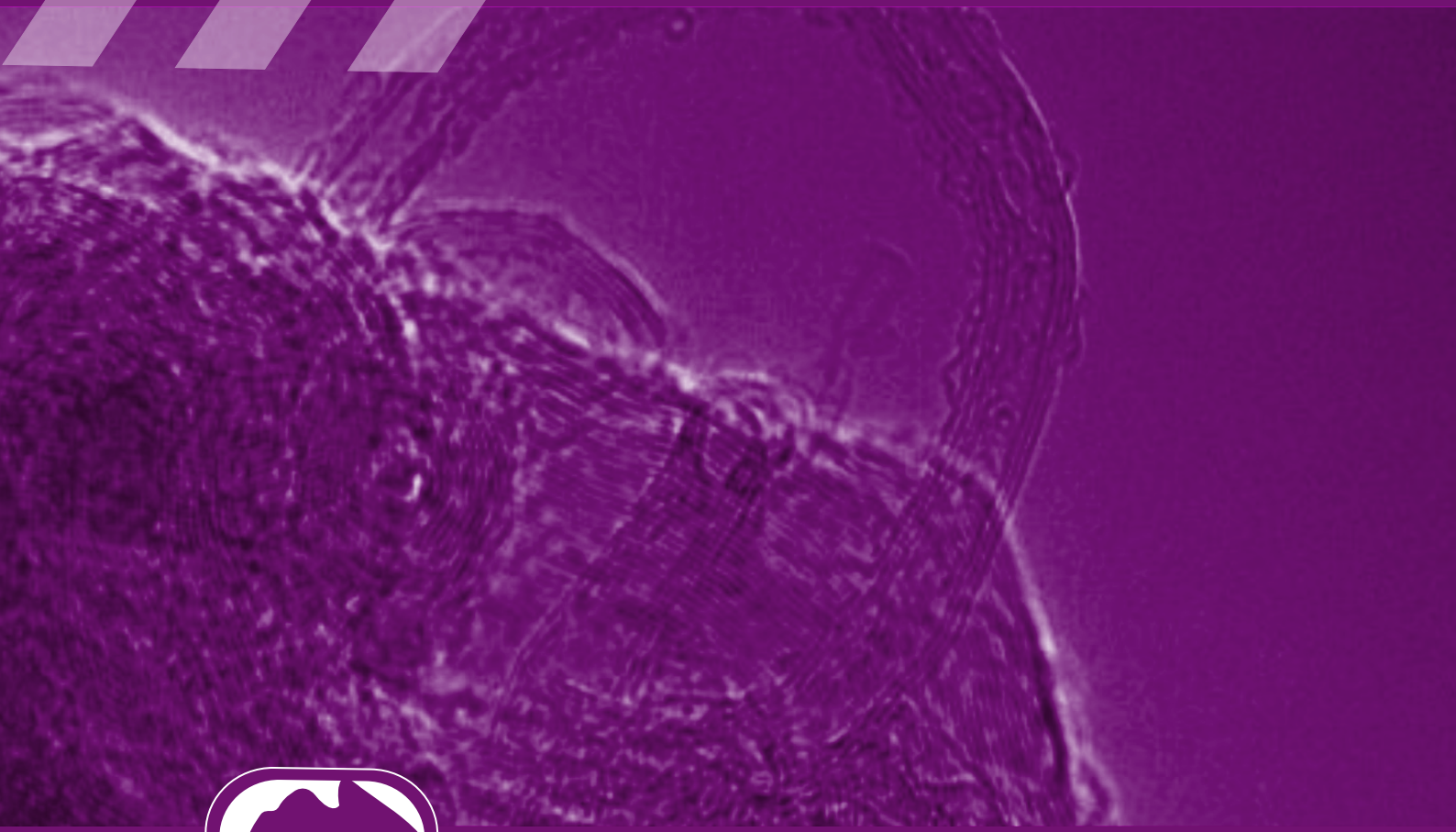
HUMAN HEALTH HAZARD ASSESSMENT AND CLASSIFICATION OF CARBON NANOTUBES



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National Industrial Chemicals Notification and Assessment Scheme

Australian Government Department of Health and Ageing

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SURRY HILLS, NSW 2010

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Overview

Carbon nanotubes (CNTs) are carbon sheets rolled to form either a seamless cylinder, known as single-walled CNTs (SWCNTs), or many cylinders stacked one inside the other, known as multi-walled CNTs (MWCNTs). CNTs have attracted a great deal of attention due to their unique structural, physical and chemical properties that lend their use to a variety of industrial and biomedical applications including fillers in composites for anti-static applications, catalysis, biosensors, composite materials with improved structural and electrical properties and drug carriers. The global production capacity for CNTs is expected to reach 1000 tonnes in 2014 with an estimated value of US$1 billion. However, there are concerns that the properties of the CNTs might lead to adverse health effects. In particular, some forms of CNTs have come under scrutiny due to their similarity to asbestos fibres in terms of their size and shape. Particle size (length and diameter), state (agglomerates and dispersions), functional groups and impurities may contribute to varying toxicological effects of CNTs.

Given the health concerns relating to CNTs and their rapid development and use in many applications, Safe Work Australia commissioned National Industrial Chemicals Notification and Assessment Scheme (NICNAS) to conduct a health hazard assessment and hazard classification on SWCNTs and MWCNTs. CNTs (single or multi wall) are not listed in the Australian Inventory of Chemical Substances (AICS) and therefore, they are considered as new industrial chemicals in Australia. If CNTs are manufactured in Australia or imported into Australia, they should be notified to NICNAS.

NICNAS predominantly used reviews and journal articles on CNTs published from January 2007 to end of June 2010 to determine the health hazards. In addition, a few key articles post June 2010 were included during document review and revision.

The *Approved criteria for classifying hazardous substances* (Approved Criteria) (NOHSC, 2004) and the Globally Harmonised System of Classification and Labelling of Chemicals (GHS) (United Nations, 2009) were used to determine the appropriate classification for CNTs. The following approach was used to describe the classification based on the information available for each health end point:

- Not classified as hazardous - OECD test guideline studies and/or other suitable scientific data acceptable for regulatory decision making are available for SWCNTs/single-walled carbon nanohorns (SWCNHs) and/or MWCNTs, however the data do not meet the criteria for classification specified in the Approved Criteria or the GHS.

- Cannot be classified - Guideline studies or other suitable scientific data acceptable for regulatory decision making (i.e. administration route relevant for human exposure) are not available for SWCNTs/SWCNHs or MWCNTs, or the available data are not sufficient to make a classification decision.
- Classified as hazardous –At least one guideline toxicity study or other suitable data for SWCNTs/SWCNHs and/or MWCNTs are available for which the outcomes meet the criteria for classification described in the Approved Criteria or the GHS.

The impact of the wide range of variables on the hazard classifications is not clear, and therefore the read-across of data from one negative test to all CNTs (and thus the outcome "not classified as hazardous") may need to be revisited if data for any given endpoint indicates that the toxicity for this endpoint covers a wider range than is implicit in the read-across assumption. Based on the limited studies conducted to OECD test guidelines, CNTs are determined to be of low acute oral and dermal toxicity, they are not irritating to skin and eyes and do not have the potential to cause skin sensitisation. Therefore, CNTs are not classified as hazardous according to the Approved Criteria (NOHSC, 2004) or the GHS (UN, 2009) for these health endpoints.

No acute inhalation toxicity studies are available for SWCNTs. The limited number of acute inhalation toxicity studies available on MWCNTs in mice demonstrated no deaths after 6 hr exposure to doses of 241 mg/m^3 (0.241 mg/L). However, it is not possible to conclude the acute inhalation toxicity potential of CNTs based on the low maximum dose tested. Limited data on intranasal or intratracheal instillation of CNTs indicated that an allergen booster is required to promote allergic responses in mice. There were no appropriate studies to make a conclusion about the respiratory sensitising potential of CNTs. Based on the limited information available CNTs cannot be classified using the Approved Criteria or the GHS for acute inhalation toxicity and respiratory sensitisation.

Based on the transient nature of the pulmonary effects reported in the three acute inhalation toxicity studies available, CNTs are not classified as hazardous according to the Approved Criteria in terms of irreversible effects after a single exposure. As the pulmonary effects reported in mice/rats subsided following exposure and there were no functional impairments reported CNTs are not classified as hazardous according to the GHS in terms of specific target organ toxicity following a single exposure.

No repeat dose oral toxicity studies have been reported to date on CNTs. The only study available on acute dermal toxicity of SWCNTs is of limited value due to only local effects being reported and the high iron (Fe) impurity content (30%) in the test material.

Two 90-day repeat dose inhalation toxicity studies in rodents have shown that MWCNTs can cause fibrosis and granulomas which, although occurring at very low doses, are postulated to be the result of lung overloading due to the high displacement volume of the low-density MWCNT assemblage structure. Although acknowledging the adverse effects are likely due to lung overload, the doses at which these effects were observed are significantly below the classification cut-off under both the Approved Criteria and the GHS, and therefore can be considered to be an intrinsic property of these particles. Hence, it is considered that inhaling MWCNTs repeatedly even at very low doses could be at least harmful to humans and as such, MWCNTs should be classified with the following provisional hazard classification for repeated or prolonged inhalation exposure in accordance with:

the Approved Criteria (3rd Edition, 2004)

Xn; R48/20 Harmful: Danger of serious damage to health by prolonged exposure through inhalation;

and

the GHS (UN, 2009)

Specific target organ toxicity following repeated exposure Category 2

Warning: May cause damage to lungs/respiratory system through prolonged or repeated inhalation exposure

Although there is no supportive data for SWCNTs, given the adverse effects have been postulated due to lung overloading, SWCNTs are not expected to behave differently. Therefore, the above provisional classification should also be applied to SWCNTs until data to the contrary becomes available, particularly as the applicability of the pathogenic fibre hypothesis to granuloma and fibrosis induction is not clear.

A few *in vitro* studies have shown positive results indicating SWCNTs and MWCNTs may have some genotoxic potential under specific circumstances. Although these studies have demonstrated that CNTs can cause oxidative stress and DNA damage, the results are inconclusive. Due to lack of sufficient *in vivo* data, CNTs cannot be classified for genotoxicity according to the Approved Criteria or the GHS.

MWCNTs have been shown to induce mesothelioma in rodents after a single intraperitoneal or intrascrotal exposure. Furthermore, recent studies have shown that MWCNTs can reach the mesothelial region. In studies conducted in mice MWCNTs have been shown to rapidly translocate to the pleura and enter the intrapleural space and be persistent after aspiration exposure, depending on the dose administered, and to reach the sub-pleural region after inhalation exposure. There is evidence to suggest a correlation between length and rigidity

(based on the diameter) of MWCNTs and a pathogenic response. Considering the limited number of studies available, it is not possible to definitively conclude the minimum length, thickness and aspect ratio of MWCNTs that contribute to the carcinogenic potential although the tests to date which have indicated mesothelioma induction have been carried out on MWCNTs having lengths of pathogenic fibre dimensions as defined by WHO (length > 5 µm). This is evidence that MWCNTs conform to the pathogenic fibre toxicity paradigm. However, as CNTs not intrinsically meeting the pathogenic fibre criteria have been shown to present as fibre-like structures of pathogenic dimensions through aggregation, it is not sufficient to determine the carcinogenic potential of CNTs on length of the individual CNT fibres alone but rather on their ability to present as a fibre with pathogenic dimensions i.e. either as the individual fibre or through aggregation.

Based on the limited data available on mesothelioma formation in animal studies and difficulty in conclusively determining whether a specific MWCNT can present as a fibre of pathogenic dimensions, all MWCNTs should be considered as hazardous and classified for carcinogenicity as follows in accordance with:

the Approved Criteria (3rd Edition, 2004)

Xn; R40	Harmful: Limited evidence of a carcinogenic effect;

and

the GHS (UN, 2009)

Carcinogen Category 2	Warning: Suspected of causing cancer

There are no studies demonstrating that SWCNTs cause mesothelioma. Neither is there evidence to suggest that SWCNTs will behave any differently with respect to the potential to form granulomas or mesotheliomas given they have been shown to be durable and have shown to elicit a fibre pathogenic response through the ability to form rigid fibre-like structures through aggregation inside the body. Hence it is prudent to consider the above classification according to the Approved Criteria or GHS as also being applicable to SWCNTs.

Based on only limited data available on reproductive/developmental toxicity, CNTs cannot be classified for reproductive/developmental toxicity according to the Approved Criteria or the GHS.

