracheal administration, i.e. ENPs can cause lung toxicity (characterised as inflammation, fibrosis and granuloma) if tissue burdens exceed or perturb defence mechanisms.

ENPs, particularly when in high concentrations form agglomerates whose size is much larger than the individual ENP. Well dispersed ENPs have greater biological effects than aggregated material. The dispersion and hence effects of ENPs in *in vitro* and *in vivo* experiments is markedly affected by the dispersion agent used to administer the material into the experimental system. Criteria for assessing experimental data and

whether it could be given a high weighting in an evaluation data set should include whether the ENP has been physically characterised in the medium used in the experimental system.

There is a dearth of information on occupational ENP exposures and characterisation of airborne ENPs. It is therefore uncertain if the ENP material used in experimental tests is the same as that to which workers may be exposed.

Biopersistence is an important determinant of the pulmonary response to particulates and ENPs since it determines whether they will accumulate to a level that may potentially give rise to chronic adverse health effects. Unfortunately agreed criteria for judging biopersistence of ENPs has not been established and not many ENPs have been characterised in this regard. Nevertheless the criteria and tests formulated for biopersistence of synthetic mineral fibres by the European Union is a good starting point for evaluating ENPs.

Many experiments have shown nanoparticles can be absorbed from the lungs into the systemic circulation. Limited experiments with intravenously administered ENPs show avid uptake by the tissue reticuloendothelial system and long retention by tissues, particularly the liver, spleen, lungs and kidneys. The toxicological evaluations undertaken in these studies are limited but at this time serious systemic effects have not been observed. It is emphasised however the number of ENPs tested is small, have involved only single dose exposures, and observational periods are short (up to approximately 3 months). Similar outcomes have been observed when 'traditional' tests for evaluating the short term safety of chemicals have been applied to ENPs (i.e. acute oral toxicity, skin and eye irritation, dermal sensitisation, bacterial mutagenicity and *in vitro* mammalian chromosomal aberration tests).

Due to their physical similarity to pathogenic mineral fibres, SWCNTs and MWCNTs have been investigated with respect to their ability to cause fibre like reactions (fibrosis, granuloma and mesothelioma).

For MWCNTs the following appears evident:

- In cultured mesothelial cells MWCNTs are activators of molecular events associated with mesothelial development and can stimulate mesenchymal cell growth

- In the mouse intraperitoneal fibrogenic screening assay long straight thin MWCNTs (i.e. pathogenic fibre like according to WHO definition) caused an inflammatory and granuloma response typical of pathogenic fibres. In a modification of the assay that made it more sensitive, and letting the animals live long enough to allow development of tumours, MWCNTs resulted in deaths due to mesothelioma. These effects did not occur with CNTs that were not long and thin, or with fullerenes

- In mice, pharyngeal aspiration of long thin MWCNTs resulted in sub-pleural lesions and penetration of the pleura by MWCNTs (preliminary unpublished data)

- Inhalation exposure of mice to aerosols of MWCNTs resulted in inflammatory foci on the pleural surface and some MWCNTs embedded within the pleural wall (preliminary unpublished data).

Each of the above pieces of information individually has experimental uncertainty associated with them. Nevertheless they collectively form a weight of evidence that suggests long thin MWCNTs (i.e. those of pathogenic fibre dimensions) present a mesothelioma hazard to workers if they are inhaled. Data to date indicate 'non-pathogenic fibre' (i.e. those less than 5 μm long and with an aspect ratio <3:1) MWCNTs do not have this hazard. Although SWCNTs have not been subjected to the same tests as MWCNTs, at this time it would be prudent to assume that if they are of pathogenic fibre dimensions, and are biopersistent, then they may also present fibrogenic and mesothelioma hazards as do MWCNTs with these physical properties.

A risk assessment issue for fibre-like CNTs is whether, or if, they can become airborne in the workplace, and if they retain their fibre-like characteristics and can be inhaled by workers.

In summary, the understanding of the biological and toxicological effects of ENPs has significantly advanced in the last few years. Much of this has been in relation to working out what type of physical characterisation and toxicological data is required for hazard and risk assessment, and how to go about obtaining it. Serious adverse effects have not been observed in limited applications to ENPs of 'traditional' tests for assessing the acute toxicity of chemicals. The toxicological data sets available for ENPs remain rudimentary, for example long term inhalation studies, reproductive or developmental studies are not available. The fact that, if NPs are absorbed into the systemic circulation they may be retained within cells for long periods makes it imperative that chronic studies be undertaken for hazard and risk assessment of ENPs.

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Appendix 1: Experimental considerations

1.1 Intratracheal instillation

Current understanding of the mode of action of fine particulates and paradigms for assessing their health risks revolve around two central elements; persistence of the particulates in the lung and the production of sustained inflammation and oxidative stress. Since contemporary understanding indicates chronic pulmonary effects are more likely to be due to retained particulate dose, it is important, as part of the hazard characterization of nanomaterials to understand their deposition and retention in the lung.

There have been few repeat inhalation exposure studies with engineered nanoparticles. On the other hand many rat intratracheal experiments have been reported for a wide variety of nanomaterials. In order to properly evaluate, and place in context, data derived from intratracheal experiments it is important to understand particular aspects of lung physiology and the constraints placed on interpretation by the technique.

Advantages of intratracheal installation include:

- Simplicity of technique
- Specialised equipment is not needed
- Cost is lower
- Relatively low amounts of material are required. For engineered nanoparticulates not only may there not be enough material to generate sufficient air concentrations for an adequate duration, procedures for consistently producing an atmosphere of individual nanoparticles for inhalation studies are not widely available
- The actual dose(s) delivered into the lungs is known
- Exposure of skin and fur, with subsequent oral exposure via grooming, is avoided
- Long fibre-like structures like asbestos and such as can occur with aggregates of carbon nanotubes, which are respirable by humans, may be removed by the relatively convoluted nasal turbinates of rodents. Especially rats which are obligate nasal breathers
- If conducted appropriately:
    - qualitatively similar results are obtained as with inhalation studies.
    - intratracheal studies can inform on dose ranges for subsequent inhalation studies
- It is a suitable screening tool in a tiered program of hazard identification.

