A review of the toxicity of particles that are intentionally produced for use in nanotechnology applications, seen from an occupational health perspective

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Contents

Introduction

Nanotechnology is the production and application of structures, devices and systems by controlling shape and size at nanometre scale (The Royal Academy of Engineering & The Royal Society, 2003). A nanometre is one millionth of a millimetre. The applications to which nanotechnology can be applied are diverse and include electronics, precision engineering and biomedical applications. Similarly, the range of materials that is encompassed under the nanotechnology definition is extensive, and includes nanoparticles, nanocrystals, nanodots, self-assembly monolayers, nanotubes etc.

Two broad categories of nanotechnology can be described:

1) 'top-down' nanotechnology involves creating nanoscale structures by machining and etching techniques;

2) 'bottom-up' technology is the novel synthesis of organic and inorganic structures, atom by atom, or molecule by molecule.

From an occupational health perspective, one key issue that arises from the development of nanotechnology is the potential toxicological properties associated with exposure to materials in the nanometre size range. In this context, exposure to nanometre particulate or fibrous material that has the potential to become airborne is of particular relevance. This review therefore focuses on particles in the nanometre size range that are intentionally produced for application in nanotechnology. It covers existing materials that have applications in the nanometre size range, as well as novel materials. The terms 'particle' and 'nanoparticle' are used in the broadest sense, to include both non-fibrous and fibrous particles. However, where appropriate, a distinction between non-fibrous particles and fibres is made, where this is critical to the understanding of or expression of toxicity.

This review considers the available evidence on the potential health effects of particles that arise from nanotechnology or that have applications within nanotechnology. To date, extremely little has been done to evaluate the potential toxicity of novel nanoparticles arising from nanotechnology and experimental data that addresses human health endpoints (outside medical applications, which are beyond the scope of this review) is extremely sparse. Consequently, this review also draws on information from existing particulate material for which toxicity data are available for both micrometre and nanometre forms, to explore the influence of size on toxicity. It also identifies gaps in the current state of knowledge of the toxicity of nanoparticles.

In considering the toxicity of particles, the focus of this review is on particles of low water solubility, and the toxicological concerns associated with events related to clearance of such particles.

Given that the respiratory tract, and more specifically, the lung itself, is a major target organ for particle-induced effects following inhalation exposure, the potential for local effects on the lung is a key area for consideration in relation to nanoparticles. However, in addition to lung effects, within the last few decades, considerations of particle toxicology have also encompassed possible systemic effects. One key driver for this consideration has been the emergence of evidence for an association between particulate air pollution episodes and an increase in cardiovascular mortality and morbidity in susceptible individuals (EPAQS, 2001). In recent years, it has been hypothesised that it is the finer particulate fraction of air pollution, and possibly particles in the nanometre size range, that are associated with the observed

cardiovascular effects (e.g. EPAQS, 2001; MacNee *et al*, 2000). One key hypothesis that has been proposed to explain the observed link between particulate air pollution and cardiovascular effects is that blood coagulation is altered as a secondary consequence of the pulmonary events following particle exposure (Seaton *et al*, 1995). Increased coagulation would lead to an increase blood viscosity, which could enhance the potential for reduced cardiovascular blood flow and cardiac ischaemia in individuals with compromised cardiac function. Other hypotheses include an effect on neutrophil deformity (MacNee *et al*, 2000) and on atherosclerotic plaque progression and/or destabilisation (Gilmour *et al*, 2004; Suwa *et al*, 2002). However, these remain hypotheses and overall, the role of inhaled particles in cardiovascular effects remains undetermined. In addition, the available experimental evidence from studies of ambient air particles is insufficient to reach any conclusions about the role of nanometre particles in the observed ill-health consequences of air pollution episodes (Kappos *et al*, 2004).

Another aspect of systemic toxicity that has been related to inhalation exposure to nanometre particles but appears not to occur with micrometre particles is 'fume fever'. This acute systemic condition is associated with exposure to metal fumes (e.g. zinc, cadmium) or to fumes of heated polytetrafluoroethylene (Teflon, PTFE). The resultant response is typified by 'flu-like symptoms, that develop a few hours after exposure.

Whilst the mechanism underlying fume fever remains undetermined, it is clear that the effects are related to exposure to freshly formed fume and that 'aged' fume (i.e. fume that has been formed for 3-4 minutes) does not induce a response (e.g. Johnson *et al*, 2000). Such freshly formed fume consists of particles in the nanometre size range (e.g. Oberdörster *et al*, 1995; Amdur *et al*, 1982). As the fume ages, these nanometre particles agglomerate, such that aged fume is composed of micrometre particles. The hypothesis has therefore developed that the nanometre component of freshly formed fume is responsible for the effect; once the particles have agglomerated, toxicity is diminished. However, it is not yet clear whether it is the process of agglomeration itself that leads to a loss in toxicity (i.e. particle characteristics are the same, but the particles are larger) or whether there is some surface characteristic of freshly formed fume that is lost as the fume ages (i.e. nanometre particles of freshly formed fume are a different entity to micrometre particles of aged fume). One other possibility is that highly reactive freshly formed fume acts as a carrier for other gas phase materials that would not normally reach the alveoli and it is the gas-phase materials that are ultimately responsible for the 'fume fever' (Johnson *et al*, 1996).

Overall, although a causative association between inhalation exposure to nanometre particles and systemic effects has not been clearly demonstrated, either for ambient air pollution or for fume fever, nevertheless, current thinking suggests that there may be a link. Consequently, the potential systemic toxicity of inhaled nanoparticles will also be addressed in this review.

As well as inhalation exposure, occupational exposure to nanoparticles will involve the potential for dermal exposure. Thus, possible toxicological consequences of dermal exposure are also considered.

The review is structured into seven main sections. The first section provides a brief description of some novel nanomaterials, and describes some of the general physical chemical characteristics of particles in the nanometre size range. The subsequent sections consider the potential health effects of exposure to nanometre particles; each of these sections considers possible local and systemic effects following

inhalation and dermal exposure. Section two provides an overview of 'conventional' particle toxicology, and describes the known toxicological properties of poorly soluble particles in the micrometre size range. Sections three and four consider existing materials which have conventionally been used in micrometre form, but which also have nanometre counterparts: section three compares the toxicity of nanometre particles compared with micrometre particles of the same material; while section four compares the toxicity of nanometre particles of different materials. Section five considers the available information on the toxicity of novel nanoparticles. An overall conclusion, with recommendations for future requirements in terms of understanding the health effects of particles arising from nanotechnology, is then provided. Finally, an annex is also included that provides general background information on the deposition, distribution and clearance of micrometre and nanometre particles following inhalation and dermal exposure.

1. Substance identification

This review deals with solid particles and fibres with physical dimensions in the nanometre range, intentionally generated to have applications in nanotechnology. Specifically, it considers particles and fibres with diameter <100 nm (0.1 μm). To put this size range into perspective, the following table (adapted from Preining, 1998) gives some comparable dimensions:

Object	**Size/diameter (nm)**
Individual atoms	0.1-0.3
Molecules	
Simple	0.4-1.0
Large biological	70-100
DNA	117
Fine aerosol	100-1000
Human red blood cell	7000-8000
Human alveolar macrophage	21 000

Thus, the particles that have applications in nanotechnology may be smaller than some biological molecules.

Particle size and geometry are key parameters that determine their behaviour. For particles that are large enough for their surfaces and volumes to be treated as continua (larger than about 20 nm), classical measures of particle size are satisfactory. However, for smaller particles, quantum effects come into play and the standard measures of particle size are no longer adequate.

The size of the atom, and hence a molecule, is dependent on its electronic state and is defined by the size of its electron cloud. As an illustration, in its ground state, the hydrogen atom has a diameter of approximately 0.1 nm. However, if it is given sufficient extra energy (e.g. by colliding with ions) it can move into an excited state and the electron 'jumps' to the next quantum level. In this excited state the physical size of the atom is increased to 0.6 nm.

For particles in the nanometre size range, the number of atoms in the particle is also very small. Consider that 1 g of gold (1 small earring) contains 3×10^{20} atoms; in comparison, a 5 nm gold particle contains roughly 1000 atoms. One consequence of having so few atoms is that a high proportion of them are at the particle surface. In a 5 nm particle, 40-50% of the atoms are at the surface. These surface atoms behave like individual atoms. Thus, for small nanoparticles, with many atoms at the surface,

surface reactivity will be high. This has implications for the structure adopted by such particles. It has been shown that for some metals and metal oxides the nanometre particles adopt structures that differ substantially from the bulk form (Jefferson, 2000). It is also possible that the physical and chemical properties of nanoparticles will differ from the bulk form.

As particle size decreases towards the molecular level, their behaviour is more like that of a vapour (ICRP, 1994). The kinetic behaviour of nanoparticles follows basic laws of gaseous diffusion. It can be calculated that a 5 nm particle will undergo 8.2 collisions per nanosecond, or 8.2×10^9 collisions per second (Preining, 1998). Consequently, interactions between particles are extensive. It is likely that each collision will lead to attachment and agglomeration. This is a reasonable assumption to make as the surface of each particle is highly reactive and agglomeration will lead to a reduction in the number of atoms or molecules at the surface with a reduction in the surface energy. Table 1 shows the agglomeration half-life of different concentrations of nanoparticles of various sizes (from Preining, 1998).

Table 1 Coagulation half-life

Particle diameter (nm)	**Half-life**			
	1 g m^{-3}	**1 mg m^{-3}**	**1 μg m^{-3}**	**1 ng m^{-3}**
0.5	0.39 μs	0.39 ms	0.39 s	6.5 min
1	2.2 μs	2.20 ms	2.2 s	36.67 min
2	12 μs	12.00 ms	12 s	3.34 hrs
5	0.12 ms	0.12 s	2 min	33.34 hrs
10	0.7 ms	0.7 s	11.67 min	8.1 days
20	3.8 ms	3.8 s	63.34 min	43.98 days

It can be seen from this that it is not possible to maintain a significant concentration of aerosolised individual nanoparticles for any appreciable length of time. However, it is possible that particle agglomerations may also be in the nanometre range.

As well as inter-particle interactions, some collisions will occur between the nanoparticles and other airborne molecules, such as water or pollutants. Given the high collision rate, these gas molecules will spend a relatively long time, i.e. longer than the time between collisions, adsorbed on the surface. This means that there is a significant likelihood of a reaction between the adsorbed molecule and the nanoparticle.

1.1. The nature of novel nanoparticles arising from nanotechnology

The nature and range of novel materials arising from nanotechnology is diverse. This section provides a brief description of some of the novel nanoparticles that are currently known, although not all yet have commercial applications.

1.1.1. Carbon nanotubes

Carbon nanotubes resemble rolled up sheets of graphite, with one end capped. The carbon atoms in nanotubes configure in a hexagonal pattern, as they do in graphite. However, their physical structure confers properties of extreme strength and electrical conductivity. Carbon nanotubes can have single or multiple walls. The single-walled variety has been most studied in terms of its physical and electrical properties, because its behaviour is more easily predicted in this form. An individual nanotube is about 1 nm in diameter and several micrometres in length. However,

nanotubes are highly electrostatic and generally agglomerate into bundles or nanoropes of about 20-50 nm in diameter. The aspect ratios (length:diameter) of nanotubes and nanoropes are such that they fall under the conventional definition of a fibre (aspect ratio > 3).