Toxicological properties of CNTs may vary due to impurities and their concentrations, dimensions, state (agglomerated/aggregated or dispersed) and surface functionality. Based on limited data available, a correlation between toxicity and any intrinsic properties cannot be

determined at this stage. Therefore, when specific toxicity data are available, a case-by-case hazard assessment of CNTs is recommended.

Abbreviations

ACP	acid phosphatase
AICS	Australian inventory of chemical substances
ALP	alkaline phosphatase
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BP	bucky paper
BSA	bovine serum albumin
bw	body-weight
c-MWCNT	carboxylic functionalized MWCNT
CNHs	carbon nanohorns
CNS	central nervous system
CNTs	carbon nanotubes
DC	dendritic cells
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPL	dipalmitoyl lecithin
FBGCs	foreign body giant cells
GHS	Globally Harmonised System of Classification and Labelling of Chemicals
IFN	interferon
IgE	immunoglobulin E
IgG	immunoglobulin G
IL	interleukin
LALNs	lung associated lymph nodes
LDH	lactate dehydrogenase
LC50	median lethal concentration
LD50	median lethal dose
LFA	long fibre amosite
LOAEC	lowest observed adverse effect concentration
LOAEL	lowest observed adverse effect level
Mcp	monocyte chemoattractant protein
MDA	malondialdehyde
Mip	macrophage inflammatory protein
MMAD	mass median aerodynamic diameter
MPO	myeloperoxidase
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWCNTs	multi-walled carbon nanotubes
NK cell	natural killer cell
NICNAS	National Industrial Chemicals Notification and Assessment Scheme
NOAEC	no observed adverse effect concentration
NOAEL	no observed adverse effect level
NPCB	nnoparticulate carbon black
NQO1	NAD(P)H:quinone oxidoreductase 1
NR	Neutral red (3-amino-7-dimethylamino-2 methylphenazine hydrochloride)
OECD	Organisation for Economic Co-operation and Development
OVA	ovalbumin
PAHs	polycylic aromatic carbons
PBS	phosphate buffered saline
PF127	polyoxyethylene-polyoxypropylene block co-polymer
PDGF	platelet derived growth factor
PMN	polymorphonuclear leukocytes
PNS	peripheral nervous system
RNA	ribonucleic acid

ROS	reactive oxygen species
SFA	short-fibre amosite
SWCNHs	single-walled carbon nanohorns
SWCNTs	single-walled carbon nanotubes
TG	technical guidance
TGF	transforming growth factor
Th cytokines	T-helper cytokines
TNF	tumour necrosis factor
WST-1	water soluble tetrazolium salt (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium)
XTT	3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate

Glossary

agglomerate: Collection of loosely bound particles or aggregates or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components. The forces holding an agglomerate together are weak forces, for example van der Waals forces, as well as simple physical entanglement.

aggregate: Particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components. The forces holding an aggregate together are strong forces, for example covalent bonds, or those resulting from sintering or complex physical entanglement.

apoptosis: A form of regulated cell death.

blood-brain barrier: A central nervous system epithelial cell barrier that is impermeable to all except lipophilic molecules (such as oxygen, carbon dioxide, and ethanol) and those with specific transporters (such as sugars and some amino acids).

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

comet assay: a single cell electrophoresis assay to assess DNA damage.

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

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

granuloma: Small nodules usually consisting of epithelioid macrophages surrounded by lymphocytes.

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

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

LDH assay: Cell viability assay that measures cell membrane integrity based on the release of lactate dehydrogenase (LDH), a stable cytoplasmic enzyme present in most cells.

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

MTT assay: Cell viability assay based on conversion of the water soluble tetrazolium salt MTT to insoluble purple formazan in live cells by action of succinate dehydrogenase within the mitochondria.

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

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

necropsy: The procedure of post-mortem examination.

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

neutrophil: A type of leucocyte or white blood cell.

NR assay: Cell viability assay based on the ability of viable cells to take up Neutral Red dye in the lysosomes of the cells.

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

WST-1 assay: Cell viability assay based on the conversion of the water-soluble tetrazolium salt (WST-1) to a coloured formazan product by mitochondrial dehydrogenases.

XTT assay: Cell viability assay based on the conversion of the water-soluble XTT sodium salt to an orange formazan product by mitochondrial dehydrogenases.

1. Introduction

1.1 Objectives

The National Industrial Chemicals Notification and Assessment Scheme (NICNAS) has been commissioned by Safe Work Australia to conduct a health hazard assessment of carbon nanotubes (CNTs) for all health endpoints in order to determine hazard classification, with the focus on carcinogenicity.

Classification is to be undertaken according to the *Approved criteria for classifying hazardous substances* [NOHSC:1008(2004)] (Approved Criteria) and the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) third revised edition (United Nations, 2009). Key variables to be considered in the hazard assessment, where applicable, include:

- type of carbon nanotubes, including a distinct assessment of both multi-walled carbon nanotubes (MWCNTs) and single-walled carbon nanotubes (SWCNTs);
- length or structure of carbon nanotubes;
- the influence of agglomeration state, and
- the effect of impurities

1.2 Sources of information

The hazard assessment is based on information from journal articles, including reviews, published since 2007 up to end of June 2010, although articles published pre-2007 have been included where relevant. In addition, a few key articles since June 2010 have also been included during document review and revision. Information for this hazard assessment was also obtained from a report by Safe Work Australia on 'Engineered Nanomaterials: A Review of the Toxicology and Health Hazards' (Safe Work Australia, 2009).

Due to the limited data available on SWCNTs, studies investigating health effects of single-walled carbon nanohorns (SWCNHs) have been included where relevant.

1.3 Report structure

Section 1 provides the objectives and sources of information for this report. Section 2 provides a brief background on the drivers for the health hazard assessment of carbon nanotubes. Section 3 provides information on chemical identity, synthesis and general physical properties of carbon nanotubes. Section 4 summarises the published literature on CNTs under specific health end points including a section on toxicokinetics and other toxicity studies that have investigated potential toxicity. The study details are included in Appendix 1 under each health end point. Studies on SWCNTs (with supporting data from SWCNH studies) and MWCNTs have been reviewed separately. Section 5 provides information considered in the determination of the hazard classification of CNTs for each health end-

point according to the Approved Criteria (NOHSC, 2004) and the GHS (UN, 2009). Section 6 details the recommendations to Safe Work Australia for the classification of CNTs against the Approved Criteria (NOHSC, 2004) and the GHS. Publications reviewed but not considered relevant for the health hazard classification of CNTs are included in Appendix 2.

2. Background

Carbon nanotubes are graphite sheets rolled to form either a seamless cylinder, known as SWCNTs, or many cylinders stacked one inside the other, known as MWCNTs. The lengths of these tubes can range from several hundred nanometers (nm) to several micrometers (µm) (Simeonova, 2009), but longer CNTs up to 3 mm in length have also been reported (CSIRO, 2010). However, their diameter is usually less than 100 nm (Simeonova, 2009).

Carbon nanotubes have attracted a great deal of attention due to their unique structural, physical and chemical properties and show promise for a wide array of applications in fields as diverse as electronics and medicine. However, concerns have been raised over the safety of CNTs. In particular, CNTs have come under scrutiny due to their thin fibre-like structure and presumed insolubility in the lungs, both attributes of harmful asbestos fibres (Donaldson and Poland, 2009).

Recent research in rodents has shown a variety of outcomes ranging from no response in the lungs to severe lung injury with CNTs delivered using chemical or physical dispersions. These disparities have been attributed to intrinsic and extrinsic properties of the specific CNTs used in rodent studies and to the method of administration.

CNT cytotoxicity has been attributed to a range of issues such as metal impurities, length and size distribution, surface area, dispersion and aggregation status, coating or functionalisation, immobilisation (i.e. tested as a suspension in cell culture media or immobilised to a matrix or to a culture dish), cellular uptake or internalisation and cytotoxic response of different cell lines to CNTs (Hussain et al., 2009). Nano particulate impurities, such as catalytic trace metals, can remain on the surface of CNTs even after several post-purification treatments and can influence their toxicity (Tejral et al., 2009). In addition, the post-purification methods can alter properties of CNTs such as degree of aggregation, wall structure and surface functionalisation (Tejral et al., 2009).

CNTs are not on the Australian Inventory of Chemical Substances (AICS) and are therefore considered as new industrial chemicals in Australia. Since there have been no notifications to NICNAS to date, CNTs have not been assessed by NICNAS previously.

3. Identity, synthesis and properties

3.1 Chemical identity of carbon nanotubes

Chemical Name Fullerenes, tubular

CAS No. 308068-56-6

Presently, only one chemical name and CAS No. has been assigned to CNTs. It is expected that further CAS numbers will be assigned in the future to specify the number of walls and functionalisation, and possibly further parameters such as diameter, length and description of morphology.

CNTs belong to the family of fullerenes, the third allotropic form of carbon along with graphite and diamond (Lacerda et al., 2006). The lattices of carbon atoms remain continuous around the circumference and parallel to the axis of the nanotube (Lison and Muller, 2008).

3.2 Synthesis

There are several major techniques of CNT synthesis the most common of which is the chemical vapour deposition method, where nanotubes are formed by the decomposition of a carbon containing gas with the use of nano-sized catalytic particles, usually iron (Fe), cobalt (Co), yttrium (Y) or nickel (Ni). The most commonly used production technologies are the HiPco process, and the CoMoCAT process. Other methods include the arc-evaporation method that involves passing a current between two graphite electrodes in an atmosphere of helium in the presence of metal catalysts (Co or Ni) and laser ablation that involves employment of a powerful laser to vaporise metal (Co and Ni) graphite target (Shvedova et al., 2009).

After synthesis, CNTs are usually purified by washing with dilute acid to remove amorphous carbon (soot), free residual catalyst and any support material (silica, alumina, and magnesium oxide) (Donaldson et al., 2006). Washing with concentrated acids is required to remove some support materials like silica and alumina. Highly purified tubes may have additionally undergone some form of oxidation and the purification procedures can potentially introduce defects in the tubes.

3.3 Physical properties

SWCNTs have a diameter ranging from 0.4-2.0 nm and a length of 20-1000 nm. MWCNTs have larger diameters (1.4-100 nm) as they consist of several layers of carbon cylinders and have lengths up to several microns (Lacerda et al., 2006), or millimetres (CSIRO, 2010). CNTs have a strong tendency to bundle together in ropes (nanoropes) as a consequence of attractive van der Waals forces. Bundles of nanotubes can be considerably longer and wider than the nanotubes from which they are formed (Donaldson et al., 2006). SWCNT and

MWCNT bundles or nanoropes can be up to 500 nm or 3 µm in diameter, respectively (Sanchez et al., 2009). In turn, agglomerates can form from these nanoropes generating a wide range of particle-sized structures in the micrometre range (Mercer et al., 2008). The degree and kind of aggregation of MWCNTs is determined by the rigidity of nanotubes and whether their diameters are thin enough to allow their buckling and self-aggregation into low-density, particle-like, intertwined and coiled assemblages. Also the production method may impact the type of assemblage structure and whether it is stabilised by mere agglomeration or some kind of intertubular aggregation (physical entanglement). Hence, depending on these characteristics, agglomerate structures of nanotubes may differ appreciably from thin-walled MWCNT to thick-walled, rigid MWCNT (Pauluhn, 2010a).

CNTs have very high surface areas. Theoretically, discrete SWCNTs have surface areas of ~1300 m^2/g, whereas MWCNT generally have surface areas of a few hundred m^2/g. As a result of the strong tendency of CNTs to bundle, the available surface area is lowered in the case of SWCNTs to ~300 m^2/g (Donaldson et al., 2006). In addition to high surface area, CNTs have high mechanical strength, high electrical conductivity and high thermal conductivity (Lacerda et al., 2006).

Depending on the purification procedure, CNTs can contain varying amounts of residual metals (Co, Fe, Ni, Mo), amorphous carbon and residual support material (alumina, magnesium oxide or silica) (Donaldson et al., 2006). Purification can lead to breakages, resulting in defective or shorter tubes and surface functionalisation (Sanchez et al., 2009).

Pristine CNTs (as prepared, non-functionalised) are inherently hydrophobic. Modification of the surface with different molecules can render CNTs more hydrophilic. Functionalisation also reduces bundling or aggregation of individual tubes (Lacerda et al., 2006).