Some disadvantages of intratracheal installation are:

- Nonphysiologic dose route
- It is invasive, requiring light anaesthesia
- Various aspects of the technique potentially affect routes and rates of clearance from the lungs:
    - Dose, and dose rate (i.e. bolus), are usually greater than what would occur with inhalation.
    - Material in liquid dispersion is not in the form of potential human exposure and will likely result in different pulmonary distribution compared to that from inhalation
- The technique only addresses potential effects on the lower respiratory tract (the nasal, oral, pharynx and larynx areas are bypassed)
- The liquid vehicle may have effects on its own, as well as influence the distribution and effects of the test nanoparticles.

Since many studies evaluating the impact (hazards) of different substances employ direct intratracheal instillation, the Inhalation Specialty Section of the Society of Toxicology produced a document summarising some key issues concerning the technique (Driscoll et al. 2000).

The persistence of particles and fibres in the lung is considered to be a prerequisite for induction of adverse pulmonary effects. Persistence can be an innate property of the NP, or it can be achieved artificially in the experimental protocol by instilling large amounts of NP so lung clearance mechanisms are overwhelmed and the material remains in the lung for a long time. This phenomenon is referred to as particle overload. The rationale for such large exposures is they potentially mimic the lung particulate burden that may accumulate with repeated exposures over a long period, say a working lifetime. It should be remembered however that clearance is a continuous process and that accumulation is not an inevitable outcome of repeated low level exposures.

In assessing the potential carcinogenicity of carbon black and TiO_2 IARC considered the relevance of rodent particle overload mechanisms for humans. They stated (Baan 2007):

"*Among rodents, species differences have been observed in the effectiveness of lung clearance, the degree of overloading, and the severity of disease response following inhalation exposure to fine and ultrafine poorly soluble particles: Hamsters had relatively effective lung clearance and also little overload or adverse response compared to mice or rats, whereas rats had more severe lung responses, including inflammation and tissue injury, compared with mice or hamsters (Bermudez et al. 2002, 2004; Elder et al. 2005). Induction of lung tumours has been observed in rats, but not mice or hamsters, following chronic inhalation of poorly-soluble particles, although most of the studies have been in rats (Lee et al. 1985a, 1985b, 1986; National Toxicology Program, 1993; Heinrich et al. 1995; Nikula et al. 1995).*

The rat lung response to inhaled, poorly soluble particles is more closely associated with particle surface area (Tran et al. 2000) or with particle size and volume (Pott and Roller 2005) than with particle mass. Thus, for a given particle mass lung dose, ultrafine particles would be expected to elicit a stronger response than fine particles."

It was also stated (Baan 2007) that ultrafine particles may cause impaired lung clearance at mass doses in rat lung that are much lower than those associated with classical overload (Bellmann et al. 1991 and Morrow 1992 are cited to support the conclusion[65]). Reasons for this phenomenon were not presented by the IARC evaluating group. However, for an equal mass, there will be much greater number of ultrafine particles than fine particles. Hence the reason may because a greater number of particles, or greater volume, is taken up by macrophages for ultrafine powders compared to fine powders thereby causing more ROS formation and increased/sustained inflammation.

Dose considerations are particularly important when considering the likely importance of data from intratracheal studies. The data from Li et al. (2007b) illustrates the problem. Intratracheal installation of mice with MWCNTs[66] at 50 µg/mouse lead to similar size clumps distributed in bronchi and alveoli. The clumps caused inflammation of the lining of the bronchi and severe destruction of alveolar structure. On the other hand inhalation exposure for 5, 10 or 15 days (6 hr/d) to a mean average concentration of 32.6 mg/m^3 of aerosolized MWCNTs gave smaller aggregates in the alveoli than in bronchi, and although there was proliferation and thickening of alveolar walls, the damage was significantly less than that observed with intratracheal instillation. Using a fractional deposition of 4% and **Equation 1A** in **Appendix 2**, the authors calculated the amounts deposited in the lungs for the inhalation study were 70, 140, and 210 µg/mouse respectively. The inhalation exposure comprised of 4 x 1.5 hr exposures throughout the day and although the mean MWCNT aerosol was 32.6 mg/m^3, the concentration dropped from about 80 mg/m^3 to 13 mg/m^3 over each exposure period. The authors thought the marked difference in response to MWCNT by instillation versus inhalation was the material bypassing the lung clearance mechanisms with the former, whereas the slow regular exposure by inhalation allowed some clearance to proceed. Their overall conclusion was that the different pathological lesions of MWCNT after instillation and inhalation may be due to different size aggregations and distribution in the lung.

Conducting inhalation studies with NPs is difficult for a number of reasons, not the least of which is that it is not easy to dependably generate NP aerosols to achieve a constant target concentration of NPs. Ostraat et al. (2008), from DuPont engineering, describe a 'reactor' that can be used generate a continuous, stable supply of aerosol NPs to facilitate inhalation toxicity studies, measure explosion characteristics of

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[65] Inspection of the references cited by Baan (2007) in support of pulmonary overload occurring with lower mass amounts per lung of ultrafine particles revealed they did not discuss the influence of particle size *per se* on the overload phenomenon. It would appear that IARC base this statement primarily on the lung response and lung tumours occurring at lower airborne mass concentrations than with fine particulates. A number of studies with different insoluble particles are cited in support. For contextual information ultrafine TiO_2 has primary size of 10 – 50 nm but fine TiO_2 200 – 300 nm (Baan 2007).

[66] The MWCNTs had an average diameter of 50 nm, length 10 µm, surface area 280 m^2/g, purity >95%. They were used as purchased from the Chinese manufacturer. MWCNTs were suspended and sonicated in saline containing 1% Tween-80, after a single dose of 50 µg/mouse pathological lesions were evaluated 8, 16 & 24 days post installation – suspensions of MWCNT were not characterised.

aerosol NPs, and to test the barrier efficiency of respirator filters and personal protective clothing and bag house exhausts. Baron et al. (2008) described a complicated apparatus for aerosolisation of SWCNTs.

1.2 Intraperitoneal exposures

Intraperitoneal administration of insoluble mineral fibres has been used for many decades to probe the processes of mesothelioma (e.g. Davis 1974). Moalli et al. (1987) used the technique to show that thin long asbestos fibres (<0.25 μm diameter and >8 μm long) were highly tumourigenic whereas shorter asbestos fibres or spherical mineral particles (toxic silica and non-toxic TiO_2 particles) were not. Silica and TiO_2 were cleared from the peritoneum without provoking an inflammatory reaction or mesothelial injury whereas the long asbestos fibres were trapped on the peritoneal membrane surface and there was an intense inflammatory reaction with accumulation of activated macrophages.