There are various current methods of production of nanotubes, all of which result in the presence in the material of variable amounts (up to about 30% by mass) of catalytic transition metals such as iron, nickel or cobalt. Secondary processing of the material is undertaken to remove as much of the metal catalyst as possible; however, complete removal is difficult, as the metal is encased within the carbon.

Helical tubular structures also exist, again with the carbon atoms configured in a hexagonal pattern, as in graphite.

Nanotubes are completely insoluble in water and are biologically non-degradable.

1.1.2. Fullerenes

Fullerenes are molecules of carbon formed into hollow, cage-like structures. There is an extensive family of fullerenes, the most well-known of which is C_{60}, or Buckminsterfullerene ('buckyballs'). C_{60} is made up of 60 carbon atoms arranged in a ball shape of hexagonal and pentagonal panels (buckyballs are named after the architect Buckminster Fuller, who designed the geodesic dome structure that gave the discoverers of the C_{60} structure the clue to the arrangement of the carbon atoms). Fullerenes with different numbers of carbon atoms (e.g. C_{70}, C_{76}, C_{84}) and fullerene derivatives (with other atoms inserted within the structure; endohedral fullerenes) also exist, as do buckyballs with a shell around them – bucky-onions.

1.1.3. Nanodots

Nanodots are crystalline structures of compounds such as cadmium, selenium, tellurium and sulphur. Their nominal diameter is in the order of several nanometres and they are available as suspensions in a carrier or integrated into solids (such as polystyrene, polyurethane, polycarbonate or silica).

1.1.4. Carbon nanofoam

Carbon nanofoam is the fifth known allotrope of carbon. Clusters of carbon atoms (with an average diameter of 6-9 nanometers) are randomly interconnected to form a web-like structure. It is an extremely lightweight, spongy solid, which can act as a semiconductor. However, the property that sets it apart from other forms of carbon is its magnetic properties. Although it has been found to contain some iron and nickel as trace impurities, their presence does not account for the magnetism observed. Rather, it is the heptagonal arrangement of the carbon atoms that is believed to confer the magnetic behaviour.

2. Toxicology of particles in the micrometre size range

This section describes in general terms the toxicity of poorly soluble particles in the micrometre size range. It provides a background picture against which to set considerations of the potential toxicity of nanoparticles. It begins with a description of the human diseases associated with exposure to particles and then provides an overview of what is known about the mechanisms of particle toxicity based on studies in animals. An overview of the toxicokinetics of particles relevant to their toxicity is

attached as an appendix.

2.1. Human health effects

2.1.1. Inhalation

Effects on the respiratory tract

An immediate consideration of inhalation exposure to poorly soluble particles is for the consequences to the respiratory tract and lung. The broadest group of lung diseases attributable to particle exposure is pneumoconiosis. The term literally means ‘dusty lungs’; however, medically, pneumoconiosis is defined as the non-neoplastic reaction of the lungs to inhaled mineral or organic dust and resultant alteration in their structure excluding asthma, bronchitis and emphysema (Parkes, 1982). Pneumoconiosis can develop as a result of exposure to fibrous or non-fibrous particles.

The severity of pneumoconiosis can range from very mild to severe. In its mildest form, it essentially represents dust accumulation, with minimal lung effects and only very minor changes in lung structure without adverse functional consequences (e.g. siderosis associated with iron exposure; stannosis associated with tin exposure). In its most severe form, fibrotic changes in the lung can lead to deficiencies in gas exchange and impaired lung function and can be fatal (e.g. silicosis associated with silica exposure; asbestosis associated with asbestos exposure).

In some cases, those exposure situations eliciting the most agressive forms of pneumoconiosis have also been causally linked to the development of lung cancer e.g. asbestos and silica. In addition to lung cancer, exposure to asbestos is causally associated with the development of malignant mesothelioma. However, it is clearly not the case that severe pneumoconiosis is generally associated with progression to lung cancer – for example, there is no evidence for an increased risk of lung cancer in coal miners with fibrotic forms of pneumoconiosis.

In addition to pneumoconiosis, there are other types of lung toxicity that merit consideration in terms of potential consequences of particle exposure: namely, bronchitis, emphysema and asthma.

Bronchitis, a condition often associated with ‘dusty’ occupations, is inflammation of the bronchi and is characterised by increased mucous secretion in the bronchial tree. It can produce airflow obstruction.

Emphysema is a condition that affects the alveolar sacs, causing a break down of the alveolar walls, resulting in fewer, larger air spaces. Emphysema has also been associated with ‘dusty’ occupations, although the main cause is cigarette smoking.

Asthma is a disease in which the airways become hypersensitive and are prone to constriction, with swelling of the airway lining, leading to airflow obstruction. It is often presented as an allergic response; however, for a number of causes of asthma the mechanism(s) involved have not been clearly established.

Systemic effects

There are two aspects to consider in relation to the potential systemic effects of exposure to poorly soluble particles. The first is the ability of inhaled particles to become systemically available, leading to adverse systemic consequences. The

second is the potential for materials to leach from particles contained within the lung, so that although the particle itself does not become systemically available, its leached components may.

In relation to the first aspect, inhalation exposure to poorly soluble micrometre particles is not generally associated with systemic effects of material that remains in particulate form. There are certain examples of particle exposures where systemic consequences of inhalation exposure have been proposed (for example for particulate air pollution and for silica exposure), but a causal link between these exposures leading directly to particle-induced systemic effects has not been clearly established. One possible reason for a lack of systemic toxicity could be limited systemic availability of micrometre sized particles.

There are considerably more examples of inhaled particles producing systemic toxicity as a consequence of slow dissolution of the particle or leaching of its components from the lungs into the systemic circulation over a long period of time – for example, the systemic toxicity associated with cadmium or lead exposure is due to systemic availability of leachates.

2.1.2. Dermal

Local effects

Generally speaking, dermal exposure to micrometre sized particulate material is not associated with chemically mediated effects on the skin, although leaching of components of the material following prolonged skin contact can lead to local skin irritation or sensitisation (e.g. sensitisation to nickel in jewellery arises as a result of leaching of nickel ions).

Skin irritation as a consequence of mechanical abrasion has been noted for a few particle types, e.g. for man made mineral fibres. This effect is greater for larger fibres compared to smaller fibres.

Another nuisance phenomenon is observed in carbon black workers, with discolouration of the skin (carbon black ‘tattoos’) due to retention of particles in hair follicles.

Systemic effects following dermal exposure

The lack of any significant dermal absorption of insoluble micrometre particles (see appendix) means that in general, dermal exposure is not associated with systemic toxicity. However, if there is prolonged contact with the skin, leaching of components from the particle over a long period of time could give rise to systemically mediated effects.

2.2. Mechanistic basis for local toxicity to the lung

This section describes the current understanding for how particle exposure produces adverse effects on the lung. This understanding has been developed from studies in animals, primarily the rat. Some particles have appreciable inherent cytotoxicity towards lung cells, whereas others do not; particle shape (fibrous or non-fibrous) is another important consideration here.

2.2.1. Non-fibrous, non-cytotoxic particles

For poorly soluble particles of low inherent cytotoxicity (e.g. carbon black, titanium dioxide, coal dust, talc), exposure to high concentrations leads to a common pattern of pathological effects in the rat. Single high exposures produce transient pulmonary inflammation, measured as an immediate influx of inflammatory markers in the lung. Following repeated exposure, sustained inflammation and lung damage is observed, with hypertrophy, epithelial hyperplasia and interstitial fibrosis, which ultimately can lead to lung tumours (Driscoll, 1996). These substances, although difficult to test in standard mutagenicity assays due to their poor solubility, appear not to be mutagenic. The tumours are believed to arise via a non-genotoxic mechanism, as a secondary consequence of the sustained inflammatory lung response.

A good understanding of the underlying mechanisms for this characteristic pulmonary response in rats has been developed. Critical to the expression of pulmonary toxicity is the ability of the lung defence mechanisms to actively clear deposited particles. The normal processes for clearance of particles that deposit within the respiratory tract are described in the appendix. The pattern of inflammation and lung toxicity described above develops in situations where these normal lung clearance mechanisms are overwhelmed and fail: a process termed ‘overload’. Overload refers to the loss of mobility of alveolar macrophages (AMs) when their capacity to phagocytose and remove particles is exceeded (Morrow, 1988). Morrow proposed that the ability of AMs to migrate to the mucociliary escalator is inversely related to their volumetric loading of phagocytosed particles (the volumetric overload hypothesis). According to this hypothesis, the onset of clearance inhibition occurs at a volumetric loading of about 60 μm^3 (6% of macrophage volume); complete immobilisation of AMs occurs at a loading of about 600 μm^3 (60% of macrophage volume). These predictions are supported by some experimental evidence (Oberdörster *et al*, 1992).

Such an overload phenomenon is associated with inflammation because particle laden AMs cannot migrate effectively to the mucociliary escalator, but remain in the alveolar regions where they secrete inflammatory mediators.

Continued exposure in circumstances where clearance rates are reduced due to overload leads to an increase in the lung burden of particles. The resultant interaction between particles and the lung epithelium, and the high particle load of AMs, are both thought to lead to the release of inflammatory mediators and generation of reactive oxygen species. These in turn can produce epithelial cell damage, including DNA damage; cell proliferation can ensue as a means of repairing areas of damaged epithelium. Thus, under conditions of overload and sustained inflammation, a process of hyperplasia, metaplasia and ultimately tumour formation can develop (Driscoll, 1996; Faux *et al*, 2003). A schematic description of the stages thought to be involved in the pulmonary toxicity of poorly soluble particles is shown in Figure 1.