SWCNHs although not part of the scope of the assessment report, have also been considered in support for the hazard assessment (see Section 4). SWCNHs are similar in structure to SWCNTs having diameters of 2-5 nm. Unlike the single-walled and multi-walled CNTs, SWCNHs are free of metal impurities because they are produced by laser ablation of a pure graphite target. SWCNHs typically form aggregates which have a spherical structure of 50-100 nm (Miyawaki et al., 2008).

4. Toxicology

Toxicology studies conducted on laboratory animals are summarised under each health end point. Details of these studies with information used for health hazard classification in Section 5 are provided in Appendix 1.

Studies on SWCNHs have also been considered in support for the hazard assessment where acute health end points are data poor for SWCNTs (see Section 3.3 on description of SWCNHs).

4.1 Toxicokinetics

No information is available on gastrointestinal (GI) tract absorption or dermal absorption of unfunctionalised CNTs. Studies on toxicokinetics of unfunctionalised CNTs were conducted by intravenous administration or intratracheal instillation.

CNTs that entered the lung through the respiratory route were not distributed to the systemic circulation or central nervous system (CNS) (Simeonova, 2009). Yang et al. (2007 & 2008) reported biodistribution of intraveneously injected SWCNTs in mice with accumulation in the liver, lungs and spleen, and little excretion via urine and faeces. Up to ~3% of the injected dose was found in the brain, indicating that SWCNTs can cross the blood brain barrier.

It has been demonstrated that MWCNTs can reach the sub-pleural tissue and even penetrate the pleura and enter the intrapleural space after inhalation or aspiration exposure (Ryman-Rasmussen et al., 2009b, Porter et al., 2010 and Mercer et al., 2010). Furthermore, the translocation to the sub-pleural tissue and intrapleural space was demonstrated to be rapid. The rate of translocation and their persistence may be dose dependent (Mercer et al., 2010).

Functionalisation of CNTs may change toxic properties of CNTs. Intravenously injected ammonium functionalised SWCNTs cleared rapidly through the renal route (3.5 h maximum half-life in blood) without accumulation in tissues (Singh et al., 2006). Hydroxylated SWCNTs also cleared via the renal route without accumulation (Lacerda et al., 2006). However, polyethylene glycol functionalised SWCNTs persisted in the liver and spleen of mice for 4 months (Schipper et al., 2008). These results indicate that accumulation and/or elimination of SWCNTs may depend on their surface chemistry. Further studies are required to investigate biopersistence of CNTs in tissues.

Due to the limited studies available, it is unclear to what extent the various properties of CNTs (dimensions, agglomerate/aggregate status, surface characteristics and impurities) affect their absorption, distribution, metabolism and excretion. However, there is some

evidence to suggest that functionalisation with polar groups such as ammonium and hydroxyl groups may aid in the elimination of CNTs from the body.

4.2 Acute toxicity

4.2.1 Acute oral and dermal toxicity

Three acute oral toxicity studies have been conducted in rats and mice for SWCNTs, SWCNHs and MWCNTs.

The acute oral median lethal dose (LD50) was determined to be > 1000 mg/kg bw for SWCNTs and reported as > 5000 mg/kg bw for MWCNTs (Table 1).

Table 1: Summary of acute oral toxicity studies

Test material	LD50	Remarks / Reference
SWCNT (1) Ultrashort (diam. ~ 1 nm, length ~20-80 nm, Fe < 1.5%); (2) Raw (diam. ~ 1 nm, length ~1-2 µm, Fe 25%); (3) Purified (diam. ~ 1 nm, length ~1-2 µm, Fe < 4%)	> 1000 mg/kg bw in mice	Limit test; No deaths; Kolosnjaj-Tabi et al, 2010
SWCNH	> 2000 mg/kg bw in rats	No deaths; Miyawaki et al., 2008
MWCNT (mean diameter 10-15 nm; mean length ~200-1000 nm; Co 0.53%)	> 5000 mg/kg bw in rats	OECD TG 423; No deaths; Pauluhn et al., 2010b

Based on a single study SWCNTs are of low acute oral toxicity. SWCNHs were also found to be of low acute oral toxicity providing further support that SWCNTs are of low acute oral toxicity.

There was no published acute dermal toxicity data on SWCNTs. In a single acute dermal toxicity study conducted according to OECD test guidelines with MWCNTs in rats a LD50 > 2000 mg/kg bw was reported.

Based on studies conducted according to OECD test guidelines MWCNTs are of low acute oral and dermal toxicity. Given only one type of MWCNT has been studied with no reported toxicity, the impact of dimensions (length and width), effect of impurities and agglomeration/aggregation state on oral and dermal toxicity of MWCNTs cannot be determined.

4.2.2 Acute inhalation toxicity and pulmonary effects

Pulmonary inflammation (irritation and swelling) and fibrosis (increased collagen content and/or structural alterations) are well-known consequences of particulate exposure. Nanoparticles have shown a greater influence on these effects compared to submicron size particles of the same material. Lung inflammation is often due to oxidative stress. Reactive oxygen species generation at the surface of nanomaterials release free radicals leading to the depletion of antioxidants and initiate lipid peroxidation in cells which triggers a series of events that result in inflammation. Chronic inflammation may cause lung disease and/or cancer (Lewinski et al., 2009).

Susceptibility of the pleural mesothelium to fibrous dusts is well known. Compared with larger particles, nanoparticles have a higher deposition rate in the peripheral lung and therefore may cross the pulmonary epithelium and reach the interstitium to be circulated in the blood stream.

The fraction of inhaled nanoparticles likely to be deposited in the alveolar region of the lung varies considerably according to the particle characteristics (size, shape) and their behaviour in air (Safe Work Australia, 2009). Once inhaled, CNTs can interact with internal fluids and agglomerate into larger sized structures. The size of the agglomerate may affect its final deposition site within the lungs and also the efficiency of the clearance mechanism. Fractional deposition in the alveolar region of the respiratory tract for healthy individuals is greatest for particles in the size range of ~10-30 nm (Safe Work Australia, 2009).

Pulmonary toxicity of CNTs has been evaluated using a number of different administration routes (inhalation, pharyngeal aspiration and intratracheal instillation). Inhalation exposures are considered to more closely mimic occupational exposures and avoid the bolus effects that can occur from intratracheal instillation and pharyngeal aspiration. Indeed researchers have observed different pulmonary effects dependent on the method of delivery. For example, Li et al., (2007) reported that the lesions induced by intratracheal instillation and inhalation exposure were different. This may be due to the differences in the size of MWCNT aggregates formed and their distribution in the lung from the two administration methods.

Therefore results from administration routes other than inhalation must be treated with caution.

No acute inhalation toxicity studies have been reported for SWCNTs, but there are studies reported on MWCNTs.

Table 2: Acute inhalation toxicity studies with MWCNTs

Test material	Study details	Key Findings	Reference
MWCNT *Bulk*: L: 0.3-50 µm D: 30-50 nm > 94% pure Ni 0.34%; La 0.03% *Aerosol:* Aerosol consisted of amorphous aggregates 2 µm or less with protruding tubes extending beyond the nexus. Smaller aggregates and individual MWCNTs were also present. MMAD 714 ± 328 nm.	Nose-only 6 h inhalation exposure to normal and ovalbumin sensitised C57BL/6 mice. Dose: 100 mg/m^3 Observation period: 1 and 14 d	Significant airway fibrosis observed in ovalbumin sensitised mice only. No deaths up to 100 mg/m^3 (NOAEC > 100 mg/m^3)	Ryman-Rasmussen et al (2009a)
MWCNT *Bulk*: L: 0.3-50 µm D: 30-50 nm > 94% pure Ni 0.34%; La 0.03% *Aerosol:* Aerosol consisted of agglomerated and individual nanotubes with lengths from less than 100 nm to more than 10 µm. MMAD 183 nm (high dose), 164 nm (low dose).	Nose-only 6 h inhalation exposure to C57BL/6 mice Doses: 1 or 30 mg/m^3 (estimated deposition of agglomerated and individual nanotubes was 0.2 or 4 mg/kg bw) Observation period: 1 d, 2, 6 and 14 wks	Sub-pleural fibrosis after 2 and 6 weeks in 30 mg/m^3 dose group. Nanotubes in sub-pleural wall and incidences of fibrosis diminished after 14 weeks. No deaths up to 30 mg/m^3.	Ryman-Rasmussen et al (2009b)
MWCNT (Baytubes®) *Bulk*: D: 10-16 nm L: not specified Co 0.53% *Aerosol:* MMAD 2.2-2.9 µm	Nose-only 6 h inhalation exposure to Wistar rats Dose: 241 mg/m^3 Observation period: 90 d	Pulmonary inflammation subsided with time, following exposure, in contrast to quartz exposure. No deaths up to 241 mg/m^3. In a comparative study at a dose of 11 mg/m^3 using MWCNTs containing 0.12 and 0.53% Co, showed pulmonary inflammation had no correlation with Co content.	Ellinger-Ziegelbauer and Pauluhn (2009)

L: Length D: diameter

The median lethal concentrations (LC50s) have not been reported in any of the acute inhalation toxicity studies using MWCNTs. However as no deaths were observed in any of the studies this indicated LC50 values are greater than the highest tested dose (i.e. LC50 > 241 mg/m^3).

A no observed adverse effect concentration (NOAEC) (reported as the no observed adverse effect level (NOAEL) by the study author) of 100 mg/m^3 has been established based on no adverse effects observed in non-ovalbumin sensitised mice exposed to MWCNTs for 6 h (Ryman-Rasmussen et al., 2009a). Given only the sensitised mice demonstrated airway fibrosis after a 6 h exposure to MWCNT, indicating that pre-existing inflammation was required to cause airway fibrosis after a single exposure to MWCNTs, this finding suggests that individuals with asthma may be more susceptible to the effects of inhaled MWCNTs (Ryman-Rasmussen et al., 2009a). However the test samples used in these two studies contained 0.34% nickel which is known to elicit immune responses and pleural fibrosis (Ryman-Rasmussen et al., 2009a).

Macrophage embedded nanotubes in the sub-pleural wall after 6 h exposure to 1 or 30 mg/m^3 MWCNTs have been reported in mice (Ryman-Rasmussen et al., 2009b). Sub-pleural fibrosis was observed after 2 and 6 weeks following inhalation of the high dose, but the incidence diminished and the number of nanotubes remained in the sub-pleural wall decreased over 14 weeks.

In another acute inhalation toxicity study (Ellinger-Ziegelbauer and Pauluhn 2009), no deaths have been reported in rats with MWCNT doses up to 241 mg/m^3. Pulmonary effects that subsided during the 3 month post exposure period were observed in mice indicating the reversible or transient nature of these effects.

Although the exposure doses are reported in these acute inhalation toxicity studies, the actual deposited doses are not known, limiting a comparison of toxicity effects across the different studies.

Studies have been conducted using intratracheal instillation (doses up to 17.3 mg/kg bw in rats) or pharyngeal aspiration (doses up to 80 µg/mouse) of CNTs in rats and mice to investigate pulmonary effects following a single exposure. Although these administration methods are not representative of occupational exposure due to the bolus doses that can lead to lung overloading, the methods do allow for the actual delivered dose to be determined. However, despite many of the drawbacks of these administration methods, the results of these studies can provide some insight into the toxic effects of CNTs noting the limitations of these studies. For example, although there are issues associated with physical changes occurring during dosing of CNTs by these administration routes, such as

aggregation/agglomeration, the results of the studies by Shvedova et al. (2005) and Mercer et al. (2008) with SWCNTs administered to mice by pharyngeal aspiration indicate that the particle status (i.e. agglomerates /aggregates or dispersed) can lead to different types of pulmonary lesions and can determine where these lesions may occur. A rapid progressive fibrosis with two distinct morphologies specific to aggregates (discrete granulomas) and dispersed SWCNTs (diffuse interstitial fibrosis and alveolar wall thickening) was reported by Shvedova et al. (2005). This finding was similarly observed by Mercer et al. (2008) in a study that showed improved dispersion of SWCNTs altered the distribution into the alveolar interstitium, inducing increased collagen production that progressed in the absence of persistent inflammation (Mercer et al., 2008).

The findings of Miyawaki et al. (2008) suggest the role metal impurities play in pulmonary toxicity. Equal doses of SWCNTs with 5-10% Fe and SWCNHs without metal impurities (see Table 3 for dimensions of test material) have shown foamy macrophages in rats after intratracheal instillation. However, granulomas were only observed in rats receiving Fe containing SWCNTs, suggesting the Fe impurities may enhance toxic effects. Given mice treated by pharyngeal aspiration with both dispersed and aggregated SWCNTs containing only 0.23% Fe developed rapid progressive fibrosis (Shvedova et al, 2005), a correlation with metal impurities and their concentration cannot be made when all available studies are taken together.