Roller et al. (1997) investigated tumour incidence dose-response relationships of 13 fibrous dusts[67] in intraperitoneal studies. Although there was a wide range of potencies, the dose response curves for different fibres were parallel when dose was defined as number of fibres with certain dimensions. The authors considered the experimental evidence was consistent with the assumption that the mechanism responsible for the mesotheliomas in this experimental system is specific for the fibrous shape of the particles administered.

It is easy to question the relevance to humans of data that has been produced by injecting a bolus mass of fibre into the abdominal cavity of a rodent; there are concerns about the unphysiological nature of the exposure and possible 'overload' perturbation of normal physiology.

Weighing against the above apprehensions are the following:

- The mesothelial tissue within the abdominal cavity is the same as that in the pleural cavity where fibre-induced tumours occur in humans after prolonged inhalation of fibrogenic fibres. The responses of the two tissues would be expected to be the same

- The test allows early fibrogenic events to be evaluated within a short time after dosing; inflammation with days and granuloma within weeks. Tumour formation can be assessed if the animals are kept, with or without further dosing, for about two years

- In this test when the fibre structure disappears by dissolution or breakage the carcinogenic properties also disappear

[67] One granular dust (silicon carbide), two asbestos dusts (crocidolite and tremolite) and 11 dust samples from man-made vitreous fibres. Dose was defined as the number of fibres with length>5 μm, diameter<2 μm and length/diameter>5/1. Tissue samples were examined histologically and animals counted positive (incidence used for probit dose response evaluation) if at least one tumour was found in the abdominal cavity and evaluated as a mesothelioma.

- The test model clearly differentiates the carcinogenic potency of long vs short, and between durable and nondurable fibres. In this regard it is consistent with other data, including human experience

- It is argued that the rat inhalation model for asbestos fibres is relatively insensitive (by approximately 200 times) compared with their carcinogenicity in humans. The rat inhalation model may therefore not be appropriate for identification of biopersistent fibre hazards for man

- The rat respiratory anatomy is very different from humans such that inhaled long thin fibres may not reach the tissue site of action in rats unless high airborne concentrations are used

- Inhalation studies in rats are difficult and expensive; in contrast the intraperitoneal model is quick and economical.

p53 knockout enhancement

Expression of p53 protein is an early cellular response to DNA damage, the protein induces transcription of genes that activate cell cycle checkpoints and direct a cell with DNA damage to self destruct via apoptosis. There are many studies showing mice deficient in p53 are very susceptible to chemical carcinogens and also to cancer induced at the result of chronic inflammation. In addition p53-deficient mice ($p53^{-/-}$ or $p53^{+/-}$) show a predisposition to spontaneous development of various tumours (Donehower et al. 1992, Harvey et al. 1993).

Marsella et al. (1997) injected[68] wild type or p53 deficient mice ($p53^{-/-}$ or $p53^{+/-}$) weekly with crocidolite asbestos. Unlike the wild type and heterozygous ($p53^{+/-}$) mice, many of the $p53^{-/-}$ mice died of spontaneous lymphomas or hemangiosarcomas before developing mesotheliomas. However, spontaneous mesothelioma is rare in these mice. On the other hand with crocidolite 76% p53 heterozygous mice and 32% wild type developed mesothelioma. The authors referred to the literature to support a statement that spontaneous mesotheliomas had not been observed in these mice. Using the same dose regime, Vaslet et al. (2002) showed crocidolite induced malignant mesotheliomas developed much earlier in $p53^{+/-}$ mice (32 weeks vs 44 weeks latency).

Chronic inflammation set up around a foreign body is a major factor for the development of 'foreign body' induced sarcomas (Brand et al. 1977). Tazawa et al. (2007) subcutaneously implanted plastic discs in $p53^{+/-}$ mice. A large proportion (79%) of $p53^{+/-}$ mice developed sarcomas around the implant compared to 10% of $p53^{+/+}$ mice, also the time for tumour development was shorter. Spontaneous sarcomas were observed in $p53^{+/-}$ mice with no plastic disc implant with an incidence of 20%. Inflammatory markers were significantly increased around implant tumours compared to spontaneous tumours.

It is evident $p53^{+/-}$ mice are very sensitive to cancer formation and are useful for studying carcinogenic mechanisms of substances. However, if they are applied as a

[68]Treatment was weekly intraperitoneal injections of crocidolite 200µg (5.8×10^8) for 35 weeks.

screening test for identifying fibrogenic hazards of NPs after intraperitoneal administration of NPs it is unknown whether the heightened sensitivity may result in a significant number of false positive results. Donaldson et al. (2008a) have questioned whether responses seen with massive doses of CNT (3,000 µg/mouse) reflect fibre responses or a general solid state carcinogenic response mediated by non specific inflammation. This is discussed further in **Section 7.5.2**.

1.3 Dispersal, agglomeration and biological dose

Generally agglomerates/aggregates appear to be less toxic in *in vitro* systems than well dispersed nanoparticles (Johnston et al. 2000, Gordon et al. 2008). However this is not always the case; the cytotoxicity of purified SWCNTs was significantly less[69] when they were well dispersed compared to aggregates (Wick et al. 2007). The authors concluded the quality of the dispersion of SWCNTs is of extreme importance and is needed to be known to make accurate interpretation of *in vitro* cytotoxic data.

It had previously been shown that the type of pulmonary response was influenced by the extent of dispersion of NPs in the lung (Shvedova et al. 2005a); patently the *in vivo* dispersion is in part determined by the extent the administered NP is dispersed in the dosing media. This has recently been confirmed by the demonstration that the toxicological response of mice to 10 µg of aspirated well dispersed SWCNTs was different from that of less well dispersed SWCNTs (Mercer et al. 2008). For the latter, light microscope investigation of lungs showed large aggregates 2 – 20 µm in diameter. The lung response was recruitment of macrophages which encased the agglomerates and formed a connective-rich granulomatous inflammation, typical of responses to insoluble particles. On the other hand dispersed SWCNTs resulted in a diffuse interstitial distribution; macrophage phagocytosis, epithelioid macrophages or granulomatous lesions were not observed but there was a more general connective tissue response seen by increased alveolar thickness.