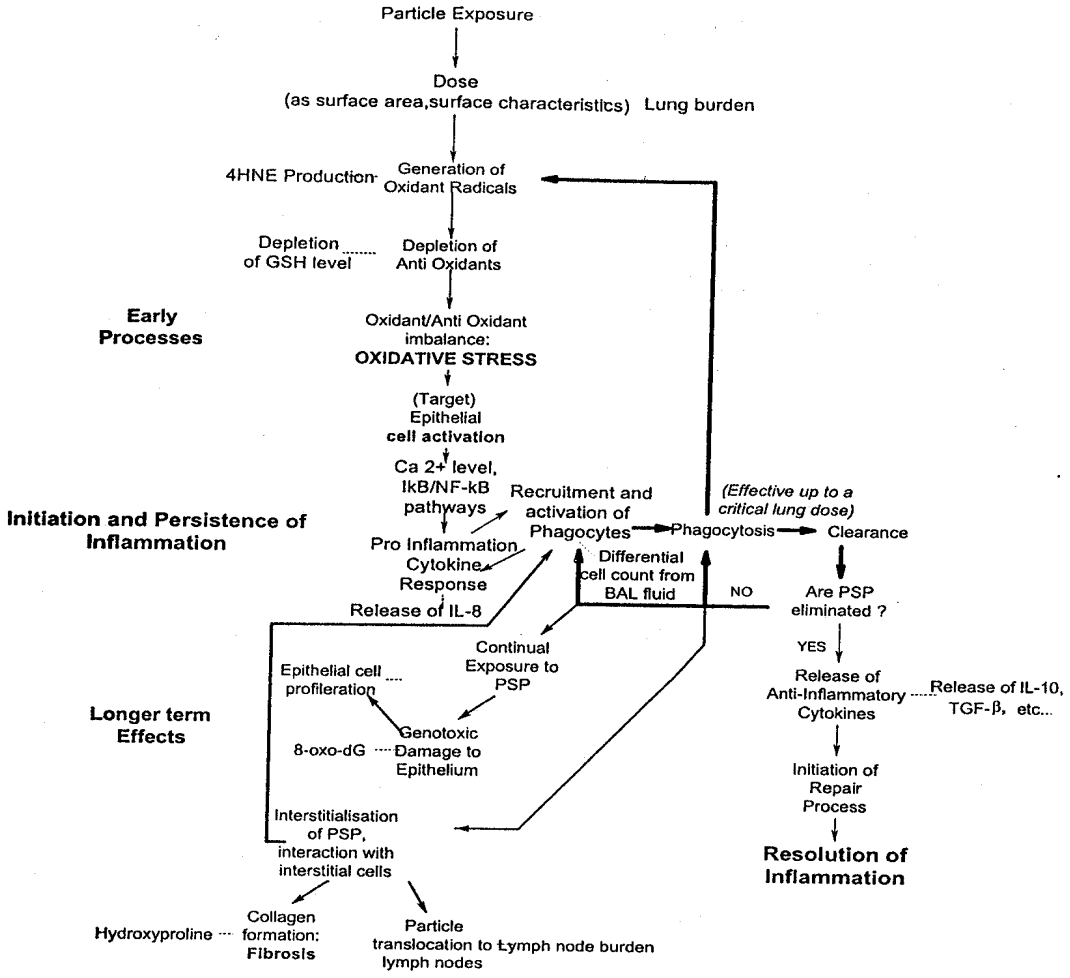

Figure 1: The hypothesised stages in the pathway leading to inflammation and longer term effects as a result of sustained inhalation exposure to poorly soluble particles (PSP) (From Faux *et al*, 2003).

Impairment of clearance also results in prolonged contact time between particles and the lung epithelium. This, possibly together with epithelial damage as a result of the inflammatory processes, enhances the potential for particles to enter the pulmonary interstitium, a site where lung disease has been observed for some particles.

2.2.2. Non-fibrous, cytotoxic particles

Some inhaled poorly soluble particles (e.g. silica) are directly toxic to alveolar macrophages. Phagocytosis of inherently cytotoxic poorly soluble particles can cause AM necrosis, with release of inflammatory mediators. In addition, cytotoxic particles can produce direct damage to the pulmonary epithelium. The cytotoxicity of particles such as silica appears to be related to the surface chemistry and free radical

generating potential (e.g. Donaldson *et al*, 1996). Such surface reactivity can be conferred by the presence of metals and surface radicals. One possibility is that the surface properties of these particles are such that they can cause oxidative stress, which can produce direct damage to the lung epithelium; or can induce expression of inflammatory genes, leading to an enhanced lung inflammatory response. Ultimately, the sustained inflammatory and proliferative response can lead to the long-term damage described above for particles of low cytotoxicity.

2.2.3. Fibrous particles

The mechanisms of toxicity of fibres share many similarities with that for non-fibrous particles i.e. pulmonary toxicity can arise as a secondary consequence of impaired clearance and/or as a result of direct cytotoxicity. A review of the toxicology of fibres has been produced by HSE (HSE, 1996). Thus a detailed description of the mechanisms underlying pulmonary effects of fibres is not provided here. However, it is important to note that, in addition to the lung, the pleural mesothelium is a potential target tissue of concern for inhaled fibres. This is exemplified by the human experience of mesothelioma from asbestos exposure (see HSE, 1996).

2.2.4. Species differences

Given that the understanding of the mechanisms underlying the pulmonary response to inhaled particles is based very heavily on observations in experimental animals, and particularly the rat, consideration must be given to the relevance of animal data to human health. The available evidence for the poorly soluble non-fibrous particles of low cytotoxicity clearly indicates that quantitatively, the rat is more sensitive to the pulmonary toxicity of inhaled particles than other experimental animal species (e.g. mice or hamsters). The best comparable human experience available, certainly in terms of the consequences of overload as a result of high exposures to particles of low cytotoxicity, is that from coal-mine workers. This experience suggests that humans are quantitatively less susceptible than rats to the toxicological consequences of overload; in particular, there is no evidence for an excess of lung cancer in cohorts of coal-mine workers, even under circumstances of high lung dust burdens leading to severe pneumoconiosis.

However, it is also an interesting observation that other non-fibrous poorly soluble particles that are known to be carcinogenic towards the respiratory tract in humans (e.g. silica, nickel subsulphide), to the extent that they have been tested in animals, have produced lung tumours in the rat but not in mice and/or hamsters.

The experimental animal evidence for the effects of exposure to fibres also shows differences in response between species, both in terms of the development of lung cancer and of mesotheliomas. Fibres that are known to produce lung cancers in humans have also produced lung cancers in the rat, but not in the hamster (to the extent that testing has been done in this species); the available exposure response data for humans does not allow any conclusions to be drawn in relation to the relative susceptibility of humans compared with the rat. In relation to mesotheliomas, the pattern of evidence available from studies in rats, when compared with human experience, does not allow any clear conclusions to be drawn about the relative susceptibility of rats and humans. It appears that the hamster may be more susceptible than the rat to the development of mesothelioma; again, the quality of dose-response data precludes comparisons with human susceptibility.

Overall, it is clear that considerations of possible species differences must be included in any consideration of the testing requirements for novel nanoparticles, and

in the interpretation of pulmonary toxicity data generated.

3. Micrometre versus nanometre: a comparison of the toxicity of nanometre particles and micrometre particles of the same substance

This section describes the known differences in toxicity between micrometre-sized particles and nanometre-sized particles of the same material. Although the information presented in this section does not relate to novel nanoparticles, knowledge about the comparative toxicity of micrometre and nanometre particles of the same material may be useful in relation to making predictions about the potential toxicity of novel nanoparticles. In addition, some of the materials covered in this section have, or could have, applications as a consequence of their nanometre size (e.g. nanometre titanium dioxide has some cosmetic applications)

3.1. Inhalation exposure

3.1.1. Effects on the respiratory tract

As described in the previous section, the main expression of toxicity following repeated inhalation exposure to poorly soluble particles in the micrometre range is to the respiratory tract. There is an extensive body of evidence from studies in rats, which indicates that for the same material, nanometre particles are more potent (in mass terms) than micrometre particles in inducing pulmonary toxicity. One of the first studies to demonstrate this was that by Ferin *et al* (1992). (Also reported by Oberdörster *et al*, 1994). In this study, rats were exposed to 0 or 23 mg/m^3 of titanium dioxide in either the nanometre size range (primary particle size 21 nm) or micrometre size range (250 nm). Agglomeration of the particles resulted in similar aerodynamic diameters of the two particle types; consequently, deposition patterns within the lungs were expected to be comparable for both particle sizes. Exposures were for 6 hours/day, 5 days/week for up to 12 weeks, after which rats were maintained in filtered air for up to 64 weeks. Rats were sacrificed at intervals up to 64 weeks after the start of exposure for analysis of bronchoalveolar lavage fluid (BALF) and microscopic examination of the lungs.

Both particle size fractions elicited an inflammatory response in the alveoli and interstitium, as indicated by an influx of polymorphonuclear leucocytes (PMN). However, the response elicited by nanometre TiO_2 was markedly greater and more persistent than that produced by micrometre TiO_2. The magnitude of the alveolar inflammatory response to nanometre particles was up to 43-fold higher than that to micrometre particles and persisted up to 1 year post-exposure (64 weeks from the start of exposure), whereas the less marked inflammatory response to micrometre TiO_2 had resolved almost to control levels by 41 weeks.

Histopathological examination of the lungs at week 41 (29 weeks post-exposure) showed a fibrotic response in animals exposed to both particle sizes of TiO_2, but again, the severity of the response was greater in animals exposed to nanometre sized particles. Type II alveolar cell hyperplasia, indicative of epithelial damage, was observed in animals exposed to nanometre TiO_2 but apparently not in animals exposed to micrometre TiO_2.

In parallel with the differences in inflammatory and pathological response seen with the two different particle sizes, there were differences in the distribution of particles in the lung. Nanometre particles were interstitialised to a significantly greater extent than micrometre particles. Post-exposure, clearance of both micrometre and nanometre particles was impaired compared with normal clearance rates. However,

on a mass basis, clearance of nanometre particles was approximately 3 times slower than that for micrometre particles. The effect on clearance of nanometre particles was not associated with volumetric overloading of the alveolar macrophages; volumetric loading of macrophages with nanometre TiO_2 reached only 2.6%, which is less than the 6% volume threshold for clearance impairment proposed by Morrow (1988). In animals exposed to micrometre particles, clearance rate had returned to normal by week 41, but remained impaired in animals exposed to nanometre TiO_2.

Overall, therefore, this study demonstrated that nanometre particles induce a greater inflammatory response and a more marked effect on clearance than micrometre particles, on a gravimetric basis. However, when the effects on pulmonary inflammation and clearance were considered with respect to particle surface area rather than mass dose, the two particles sizes exhibited a similar degree of potency. Thus, particle surface area rather than mass dose appeared to be a critical determinant of pulmonary response. The smaller particles had a greater surface area per unit mass, and this appeared to be the basis for their greater response per unit mass.

The finding that toxicity per unit mass of the same substance is enhanced as particle size decreases has subsequently been confirmed consistently across a range of exposure situations (single, short-term and long-term repeated exposure, using either intratracheal instillation or inhalation exposure) for a range of poorly soluble substances - aluminium trioxide (Oberdörster *et al*, 1990), carbon black (e.g. Gallagher *et al*, 2003; Gilmour *et al*, 2004; Li *et al*, 1996; Renwick *et al*, 2004), metallic cobalt (Zhang *et al*, 2000), metallic nickel (Zhang *et al*, 2003) and titanium dioxide (e.g. Borm *et al*, 2000; Heinrich *et al*, 1995; Lee *et al*, 1985; Oberdörster *et al*, 1990, 1992; Renwick *et al*, 2004). In addition to the potential explanatory hypothesis that toxicity is related to particle surface area, a number of other hypotheses have emerged to explain why nanometre particles should exert a greater pulmonary toxicity than micrometre particles on a mass basis. Each of these various hypotheses is discussed below.

It is also worthy of note that the species differences in susceptibility to the pulmonary effects of inhaled micrometre sized particles (i.e. rat more susceptible than mouse or hamster) have been confirmed for nanometre titanium dioxide, in a repeated exposure inhalation study (Bermudez *et al*, 2004).