Table 3: Pharyngeal aspiration and intratracheal instillation studies with CNTs

CNT type tested	Study details	Key Findings	Reference
Pharyngeal aspiration			
SWCNT D: 1-4 nm > 99% pure with Fe 0.23%	C57BL/6 female mice 0, 10, 20 or 40 µg/mouse Necroscopy: 1, 3, 7, 28, 60 d after administration Reference materials: ultrafine carbon black or SiO_2 at 40 µg/mouse	Rapid progressive fibrosis with two morphologies: (i) aggregates - granulomas mainly associated with hypertrophied epithelial cells (ii) dispersed - diffuse interstitial fibrosis and alveolar wall thickening. Persistent changes in pulmonary function and decreased bacterial clearance. Reference materials did not induce granulomas or alveolar wall thickening and demonstrated a weaker pulmonary inflammation.	Shvedova et al (2005)
Dispersed SWCNT D : 0.69 µm (agglomerate size) Non-dispersed	C57BL/6 mice 10 µg/mouse Observations: 1 and 7 d and 1 month	Dispersed SWCNTs more prone to enter alveolar walls and more potent in producing interstitial fibrotic reaction in absence of granulomas.	Mercer et al (2008)

CNT type tested	Study details	Key Findings	Reference
D : 15.2 µm (agglomerate size) Primary particle size of SWCNTs not reported.	after exposure	The non-dispersed largely deposited (~80%) in alveolar region proximal to small airways as large agglomerates rapidly forming granulomatous lesions.	
SWCNT D 1-4 nm L ; 1-3 µm C 99.7% ; Fe 0.23%	C57BL/6 mice 0, 10 or 40 µg/mouse and exposed 3 days later to *Listeria monocytogenes* Observations: 3, 6, 8 and 10 d after LM exposure	Decreased pulmonary clearance of LM and enhanced inflammatory response.	Shvedova et al (2008)
Acid-functionalised SWCNT Non-acid functionalised SWCNT (as control)	Mice 40 µg/mouse Observations: 24 h after exposure	Acid functionalisation increased cardiopulmonary toxicity of SWCNTs. No CNTs in heart tissue.	Tong et al (2009)
MWCNT D : 20-30 nm L : 50 µm > 95% purity	C57BL/6 mice 0 or 20 µg/mouse with and without post-exposure (12 h later) to 0.5 ppm ozone (3 h; nose only) Observations: 5 and 24 h after ozone exposure	MWCNTs elicited pulmonary injury and inflammation. Post-exposure to ozone did not exacerbate the effects of the MWCNTs. In fact, some CNT-induced cytotoxic/inflammatory responses were attenuated in mice following exposure to low levels of ozone.	Han et al (2008)
MWCNT D: 31±23 nm L: 20±10 µm Fe ~3.5% (encapsulated in core) High degree of agglomeration in administered PBS suspension 30-300 nm in diameter and an average size of 98±10 nm.	C57BL mice 20 or 40 µg/mouse Observations: 1 and 7 d after exposure	MWCNTs induced a pulmonary cytotoxic and inflammatory response (measured by markers in BALF) after 1 day with some degree of resolution after 7 days.	Han et al (2010)
MWCNT Characteristics in dispersion medium: D: 49 ± 13.4 nm L: 3.86 µm Fe 0.32%; Na 0.41%	C57BL/6 mice 10, 20, 40 or 80 µg/mouse Observations: 1, 7, 28 and 56 days after exposure	Rapid onset of pulmonary fibrosis at all doses tested that persisted throughout exposure period in 20 µg and 80 µg exposure groups. MWCNTs observed in pleura in 80 µg exposure group.	Porter et al (2010) (details in Sec 4.1)
MWCNT Characteristics in dispersion medium: D: 49 ± 13.4 nm L: 3.86 µm Fe 0.32%; Na 0.41%	C57BL/6 mice 10, 20, 40 or 80 µg/mouse Observations: 1, 7, 28 and 56 days after exposure	Dose-dependent, rapid translocation to subpleural tissue and intrapleural space observed at all doses tested except for the lowest dose of 10 µg where no translocation was observed.	Mercer et al (2010) (details in Sec 4.1)

CNT type tested	Study details	Key Findings	Reference
Intratracheal instillation (IT)			
SWCNT D: 1.0 ± 0.2 nm L: several hundred nanometers to several hundred micrometers. Mostly aggregated into bundles > 90% purity with 5-10% Fe	Wistar rats 17.3 mg/kg bw Observation: 7 or 90 d Positive control: finely ground quartz	Limited incidences of foreign body granulomas and foamy macrophages. Quartz induced more severe toxicological response.	Miyawaki et al (2008)
SWCNH Average diameter: 100 nm No metal contaminants	Wistar rats 17.3 mg/kg bw Observation: 7 or 90 d Positive control: finely ground quartz	Limited incidences of foamy macrophages and anthracosis; Accumulation of particles in the lung Quartz induced more severe toxicological response.	
SWCNT Primary particle size: D : 0.9-1.7 nm L : ≤ 1µm (~95% C with 2% Fe and < 0.001% Co, Ni and Mn) Delivered as agglomerates and aggregates – size not stated.	ApoE mice 54 µg/mouse 3 and 24 h follow-up	DNA damage in BAL cells increased significantly	Jacobsen et al (2009)
MWCNT ground (D: 11.3 nm, L: 0.7 µm) and unground (D: 9.7 nm, D: 5.9 µm)	Rats Dose: 0.5, 2 or 5 mg/rat Observation: 0, 28 or 60 days	Both types caused inflammation and a dose-dependent fibrotic response. Ground MWCNTs caused higher degree of inflammation. Ground MWCNTs dispersed throughout lungs inducing formation of granulomas in the alveolar spaces or the interstitium. Unground MWCNTs induced collagen-rich granulomas around aggregates in bronchi.	Muller et al (2005)
MWCNT D : 50 nm L : 10µm (> 95% pure and < 0.2% La and Ni)	Kumming mice 0.05 mg/mouse Observation: 8, 16 or 24 d after intratracheal instillation	Deposition of clumps led to inflammation of lining of bronchi and severe destruction of alveolar netted structure around them.	Li et al (2007)
MWCNT D: 20-50 nm L: 0.5-2 µm	Rats Doses: 0, 1, 10 or 100 µg/rat	No incidences of fibrosis or signs of inflammation in lungs after 180 days; apoptosis of alveolar macrophages.	Elgrabli et al (2008a)

L: length; D: diameter

Overall, available studies (inhalation, intratracheal and pharyngeal aspiration) have reported some pulmonary effects in mice or rats when exposed to CNTs, however no deaths were reported in any of the studies. The type and location of the pulmonary lesions appear to be linked to the particle status with larger agglomerates depositing in the bronchi and forming granulomas and more dispersed CNTs reaching the alveolar region and inducing fibrosis. However, a correlation between toxicity with any other intrinsic and extrinsic properties cannot be determined at this stage based on the limited data available.

As there were no rat deaths reported at 241 mg/m^3 MWCNT exposure, the LC50 for MWCNTs should be > 241 mg/m^3. It is not considered possible to make a conclusion on the degree (low or high) of acute inhalation toxicity of CNTs based on the available studies.

4.2.3 Skin and eye irritation

Skin and eye irritation potential for SWCNTs cannot be determined as no studies are available. However, a study conducted according to OECD test guidelines but using doses significantly less than the specified dose on SWCNHs suggests that SWCNTs may not be skin or eye irritants (Miyawaki et al., 2008). As CNHs do not have impurities compared to CNTs, the impact of impurities on irritation cannot be determined. In addition it has been reported that soot comprised of a high CNT content (type not stated) has shown no skin irritation or allergic responses in human volunteers or albino rabbits (Huczko and Lange, 2001).

Based on a single study conducted according to OECD test guidelines MWCNTs are not irritating to the skin but may be mildly irritating to the eye (Pauluhn et al., 2010b). No study details were reported. Given only one type of MWCNT has been studied the effect of impurities, size and state (agglomerated/aggregated or dispersed) on skin and eye irritation potential cannot be determined.

4.2.4 Sensitisation

Dermal

Skin sensitising potential of SWCNTs cannot be determined as no studies are available. However, based on negative results seen in a modified maximisation skin sensitisation test in guinea pigs (reported to be conducted to OECD test guidelines) with SWCNHs (Miyawaki et al., 2008), SWCNTs may not be skin sensitisers. As CNHs do not have impurities compared to CNTs, the impact of impurities on skin sensitisation cannot be determined.

Based on a modified maximisation skin sensitisation test in guinea pigs conducted according to OECD test guidelines, MWCNTs do not have a skin sensitising potential (Pauluhn et al., 2010b). Given only one type of MWCNT has been studied the effect of impurities, size and

state (agglomerated/aggregated or dispersed) on skin sensitisation potential cannot be determined.

Respiratory

Substances that stimulate the immune system may cause hypersensitivity reactions or allergies. Studies indicate CNTs may promote allergic immune responses (Nygaard et al., 2009) and exacerbate airway inflammation (Inoue et al., 2009) based on research conducted in mice using intranasal or intratracheal administration respectively. However the ability of dendritic cells to activate T-lymphocytes which initiate immune responses has not been affected by carboxylic functionalised MWCNTs (c-MWCNT) in an *in vitro* study (Wang et al., 2009). Due to the variability of testing methods utilised (i.e. *in vivo* vs *in vitro*), test materials used (impurity levels, lengths, diameters, functionalisation) and the doses tested, it is not possible to make a conclusion about the respiratory sensitising potential of CNTs or to correlate any respiratory sensitisation potential with intrinsic properties of CNTs.

4.3 Repeated dose toxicity

4.3.1 Oral and dermal toxicity

No repeat dose oral toxicity studies have been reported to date on CNTs. There has been a single repeat dose dermal toxicity study reported with SWCNTs but no studies with MWCNTs.

Dermal exposure to unpurified SWCNTs with 30% Fe induced skin inflammation around or within the hair follicles of mice after daily exposure for 5 consecutive days (Murray et al., 2009). Systemic toxicity was not reported. In the absence of studies on purified SWCNTs, it is not possible to conclude whether or not the dermal effects were the result of exposure to CNTs or the Fe content of the test substance.

Given the lack of repeat dose oral and dermal toxicity studies on CNTs, systemic toxicity of SWCNTs and MWCNTs cannot be determined.

4.3.2 Inhalation toxicity

Five repeat dose inhalation toxicity studies in mice and rats have been reported for MWCNTs. However, no repeat dose inhalation toxicity studies have been reported for SWCNTs.

All repeat dose inhalation toxicity studies have shown pulmonary effects in mice and rats with MWCNT exposure (Table 4). All three mice studies were conducted using whole body exposure compared to nose only or head-nose exposure for the two rat studies.