Dispersal medium

There has been active research over the last few years seeking the most appropriate dispersal medium for *in vitro* and *in vivo* nanotoxicology experiments.

Sayes et al. (2007b) found the aggregation state of different NPs varied according to the dispersion medium. For fine ZnO and nano-ZnO, the size distribution of particles was much greater in PBS than either water or culture media. In water the average size of anatase UF TiO_2 was 129 nm but in phosphate buffered saline (PBS) it was 2,961 nm (Warheit et al. 2007b).

Tabet et al. (2009) found MWCNTs formed aggregates on top of cultured cells that were significantly larger and more numerous in PBS than in alcohol or in dipalmitoyl lecithin.

[69] Human mesothelioma cells (MSTTO-211H) were exposed for 3 days to 7.5, 15 & 30 µg non-purified commercial SWCNTs, or agglomerates, pellets or well dispersed bundles made from purified commercial SWCNTs. The sizes of SWCNTs were not provided. Dispersal was achieved with Tween 80 and sonication. All SWCNTs, except the well dispersed bundles, were aggregated after the incubation time to micron-sized structures (Wick et al. 2007).

Using Pt-NP of different shapes Elder et al. (2007) also found large aggregates/agglomerates were formed in 0.9% saline and phosphate buffer but much smaller ones in culture medium. These workers conducted mass balance calculations of the Pt-NPs but could only account for 12 – 51% of the initial dose. They concluded the losses most likely occurred prior to cell culture exposure because the amount of Pt taken up by the cells and the amount found in the supernatant were both linearly related to dose. The point was made that sources of NP loss must be considered in *in vitro* and *in vivo* studies of NP effects because this gives rise to discrepancies between actual and targeted doses. This aspect is not considered by the majority of publications investigating the toxicity of NPs.

When diesel soot agglomerates are mixed with dipalmitoyl phosphatidyl choline (DPPC), a surrogate for lung surfactant, they are observed to disperse and the expression of genotoxic activity increased (Wallace et al. 2007). After reviewing the literature dealing with preconditioning of respirable diesel exhaust particles and silicate insoluble particles, Wallace et al. (2007) concluded lung surfactant surrogate had profound effects on the expression of particulate toxicity, suggesting that interactions of respired NP with constituents of the hypophase liquid lining of the lung should be considered in the preparation and interpretation of assays for potential NP respiratory hazard.

Sager et al. (2007) were concerned that agglomeration and clumping of NP in intratracheal instillation experiments not only affected the physiological responses to NP but also influences the dose metric that most appropriately describes the response. Using SEM and TEM they thoroughly examined the dispersion and aggregation of ultra fine carbon black (UF-CB), fine CB and TiO_2 in different media (PBS, rat and mouse BAL fluid, PBS containing dipalmitoyl phosphatidylcholine (DPPC), and mouse serum albumin). PBS was found not to be a satisfactory suspension medium but BAL fluid was excellent; PBS+DPPC, or PBS+protein was not satisfactory, however PBS+DPPC+protein[70] was satisfactory but not as good as BAL fluid. These data are similar to the findings of Wallace et al. (2007). It is noted in this review that many intratracheal studies conducted over the past few years have used PBS or BSA for the instillation medium of NP.

It would seem intuitive that the best dispersal medium for delivering NPs to the lungs may be BAL fluid from rat lungs, however as pointed out by Porter et al. (2008) this has a couple of significant drawbacks; the first is the cost and the second is the fact it is not standardised, the BAL differs from rat to rat. To address these issues Porter et al. (2008) devised a dispersal medium of Ca^{2+} and Mg^{2+} free PBS supplemented with D-glucose, species specific serum albumin and DPPC. Using carbon black, TiO_2 and MWCNTs they showed this was an effective, biocompatible and economical vehicle for nanotoxicological studies.

Schulze et al. (2008) have recently provided practical advice to experimenters in overcoming the pitfalls when dispersing NPs in physiological media by rigorously characterising the interaction of metal oxide NPs with various media used for *in vitro* experiments. They emphasize the often overlooked imperative of sterility. The authors

[70]The concentrations of dipalmitoyl phosphatidylcholine (DPPC) and protein were the same as that found in BAL fluid.

provide a check list of pre-requisites for dispersion of NPs and for reliable attribution of potential toxic effects. For the risk assessor the list could contribute to a weight-of-evidence (WoE) system for evaluating *in vitro* nanotoxicology data.

The medium not only affects aggregation but may also alter surface properties of the NP. Deguchi et al. (2007) found C_{60} NPs coagulated and rapidly precipitated in PBS, but this was completely abolished by the addition of human serum albumin (HSA) or surfactant Tween 80; HSA molecules adsorbed onto the surface of the C_{60} NP, formed a protective layer and prevented salt-induced coagulation. Adsorption of substances onto the surface of NPs is most likely to occur with agglomerates or carbon based NPs. It is noted this phenomenon has been demonstrated to interfere with dye based evaluations of cytotoxicity. Stabilisation of NPs via protein coating during experimentation may also facilitate/alter cellular uptake by endocytosis (Chithrani et al. 2006, Porter et al. 2006).

As noted above, many experimenters suspend their NP preparation in a medium containing a surfactant to help minimise coagulation/agglomeration and disperse the NPs. Dong et al. (2008) studied the cytotoxicity of SWCNTs suspended in various surfactants to cultured human astrocytoma (1321N1) cells. Individual SWCNTs suspended in sodium dodecyl sulfate or sodium dodecylbenzne sulfonate were toxic due to the surfactants adsorbing onto the CNT surface. The proliferation and viability of the cells was not affected by SWCNTs alone or when suspended in sodium cholate or single stranded DNA.

A recent abstract reported a preparation of natural lung surfactant that gave excellent results for dispersing preparations of SWCNTs. Because the preparation is commercially available the authors claim it provides a simple and rapid one step method for dispersing nanoparticles that has major advantages over existing methods (Wang et al. 2009).

Overall, there have been many different types of dispersing agents used in both *in vitro* and *in vivo* experimental systems. Some are more effective than others, which in some instances is dependent upon the type of ENP under investigation. While the addition of protein and/or surfactant enhances dispersion, an agreed dispersion protocol has not been established.