1) Particle deposition characteristics

Particle deposition characteristics within the respiratory tract vary with particle size. A description of particle deposition characteristics within the respiratory tract is given in the appendix. For the same exposure in terms of mass dose, nanometre and micrometre particles distribute differently within the respiratory tract. Particles in the nanometre range are more likely to deposit in the alveoli than particles in the micrometre range. This could be a factor in the enhanced toxicity of nanometre particles (Oberdörster, 2001). However, it is also known that nanometre particles tend to agglomerate (Jefferson, 2000; Preining, 1998). As a consequence, the aerodynamic characteristics of agglomerated particles may well be similar to those of micrometre particles, with the result that in practice, there may be little difference in the deposition characteristics of nanometre and micrometre particles. This would reduce the likelihood that differences in toxicity are related to differences in deposition.

2) Particle surface area

The observation that for the same material, nanometre sized particles are more potent inducers (on a mass basis) of pulmonary toxicity than micrometre sized particles led to the hypothesis that particle surface area is a key determinant of toxicity (Driscoll, 1996; Oberdörster *et al*, 1992, 1994). Driscoll (1996) noted that for a range of insoluble, non-cytotoxic particles, pulmonary tumours observed in experimental studies in the rat were associated with lung burdens of particles with a total surface area > 2000 cm^2. Based on studies with titanium dioxide and barium sulphate, Tran *et al* (2000) suggested that a threshold for pulmonary inflammation could be identified at a total particle surface area of around 200-300 cm^2. They developed a model to explain how particle surface area might influence pulmonary toxicity. Central to their model is the generation of macrophage-attracting chemotactic factors as a result of particle contact with the lung lining fluid and epithelial cells. They propose that the magnitude of the chemotactic signal may be determined by particle surface area; particles with a high surface area per unit mass (nanometre particles) trigger a greater signal because of the greater particle-cell contact. This signal would lead to the recruitment of AMs and PMNs. However, with very large total surface areas of particles, the magnitude of the signal could be so strong as to disrupt the normal chemotactic gradient, thereby preventing AM migration to the mucociliary escalator, regardless of their particle burden. Impaired AM clearance would result in prolonged contact between unphagocytosed particles and the alveolar epithelium and lead to an enhanced inflammatory response. This situation would also tend to favour interstitialisation of the unphagocytosed particles.

In further support of this hypothesis, Renwick *et al* (2004) demonstrated increased migratory activity of AMs towards the chemotaxin C5a, following intratracheal instillation of nanometre carbon black and nanometre titanium oxide in rats; equivalent mass doses of micrometre carbon black or titanium dioxide had no effect on chemotaxis. Increased chemotactic responsiveness to C5a could act to retain AMs at the site of particle deposition in the lung.

Another factor that could be affected by particle surface area is the phagocytic efficiency of AMs. Here, the potential influence of particle surface area is through free radical generation; greater surface area could result in greater free radical generation, with consequences for AM function as a consequence of oxidative stress. However, although nanometre particles of carbon black and titanium dioxide have been shown to be more potent than their micrometre counterparts in impairing murine AM phagocytosis *in vitro* (Renwick *et al*, 2001), this effect was not demonstrated *in vivo* (Renwick *et al*, 2004). Further elucidation of the potential effects on AM phagocytosis of nanometre compared with micrometre particles *in vivo* would be useful.

Another feature of particle surface area that could be relevant to the toxicity of nanometre compared with micrometre particles, relates to catalytic efficiency. The availability of a high surface area promotes the ability of a material to function as a catalyst. This is one aspect of nanoparticles that will have applications in nanotechnology, but its consequences in biological systems are not yet understood. It may be a factor in the observations that particle surface area is a key determinant of toxicity.

3) Particle surface characteristics

It has been predicted and shown experimentally for some materials, that the surface characteristics of particles in the nanometre range are markedly different from the surface characteristics of the bulk material (Jefferson, 2000; Preining, 1998). All particles adopt structures that minimise surface energy. For nanoparticles, the

structures that would normally be adopted by the material in micrometre form are not necessarily the most energy efficient, because of the high ratio of surface to bulk atoms. In some cases, to achieve the most energy-efficient state, nanoparticles may adopt structures that do not exist in the larger scale. Consequently, the physicochemical properties of nanoparticles may be different from particles of the same material in the micrometre range. On this basis, it might be expected that the difference in the surface properties of nanometre particles compared with micrometre particles could be a significant factor in the differences seen in their pulmonary toxicity.

Some experimental evidence for this is available. Donaldson *et al* (1996) measured free radical generating activity for a range of non-fibrous and fibrous particles. They proposed that the ability of particles to generate free radicals could be critical to their toxicity. They showed that free radical generating ability was greater for nanometre titanium dioxide compared with the same mass of micrometre titanium dioxide. Subsequently, Zhang *et al* (1998) also showed that for the same material, particles in the nanometre size range produced greater free radical generating activity than particles in the micrometre range.

Oberdörster (2001) also reported on the importance of particle surface properties, based on a study using nanometre TiO_2 (primary particle size 20 nm). Native TiO_2 has a hydrophilic surface; however, the surface can be rendered hydrophobic by application of an appropriate surface coating, in this case a silane compound. Both uncoated and coated TiO_2 was instilled into rat lungs. At 24 hours following instillation, a clear difference in the inflammatory response was seen; the hydrophobic, coated particles elicited a much lower inflammatory response than the hydrophilic particles.

Höhr *et al* (2002) looked at the relative importance of particle surface area versus particle surface characteristics on the pulmonary inflammatory response. This study compared the inflammatory response elicited in the rat lung by a single intratracheal instillation of methyl-coated (hydrophobic) or uncoated (hydrophilic) micrometre and nanometre TiO_2. The dose levels (1 and 6 mg) TiO_2 were chosen to allow comparisons based on equivalent mass and equivalent surface area. There was no evidence for any consistent effect of particle coating on the inflammatory response, although there were some discrepancies between the text and the data presented, which reduces the confidence in the reliability of this study.

Overall, although limited, the available experimental evidence points towards particle surface characteristics as having a potentially important role in the observed differences in toxicity between nanometre and micrometre particles. Further studies would be needed to demonstrate this conclusively, and to investigate the mechanistic basis for such an effect.

3.1.2. Summary of effects on the respiratory tract

There is a considerable body of evidence that demonstrates an enhancement of pulmonary toxicity for particles of the same material in the nanometre size range compared with the micrometre size range, on a mass dose basis. One key factor that seems to be involved in this enhancement is the increase in particle surface area that is concomitant with the reduction in particle size. However, although various hypotheses have been developed, and tested, to elucidate the mechanistic basis for the influence of particle surface area on toxicity, many gaps in knowledge remain.

3.1.3. Systemic effects

There is no useful experimental evidence that compares the systemic toxicity of nanometre and micrometre particles of the same material. There is some information on (and some predictions can be made about) the relative systemic availability of nanometre compared with micrometre particles. The systemic availability and clearance of inhaled micrometre and nanometre particles is discussed in the appendix. The available information suggests that there would be differences in systemic availability between micrometre and nanometre particles following inhalation exposure. It is predicted that uptake across the lung epithelium will be size dependent, with nanometre particles more readily taken up across the lung than micrometre particles; the limited experimental evidence supports this. Similarly, once absorbed, it appears that recognition of particles by systemic clearance mechanisms is size dependent, such that nanometre particles could be cleared less rapidly than micrometre particles. Another possibility is that nanometre particles could escape the systemic circulation more readily than micrometre particles, by virtue of their very small size. Their distribution and localisation within different organ systems within the body is therefore likely to differ from that of micrometre particles, although the consequences of this, in terms of expression of any toxicity, are not automatically evident.

3.2. Dermal exposure

3.2.1. Local effects

There is no useful published information on the comparative toxicity towards the skin of nanometre compared with micrometre particles, neither in terms of local irritation to the skin, nor possible skin sensitisation.

Effects such as mechanical abrasion and discolouration seen with some micrometre particles could also be considerations for their nanometre counterparts. Certainly, discolouration could be of greater concern for some nanometre particles than for micrometre particles, given the evidence that nanometre particles can penetrate the outer layers of the skin via hair follicles (see below). Mechanical abrasion may be of lesser concern for nanometre particles, because it appears that abrasion potential is greater for larger particles.

The other aspect of dermal exposure that could lead to local effects is the possibility of leaching of components of the particle when there is prolonged exposure. The potential for leaching is likely to apply to nanometre particles in which there are leachable constituents (such as metal ions in single-walled carbon nanotubes – see section 5). It is also possible that there could be greater opportunity for prolonged exposure to nanoparticles if there is retention in hair follicles, as this could enhance the capacity for leaching.

3.2.2. Systemic effects

There is no information on the systemic effects of nanometre particles compared with micrometre particles of the same material, following dermal exposure. As a surrogate, the uptake of nanometre particles across the skin compared with that of micrometre particles could be considered. There is very little published experimental information on the dermal absorption of particles in the nanometre size range compared with those in the micrometre range. A small number of published studies have investigated the dermal penetration of different particle sizes of titanium dioxide, specifically because of its use in the nanometre size range in sunscreen

formulations. The application method used in these studies was designed to mimic sunscreen use, and therefore is not directly reflective of the occupational situation. Nevertheless, the methods used have maximised the potential for skin contact with the material and therefore should have adequately explored dermal penetration potential.

Tan *et al* (1996) first reported in a pilot study that 10-50 nm particles of titanium dioxide could penetrate the stratum corneum to the dermis following repeated application in volunteers. However, the study was limited, particularly as the study volunteers were undergoing surgery for skin lesions, and therefore the dermal barrier may already have been compromised.

Lademann *et al* (1999) investigated the dermal penetration of 20 nm (assumed particle size, based on description of product used) titanium dioxide particles in a sunscreen formulation. The sunscreen was applied repeatedly over 4 days to the forearm skin of human volunteers. The only evidence for penetration of TiO_2 beyond the upper skin layers was via single follicle channels. The concentration of titanium in these channels was two orders of magnitude lower than in the upper skin layers.

In the third and most detailed study, Schulz *et al* (2002) (also reported by Pflücker *et al*, 2001) investigated the influence of particle size on the dermal absorption of three titanium dioxide preparations. Each had a different primary particle size (10-15 nm, 20 nm and 100 nm), shape (cubic or needles) and hydrophobic/hydrophilic characteristics. The preparations were topically applied unocclusively in an oil-in-water emulsion to the forearm skin of human volunteers for 6 hours. Skin biopsies were examined by scanning electron microscopy to visualise the distribution of particles within the skin layers. None of the particles penetrated beyond the outer layer of the stratum corneum.

Taken together, these findings suggest a lack of significant dermal penetration for nanometre particles, regardless of size or hydrophilicity. Although not conclusive evidence itself for a generic property, the results do not point to any enhancement of dermal absorption potential associated with a reduction in particle size from the micrometre to the nanometre range. Two conclusions can be drawn from these data: systemic toxicity as a result of particle uptake across the skin is unlikely to be a significant concern for insoluble particles in general; and there are unlikely to be significant differences between nanometre and micrometre particles in terms of systemic effects following dermal exposure.