Table 4: Repeat dose inhalation toxicity studies on MWCNTs

Test material	Study details	Key Findings	Reference
MWCNT D ; 50 nm L : 10µm > 95% pure and < 0.2% La and Ni Aerosol consisted of small size clusters of MWCNTs reported to be mostly in the respirable range (no further details reported).	Kumming mice 0.05 mg/mouse Whole-body exposure to aerosol for 5, 10 or 15 d (6 h/d) Dose: 32.6 mg/m^3 Estimated lung deposition: 0.07, 0.14 and 0.21 mg for 5, 10 and 15 d exposure groups, respectively. Observation: 8, 16 or 24 d respectively after first exposure	Aggregations in lining wall of bronchi and alveoli wall. Smaller aggregations in alveoli. No inflammatory cells around aggregates in bronchi. Proliferation and thickening of alveolar wall. No deaths.	Li et al (2007)
MWCNT D: 10-20 nm L: 5-15 µm > 98% C with 0.5% Ni and 0.5% Fe Aerosol consisted of agglomerates including some free tubes. MMAD 0.7-1 µm for 0.3 and 1.0 mg/m^3 and 1.8 µm for 5 mg/m^3.	C57BL/6 mice Whole body exposure to aerosol for 7 or 14 days, 6 h/day Doses: 0, 0.3, 1.0 and 5.0 mg/m^3	Did not cause any significant lung damage, but did suppress immunity. Most MWCNTs taken up by macrophages.	Mitchell et al (2007)*
MWCNT D : 50 nm L : 10 µm > 95% purity with La and Ni < 2% Aerosolised MWCNTs were reportedly to be predominantly in respirable sizes (no further details reported).	Kumming mice Whole-body exposure to aerosol, 6 h/day, once in every 2 days for 30 and 60 days. Dose: 32.6 mg/m^3 Estimated lung deposition 0.21 mg (30-day) and 0.42 mg (60-day).	Induced severe pulmonary toxicity (aggregates of CNTs on bronchi and alveoli walls and thickening of alveoli wall) in 60-day exposure group but not in the 30-day exposure group. Higher deposition in 60-day group may have exceeded lung clearance threshold.	Li et al (2009)
MWCNT (with relatively low dust forming potential) D : 5-15 nm L : 0.1-10 µm 90% C and 10% metal oxides (9.6% Al_2O_3 + Fe + Co) Aerosol: 0.7- 2 µm wool-like agglomerates	Wistar rats Head-nose exposure to aerosol for 90 days, 6 h/day, 5 days per week. Doses: 0, 0.1, 0.5 or 2.5 mg/m^3	Induced a dose dependent inflammation and granuloma formation in the lung and associated lymph nodes. No sign of pulmonary fibrosis, but pronounced alveolar lipoproteinosis. LOAEC = 0.1 mg/m^3 based on single granulomas observed at this dose level.	Ma Hock et al (2009)

Test material	Study details	Key Findings	Reference
MWCNT D : 5-15 nm L : 0.1-10 µm > 98% C; ~0.5% Co MWCNTs micronised to increase dustiness MMAD: 2.7-3.4 µm Coiled, tangled agglomerates	Wistar rats Nose-only exposure to aerosol for 90 days (6 h/day, 5 days per week) Doses: 0, 0.1, 0.4, 1.5 or 6.0 mg/m^3 Post exposure observation period: 6 months	NOAEC = 0.1 mg/m^3 based on no treatment related effects at this dose. At 0.4 mg/m^3 and above exposure related lesions were observed in the upper respiratory tract (goblet cell hyper- and/or metaplasia, eosinophilic globules, and focal turbinate remodelling) and lower respiratory tract (inflammatory changes in the bronchioloalveolar region and increased interstitial collagen staining adjacent to deposited particles). Reduction in the severity of the inflammatory response was seen at 0.4 mg/m^3 and above during the post-exposure observation period. The post-exposure observation period was too short to reveal any appreciable reversibility of the effects.	Pauluhn (2010a)

D: diameter; L: length

* The test material used in this study was later confirmed as a mixture of carbon nanofibres and carbon nanotubes, with the majority being nanotubes (McDonald and Mitchell, 2008)

The two 90-day inhalation toxicity studies conducted in rats using MWCNTs of similar dimensions (same length and diameter tubes) have established a lowest observed adverse effect concentration (LOAEC) of 0.1 mg/m^3 for 0.7-2 µm agglomerates (Ma Hock et al., 2009) and a NOAEC of 0.1 mg/m^3 for 2.7-3.4 µm agglomerates (Pauluhn, 2010a). The purity of MWCNTs used was higher in the test which established the NOAEC (> 98% C), compared to the study in which a NOAEC could not be determined (90% C). The high aluminium oxide (Al_2O_3) level (9.6%) in the test material may have contributed to the effects seen at 0.1 mg/m^3 in the Ma Hock study, compared to the Pauluhn study which did not show any effects at the same dose level. The exposure of animals to MWCNTs in these two studies was described differently i.e. head-nose (Ma-Hock et al, 2009) vs nose only (Pauluhn, 2010a), hence it is also not clear if the effects reported at 0.1 mg/m^3 (Ma-Hock et al., 2009) were due to the possible increased exposure or due to the size difference of agglomerates. Kuempel (2011) used animal dose response data from Ma-Hock et al. (2009) and Pauluhn (2010) studies to extrapolate risk in workers if exposed to MWCNTs for a 45-year working lifetime. The human exposure equivalent was estimated as 6.8 µg MWCNT/m^3 (8-h in air) for 5.6 years based on the rat LOAEL of 0.1 mg/m^3 for granulomatous inflammation of minimal or greater severity in the 13-week study.

As there were no repeat dose inhalation toxicity studies on SWCNTs, the information available is not sufficient to conclude that inhalation hazards of SWCNT could be any different to MWCNTs.

As four (out of 5) repeat dose inhalation toxicity studies conducted on MWCNTs did not include post exposure recovery periods, it is not possible to make a conclusion about the reversibility of the pulmonary effects observed. The only study that had a 6-months post exposure observation period (Pauluhn, 2010a) indicated a slight reduction of the inflammatory response during this period, however the length of the post exposure observation period was insufficient to observe complete reversibility of the effects. Further repeat dose inhalation toxicity investigations with longer post exposure recovery periods are required to test the sustainable or transient nature of the pulmonary effects.

Furthermore, the two 90 day repeat dose inhalation studies use morphologically similar CNTs, specifically highly curled, wool-like bundles. A more pronounced repeat dose response may be seen if testing was conducted on rigid, straight CNTs of pathogenic fibre dimensions (see Section 4.5). This is a deficiency in the literature that warrants further investigation.

MWCNTs used in these studies had various impurities such as Ni, La, Co, Fe and Al_2O_3 at varying percentages. Considering the variability in test material, it is not possible to conclude whether impurities (and their respective concentration) influenced the toxicity. As all studies were conducted with MWCNTs, up to ~10 µm length, it is not possible to determine the effects of nanotube length on the inhalation toxicity. Kuempel (2011) suggested that until specific particle size characteristics are linked to qualitative and quantitative differences in toxicity, it would be prudent to apply the available data to risk assessment and management of other CNTs.

4.4 Genotoxicity

There have been limited *in vitro* and *in vivo* genotoxicity studies conducted on CNTs. SWCNTs, SWCNHs and MWCNTs were all found to be negative in bacterial reverse mutation and chromosomal aberration assays (Kisin et al., 2007; Wirnitzer et al. 2009; Di Sotto et al., 2009 and Miyawaki et al., 2008). However, SWCNTs did induce the frequency of DNA damage in a dose-dependent manner in Chinese hamster lung fibroblast V79 cells (Kisin et al., 2007), mouse embryo fibroblast cells (Yang et al., 2009) and human epithelial BEAS 2B cells (Lindberg et al., 2009). Comet assay has shown positive results in two cell lines for SWCNTs (> 99% and > 95% pure). DNA damage in mouse embryonic stem cells exposed to MWCNTs has also been reported but this study is of limited value due to single dose tested and lack of positive controls (Zhu et al., 2007).

Table 5: Summary of *in vitro* and *in vivo* genotoxicity studies

Test material	Test type	Result	Reference
SWCNT			
SWCNT (> 99%) D: 0.4-1.2 nm Fe 0.23%	Bacteria reverse mutation assay (Ames test) in *Salmonella typhimurium* (YG1024 and YG1029).	Not mutagenic in 2 strains tested without metabolic activation.	Kisin et al (2007)
	Chromosomal aberration (Chinese hamster lung fibroblast V79 cell line)	Not genotoxic	
	Comet assay (Chinese hamster lung fibroblast V79 cell line)	Genotoxic. DNA damage at 24 hr in a dose dependent manner at 24, 48, 96 µg/cm^2.	
SWCNT (99.9% pure) D: ~ 8 nm L: < 5 µm	Mouse embryo fibroblast cells	DNA damage by mechanical injury to cell nucleus.	Yang et al (2009)
SWCNT (> 50% SWCNT and ~40% other CNTs) with < 5% catalyst metals (Co and Mo) D: 1.1 nm L: 0.5-100 µm	Comet assay (human epithelial BEAS 2B cells)	Genotoxic. Dose dependent increase in DNA damage.	Lindberg et al (2009)
	Micronucleus assay (human epithelial BEAS 2B cells)	Genotoxic. Significant increase in micronucleated cells after 48 h, but no dose response observed.	
SWCNH			
SWCNH Dimensions not reported	Bacterial reverse mutation assay in *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* (WP2uvrA) with or without metabolic activation.	Not mutagenic	Miyawaki et al (2008)
	Chromosomal aberration (Chinese hamster lung fibroblast cell line)	Not genotoxic	
MWCNT			
MWCNT (purified) Free of metal catalysts	Mouse embryonic stem cells (ES)	DNA damage Study is of limited value (single dose tested; no positive control)	Zhu et al (2007)
MWCNT (Baytubes®) agglomerates (0.1- 0.3 mm); > 95% C; Co < 1%; amorphous C not detectable.	Bacterial reverse mutation test (Ames test)	Not genotoxic	Wirnitzer et al (2009)
	Chromosomal aberration test (Chinese hamster fibroblast V79 cells)	Not genotoxic	
MWCNT (> 90% pure) D: 110-170 nm L: 5-9 µm Metal impurities < 0.1%	Bacterial reverse mutation assay in *Salmonella typhimurium* (TA98 and TA100) and *Escherichia coli*	Not mutagenic	Di Sotto et al (2009)

Test material	Test type	Result	Reference
	(WP2uvrA) with and without metabolic activation.		
MWCNT bucky paper (similar to woven asbestos)	In vitro and in-vivo implantations 1. *In vitro*: 5 types of human cell cultures. 2. *In vivo*: Patch of BP on male rats	Effects specific to transformed cells (eg cancer cell lines). No effects in normal or healthy animals	Belluci et al (2009)

D: diameter; L: length

Considering all available information, it is possible to conclude that SWCNTs may have some potential to cause DNA damage or genotoxicity. However SWCNHs without metal impurities have shown negative results in the Ames and chromosomal aberration test indicating the need for further investigation on impurities of CNTs and their impact on genotoxicity/mutagenicity potential. MWCNTs may also induce DNA damage under some circumstances (Belluci et al., 2009; Zhu et al., 2007).

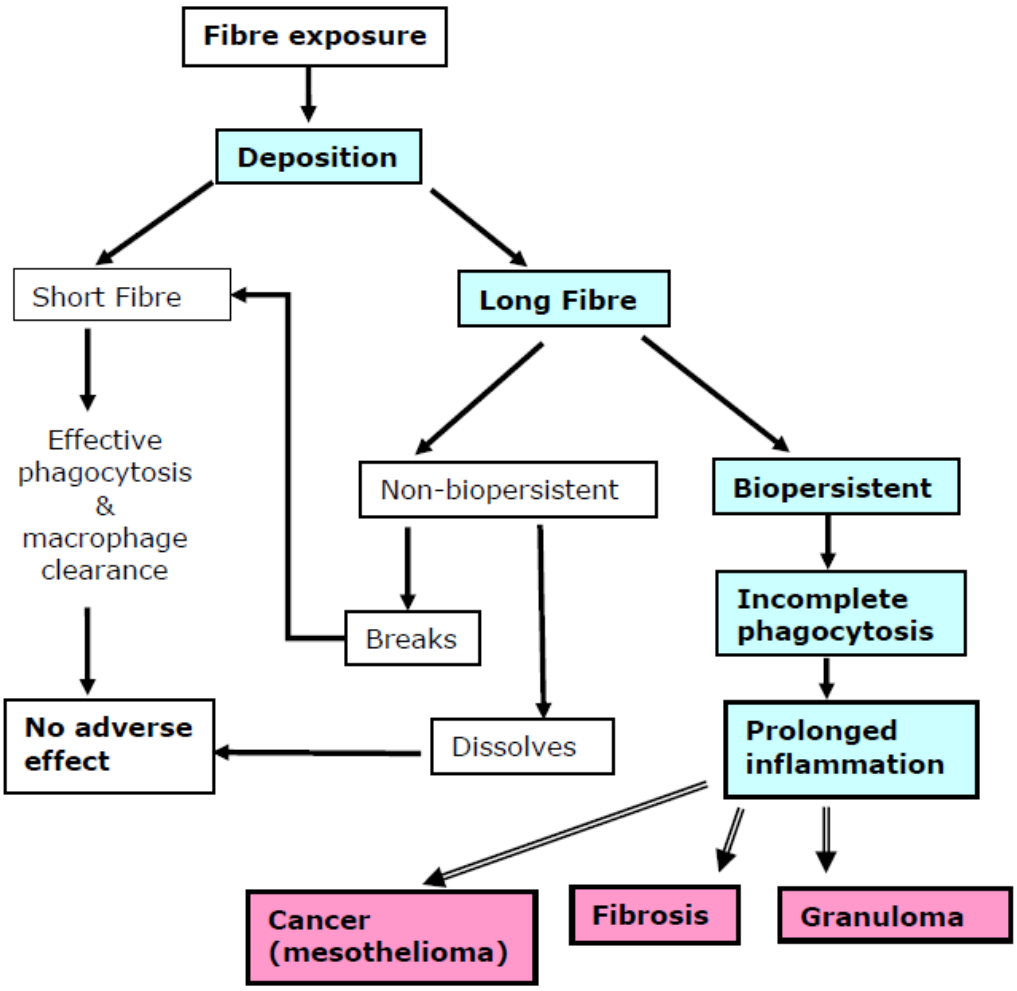
The CNTs tested for genotoxicity had different dimensions and impurities. Some studies have used CNTs with a wide range of lengths (0.5-100 µm; Lindberg et al., 2009). Therefore, it is not possible to correlate the genotoxic potential of CNTs to impurities and/or dimensions of the CNT based on the limited data available.