Aging and agglomeration

It has been known for some time that aged crystalline silica has significantly less biological activity than does freshly cleaved alpha silica. This is due to weathering of the catalytic surfaces of the crystal structure. With ENPs, aging may not only change the surface characteristics but also the ENP size, distinguishing between the two is not easy. When mice were exposed by inhalation and evaluated for lung injury and inflammation 24 hours later, freshly generated carbon nanoparticles (10 – 50 nm) were significantly more toxic than aged nanoparticles (160 – 250 nm); a similar trend was observed for fresh and aged copper and zinc nanoparticles (Gordon et al. 2008). In rats a 15 minute inhalation of freshly prepared Teflon fume particles (50 $\mu g/m^3$) with a mean diameter of 16 nm were much more toxic than particles that had been aged for 3.5 minutes to allow coagulation to particles with mean diameters >100 nm (Johnston et al. 2000). While consistent with the notion of lesser toxicity associated with the larger particles, it is also possible the surface chemistry of the Teflon particles may have changed. This study also demonstrated that the pulmonary toxicity could be

prevented by adapting the animals with short 5 minute exposures for 3 days prior to the 15 minute exposure.

Aging, or agglomeration/aggregation, of nanoparticles appears to give rise to lesser pulmonary toxicity than dispersions of the original nanoparticle. This has obvious implications for measurements of ENPs in the workplace and for occupational risk to ENPs. No specific additional information on protection from the pulmonary effects of nanoparticles with short exposures was found for this review. Despite the relevance for workplace exposures this concept does not appear to have been picked up by investigators and therefore remains an open question regarding its reality.

Appendix 2: Experimental dose conversions

Converting a human inhalation exposure to an instilled animal dose

The calculation for converting a human inhalation exposure for time T minutes into an experimental intratracheal dose per animal is provided as Equation A1 below (Whalan et al. 2006, Valentine and Kennedy 2001).

Equation A1

$$D = E_D \times V_m \times C \times T$$

Term	Description
D	Intratracheal animal dose (mg/animal) equating to the deposited amount in human lungs when exposed to workplace concentration C (mg/m^3) for time T (min).
E_D	Deposit efficiency for the substance within the respiratory tract
V_m	Minute volume (litres/min) for given age (i.e. weight) of animal
C	Concentration of the test substance (mg/m^3)
T	Time of exposure (min)

Converting an instilled dose to an equivalent inhalation concentration for human exposure

Rearranging Equation A1 gives Equation A2 below:

Equation A2

$$C = \frac{D}{E_D \times V_m \times T}$$

Example calculations

Intratracheal dose equivalent to human exposure to 10 mg/m^3:

Symbol	Value	Description
D	To solve	Equivalent intratracheal dose (mg/animal) for human lung deposition at exposure of C mg/m^3 for time T (min).
E_D	0.55 (human)	Fractional deposition in humans - size dependent (see Figure A1). For particles of 1-100 nm the maximum fraction deposited in the alveolar region is estimated to be 60% based on conventional models (maximum fraction deposited is 50% for 20 nm particles) however nanoparticle specific considerations could increase alveolar deposition by up to 10%. For the example calculation below a value of 55% for humans has been used. For CNT the fractional deposition in the alveolar may only be 1 – 4%
V_m	0.03/mouse 0.15/rat	Minute volume (litres/min). This parameter depends on the exposed experimental animal and its age. The value used in the calculation should match the species and weight of the animals in the experiment. Minute volume for a 25 g mouse is approximately 0.03 L/min and for a 200 g rat is 0.15 L/min (Whalan et al. 2006).
C	10	Concentration (mg/m^3) of the test substance giving rise to same deposited dose in human lungs as that given experimentally to an animal by intratracheal administration.
T	360	Time of exposure (min/d) in a day that the assumed inhalation exposure occurs. While the work day is 8 hours not all this time is spent in the area were exposure may occur. For the calculations the actual exposure time is assumed to be 6 hours per working day.

For rats

$$D = E_D \times V_m \times C \times T$$

D = 0.55(unitless) x 0.15(L/min) x [10(mg/m^3)/10^3(L/m^3)] x 360(min)

= 0.3 mg per rat (of 200g)

= 1.5 mg/kg

For mice

$$D = E_D \times V_m \times C \times T$$

D = 0.55(unitless) x 0.03(L/min) x [10(mg/m^3)/10^3(L/m^3)] x 360 (min)

= 0.06 mg per mouse (of 25 g)

= 2.4 mg/kg

Human exposure equivalent to intratracheal dose of 5 mg/kg:

Symbol	Value	Description
C		Concentration (mg/m^3) of the test substance giving rise to same deposited dose in human lungs as that given experimentally to an animal by intratracheal administration.
D	0.15mg/ mouse 1.0mg/rat	Intratracheal dose (mg/animal). For the example calculations this is assumed to be 5 mg/kg given to a 30 g mouse or a 200 g rat.
E_D	0.55 human	Fractional deposition in humans - size dependent (see Figure A1). For particles of 1-100 nm the maximum fraction deposited in the alveolar region is estimated to be 60% based on conventional models (maximum fraction deposited is 50% for 20 nm particles) however nanoparticle specific considerations could increase alveolar deposition by up to 10%. For the example calculation below a value of 55% for humans has been used. For CNT the fractional deposition in the alveolar may only be 1 – 4% of the respired dose (Lam et al. 2006, Li et al. 2007)
V_m	0.03 (mouse) 0.15 (rat)	Minute volume (litres/min). This parameter depends on the exposed experimental animal and its age. The value used in the calculation should match the species and weight of the animals in the experiment. Minute volume for a 25g mouse is approximately 0.03 L/min and for a 200 g rat is 0.15 L/min (Whalan et al. 2006).
T	360	Time of exposure (min/d) in a day that the assumed inhalation exposure occurs. While the work day is 8 hours not all this time is spent in the area were exposure may occur. For the calculations the actual exposure time is assumed to be 6 hours per working day.