However, there remains the potential for leaching of components from particles, if there is prolonged contact with the skin. This could give rise to systemically mediated effects. It is possible that nanoparticles could be retained for long periods in the skin, if there is retention in hair follicles, and thus there could be an enhanced potential for systemic availability of leachates from nanoparticles compared with micrometre particles. The need to consider such a hypothesis would have to be determined for each nanoparticles type.

4. Differences/similarities in toxicity between nanoparticles of different existing materials

4.1. Inhalation exposure

4.1.1. Effects on the respiratory tract

The importance of particle surface area in the toxicity of inhaled poorly soluble

particles has been clearly established, as described in the previous section. The information presented in that section shows that for the same material, smaller particles elicit a greater pulmonary inflammatory response than do larger particles, on a mass basis. In addition, it has been shown that particle surface area can provide a unifying dose metric for a range of different insoluble particles of low cytotoxicity – regardless of the particle type, toxicity to the respiratory tract (in terms of rat lung tumour response) is related to particle surface area (Driscoll, 1996).

However, two other factors have been proposed to be involved in determining the respiratory tract response to particle exposure:

1. particle surface activity – specifically, the ability of the particle surface to generate free radicals (Donaldson *et al*, 1996);

2. particle agglomeration/disagglomeration – a determinant of the availability of individual particles to the lung surface once the material has entered the lung (Oberdörster, 1996).

These factors may well be related to particle surface area, but they are also particle-specific and may be an important determinant of inherent toxicity towards the respiratory tract. The available data that explore how these factors may lead to differences in pulmonary toxicity between different existing nanometre materials are summarised below.

Different particle surface activities

A single study has investigated the association between particle surface activity and pulmonary toxicity for nanometre particles of different materials (Zhang *et al*, 1998). The study explored the potential of three different materials in the nanometre size range to produce pulmonary inflammation following a single intratracheal instillation in the rat. Metallic cobalt (20 nm particles, specific surface area 47.9 m^2/g), metallic nickel (20 nm particles, specific surface area 43.8 m^2/g) and titanium dioxide (28 nm particles, specific surface area 45 m^2/g) were instilled in equal mass doses (1 mg).

Analysis of BALF 1-30 days post-exposure indicated clear differences in the inflammatory responses elicited by the three particle types. Nickel demonstrated statistically significantly greater inflammatory responses than either cobalt or titanium dioxide and cobalt was more inflammogenic than titanium dioxide. Nickel also produced a marked increase in lymphocytes in BALF, with a pattern of response different to that produced by the other particles. Nickel and cobalt but not titanium dioxide caused lipid peroxidation. The particle types were also assessed for their ability to produce free radicals *in vitro*. In this respect, titanium dioxide showed little free radical generating activity, whilst nickel and cobalt showed similar free (hydroxyl) radical production ability.

The pattern of results supports the hypothesis that free radical generating potential may be one element that determines inflammatory potential and pulmonary toxicity. However, clearly other factors are involved, since the degree of inflammation *in vivo* did not correlate directly with free radical production ability: nickel and cobalt had similar free radical production potential *in vitro*, whereas nickel produced considerably more inflammation *in vivo* than did cobalt.

This study demonstrates clear differences in pulmonary toxicity between different materials (with similar surface areas) in the nanometre size range. The reasons for the differences in toxicity are not fully understood. However, one factor that may be

involved is free radical production ability, possibly mediated by the transition metal component of the particles.

Differences in disagglomeration

As described previously, particles in the nanometre range are unlikely to remain as singlet particles in the atmosphere for any length of time, but will tend to agglomerate (Preining, 1998). The extent to which disagglomeration occurs once particles enter the lung may influence the subsequent toxicity.

One hypothesis that has emerged from studies with different particles in the nanometre size range is that differences in pulmonary responses could be related to the extent of disagglomeration (e.g. Oberdörster *et al*, 1992; Takenaka *et al*, 1986).

The first evidence for this hypothesis emerged from a study using nanometre particles of titanium dioxide and carbon black (Oberdörster *et al*, 1992). In this study male rats (4 per group) were instilled with saline or 500-1000 μg rutile TiO_2 (particle diameter 12 or 250 nm), anatase TiO_2 (particle diameter 20 or 250 nm) or carbon black (particle diameter 20 nm). For all types of particle in the nanometre size range, administration was as aggregates of particles. Assessment of inflammatory changes and lung dosimetry were determined 24 hours post-instillation.

The inflammatory response seen in the alveolar space was dependent on the location of particles within the lung. The highest inflammatory response was seen for carbon black; however, carbon black particles showed relatively little translocation to the pulmonary interstitium. In contrast, the two highest doses of nanometre titanium dioxide elicited relatively mild inflammatory responses; however, these particles also showed the greatest degree of translocation to the pulmonary interstitium.

The authors suggested that one explanation for this result could be a difference in the disagglomeration rate of the two particle types. If TiO_2 particles disagglomerate more rapidly into singlet particles than carbon black particles of the same primary diameter, AM clearance would be slower for TiO_2 than for carbon black. The resultant increase in contact time between the particles and the epithelial surface would enhance interstitial uptake.

In support of this, other studies have shown that not all nanometre particles undergo interstitialisation to the same extent. For example, 15-30 nm gold particles were rapidly phagocytosed by alveolar macrophages, with limited interstitialisation (Patrick and Stirling, 1988). In comparison, 20 nm particles of Al_2O_3 were interstitialised similarly to 30 nm particles of TiO_2 (Ferin *et al*, 1990).

Overall, some studies point to the possibility of an effect of disagglomeration on the behaviour of nanoparticles. However, there is no experimental evidence to show whether or not such an effect actually occurs in practice. Nevertheless, the possibility remains that disagglomeration is an important feature of particle toxicity to the lung.

4.1.2. Systemic effects

The only relevant information in relation to comparisons of the systemic toxicity potential of different nanometre particles comes from studies that have looked at their systemic distribution (see appendix for details of individual studies). The studies that have been performed to date have given conflicting results in terms of the extent of extrapulmonary translocation following inhalation exposure. For example, significant translocation to the liver and other organs has been reported by a number of authors,

for a variety of particle types, in animals and human volunteers (Nemmar *et al*, 2001, 2002; Oberdörster *et al*, 2002; Takenaka *et al*, 2001); whereas other authors have found no significant systemic distribution of nanoparticles (Brown *et al*, 2002; Kreyling *et al*, 2002, 2004; Semmler *et al*, 2004). One possible explanation for the observed differences in systemic translocation may be related to the exposure conditions, chemical composition and particle size of the different types of nanometre particle used in these studies. Kreyling *et al* (2002) proposed two hypotheses that may explain differences in the results they saw for iridium nanoparticles (negligible systemic translocation) and those reported by Oberdörster *et al* (2002) (significant systemic translocation of ^{13}C nanoparticles). The first hypothesis related to possible differences in disagglomeration. If ^{13}C nanoparticles dissagglomerate in the lung more to a greater extent than ^{192}Ir nanoparticles, this could enhance the direct passage of singlet particles across the lung epithelium. The second hypothesis concerned the tendency of particles to bind to high molecular weight proteins, which would then influence their subsequent fate. Either hypothesis could explain the observed differences.

Clearly there remain uncertainties surrounding the systemic availability of nanometre particles in general, and how different nanoparticles might behave in terms of their systemic distribution following inhalation exposure. If there are differences in systemic availability according to individual particle characteristics, then it would be also predicted that this could have consequences for the expression of any systemic toxicity; different materials in nanometre form could have different systemic effects.

4.2. Dermal exposure

There is no information on the differences or similarities between nanometre particles of different existing materials in terms of their health effects, either local or systemic, following dermal exposure. It seems feasible that such differences could occur, as a result of some of the particle-specific factors described above.

Another factor is the potential for leaching of components, which could have consequences for local and systemic effects. Differences in the composition of different nanoparticles could result in differences in leaching potential (both in terms of rate and identity of leachates) and thus could influence toxicity. However, in the absence of information on leaching potential for nanoparticles, this remains speculative.

5. Novel nanoparticles

There is very little toxicological information on novel nanoparticles. This section summarises the information that is known, and identifies the gaps in current knowledge.

5.1. Carbon nanotubes

A description of carbon nanotubes is given in section 1.1.1.

5.1.1. Single exposure toxicity to the respiratory tract

There are no studies using the inhalation route of exposure. The only information comes from three intratracheal instillation studies in rodents. These studies were performed for preliminary screening purposes only, and each included a comparison with reference particulate materials. By their nature, as screening studies, they provide extremely limited information in terms of the likely health effects of exposure

to carbon nanotubes (CNT) in the occupational setting.

The first of the studies investigated the pulmonary toxicity of single-walled CNT (SWCNT) soot in the rat (Warheit *et al*, 2004). The soot consisted of SWCNT agglomerates (~30 nm diameter; 50-60% by weight), amorphous carbon (30-40%), nickel (5%) and cobalt (5%). Male rats (numbers not specified) were exposed to 1 or 5 mg/kg SWCNT by intratracheal instillation. Additional groups of control male rats (numbers not specified) were similarly exposed to 1 or 5 mg/kg of the following particles: a graphite/catalyst mixture (graphite particle size 3-10 µm; cobalt and nickel particle size 2-3 µm; metal content the same as for SWCNT); crystalline silica (Mil-U-Sil 5; positive control; particle size 1-3 µm); and carbonyl iron particles (negative control; particle size 0.8-3 µm).

Pulmonary toxicity was assessed by analysis of bronchoalveolar lavage fluid (BALF) for indicators of cell damage and inflammation (lactate dehydrogenase (LDH), alkaline phosphatase (ALP), protein concentration and neutrophil numbers (PMN)); by investigation of alveolar macrophage (AM) chemotaxis; measurement of pulmonary cell (tracheobronchial and lung parenchymal) proliferation; and by histopathological examination of the lungs. All examinations were performed at 24 hours, 1 week, 1 month and 3 months post-exposure. Graphite-exposed animals were examined only for histopathological and cell proliferation changes.

Mortality (15%, actual numbers not stated) was seen within 24 hours of instillation of 5 mg/kg SWCNT. Cause of death was suffocation due to blockage of the upper airways by agglomerated SWCNT. This is most likely to be an artefact of the dosing method, and not relevant to inhalation exposure. Surviving animals in this group showed no outward signs of toxicity and had normal weight gain. No other mortalities occurred.

An immediate (24 hours) inflammatory response with cell damage was seen in animals exposed to 5 mg/kg SWCNT, evidenced by increases in PMN, LDH and protein. This response was transient, and values were similar to control levels at all other time points. No treatment-related effects were seen at 1 mg/kg SWCNT. Chemotaxis was unaffected by SWCNT exposure.