4.5 Carcinogenicity

No 2-year carcinogenicity studies conducted according to the OECD test guidelines are available for SW- or MWCNTs. Given some CNTs have attributes similar to asbestos (high aspect ratio and possibly biopersistent), the focus of available studies has been on assessing the potential of CNTs to cause asbestos-like carcinogenesis i.e. mesothelioma. The studies available have used either intraperitoneal, intrapleural or intrascrotal instillation methods to investigate the potential for mesothelioma formation. In addition, an inhalation study has been conducted to investigate whether CNTs delivered to the lung reach the mesothelial region.

A report by Safe Work Australia have presented a flow chart for the fibre toxicity paradigm (Figure 1) which shows the pathological response (granuloma formation, fibrosis and cancer) associated with long, thin biopersistent fibres (Safe Work Australia, 2009). Long fibres are defined by Donaldson et al. (2006) as fibres that significantly exceed the size of macrophages and are taken to be 10-20 µm long. The World Health Organization (WHO 1985) defines pathogenic fibers as being greater than 5 µm long and thinner than 3 µm, with the important feature of an aspect ratio of > 3:1 (Safe Work Australia, 2009). Biopersistent fibres do not dissolve or break into shorter fibres in the lung (Poland et al., 2008).

Figure 1: Summary of the fibre toxicity paradigm (reproduced from a report by Safe Work Australia (Safe Work Australia, 2009))



Jaurand et al (2009) has compared the physical and chemical parameters of asbestos and CNTs (see Table 6 and 7).

Table 6: Comparison between physical and chemical parameters of asbestos and fibre-like CNTs*

Parameter	Comparison
Shape	Both are elongated particles; fibre shaped
Dimensions	Asbestos fibre diameter: range of 100 nm. Chrysotile fibrils: ~50 nm diameter. Same order as MWCNTs.
Structure	Chrysotile: multi-walled rolled sheets of brucite ($Mg(OH)_2$) and silicon oxide (SiO_2). Important aggregation with CNTs, which may form more entangled bundles, ropes, than asbestos.
Chemistry	Different chemistry. Possibility of metal impurities in both asbestos and CNTs.
Surface reactivity	Both show sorptive properties to biological molecules. Reactive oxygen species (ROS) production: no definite answer for CNTs

*Reproduced from Jaurand et al., 2009.

Table 7: Comparison between biological effects of asbestos and fibre-like CNTs*

Cell/tissue response	Comparison
Particle uptake	Demonstrated with both types. Conflicting results with CNTs. Exocytosis found with CNTs, so far not investigated with asbestos.
Cytotoxicity	Both cytotoxic.
DNA damage, mutation, gene interaction	Found with both asbestos and CNTs
Transfection	Gene transfer is with asbestos. CNT gene knockdown.
Biodistribution	Both types are cleared via the lymphatic system and found in different organs
Inflammation, granulomas, fibrosis	Found with asbestos and CNTs. Both types show dependence of biological effects with fibre dimensions: bioactivity of long fibres
Cancer	Mesotheliomas found with both asbestos and CNTs by peritoneal exposure

*Reproduced from Jaurand et al., 2009.

Biopersistence

The potential for biopersistence of CNTs in the lung has been demonstrated in a study by Muller et al. (2005) that showed a significant fraction of the administered dose of MWCNTs after intratracheal instillation still remained in the lung after 60 days and that the rate of clearance was dependent on length (i.e. 81.2% remained for 5.9 µm long MWCNTs and 36% remained for 0.7 µm MWCNTs). In addition, in a durability study using an *in-vitro* treatment/incubation in a simulated biological fluid as a prediction of biopersistence, three out of four types of carbon nanotubes tested were shown to be durable i.e. they showed no or minimal loss in mass and no change in fibre length or morphology (Safe Work Australia, 2011a). The CNTs tested in the study are given in Table 8. Two types of asbestos fibres and one type of glass wool fibres were used as controls.

Table 8: Physico-chemical characteristics of CNTs used in Safe Work Australia (2011a) study

Type of CNT	Diameter (nm)	Length (µm)	Soluble metals > 5 µg/g	Description of morphology
SWCNT	5±2	4±2	Fe 185, Co 442, Ni 47.4, Mo 144, Mn 15.7, Al 6.2	Bundles of tightly agglomerated SWCNTs in which the presence of individual CNTs could not readily be determined.
$MWCNT_{spin}$	9±3	200-300*	Fe 50.1, Sr 48.4	Agglomerated sheets of very long fibres with a hair-like appearance.
$MWCNT_{tang2}$	10.3±5	5-20*	Fe 606, Mo 655, Al 41.6, Zn 9.5	Bundles of intermediate length MWCNTs. Often stellate in form with longer fibres protruding from the central tangled agglomerate, a large proportion of which are in respirable size range < 5 µm.
$MWCNT_{long1}$	64±16	12±6	Fe 15.6	Dispersed bundles and singlets of long and intermediate-length MWCNTs, many in the range 10-20 µm and longer. Many very short fibres often decorate the long fibres.

*Length as supplied by manufacturer

In the study, samples were incubated in a simulated biological fluid (Gambles solution) for up to 24 weeks, with samples removed, filtered, dried and weighed at defined time points (0, 3, 6, 10 and 24 weeks). SWCNT and $MWCNT_{spin}$ showed no loss of mass and no change in morphology or average fibre length. $MWCNT_{tang2}$ showed ~25% loss of mass after 24 weeks incubation with no change in morphology. This loss of mass was only observed at the 24 week time point. $MWCNT_{long1}$ showed a ~30% loss of the original mass at all time points from week 3 onwards except for week 10 where a mass loss of ~20% was observed, with slightly decreased fibre length and decreased proportion of long fibres (>20 µm fibres decreased from 10% to 8% by week 3, to 4% by week 10; >15 µm fibres decreased from 30% to 18% by week 3 and to 13% by week 10), indicating that they had undergone fibre dissolution and/or breakage. Although the $MWCNT_{tang2}$ showed a mass loss of ~25%, the study authors have considered that this mass loss is not significant as it reflects experimental error (~20%) experienced in the study for recovery of the CNTs and a consistent trend was not evident across all time-points. However, the study authors did not rule out that the $MWCNT_{tang2}$ may have experienced a small mass loss. In contrast, the study authors considered the loss of mass observed for $MWCNT_{long1}$ to be consistent and statistically significant across all time-points.

The result is somewhat surprising as the only CNT found to be not durable was the $MWCNT_{long1}$ having the thickest diameter (~64 nm) in contrast to the SWCNT having only a diameter of ~5 nm. The study authors suggest that the loss of mass and fibre shortening observed for the $MWCNT_{long1}$ could be attributable to the dissolution of a potentially soluble component and/or defects in the CNTs resulting from synthesis, or removal of impurities that could act as points of weakness for mechanical or chemical attack. Given only one type of CNT showed a statistically significant loss in mass and change in fibre length, this finding also illustrates that durability is not consistent across all types of CNTs.

Studies investigating the potential for CNTs to cause mesothelioma

There are seven studies that have assessed the potential for CNTs to cause mesothelioma.

MWCNTs

Mice administered a single intraperitoneal dose of MWCNTs at 3 mg/animal (equivalent to 1 x 10^{10} particles) had large mesothelial tumours after 25 weeks that were invasive to several tissues but no metastases were detected. The overall incidence of mesothelioma was greater for the MWCNT treated animals (14/16) compared to the positive control group that were dosed with crocidolite (14/18; 1 x 10^9 particles) (Takagi et al., 2008). The study however has been criticised for the use of extraordinarily high doses (Donaldson et al., 2008). The high doses may have resulted in extensive abdominal fibrosis causing death by constriction ileus. In addition concerns were raised whether the p53 mice were too sensitive (Donaldson et al., 2008).

In another study, rats administered MWCNTs at 1 mg/kg bw by a single intrascrotal injection developed mesothelioma and died before the end of the study period of 52 weeks. However, no mesothelioma was found in crocidolite treated rats (at 2 mg/kg bw) in this study (Sakamoto et al., 2009), although the study authors suggested that the dose used may have been too low to induce mesothelioma compared to previous studies. The MWCNTs used in this study were reported to be the same as that used by Takagi et al (2008), however the dose used was estimated to be 120 times less.

Three other intraperitoneal studies showed inflammatory responses and/or granuloma formation with single injections of MWCNTs in rats and mice, however, mesothelioma formation was not observed in the post-exposure observation periods (Muller et al., 2009; Poland et al., 2008; Safe Work Australia, 2011a). Although the post exposure observation period in the Muller study was 24 months, it was only 7 days in the Poland and Safe Work Australia studies. Therefore it is not known if the inflammatory and granulomatous changes would develop into mesotheliomas in the latter studies.

In a recent study, single injection of MWCNTs into the pleural cavity of mice elicited formation of fibrotic lesions along the parietal pleura and proliferation in the mesothelial layers that increased with time (Murphy et al., 2011). However, given the relatively short observation period of 24 weeks, it is not known if these fibrotic changes would develop into mesothelioma.

SWCNTs

Three types of SWCNTs, ultrashort (~20-80 nm), raw (~1-2 µm) and purified (~1-2 µm), administered as a single intraperitoneal dose to mice at 50-1000 mg/kg bw induced granuloma formation in a dose dependent manner through coalescing inside the body to form large (> 10 µm) fibre-like structures (Kolosnjaj-Tabi et al., 2010). In contrast to the large flexible bundles of the raw and purified SWCNTs, the short, compact bundles of ultrashort SWCNTs were able to diffuse into the organs resulting in the formation of granulomas inside the organs as well as on the surface of organs. No granuloma formation was observed with aggregates < 10 µm. Thus the ability to form fibre-like structures through aggregation was relevant for potential granuloma formation. No mesotheliomas were observed after the relatively short 150 day observation period. Although high doses were used in this study increasing the likelihood of the CNTs to coalesce inside the body to form fibre-like structures, it is not possible to exclude that repeated exposure to low doses of SWCNTs over a long period of time will not coalesce inside the body to form fibre-like structures.

In the only other study conducted with incubated and non-incubated SWCNTs of slightly longer length (4±2 µm), no inflammatory response was observed after a single intraperitoneal administration in mice with a post-observation exposure period of 7 days (Safe Work Australia, 2011a).