From mice

$$C = \frac{D}{E_D \times V_m \times T}$$

C (µg/m^3) = (0.15 mg)/[(0.55 x 0.03 L/min x 360 min)] x 10^3 (L/m^3)

= 25 mg/m^3

From rats

$$C = \frac{D}{E_D \times V_m \times T}$$

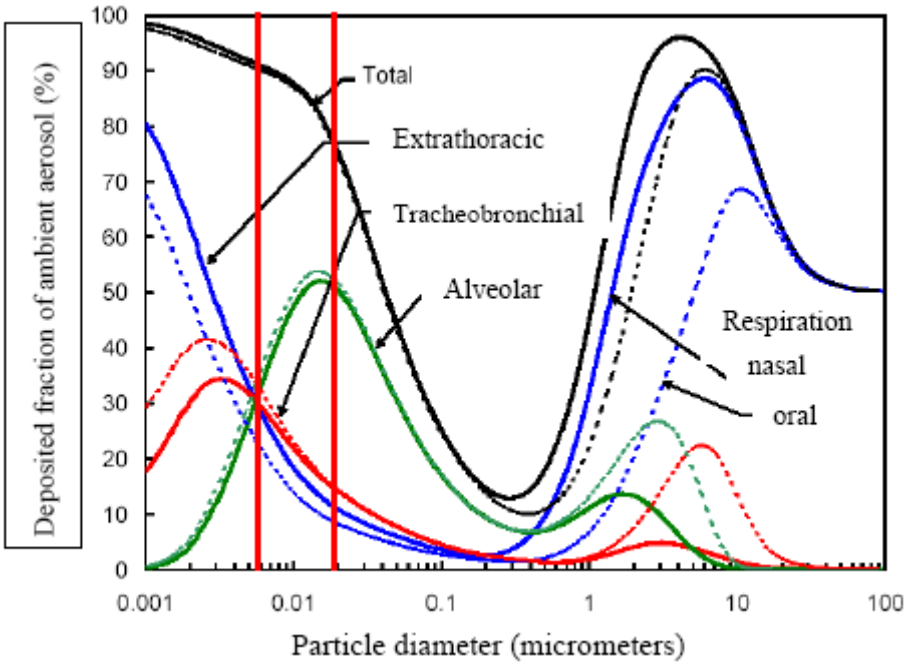
C (µg/m^3) = (1.0 mg)/ (0.55 x 0.15 L/min x 360 min) x 10^3 (L/m^3)

= 34 mg/m^3

Note:

The example calculations assume the deposited dose is due to a single inhalation exposure over a 6 hour period. A similar result can be calculated for a repeated exposure over several days for shorter/longer periods of time per day.

Figure A1: Modelled total and regional deposits of particles in the airway according to particle size



Source: Modelled by Institut National de Recherche Scientifique (cited in IRSST 2007).

The fraction of inhaled nanoparticles that deposits to the alveolar region of the lung varies considerably according to the particle characteristics (size, shape) and their behaviour in air. Understanding the regional deposition of nanoparticles in the lung is important for human health risk assessment and dose conversions. Mathematical models have been developed to predict deposition of inhaled particles (ultrafine dusts) in the lungs of humans and animals. These are largely based on experimental data for ultrafine dusts. There are many deposition models available [Price 2002 (Dutch RIVM), Witschger and Fabries 2005 (French INRS), USEPA 2004, Nordic Council 2007]. These models consistently show that fractional deposition in the alveolar region of the respiratory tract for healthy individuals is greatest for particles in the size range of approximately 10-30 nm as shown in **Figure A1**. The existing mechanistic models may need to be refined for nanosize materials as there are nanoparticle-specific mechanisms of transport and deposition that can alter the deposition efficiency. For example, Asgharian and Price (2007) modelled fraction deposition including nanoparticle specific considerations and reported that fractional deposition in the alveolar region may increase up to 10% depending on particle size.

Lung dosimetry modelling provides a biologically-based method to extrapolate doses from animals to humans. However, current mass-based lung dosimetry models may not fully account for differences in the clearance and translocation of nanoparticles. Using dose-response data in rats chronically exposed to fine or ultrafine titanium dioxide, carbon black, or diesel exhaust particulate, Kuempel et al. (2006) showed the dosimetry models predicted reasonably well the retained mass lung burdens. They note however additional model validation is needed for NPs of varying characteristics,

as well as extension of the models to include particle translocation to organs beyond the lungs.

Kuempel et al. (2006), in a review discussing human dosimetry of NPs, also made the point that fractional deposition of nanoparticles in the alveolar and tracheobronchial regions can be several times higher than that for larger respirable particles. Total NP deposition increases with exercise and is higher in individuals with chronic obstructive lung disease or active asthma. Furthermore, according to Kuempel et al. (2006) several human lung deposition models underestimate NP deposition in exercising individuals. It was pointed out that current human lung models have had limited evaluation of the deposition of NPs <10 nm and of charged particles of nanoparticle size. Unfortunately, although the author discusses the pitfalls, quantitative information on deposition efficiency is not provided.

Research needs for aerosol dosimetry of NPs has been briefly presented by Phalen and Hoover (2006).

Appendix 3: DNA damage by EPs

For organic chemicals, *in vitro* and *in vivo* genotoxicity tests have contributed towards understanding the mechanisms involved in carcinogenesis, and for risk assessment purposes they assist to distinguish between genotoxic and non-genotoxic carcinogens. This has practical risk assessment implications since exposure thresholds are considered to exist for the latter but perhaps not the former. These broad carcinogenic modes of action provide a convenient regulatory risk assessment process separation of carcinogens. For non-genotoxic carcinogens risk assessment is facilitated by identification of a no observed effect level for the key event leading to the tumour response and subsequent application of safety (uncertainty) factors to derive a 'safe' level of exposure. For genotoxic carcinogens, where some risk is assumed to theoretically exist at any exposure level, no matter how low, the experimental (or occupational) dose response for cancer is mathematically extrapolated to derive a statistical cancer potency factor specific for the agent. This can then be used to calculate the potential risk of developing cancer associated with a particular level of environmental or occupational exposure.

The physicochemical properties of ENPs, and particulates in general, are different from other toxicants suspected of being carcinogenic and tested for their ability to interact with genetic material. As with other cancer suspect agents there are primary and secondary pathways that may lead to genotoxicity. Particle size, shape, degree of crystalline structure, solubility, impurities and interaction with cell division may contribute to direct (primary) genotoxicity pathways. These may or may not have a threshold. Excessive and persistent formation of ROS is considered a hallmark of the secondary genotoxicity of poorly soluble non-fibrous and fibrous particles. Since lung inflammation is known to occur and persist only at sufficient particle dose, or lung burden, this secondary genotoxicity pathway is considered to contain a threshold (Greim et al. 2001).