Histopathological examination of the lungs of rats exposed to both dose levels of SWCNT showed multifocal granulomas, distributed diffusely and randomly in the lung. The appearance of the granulomas was not dose-related (although the lack of a dose-response could be related to the non-uniform dosing pattern associated with the exposure route) and was first observed at 1 week post-exposure. There was no apparent progression of these lesions with time. Some SWCNT agglomerates that deposited in the airways were also surrounded by granulomas, an unusual response to see outside the alveoli.

A non-statistically significant increase in tracheobronchial cell proliferation rate was seen at 24 hours in animals exposed to 5 mg/kg SWCNT. Lung parenchymal cell turnover was unaffected by SWCNT exposure.

In comparison, exposure to 1 and 5 mg/kg silica produced an immediate and sustained inflammatory lung response, with evidence of sustained cell damage also seen at 5 mg/kg. Transient increases in BALF ALP values (indicating toxicity to the surfactant secreting cells) were seen at both dose levels and impairment of AM chemotaxis was seen at 5 mg/kg.

Histopathological examination of silica-exposed animals revealed a dose-related

inflammatory response, characterised by neutrophils and foamy (lipid containing) AM accumulation, with lung tissue thickening, indicative of progression to fibrosis. There was an increase in tracheobronchial cell proliferation rate at 1 and 5 mg/kg silica (not statistically significant). Lung parenchymal cell turnover was statistically significantly increased in animals exposed to 5 mg/kg silica at 24 hours and 1 month.

The only finding in animals exposed to carbonyl iron was an immediate (24 hours), transient influx of PMNs. There were no other changes in BALF parameters, and neither carbonyl iron exposure nor graphite exposure produced any adverse histopathological findings or changes in cell proliferation.

Overall, this preliminary screening study indicates an immediate, transient inflammatory lung response to instilled SWCNT. Histopathologically, SWCNT produced granulomas within 1 week post-exposure, although unusually, this was in the absence of sustained inflammation or cell damage. The pulmonary response to SWCNT was closer to (but not as pronounced as) that produced by silica than by carbonyl iron or graphite, which suggests that SWCNT has some inherent cytotoxicity.

The second screening study investigated the toxicity of three different types of SWCNT, produced by different methods and with different metal contents (Lam *et al*, 2004). Raw (RNT), purified (PNT) and nickel-containing (CNT) nanotubes were administered in a single dose to mice by intratracheal instillation. RNT contained 27% iron (by weight). PNT was treated to remove residual metal and contained 2% iron. CNT contained 26% nickel and 5% yttrium. Other metals in RNT and CNT were present at <1%. Carbon black and silica (Mil-U-Sil-5), administered at the same dose levels as SWCNT, were included as reference dusts and mouse serum was used as the vehicle control. The particle size of the nanotubes and of the reference dusts was not stated. Nanotubes were sonicated prior to administration to reduce agglomeration.

Groups of male mice were intratracheally instilled with 0.1 or 0.5 mg of each particle type (approximately 3.3 or 16.7 mg/kg respectively, for a 30 g mouse). Based on the assumption that a mouse breathes 30 ml air per minute, and that 40% of respirable nanoparticles deposit in the pulmonary region, instillation of 0.5 mg SWCNT equates to an inhalation exposure of 5 mg/m^3, 8 hours/day for 17 days. Animals were sacrificed at 7 or 90 days post-instillation and the lungs were examined histopathologically. Body weight was measured in animals sacrificed at 90 days.

Deaths (5/9) occurred within 4-7 days post-instillation in animals treated with 0.5 mg CNT. Animals in this group were lethargic and inactive and showed a bodyweight loss of about 27% within the first week post-exposure. Survivors showed no clinical signs after one week, and gained weight. No deaths, clinical signs or bodyweight losses occurred at 0.1 mg CNT or in any other treatment group or controls. Exposure to 0.5 mg RNT produced some clinical signs (some inactivity, hypothermia, piloerection and occasional shivering), 8-12 hours following treatment, but animals were normal after this time and no weight loss occurred. No clinical signs were seen with 0.1 mg RNT, or in animals treated with PNT, carbon black or silica.

Gross examination of the lungs at 90 days post-exposure showed abnormalities in some animals exposed to 0.5 mg CNT, PNT or RNT. Histopathological examination showed granulomas, often in the interstitium. There was some evidence of necrosis and interstitial and peribronchial inflammation. The lesions seen at 90 days were generally more pronounced than those seen at 7 days. Granuloma formation and inflammation was seen in some animals exposed to 0.1 mg RNT and PNT but not

0.1 mg CNT; the lesions were less severe than seen with the higher dose.

Exposure to carbon black did not produce any inflammatory or granulomatous changes in the lungs. Exposure to 0.1 mg silica produced an inflammatory response in a single animal sacrificed at 90 days; 0.5 mg silica produced a mild to moderate inflammatory response in the alveoli and interstitium at 7 and 90 days and one mouse showed a slight granulomatous response at 7 days.

Overall, this study demonstrates a toxicity response to three different types of intratracheally instilled SWCNT in the mouse lung. Quantitative differences in response were seen between the three SWCNT materials. One form of SWCNT produced mortality; this suggests a specific effect of the particular material, perhaps associated with its metal content and the dosing method, rather than a generic effect of SWCNT. However, lung damage (granuloma formation in the pulmonary interstitium, progressing to necrotic damage in some cases) was seen with all three SWCNT materials, regardless of metal content. This, together with the results of the screening study by Warheit *et al* (2004) in which granuloma formation was observed, suggests that granuloma formation is associated with the nanotubes themselves. The pulmonary response to SWCNT was closer to that produced by the same mass dose of silica rather than carbon black, again suggesting inherent cytotoxicity of SWCNT.

In an earlier study, that was very briefly reported, guinea pigs were administered a single intratracheal dose of 25 mg of soot containing CNT (Huczko *et al*, 2001). The composition of the CNT material was not further defined, other than it was synthesised using a Co/Ni catalyst, and therefore is likely to have Co and Ni as impurities. It also appears that the material was likely to contain a mixture of single- and multi-walled CNTs. Control animals were administered soot without CNTs. Four weeks after administration, the animals were subject to pulmonary function tests (measurement of tidal volume, respiratory frequency and lung resistance) and BALF analysis. No differences between CNT-exposed animals and controls were found in any of the measured parameters.

5.1.2. Irritation

Skin and eye

In a very briefly reported study, CNT soot (not further defined) was applied as an aqueous suspension to the skin of 40 volunteers (Huczko and Lange, 2001). The exposure period was not clear. No skin reactions were observed at 96 hours.

In the same study, the CNT soot was instilled into the eye of 4 rabbits (0.2 ml suspension in water). No abnormalities were observed at 24, 48 or 72 hours.

Overall, the limited information available does not suggest any local irritancy potential of CNT.

5.1.3. Additional information

One study has investigated the toxicity of SWCNT in cultured human epidermal keratinocytes (HaCaT) (Shvedova *et al*, 2003). Cells were exposed to SWCNT (30% iron content by mass; iron valency not stated) for up to 18 hours. Exposure produced clear indications of hydroxyl (·OH) radical production with associated oxidative damage (lipid peroxidation, antioxidant depletion), and a reduction in cell viability. Addition of a metal chelator suppressed ·OH generation and improved cell viability. Ultrastructural cell changes were seen, including changes to cytoplasmic organelles

and disruption of the monolayer structure. These observations are consistent with the ability of ferrous iron (Fe^{2+}) to catalyse hydroxyl radical generation from hydrogen peroxide. However, knowledge about the valency of the iron in the SWCNT material, and on its availability to catalyse any such reactions within the skin *in vivo* is required before any reliable conclusions can be drawn in relation to the potential *in vivo* dermal toxicity of SWCNT.

Derivatives of SWCNT have been shown to cross cell membranes, and in some cases, to enter the nuclei of human and mouse fibroblasts and human keratinocytes (Pantarotto *et al*, 2004). The study was performed to determine the potential for carbon nanotubes to deliver biologically active molecules into cells, for therapeutic applications. Carbon nanotubes (1 nm diameter, 0.1-3 μm length) were labelled either with fluorescein isothiocyanate (FITC), or with an FITC-labelled peptide. These SWCNT derivatives were very water soluble and in the dilutions used in the test system, did not aggregate. The FITC-labelled SWCNT readily crossed the cell membrane and was located mainly in the cytoplasm; the peptide-SWCNT conjugate reached the cell nucleus. Fluorescein alone and FITC-peptide did not cross the cell membrane in the same test system. The mechanism whereby the SWCNT derivatives entered the cells was not elucidated, but was shown not to be via endocytosis. However, the results from these very specialised SWCNT derivatives are unlikely to be relevant to exposures to SWCNTs themselves in the occupational setting.

5.1.4. Summary of health effects of carbon nanotubes

There is a paucity of information about the potential health effects of exposure to carbon nanotubes. There are no studies that utilise the inhalation route of exposure and no studies that investigate the effects of repeated exposure. The only information is from single-exposure, screening-type studies.

In the three available studies that investigate potential toxicity to the rodent respiratory tract, it is apparent that single intratracheal exposure to SWCNT produces an immediate but transient inflammatory response, with subsequent lung pathology characterised by granuloma formation. The granulomatous response was seen for different SWCNT types, produced by different processes and with different metal compositions, suggesting that this is a generic lung response to carbon nanotubes. In the two studies that compared SWCNT with cytotoxic and non-cytotoxic reference dusts, the pulmonary responses to SWCNT were closer to those induced by silica (cytotoxic) than by either graphite or carbon black (non-cytotoxic). No effect was seen on pulmonary function parameters measured four weeks after exposure in one study.

The limited information suggests that carbon nanotubes do not have irritancy potential to the skin or eyes.

It is noteworthy that CNT produced now or in the future may fall under the definition of a ‘countable’ fibre for regulatory purposes (length > 5 μm, diameter < 3 μm, aspect ratio > 3:1 (HSE, 1996)). Given this, the toxicological concerns associated with such fibres, influenced by fibre properties such as chemical composition, surface properties and durability, must also be considered in relation to CNT.

5.2. Fullerenes

As with carbon nanotubes, there is a dearth of information about the potential toxicity of fullerenes. There is no useful information from standard toxicity studies. The

limited information that is available is summarised below.

In a very briefly reported study, fullerene soot, (C_{60} content between 0 and 15% by weight) was tested for its potential to cause skin reactions in 30 volunteers (Huczko *et al*, 1999). A soot suspension in water was placed in contact with the skin; the exposure time was unclear. No skin reactions were observed when assessed after 96 hours. In the same study, soot samples (0-15% C_{60} by mass) were instilled into the eye of 4 rabbits (0.2 ml fullerene suspension in water). No abnormality was observed at 24, 48 or 72 hours. Overall, although limited, these data suggest that fullerenes are not irritating to the skin or eye.