Table 9: Studies investigating the potential of CNTs to cause mesothelioma

Test Material	Study details	Key Findings	Reference
Intraperitoneal injection			
SWCNT D: ~1 nm for all CNTs Ultrashort L: ~20-80 nm; Fe (<1.5%) Short, compact bundles of aggregates (< 300 nm) that were difficult to disperse in administered suspension Raw L: ~ 1- 2 µm; Fe (25%) Tangled, flexible bundles (< 5 µm)	Swiss mice For ultrashort: single dose of 50, 300 or 1000 mg/kg bw For raw and purified: single dose of 50, 300 or 500 mg/kg bw Observation: 14 or 150 d after administration	All SWCNTs tested induced the formation of granulomas in a dose-dependent manner. SWCNTs induced granuloma formation by coalescing inside body to form large (> 10 µm) fibre-like structures. Granulomas observed only on the surface of the organs for the raw and purified SWCNTs in contrast to the ultrashort SWCNTs where granuloma formation also occurred inside the organs.	Kolosnjaj-Tabi et al (2010)

Test Material	Study details	Key Findings	Reference
Purified L: ~ 1- 2 µm; Fe (< 4%) Tangled, flexible bundles (< 5 µm) in administered suspension		Small aggregates (< 10 µm) phagocytosed without granuloma formation. Short (< 300 nm) excreted through kidneys and bile.	
MWCNT <u>Tangled (x2)</u> D: ~15 nm, L: 1-5 µm D: ~10 nm, L: 5-20 µm Consists of agglomerates, a large proportion of which are in the respirable range < 5 µm. <u>Long straight (x2)</u> D: 84 nm, L: 13 µm (12% > 20 µm; 24% > 15 µm) Dispersed bundles and singlets D: 165 nm, L: 56 µm (76% > 20 µm; 84% > 15 µm) Regular bundles and ropes	C57BL/6 mice A single dose of 50 µg/mouse Observations: 24 h & 7 d after administration	Tangled MWCNTs did not cause any significant inflammation or giant cell formation. A small non-significant granuloma response observed in one animal only. Long straight MWCNTs produced inflammation, foreign body giant cells (FBGCs) and granulomas similar to that observed with long-fibre amosite.	Poland et al (2008)
MWCNT D : 100 nm L : 27.5% > 5 µm (100% < 20 µm) Fe 0.35% Aggregates among dispersed rod-shaped or fibrous particles in administered suspension (0.5% methyl cellulose).	P53 heterozygous mice A single dose of 3000 µg/mouse Observations: 25 weeks after administration (due to 100% mortality) Positive control: crocidolite (3000 µg/mouse)	MWCNTs induced mesothelioma (invasive but no metastasis). All MWCNT-treated animals died before the end of the observation period. Mesothelial response in MWCNT treated animals was greater than crocidolite treated animals.	Takagi et al (2008)
MWCNT with or without defects D: 11 nm L: 0.7 µm - with defects (dangling bonds and products of reaction of dangling bonds with air; Al 0.37%, Fe 0.49%, Co 0.48%); - without defects (low level of structural defects and depleted metal content; Al 0.37%, Fe < 0.01%, Co < 0.01%) (Both types formed agglomerates)	Wistar rats Single dose of 2 or 20 mg/animal MWCNT (with defects) or 20 mg/animal MWCNT (without defects) Positive control: 20 mg/animal crocidolite Vehicle control: Phosphate buffered saline Observation period: 24 months	MWCNT with or without structural defects did not induce mesothelioma. MWCNTs with structural defects elicited inflammatory responses similar to crocidolite, but not sustained to have further activity. Crocidolite induced a carcinogenic response in 34.6% of animals treated. Vehicle control 3.8%.	Muller et al (2009)

Test Material	Study details	Key Findings	Reference
1. SWCNT (without incubation) D: 5±2 nm L: 4±2 µm Tightly agglomerated bundles, presence of individual CNTs could not readily be determined; and SWCNTs (after incubation in Gambles solution for 10 weeks) There was no change in the dimensions or morphology compared to the non-incubated SWCNTs. 2. MWCNT (without incubation) D: 64±16 nm L: 12±6 µm (10% > 20 µm; 30% > 15 µm); and MWCNT (after incubation in Gambles solution for 10 weeks) There was no decrease in average fibre width but there was a statiscally significant decrease in the average length compared to MWCNTs non-incubated. Additionally there was a decrease in the proportion of long fibres. L: (4% > 20 µm; 13% > 15 µm)	C57BL/6 mice A single dose of 50 µg/mouse Observations: 24 h & 7 d after administration	SWCNTs did not induce an inflammatory response before or after incubation. MWCNTs (not incubated) induced an acute inflammatory response at 24 h that did not subside by 7 d, and also induced a strong fibrotic response at 7d. MWCNTs (incubated) elicited a reduced inflammatory and fibrotic response compared to non-incubated MWCNTs.	Safe Work Australia (2011a)
Intrapleural injection			
MWCNT <u>Tangled</u> (x2) D: ~15 nm, L: 1-5 µm D: ~10 nm, L: 5-20 µm <u>Short straight</u> D: 20-30 nm, L: 0.5-2 µm <u>Long straight</u> (x2) D: 84 nm, L: 13 µm (12% > 20 µm; 24% > 15 µm) D: 165 nm, L: 56 µm (76% > 20 µm; 84% > 15 µm) Negative control: Short fibre amosite (SFA) (1% > 20 µm)	C57BL/6 mice A single dose of 5 µg/mouse Observations: 24 h, 7 d, 4 wks, 12 wks and 24 wks	Long straight MWCNTs showed an early acute inflammatory response in the first 7 days followed by the progressive development of fibrotic lesions along the parietal pleura and proliferation in the mesothelial layers. The positive control LFA also showed an early acute inflammatory response whereas the short straight and tangled MWCNTs did not elicit a significant inflammatory reaction at any time point examined.	Murphy et al (2011)

Test Material	Study details	Key Findings	Reference
Positive control: Long fibre amosite (LFA) (35% > 20 µm) The short and tangled MWCNTs and SFA were reported to be of respirable size (< 5 µm)			
Intrascrotal injection			
MWCNT Reported to be exactly the same test material as used by Takagi et al (2008). D : 82% in range 70-110 nm L : 72.5% in range 1-4 µm Fe 3500 ppm (0.35%) Agglomerates and dispersed as multi-sized rod-shaped or fibrous particles in administered suspension (2% carboxymethyl cellulose)	Fischer rats A single dose of 1 mg/kg bw Positive control: crocidolite Observations period: 52 week	MWCNTs induced mesotheliomas in 6 of 7 treated rats that died prior to the end of the study. No mesothelioma found in vehicle or crocidolite-treated (2 mg/kg bw) rats.	Sakamoto et al (2009)

D : diameter; L : length

Based on the results of the intraperitoneal and intrascrotal studies (Takagi et al., 2008; Sakamoto et al., 2009) it is possible to conclude there is potential for MWCNTs to cause mesothelioma formation.

Based on the results of two intraperitoneal studies for SWCNTs, the potential for SWCNTs to cause mesothelioma cannot be determined. Although one short term study showed that SWCNTs did not elicite a fibre pathogenic response despite their durability in body fluids for 10 weeks (Safe Work Australia, 2011a), another longer term study (150 days) showed that SWCNTs can form fibre-like structures *in-situ* once inside the body that induced granuloma formation (Kolosnjaj-Tabi et al., 2010).

Key intrinsic factors for inducing mesothelioma

There is evidence to suggest that length may be one of the key factors for inducing a pathogenic response that may lead to mesothelioma formation. Currently, it is not possible to definitively conclude the minimum length at which CNTs may induce mesothelioma formation. The WHO definition for pathogenic fibres as being greater than 5 µm may also apply to CNTs. This is supported by the observation that mesothelioma developed in both studies where some proportion of the CNTs had lengths exceeding 5 µm (Takagi et al., 2008 and Sakamoto et al., 2009), whereas mesothelioma was not observed in the two year study

using shorter fibres of 0.7 µm (Muller et al., 2009). In addition, after direct intraperitoneal or intrapleural injection of CNTs in mice, granuloma formation or fibrosis occurred with long straight CNTs (length > 10 µm) whereas a negligible response was observed with short (< 5 µm) CNTs (Poland et al., 2008; Murphy et al., 2011; Safe Work Australia, 2011a). The Safe Work Australia study also found that the pathogenicity of long MWCNTs decreased after incubation in Gambles solution reduced the proportion of fibres > 15 µm and > 20 µm by ~50%.

In addition to length, the rigidity of fibres (based on their diameter) is also likely to be an important parameter for mesothelioma formation. Diameters less than a certain limit may not create rigid straight fibres to cause toxicity effects similar to asbestos. Jaurand et al. (2009) has reported that MWCNTs can have diameters in the same range of chrysotile (~50 nm) (Table 6). Fragile long thin fibres (less rigid) may break into smaller strands or buckle and entangle to form agglomerates that can be engulfed by macrophages, without leading to mesothelioma formation. The MWCNTs used in the study by Mitchell et al. (2007) having diameters of 10-20 nm were described as being flexible and not rigid, and tended to coil into agglomerates. Pauluhn (2010b) reported that thin MWCNTs have shown to be thermodynamically more stable as tightly coiled, intertwined structures citing the test samples used by Ma-Hock et al. (2009) and Pauluhn et al. (2010a) as examples. The diameters of the MWCNTs used in these studies ranged from 5 to 15 nm. These observations perhaps give an indication that the diameter of CNTs must be at least greater than 20 nm in order to form rigid straight fibres. In support of this hypothesis, mesothelioma formation and/or significant granuloma formation has been reported with CNTs having diameters > 84 nm (Murphy et al., 2011; Poland et al., 2008; Sakamoto et al., 2009; and Takagi et al., 2008) compared to thin CNTs with diameters < 15 nm, even where some of the thin CNTs had fibre lengths up to 20 µm (Murphy et al., 2011 and Poland et al., 2008). The long, thin CNTs were reported to be tangled with aggregates/agglomerates in the respirable range < 5 µm (Poland et al., 2008; Murphy et al., 2011).

CNTs have a strong tendency to bundle together in ropes (nanoropes) as a consequence of attractive van der Waals forces. Bundles of nanotubes can be considerably longer and wider than the nanotubes from which they are formed (Donaldson et al., 2006). It has been demonstrated that short, thin CNTs (length < 2 µm; diameter ~ 1 nm) have formed pathogenic fibres through aggregation within the body (Kolosnjaj-Tabi et al., 2010). Hence the possibility that CNTs as produced form aligned bundles of pathogenic fibre dimensions held together by van der Waals forces also cannot be discounted. Therefore, determination for the potential to cause mesothelioma should not solely be based on the length and diameter of the individual CNT fibres. Rather the determination for the potential to cause

mesothelioma should be based on the ability for the specific CNT to present as a rigid, straight fibre with pathogenic dimensions as defined by WHO (i.e. length > 5 µm), either as individual fibres or through aggregation.

There was only one study that examined the role of structural defects of MWCNTs with respect to carcinogenicity (Muller et al., 2009). In this study, both types of MWCNTs (with or without defects) did not elicit a carcinogenic response. Unsubstantial inflammatory response similar to crocidolite was only observed with MWCNTs with defects.

As the two studies which induced mesothelioma in rats or mice used the same MWCNTs, it is not possible to assess the effects of dimensions and impurities in mesothelioma formation.

Summary

In summary, based on an intraperitoneal and an intrascrotal study, it is possible to conclude that there is potential for MWCNTs to induce mesothelioma formation. This is further supported by recent studies that have provided evidence that MWCNTs can rapidly translocate to the intrapleural space and be persistent after pulmonary exposure, depending on the dose administered (Mercer et al., 2010). MWCNTs have also been shown in mice to reach the sub-pleural region after inhalation exposure (Ryman-Rasmussen et al., 2009b) (see Section 4.1).

Furthermore, based on the limited studies to date, it is also possible to surmise that length, rigidity (based on their diameter) and durability are likely key factors for eliciting a pathogenic response that may lead to mesothelioma. Based on the evidence, it is reasonable to conclude that MWCNTs that can present (either as individual fibres or through aggregation) as rigid, straight fibres of pathogenic dimensions as defined by WHO (i.e. length > 5 µm) and are biopersistent will have the potential for mesothelioma formation.

There are currently no studies where SWCNTs have induced mesothelioma. However, SWCNTs have been shown to be durable in lung fluid for 10 weeks (i.e. biopersistent) and to form rigid fibre-like structures through aggregation inside the body that have resulted in granuloma formation when the structures exceeded 10 µm in length. Hence, the potential for SWCNTs to induce mesothelioma formation cannot be ruled out where they can present as rigid, straight fibres with pathogenic dimensions.

4.6 Reproductive and developmental toxicity

No reproductive and developmental toxicity studies have been reported to date on unfunctionalised CNTs. However there has been one study that evaluated the effects of water-soluble amine and carboxylate-functionalised MWCNTs on the male reproductive systems in mice (Bai et al., 2010). In this study, water-soluble MWCNTs have been

demonstrated to accumulate in the testis and cause reversible testicular damage without affecting mating and fertility after repeated intravenous injection into the tail vein of mice. Although this result suggests that CNTs may have an effect on male fertility after repeated exposure via injection, further studies are necessary to make a conclusion on the reproductive and developmental toxicity of CNTs, particularly by a more appropriate route of exposure.

4.7 Neurotoxicity

Aggregates or bundles of SWCNTs have been shown to be cytotoxic to glial and neurons *in vitro* (Belyanskaya et al., 2009). However, injected MWCNTs coated with pluronic surfactant PF127 (polyoxyethylene-polyoxypropylene block co-polymer) have not shown cytotoxic effects on brain cells, neurons or glial cells in mice after 18 days post-exposure (Bardi et al., 2009). Given the limited data it is not possible to make a conclusion about the neurotoxicity potential of CNTs.