Thus it is important to identify the relative contribution of the primary and secondary genotoxicity pathways of ENPs, under realistic exposure conditions, so a correct risk assessment can be undertaken. The importance of primary versus secondary genotoxicity has been espoused by many authors (Vallyathan and Shi 1997, MacNee and Donaldson 2003, Knaapen et al. 2004, Kisin et al. 2007, Schins and Knaapen 2007).

Current data from *in vivo* tests are usually unable to tell whether realistic ENP exposures might lead to a pulmonary cancer response, or discriminate if pre-cancerous lesions may due to primary or secondary toxicity. This is largely because the tests are short term, excessive exposures have been employed and/or insufficient data has been produced for dose-response analysis.

Suitable *in vitro* genotoxicity tests may assist in hazard identification for primary genotoxicity and thus perhaps a prima facie assumption of carcinogenic potential, as is the case for organic chemicals. However, since most ENPs at some *in vitro* concentration or other can induce oxidative stress and cytotoxicity, the challenge remains to discern whether a positive test for *in vitro* genotoxicity is a primary property of the ENP or is secondary to perturbation of cellular biochemistry and cytotoxicity.

The mechanisms of genotoxicity of particles and fibres, and the major classes of genotoxicity tests used in particle research have been reviewed by Schins (2002) and Schins and Knaapen (2007). Although ROS production is secondary to inflammation some particles, notably those containing Fe, can generate ROS and associated DNA damage in acellular systems. However care is required in interpreting these data as isolated DNA is very vulnerable to oxidative damage. In *in vitro* cell systems a primary ROS genotoxic response can be identified by enhancing genotoxicity through downgrading anti-oxidant defence systems, or conversely by amelioration by means of improving the antioxidant status of the cell.

Cunningham (2007) has reviewed the recent literature for gene-cellular interactions of nanomaterials. Several examples were provided demonstrating that the method of manufacture of the nanoparticles affected its genotoxicity, some but not all the differences were due to co-components or impurities. According to Cunningham no ENP material has been tested with a complete battery of standardized assays and the results for the same type of material using the same test sometimes differed. This signals the need, as also with *in vitro* tests of organic materials, to take a weight of evidence approach before deciding whether an ENP can cause primary genotoxicity and is therefore a candidate to be classified as such. Some of the variability in results is the lack of using standardised reference material or appropriate characterisation of the test material.

Schulze et al. (2008) examined various modifications of two OECD genotoxicity test protocols[71] for suitability for screening insoluble nanoparticles. Ten different nanomaterials were evaluated. The agglomeration state varied with the dispersion fluid (water, DMSO, foetal calf serum, or lung surfactant), in general the highest nanoparticle count was achieved with foetal calf serum. The bacterial mutation assay (i.e. Ames test) showed neither cytotoxicity nor mutagenicity; suggesting perhaps this test is not suitable for ENPs, or that ENPs are not mutagens. It was found that precipitation of agglomerated particles interfered with scoring for chromosomal breakage in the *in vitro* chromosome aberration test. This meant that extremely low concentrations had to be used which were not expected to induce any toxic effects. Overall the tests showed that preparation and characterisation of ENP dispersions is the critical step in applying standard genotoxicity tests to nanomaterials.

The study by Wang et al. (2007b) illustrates some of the difficulties in interpreting *in vitro* genotoxicity data. Ultrafine-TiO_2 was incubated with WIL2-NS cells[72]; concentrations (65 µg/ml or 130 µg/ml incubation medium) that caused genotoxic damage (increased micronucleated binucleated cells, HGPRT mutations and positive comet assay) were also associated with decreased cell viability. The relationship of genotoxicity to the cytotoxicity is unclear, the cells are not normal human cells, and the concentrations in the culture medium cannot be extrapolated to potential human exposures.

[71] The tests are the bacterial reverse mutation assay (OECD 471) and *in vitro* chromosome aberration test (OECD 473).

[72] WIL2-NS cells are human B-cell lymphoblastoid cells that have been transformed with Epstein-Barr virus. These cells are sensitive to genetic damage induced by reactive oxygen species (Umegaki and Fenech 2000) and have a point mutation in the p53 gene (Amundson et al. 1993). The reasons for using this cell line were not provided and the positive control was liquid styrene oxide.

Some studies investigating the interaction of ENPs with DNA were more interested in the phenomenology rather than creating information useful for hazard identification or risk assessment. For example, Arsac and Hikda (2007) studied the influence of salt concentration on the DNA damage caused by UV-A irradiation of nanostructured TiO_2 powder in an acellular system containing anionic vesicles (a crude representation of cell membrane). The conclusion was that the presence of salt can play a deleterious role during the photoinduced process but that the salt concentration had no influence on the DNA damage; on face value seemingly conflicting conclusions.

Schins and Knaapen (2007) have reviewed the genotoxicity of poorly soluble nano and fine particles (TiO_2, carbon black, diesel exhaust particles and crystalline silica) and concluded the available literature indicates their tumorigenesis only involves a mechanism of secondary genotoxicity.

Appendix 4: Concordance of *in vitro* and *in vivo* data

The importance of weight of evidence (WoE) for hazard classification is illustrated by the study of Sayes et al. (2007a). These workers (Sayes et al. 2004, 2005) and others (Mottrom et al. 2006) had previously reported that underivatised nano-C_{60} water insoluble fullerenes were up to orders of magnitude more toxic *in vitro* to a variety of cell types[73] when compared to identical exposures to fully derivatised, highly water soluble $C_{60}(OH)_{24}$ fullerenes, and that the water soluble nano-C_{60} aggregate was toxic *in vitro* (Sayes et al. 2005). Sayes et al. (2007a) intratracheally instilled these two types of fullerenes into rats up to a top dose[74] of 3 mg/kg. While the crystalline silica positive control produced lung inflammatory responses and early lung tissue thickening consistent with the development of pulmonary fibrosis, for both fullerenes there were:

- no alterations in lung inflammation endpoints
- no evidence of adverse biochemical endpoints, and
- no differences in lung tissue sections of exposed rats.

Thus the *in vivo* data was not consistent with that from *in vitro* studies. A similar outcome was found when the results of *in vivo* and *in vitro* data were compared following exposures to fine and/or nano-metal oxide particles (Sayes et al. 2007b).