Fullerenes have been shown to have oxidising properties and to catalyse the production of singlet oxygen following photoexcitation. Consequently, some concerns have been raised about their carcinogenic potential. In a study to investigate potential tumour-promoting activity in the mouse skin (Nelson *et al*, 1993), groups of at least 3 female mice were treated with a single dose of 200 μg fullerene (C_{60}:C_{70} ratio ~6:1; saturated solution in benzene) or 5 μg TPA (12-O-tetradecanoyl-phorbol-13-acetate; a tumour promoter) in acetone; control mice were treated with benzene or acetone only. Mice were sacrificed 0-72 hours post-exposure and the treated skin removed for analysis of ornithine decarboxylase (ODC) activity (associated with tumour promotion in mouse skin) and DNA synthesis.

Fullerene produced a slight increase in ODC activity at 6 hours post-exposure, but did not increase DNA synthesis. Fullerene treatment was reported to produce only mild effects on the skin, but no further details were provided. TPA produced a marked increase in ODC activity and DNA synthesis and marked hyperplasia. These data suggest that fullerenes do not have any significant tumour promoting activity nor produce significant local skin effects following single dermal exposure.

In the same study, the effects of repeated dermal exposure to fullerenes was also investigated. Mice pre-treated with DMBA and subsequently exposed to 200 μg fullerene twice weekly for 24 weeks did not develop skin tumours, whereas mice similarly treated with 5 μg TPA did. Fullerene treatment did not produce any bodyweight changes, nor any pathological changes such as neoplasia or dysplasia in the treated skin. Overall, however, this study is too limited to allow any conclusions to be drawn about the potential carcinogenicity of fullerenes.

The mutagenic potential of fullerene C_{60} has been assessed in a non-standard study in bacterial cells (Sera *et al*, 1996). C_{60} in polyvinylpyrrolidone induced mutations in Salmonella strains TA102, TA104 and YG3003 (a repair enzyme-deficient mutant of T102) in the presence of rat liver S9 only when it was irradiated for 20 minutes by visible light. Further investigations suggested that the mechanism for DNA damage was the generation of singlet oxygen from C_{60} following irradiation, which led to lipid peroxidation, the production of radicals and oxidative DNA damage.

Kamat *et al* (1998) investigated the potential for fullerene to induce oxidative damage following photoexcitation, using rat liver microsomes as a model biological membrane system. C_{60} (as a cyclodextrin-C_{60} complex) was incorporated into rat liver microsomes, which were then exposed to UV or visible light. Lipid peroxidation and other oxidative damage was observed, primarily due to the production of singlet oxygen. However, given the non-standard and experimental nature of this *in vitro* model, the results cannot be reliably extrapolated to the *in vivo* situation.

Another *in vitro* study investigated the effects of fullerene (C_{60}; >99% pure) and raw soot from fullerene production on bovine macrophages and macrophage-like cells

(Baierl *et al*, 1996). Cells were incubated for up to 48 hours with C_{60}, raw soot (RS) or DQ12 quartz as a positive control. Markers of cell damage (lactate dehydrogenase (LDH)), lysosomal damage (N-acetyl-β-D-glucosaminidase (NAG)), generation of reactive oxygen species (H_2O_2 and O_2^-) and markers of chemotactic activity were evaluated.

Neither C_{60} nor RS produced any significant cytotoxicity nor lysosomal damage even up to 48 hours incubation. Both particle types elicited chemotactic activity after 48 hours, although that generated by C_{60} was minimal. C_{60} also produced very little reactive oxygen species, whereas RS produced a more marked effect; however, the nature of the reactive species could not be determined.

5.2.1. Summary of toxicity of fullerenes

There are no standard toxicological studies with fullerenes and the very limited information that is available comes from non-standard studies. There is no information on the potential consequences of single or repeated inhalation exposure, neither in terms of toxicity to the respiratory tract, nor systemically. In relation to skin exposure, all the available studies have shortcomings either in their design and/or their reporting. The only reliable conclusion that can be drawn is that fullerenes appear not to be locally irritating to the skin; similarly, there was no evidence for eye irritation potential. A few *in vitro* studies are available. These focus particularly on the potential for fullerenes to produce oxidative damage in various test systems. However, given the non-standard nature of these *in vitro* systems, no reliable conclusions can be drawn from them.

Overall, therefore, there is no reliable, relevant information on the potential toxicological consequences of inhalation exposure to fullerenes, and extremely limited information on the effects of dermal exposure.

5.3. Other novel nanoparticles

No toxicological data for any other novel nanoparticles is available.

6. Summary and conclusions

There is a paucity of information and extensive gaps in our knowledge of the potential health effects of particles intentionally produced for nanotechnology applications. This lack of information and understanding applies particularly to novel nanoparticles, such as carbon nanotubes. The limited information that is available, certainly for carbon nanotubes, suggests that they do possess significant inherent toxicity, at least towards the respiratory tract.

There is an extensive body of information on the health effects of existing micrometre-sized particulate material, particularly towards the respiratory tract following inhalation exposure. Some studies have compared this toxicity with that produced when the material is rendered nanometre-sized. The general picture that emerges from experimental animal studies is that on a mass dose basis, pulmonary toxicity is enhanced when particle size is reduced from the micrometre to the nanometre range. The increase in toxicity appears to be related at least in part, to the increase in particle surface area. However, what also becomes apparent from the data is that different existing materials in the nanometre size range exhibit different degrees of toxicity towards the respiratory tract. The reasons for these differences are currently poorly understood. Consequently, it is not possible to reach generic conclusions about toxicity based on consideration of size alone; the potential toxicity

of each individual nanoparticulate material needs to be considered on a case-by-case basis.

Consideration of the potential toxicological properties of particulate materials intentionally produced for use in nanotechnology applications must address the consequences of exposure in terms of local and systemic effects, following single and repeated exposure by relevant routes. For the occupational setting, the exposure routes of relevance are inhalation and dermal.

One aspect that may be of particular importance to the novel carbon-based materials, whose production involves the use of metal catalysts, is the issue of toxicity due to the residual metal contained within the final product. Such metals might contribute to the overall expression of toxicity by the material, either from their location within the material or by leaching out from it. For example, exposure to nickel could be an issue for some carbon nanotubes, which have a relatively high (by mass) residual nickel content. Further information on the residual metal content of carbon nanotubes and other nanoparticles and leaching rates in biological systems would be required, to determine whether metal exposure is likely to be important in the expression of respiratory tract, and any other, toxicity.

Overall, therefore, there is a clear lack of information on the potential health effects of nanoparticles produced for nanotechnology applications. From the limited information that is available, the indications are that they might possess significant toxicity potential.

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Appendix

An understanding of the interactions between insoluble particles and the epithelial barriers presented by the respiratory tract and skin is fundamental to understanding mechanisms of their toxicity to and via these occupationally relevant exposure routes. This appendix therefore describes those aspects of particle behaviour that are relevant to considerations of occupational toxicity, namely respiratory tract deposition, uptake and clearance, as well as the potential for skin uptake.

Particle deposition characteristics within the respiratory tract

The site and extent of deposition of inhaled particles depends on the physical characteristics of the particle (e.g. shape, size and density) and the pattern of breathing (e.g. oral/nasal breathing, breathing frequency and volume). Deposition in the respiratory tract is related to both aerodynamic and thermodynamic characteristics of particles (ICRP, 1994). The aerodynamic (equivalent) diameter of a particle is the diameter of a unit density sphere that has the same settling velocity due to gravity. Aerodynamic diameter depends on particle size, shape and density. The thermodynamic (equivalent) diameter is the diameter of a sphere with the same diffusion coefficient as the particle.

In general, for particles with diameters in the micrometre range (> 1 μm), aerodynamic factors predominate and deposition occurs by sedimentation (deposition due to the force of gravity) or impaction (the direct collision of a particle on an epithelial surface). For particles with diameters in the nanometre range (< 100 nm), deposition occurs as a result of random diffusional movements (Brownian motion). For particles with diameters in the range 100 nm – 1 μm, both thermodynamic and aerodynamic influences are important. In addition, for fibres, interception with airway surfaces is another important mechanism for deposition.

A predictive model for deposition of particles in the respiratory tract has been developed by the ICRP (1994). The predicted regional deposition for particles of different diameters is shown in Figure 1. (Note that the x-axis scale refers simply to diameter; however, as indicated above, for particles > ~1 μm, aerodynamic diameter will predominate, whereas for particles < ~0.1 μm, thermodynamic diameter will predominate). The model demonstrates that alveolar deposition is greatest for particles in the nanometre range (maximal alveolar deposition for particles ~ 20 nm), and is appreciably greater than alveolar deposition of particles in the micrometre range.

Precise deposition patterns will vary between species and individuals due to differences in respiratory tract dynamics and breathing patterns.

Figure 1. Deposition of inhaled particles of different sizes, in the upper and lower human respiratory tract (International Commission on Radiological Protection (ICRP) model, 1994)

[SEE SEPARATE FILE FOR FIGURE]

Particle clearance from the respiratory tract

Following deposition in the respiratory tract, the subsequent fate of poorly soluble particles depends on two factors: 1) site of deposition; 2) particle size.

In general, for particles that deposit in the upper airways (the nasal passages and tracheobronchial region), the mechanism for clearance is the mucociliary escalator. Ciliated cells lining the non-gas exchange regions of the airways waft a layer of mucous upwards to the throat. The speed of mucous flow in the upper airways of humans varies widely, with a median value of about 5 $mm.min^{-1}$ (ICRP, 1994). Material trapped within the mucociliary escalator is cleared relatively rapidly (within about 1 day in humans) by swallowing or expectoration.

Ciliated cells, and thus the mucociliary escalator do not extend to the gas exchange regions of the lungs, i.e. the alveoli. For particles that reach the alveoli, the main route of clearance is via alveolar macrophages (AM). Particles that deposit on the alveolar surface are phagocytosed by AMs. AMs can encounter the particle either by chance, or for some particles, because of stimulation by the particle of the release of factors ('chemokines') derived from cells lining the respiratory tract (Warheit *et al*, 1988). Normally only a few AMs are seen in the lungs; on exposure to excessive amounts of dust, stimulation of chemokines results in attraction of AMs and neutrophils from the blood.

Following phagocytosis, the AMs move either to the mucociliary escalator for clearance or translocate to the lymph vessels and lung associated lymph nodes. Clearance of poorly soluble particles that deposit within the alveolar region of human lungs is relatively slow, with a half-life of several hundred days (ICRP, 1994).

Recent work has suggested that translocation to the brain via the olfactory nerve may be an additional path along which nanometre particles that deposit in the nasal olfactory mucosa can travel. This path circumvents the blood-brain barrier. The supporting evidence for this comes from a study by (Oberdörster *et al*, 2004). Rats (n=6) were exposed whole-body to 150 or 170 $\mu g/m^3$ ^{13}C particles (count median diameter = 36 nm) for 6 hours and sacrificed on days 1, 3, 5 or 7 post-exposure (3 rats per sacrifice time). Three unexposed rats served as controls. Lungs, olfactory bulb, cerebrum and cerebellum were analysed for excess ^{13}C. Lung burden of ^{13}C peaked at 1 day post-exposure (1.39 µg/g tissue) and declined thereafter, but was statistically significantly elevated above control at all time points. Concentrations of ^{13}C in the olfactory bulb were slightly but statistically significantly elevated at all sacrifice times (0.35-0.43 µg/g tissue). Concentrations in the cerebrum and cerebellum were statistically significantly increased on day 1 post-exposure and on day 5 (cerebellum) or 7 (cerebrum); concentrations in these tissues ranged between 0.11 and 0.44 µg/g tissue. The authors suggested that the most likely explanation for these results was axonal transport of nasally deposited particles via the olfactory nerves.