The Sayes et al. (2007b) study was designed to assess the capacity of *in vitro* screening studies to predict *in vivo* pulmonary toxicity of several fine or nanoscale particle types in rats. It was done in part to assist development and validation of *in vitro* screening protocols to enable hazard identification of ENPs as recommended by Maynard et al. (2006). Five well characterised fine or nano sized particles of characteristic *in vivo* pulmonary toxicity were intratracheally instilled into the lungs of rats at doses of 1 or 5 mg/kg. The inflammatory and cytotoxicity *in vivo* responses were evaluated at various times after instillation, and correlated with *in vitro* parameters for the same responses in three cell systems[75]. When considering the range of toxicity end points to the five different particle types, the authors concluded comparisons of *in vivo* and *in vitro* measurements demonstrated little correlation. This is of concern, given many of the *in vitro* variables assessed in the study such as cell types, culture

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[73] For example human dermal fibroblasts, lung epithelial calls, human liver carcinoma cells (HepG2), and normal astrocytes.

[74] The doses were 0, 0.2, 0.4, 1.5 or 3 mg/kg using Milli-Q water as the vehicle and evaluations conducted 1 day, 1 week, 1 month and 3 months post instillation exposure. The average particle size of the nano-C_{60} water suspension was 160 ± 50 nm, the particles were crystalline and had an average surface charge of -30 mV. The $C_{60}(OH)_{24}$ fullerenes were soluble and no other data could be obtained. Min-u-Sil crystalline quartz (3 mg/kg) served as positive control.

[75] The particles were carbonyl iron (-ve control), crystalline silica (+ve control), precipitated amorphous silica (-ve control), nano sized zinc oxide and fine zinc oxide. *In vivo* times of assessment for inflammation (neutrophil recruitment) and cytotoxicity (LDH activity in BAL) were 1 day, 1 week, 1 and 3 months. The three *in vitro* cell systems were cultures of rat lung epithelial cells, primary rat alveolar macrophages (ex BAL of untreated rats), and a co-culture of both cell types incubated for 4 time periods (1 – 48 hr) with a range of concentrations (0.052 – 520 μg/cm^2, 52 and 520 μg/cm^2 were considered to be particle-overload concentrations). The *in vitro* parameters for inflammation were the cytokines (MIP-6, TNF-α, IL-6) and for cytotoxicity were LDH leakage and the MTT assay. Rat lung epithelial cells were generally the most sensitive to the NPs.

conditions and time course of exposure, as well as the measured end points were correlates with the *in vivo* situation.

The authors also made it clear that *in vitro* cellular systems will need to be further developed, standardized, and validated (relative to *in vivo* effects) in order to provide useful screening data on the relative toxicity of inhaled particle types.

Using platinum nanoparticles of four different shapes, Elder et al. (2007) have undertaken a large comprehensive set of studies as an example of testing nanomaterials of unknown toxicity. From a hazard and risk assessment perspective the striking features of these investigations, which are briefly described below, are that doses were not unrealistic and commentary is made throughout the report on the occupational relevance of the work. The lack of reported toxicological response is in part due to the low intrinsic toxicity of the Pt-NP's, but the fact that no significant toxicity was reported removes a benchmark (the maximum tolerated dose, MTD) that has traditionally been helpful in safety assessment of chemicals. It is however questionable whether the MTD is required for safety assessment of ENPs if worker exposure relevance is inherent in the experimental designs. Given the negative results and the absence of a positive control inserts some uncertainty into the data, nevertheless this is of minor concern because the work was conducted by a group of scientists[76] who have extensively published in the field, have considerable experience with the techniques, and are regarded as leading experts in nanotoxicology research.

Elder et al. (2007) used a tiered acellular, *in vitro* and *in vivo* set of tests to evaluate the safety of Pt-NPs as was recommended by the International Life Sciences Institute (Oberdörster et al. 2005a). All the Pt-NPs were well characterised. In a cell-free assay some Pt-NPs had higher capacity to augment ROS production than others, this was attributed to greater surface area. *In vitro* studies were done with cultured human umbilical vein endothelial cells[77] (HUVEC) and A549 human lung alveolar type II epitheloid cells. In these cell systems the Pt-NPs failed to consistently demonstrate significant dose related oxidative stress or cytotoxicity at doses of 0 – 500 µg/well (0 – 132 µg/cm^2). At the highest dose, considered to be very high and of doubtful physiological relevance, small increases in parameters for cytotoxicity were observed. Agglomeration was greater in phosphate buffer than culture medium, but the lack of oxidative stress and cytotoxicity was not due to due failure of the cells to interact with and take up the Pt-NPs; by TEM both large and small agglomerates were found in the cytoplasm but not the nuclei. However mass balance calculations could only account for 12 – 51% of the *in vitro* doses, this is an issue. The Pt-NPs were also intratracheally administered to rats at 100 µg/rat in 250 µl saline. In comparison to saline exposed controls, two of the Pt-NPs produced small, but significant increases in neutrophils in BAL fluid. No other inflammatory parameters were different from saline controls. The authors considered the response was mild and may have been a function of the intratracheal dosing technique.

[76] The work has been conducted by a scientific consortium lead by Dr Gunter Oberdörster of the University of Rochester.

[77] HUVEC were chosen to model vascular endothelial tissue which might be encountered by NPs should they move out of the lung into the circulation. A549 cells are derived from human lung epithelial adenocarcinoma.

In summary, the Elder et al. (2007) study showed the Pt-NPs do not induce oxidative stress or cytotoxicity in cultured human epithelial or endothelial cells, or *in vivo* at relevant exposures. The acute intratracheal exposure resulted in approximately 10 – 20% of the administered dose being retained in lung parenchymal tissue and phagocytic cells 24 hours after dosing. The authors regarded the mild inflammatory response observed at 24 hours to be transient and would have resolved following clearance of particulates from the lungs. They do caution however that poorly soluble particles can induce severe and persistent lung inflammation following chronic high-dose exposures that overwhelm macrophage mediated particle clearance mechanisms. So, although the data indicates Pt-NPs have low toxicity the authors recommended appropriate engineering controls and personal protective equipment to prevent excessive exposures, as is done for other poorly soluble particles of low toxicity.

The Elder study demonstrates concordance between the *in vitro* and *in vivo* evaluations. But this would be expected if the test material was not intrinsically toxic and/or tested at concentrations below the biological threshold required to elicit a response.