Further investigative work to further confirm these results and to elucidate the underlying mechanisms for transport to the brain will be necessary. The toxicological significance of this finding cannot yet be determined. If confirmed in rats, other issues also require clarification: is it specific to nanometre particles, is this route also relevant to insoluble micrometre particles? How do differences in particle type and physicochemical properties (other than solubility) influence transport via this route? How should the findings in rats be extrapolated to humans? **Systemic availability of inhaled particles**

Much of the focus of attention for inhalation exposure to insoluble particles has been for effects on the respiratory tract; relatively little consideration has been given to systemic availability and distribution. However, more recently, the emergence of concerns for cardiovascular effects associated with particulate air pollution incidents has led to studies to investigate the systemic availability of nanometre particles. For example, studies are available on the systemic distribution of nanoparticles of albumin (Nemmar *et al*, 2001), carbon (Nemmar *et al*, 2002; Oberdörster *et al*, 2002), iridium (Kreyling *et al*, 2002), platinum (Oberdörster, 2000) and silver (Takenaka *et al*, 2001). Unfortunately, there are no corresponding studies with these particles in the micrometre range. However, some information on systemic distribution of micrometre TiO_2 is available for comparison.

Nanometre particles

Takenaka *et al* (2001) investigated the systemic distribution of inhaled silver particles (14 nm) in the rat. Measurable amounts of silver were found in the blood within 30 mins-2 hours following the 6 hour exposure period. Silver was also detected in the liver, kidney, heart and brain, indicating systemic distribution of the particles. When expressed as a percentage of the lung content, the silver content of these tissues was 9-21% for the liver, 3-7% for the kidney, 0.2-0.3% for the heart and 0.1-0.3% for the brain. Measurable quantities of silver remained in the liver, lung and blood at 7 days post-exposure, but not in any other organs.

Nemmar *et al* (2001) reported the passage of radiolabelled albumin particles (80 nm) from the lung to the blood, within 5 minutes of intratracheal instillation in the hamster. Radioactivity was also detected in the liver (0.06-1.24% of total radioactivity), heart (0.03-0.22% of total), kidneys, spleen and brain (levels in these organs reported to be detectable, but not quantified) at 5-60 minutes post-exposure.

Extrapulmonary translocation of nanometre particles of ^{13}C (count median diameter 22-30 nm) was investigated in the rat following whole-body inhalation exposure for 6 hours to 80 or 180 µg/m^3 (Oberdörster *et al*, 2002). Groups of 3 rats were exposed at each concentration and sacrificed at 0.5, 18 and 24 hours post-exposure. Significant accumulation of ^{13}C (indicative of particle accumulation) was seen in the liver at both exposure concentrations, by 18 and 24 hours post-exposure; at the higher exposure concentration, elevated ^{13}C in the liver was also detected at 0.5 hours. No increases in ^{13}C content were found in the heart, olfactory bulb, brain or kidney by 24 hours post-exposure. The increases in liver burden were seen without a concomitant decrease in lung burden of ^{13}C between 0.5 and 24 hours post-exposure. One possible explanation of this result is that there is an initial, rapid translocation of inhaled particles across the pulmonary epithelium, which occurs during the 6-hour exposure period and 0.5 hours post-exposure. However, it is also possible that particles entered the circulation exclusively, or at least in part, via the GI tract; GI tract exposure could have arisen as a consequence of mucociliary clearance and/or grooming. Thus, whilst this study provides evidence that systemically available nanometre particles accumulate in the liver (but not other organs), it does

not allow any conclusions to be drawn about the pathway via which particles enter the systemic circulation.

Kreyling *et al* (2002) investigated the influence of particle size on extrapulmonary distribution. This study found that particle size influenced the results. They investigated the systemic availability of iridium (^{192}Ir) particles with mean count diameters of 15 nm and 80 nm. Although the translocation of particles to extrapulmonary sites was extremely limited for both particle sizes, the translocated fraction was higher for the 15 nm particles compared with the 80 nm particles and there were correspondingly higher fractions in the extrapulmonary organs; for example, in the liver, the estimated retained fraction of 15 nm particles was almost one order of magnitude greater than that of 80 nm particles. They also showed that orally administered ^{192}Ir particles were not absorbed via the GI tract, but were excreted via the faeces within 2-3 days. This implies that any inhaled particles cleared to the GI tract via the mucociliary escalator, would not be absorbed across the gut and thus not systemically available via this route.

Subsequently, this same group confirmed limited translocation of ^{192}Ir particles (count median diameter = 15-20 nm) to extrapulmonary organs following intratracheal intubation of rats (Semmler *et al*, 2004).

Two studies have investigated the extrapulmonary distribution of inhaled nanoparticles in humans. In the first (Nemmar *et al*, 2002), male volunteers (n=5) inhaled 3-5 breaths of Technegas, an aerosol of carbon particles labelled with ^{99m}Tc. Electron microscopy of the aerosol showed individual particles of 5 to 10 nm diameter, although larger aggregates were also seen. Radioactivity was observed in the blood within 1 minute and peaked at 10-20 min, after which time levels remained relatively constant up to the final sampling point at 60 min post-exposure. Whole body radiography measurements taken 5 – 45 minutes post-exposure showed extrapulmonary distribution of radioactivity to the liver, bladder and stomach; liver radioactivity remained at a constant level of about 8% (expressed as a percentage of the initial lung radioactivity), whilst bladder radioactivity increased with time post-exposure, to about 25%. The radioactivity in the stomach is likely to have been associated with particles cleared by swallowing. Further analyses were undertaken to confirm that the radioactivity observed was associated with particles, rather than with soluble pertechnate (TcO_4^-), which can form following particle deposition in the body. The findings suggested that although some pertechnate production had occurred, at least some of the observed blood radioactivity was due to the presence of Tc-labelled particles. However, an attempt to directly observe particles in the blood was unsuccessful. These results suggest rapid translocation of nanometre particles from the lung to the systemic circulation, although the evidence is based on indirect inference, rather than direct observation.

In contrast, another study using Technegas in healthy human volunteers and COPD patients (19 subjects in total) found no evidence for distribution of particles to the liver (Brown *et al*, 2002). These authors suggested that the results reported by Nemmar *et al* (2002) were most likely to be due to distribution of pertechnate to the liver and bladder, rather than translocation of particles.

Overall, there is evidence for translocation of nanometre particles from the respiratory tract to the systemic circulation. Some studies have suggest rapid and/or significant clearance of particles from the lung to the systemic circulation, with distribution to major organs, particularly the liver, in animals and/or human volunteers. However, other groups have produced results that indicate very little, or even no translocation of nanometre particles to extrapulmonary sites. It is possible

that nanometre particles may cross the alveolar wall and enter the systemic circulation by virtue of their small size; or GI tract exposure as a consequence of mucociliary clearance, and consequent uptake from the GI tract, may play a role. More rigorous investigation is required to establish whether or not, and if so, to what extent and via which route(s), nanometre particles can enter the systemic circulation following inhalation exposure.

Micrometre particles

There is limited information on the systemic availability of inhaled micrometre particles. Some data are available for titanium dioxide (Lee *et al*, 1985a, 1985b and 1986). Rats were exposed whole-body to 0, 10, 50 or 250 $mg.m^{-3}$ coarse rutile TiO_2 (99% pure; MMAD = 1.5-1.7 µm with 84% of particles less than 13 µm) for 6 hours/day, 5 days/week for 24 months. Particles were found to accumulate in the liver and spleen, but no quantitative data were presented. In the liver, the peripheral hepatic lobules showed a more dense dust deposition than did the centrilobular region and particles were observed in Kupffer's cells and in macrophages in the portal triads; however, there was no evident tissue reaction or hepatocellular damage. In the spleen, dense particle accumulation occurred in the lymphoid tissue of the white pulp (only minimal deposition in the red pulp) with occasional aggregates of particle-laden macrophages; however, again, there was no apparent tissue reaction to the dust.

Poorly soluble polystyrene particles (about 1.1 µm diameter) instilled into the nasal passages of mice were detected in adjacent nasal associated lymphoid tissue, the draining cervical and mediastinal lymph nodes and the spleen (Eyles *et al*, 2001). The exact mechanism of distribution is unclear.

There was no evidence for transepithelial passage of uncoated polystyrene beads (0.24 µm diameter) to the pulmonary capillaries following intratracheal inhalation in rats (Kato *et al*, 2003).

For fibres, translocation to the pleura is known to occur for fibres with diameters in the micrometre range. In the absence of this type of information for fibres with diameters in the nanometre range, it should be assumed that migration to the pleura would occur.

Systemic clearance

Insoluble particles that enter the systemic circulation are generally cleared from the body by circulating macrophages. Clearance by macrophage phagocytosis is mediated by opsonisation, whereby circulating proteins are adsorbed on to the particle surface, to aid recognition of the particle by macrophages. However, the process of opsonisation is dependent on a number of factors, including particle size (Moghimi *et al*, 2001). Smaller (submicrometre) particles are less readily opsonised by circulating proteins. It is therefore possible that particles in the nanometre size range could be retained for relatively longer in the systemic circulation, compared with micrometre particles. In parallel with this, particles in the nanometre size range may escape many of the normal tissue filtration mechanisms that act to remove micrometre particles from the circulation (Moghimi *et al*, 2001). Equally, nanometre-sized particles may escape the systemic circulation via fenestrations (pores) in the capillaries. This may be particularly important in terms of particles reaching the liver, for example, where capillary fenestrae are in the region of 100 - 150 nm (Braet *et al*,

1995, cited in Moghimi *et al*, 2001).

Thus, the fate of nanometre particles that enter the systemic circulation is likely to be different to that of micrometre particles. This will almost certainly have implications for any systemic effects that could be attributed to systemic exposure.

Dermal uptake

Substances can cross the skin via three possible routes: intercellular, in which the substance penetrates the lipid medium between individual skin cells; transcellular, in which the substance enters the skin cells themselves; and trans-appendageal, in which the substance penetrates via hair follicles or sweat glands.

For absorption across the skin of particles in either the micrometre or nanometre size range to occur, dissolution into the surface moisture of the skin must occur. Absorption is therefore generally limited for poorly soluble particles, certainly via intercellular and transcellular routes. Penetration into hair follicles and sweat glands occurs for both micrometre and nanometre particles; this route has been demonstrated to occur for nanometre particles of TiO_2 (e.g. Lademann *et al*, 1999). However, the follicles and glands are also bounded by an epithelial barrier, and therefore the presence of particles at these sites does not necessarily lead to dermal uptake into the systemic circulation.

Overall, therefore, poorly soluble particles are unlikely to be absorbed across the skin. This is likely to be the case for both micrometre and nanometre particles.

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