4.8 Immunotoxicity

Adverse effects on the functioning of the immune system may lead to increased incidence of infectious diseases or cancer if the immune system's ability to respond to invading agents is suppressed. There are two publications indicating that CNTs suppressed immune function (Mitchell et al., 2007 and 2009) in mice. Concerns have been raised about the structure of the test material used in the 2007 study (a mixture of CNTs and carbon nano fibres) due to the variability of findings reported in this study compared to other similar inhalation toxicity studies conducted by different researchers. It is not clear whether the same test material was used in the Mitchell et al. (2009) study although both these studies were conducted by the same authors and test material sourced from the same manufacturer. Due to the limited number of studies available and the uncertainty of the test material used in both studies, it is not possible to make a conclusion on the immunotoxic potential of CNTs.

4.9 Cardiovascular toxicity

The results from *in vitro* and instillation studies indicate that CNTs may trigger cardiovascular effects and lead to development of thrombosis (Legramante et al., 2009; Radomski et al., 2005; Bihari et al., 2010). Simeonova and Erdely (2009) state that CNT induced lung inflammation results in a release of inflammatory mediators and activation of blood cells which can contribute to cardiovascular adverse effects. Further research is necessary to investigate effects of CNT induced inflammatory mediators and/or translocation of CNTs into systemic circulation to make a conclusion on cardiovascular effects.

4.10 Impacts of physical and chemical characteristics on potential toxicity

A number of *in vitro* toxicity studies (details in A1-10) have been conducted using different cell lines to investigate physicochemical characteristics that may influence the toxicity of CNTs (e.g. method of processing: refined or unrefined, presence of residual metal catalysts, defects, agglomeration etc.).

The stability of CNTs in different *in vitro* and *in vivo* environments depends on a wide range of parameters, such as pH and salt concentrations of the surrounding medium, and length, aspect ratio, surface charge, and functionalisation of the applied CNTs. Although many of these issues have been investigated separately, studies which examine these parameters simultaneously are unavailable. In a recent study Heister et al. (2010) demonstrated that both CNT dimensions and surface functionalisation have a significant influence on their dispersion and *in vitro* behavior. In particular, factors such as a short aspect ratio, presence of oxidation debris and serum proteins, low salt concentration, and an appropriate pH are shown to improve the dispersion stability.

Table 10: Summary of in-vitro studies investigating impact of physical and chemical characteristics on potential toxicity

Type of CNT/Dose	Cell line	Findings	Reference
Pure SWCNT (No Ni and Co, low amount of amorphous C) Dose: up to 60 µg/mL Control: graphite	Murine (J774) and human macrophages	Nitric oxide production not stimulated in murine macrophages. Uptake by human macrophages very low. Did not induce DNA damage or cytotoxicity. In contrast graphite stimulated nitric oxide production in murine macrophages and uptake in macrophages was high causing cell damage and death.	Fiorito et al (2006)
SWCNT 1. Unpurified (Fe 26%) 2. Purified (Fe 0.23%) Dose: 0.12 or 0.5 mg/mL	Murine macrophages (RAW 264.7)	ROS or nitric oxide production not stimulated. Non-purified SWCNTs more effectively generated hydroxyl radicals from superoxide radicals when cells stimulated with zymosin. Presence of Fe can enhance oxidative stress induced by other agents.	Kagan et al (2006)
SWCNT D: 1-4 nm L: 0.5 to 1-2 µm Fe 0.23% Dose: 0.1 mg/mL	Primary human monocyte-derived macrophages.	Impaired engulfment of apoptotic cells and suppressed chemotaxis. No cytotoxic effects.	Witasp et al (2009)

Type of CNT/Dose	Cell line	Findings	Reference
SWCNT **1.** "As prepared" D: ~1.5 nm, L: 400-1000 nm Ni 23.2%; Y 4.8% **2.** Purified (acid/air-oxidised) D: ~2.2 nm, L 180-400 nm Ni 5.9%; Y 0.9% **3.** Purified (acid/peroxide) D: 1.4 nm, L 13.2-33.8 nm No Ni and Y present Dose: 0.12 or 1.2 mg/mL	Human lung epithelial cells	"As prepared" less cytotoxic than acid cleaned. Increased surface oxidation correlates with increased cytotoxicity not metal catalyst content. Cytoxicity reduced after aging in saline or natural organic matter.	Panessa-Warren et al (2009)
MWCNT (highly purified) curly fibrous particles D: 67 nm Fe 2000 ppm Dose: up to 1000 µg/mL Control: crocidolite	Murine macrophages (J774.1)	Dose dependent cytotoxicity 25 times greater than crocidolite. Toxicity through disruption of macrophage plasma membrane by binding to membrane receptors (MARCO).	Hirano et al (2008)
MWCNT **1.** Long MWCNT D: 8-167 nm L: 0.11-13 µm Fe 4.24% **2.** Short MWCNT D: 8-185 nm L: 0.083-5 µm Fe 4.24% **3.** Purified MWCNT (heat treatment) L: 0.11-13 µm Fe 0.08% Dose: 0.25 to 100 µg/mL CNTs agglomerated in exposure media.	Human pneumocytes A549	Internalisaton of MWCNTs (not agglomerated) with maximal length 2-3 µm in cytoplasm of cells. CNTs not observed in cell nuclei. Cytotoxicity observed but not correlated with Fe impurities or length. However Fe was mainly encapsulated.	Simon-Deckers et al (2008)
MWCNT **1.** Unpurified D : 68 ± 30 nm L : 2-164 µm Fe 6.2% (largely internalized)	Human monocyte-derived macrophages	Purified and unpurified MWCNTs exhibited similar cytotoxicity indicating no correlation with Fe content. However, Fe in unpurified MWCNT is largely internalized and may not be bioavailable.	Cheng et al (2009)

Type of CNT/Dose	Cell line	Findings	Reference
2. Purified by high temperature annealing to minimize reactive sites D : 68 ± 30 nm L: 4-65 µm Fe 0.0005% Dose: up to 20 µg/mL		MWCNTs observed piercing cell membrane. Cytotoxicity due to incomplete phagocytosis or piercing of plasma membrane causing oxidative stress and cell death.	
MWCNT D: 12 nm L: 0.1-13 µm Al 2.4%; Fe 2% Dose: up to 100 µg/mL	Human lung epithelial (A549) and mesothelial (Met5A) cells	Decrease in metabolic activity without apoptosis. No oxidative stress or cellular internalisation of CNTs. In contrast, asbestos penetrated cells and decreased metabolic activity with increased apoptosis.	Tabet et al (2009)
Purified SWCNT and MWCNT	Human aortic endothelial cells	Dose-dependent cytotoxicity (impaired cell function and viability) on direct contact with CNT..	Walker et al (2009)
1. SWCNT D : 1-2 nm Co 2.8%, Mo 4.2%, Cu 0.03%, Ni < 0.0005% **2.** Purified SWCNT D : not specified Co 1.3%; Ni 1.2% **3.** MWCNT D : 10-20 nm No metal catalysts **4.** MWCNT D : 30-50 nm Ni 1.86%; Fe 0.55% Dose: up to 100 µg/mL	Rat alveolar macrophages (NR8383) and human lung epithelial (A549) cells	CNTs taken up by macrophages with large bundles in cytoplasm. All CNTs (except purified SWCNT) showed a dose and time dependent increase in ROS and a decrease in mitochondrial membrane potential. None of the CNTs induced inflammatory mediators or cytotoxicity.	Pulskamp et al (2007)

D: diameter; L: length

Based on the in vitro studies listed above, the mechanism of cytotoxicity has been shown largely to be related to disruption of the cellular membrane (Hirano et al., 2008; Cheng et al., 2009 and Walker et al., 2009).

There is some variation on cellular uptake of CNTs. SWCNTs were unable to induce intracellular production of superoxide or nitric oxide in murine macrophages indicating the failure of macrophages to recognise dispersed SWCNTs (Kagan et al., 2006; Fiorito et al., 2006). It is hypothesised that failure for cells, particularly macrophages, to recognise and engulf CNTs *in vivo* may result in their diffusion into systemic circulation and subsequent

effects on distant tissues. This is supported by the results of an *in vivo* study by Mercer et al. (2008) (details in Section 4.2.2) that showed SWCNTs were not avidly recognised and engulfed by alveolar macrophages and rapidly migrated into the interstitium of the alveolar septa. In contrast, SWCNTs and MWCNTs were reported to be readily taken up by rat alveolar macrophages (Pulskamp et al., 2007) and human pneumocytes (Simon-Deckers et al., 2008), respectively.

Overall, the results from the *in vitro* studies are inconclusive given the variation in cell lines used and CNTs tested. However, the studies show that metal impurities (if bioavailable), surface oxidation and surface defects may play a role in cytotoxicity of CNTs, possibly through the production of reactive oxygen species. Internalisation of the CNTs appears to be related to the cell type and not just to particle size. Furthermore, internalisation of the CNTs does not necessarily appear to be a prerequisite for toxicity as CNTs can also cause toxicity through contact with the cellular membrane.

Although there are advantages in conducting *in vitro* studies, such as focusing upon the mechanism of interest, cell cultures isolated from the *in vivo* microenvironment may behave differently from the actual *in vivo* situation (Lewinski et al., 2009). Therefore, all available *in vitro* studies should be considered together with all relevant *in vivo* studies to make a conclusion based on weight-of-evidence.

Furthermore, although standard *in vitro* cell viability assays have been found to provide accurate viability data for small molecule cytotoxicity studies, they have been proven less reliable when assessing nanomaterials largely as a result of their strong adsorptive properties (Monteiro-Riviere et al, 2009). SWCNTs have been shown to give false positive results in the MTT assay that measures mitochondrial activity due to interaction of the CNTs with the dye product formazan but not with other cytotoxicity assays WST, XTT and LDH (Wolre-Kirsch et al., 2006). The nanomaterials may also absorb the constituents of the growth media as well as proteins, thereby preventing the cells from receiving their proper nutrients and growth factors (Monteiro-Riviere and Inman, 2006). On the basis of these findings, it has been recommended that cytotoxicity data should be verified with at least two or more independent *in vitro* test systems for nanomaterials (Wolre-Kirsch et al., 2006). These findings may explain some of the conflicting cytotoxicity results discussed above.

Effect of nanotube properties on toxicity

The following summarises the information taken from the *in vitro* studies described above and other toxicology studies discussed previously in Section 4 to investigate how some of the physicochemical properties may have contributed to varying toxicity effects in CNTs.

Dimensions and wall numbers

The length and/or diameter of CNT's may have an influence on the lung clearance. From the studies that investigated carcinogenicity following intraperitoneal or intrascrotal injection, there is some indication that MWCNTs with ~100 nm diameter cause mesothelioma formation (Takagi et al., 2008 and Sakamoto et al., 2009) compared with MWCNTs with diameter up to 14 nm that did not cause mesothelioma or significant granuloma formation (Muller et al., 2009 and Poland et al., 2008). However, the test material used in the Muller (2009) study was shorter (0.7 µm) compared to the test material used in Takagi (2008) and Sakamoto (2009) studies. Therefore, it is not possible to conclude whether the diameter or the length contributed to low toxicity in the Muller study (2009) compared to the other two studies.

Studies indicate that rigid and long fibres are less prone to clearance through phagocytosis. Due to the variability of the test materials and experimental conditions used, it is difficult to make any definitive conclusions on the relationship between mesothelioma formation and diameters of CNTs. Observations from a range of toxicological studies indicate that a diameter of at least 20 nm is required to form a rigid structure (see Section 4.5 for details).

Given a pathogenic fibre is defined by the WHO as having a length greater than 5 µm and studies where mesothelioma (Sakamoto et al., 2009 and Takagi et al., 2008) or significant granuloma formation (Poland et al., 2008) was observed exceeded this length, it is reasonable to assume that the fibre paradigm will also apply to CNTs. Hence, 5 µm is hypothesised as the minimum length for mesothelioma formation.

Apart from referring to the test material as SW- or MWCNTs, most of the publications have not reported the wall numbers in MWCNTs tested. The diameter of the MWCNT may be related to the number of walls in the test material. MWCNTs with diameters ranging from 5 – 110 nm have been tested in various studies and have shown increased toxicity with increased diameters (Poland et al., 2008).

Structural defects

Muller et al. (2009) also compared MWCNTs with or without structural defects for mesothelioma formation, but found no difference apart from the CNTs with defects showing unsustained inflammatory response. The test materials used in this study had different impurity levels and formed agglomerates, therefore the inflammatory response observed cannot be attributed to the defects in CNTs alone.

Agglomeration / aggregation

Some studies have provided dimensions of agglomerates/aggregates of the test material instead of the primary particle sizes (Mitchell et al., 2007). Agglomerated/aggregated or

dispersed CNTs have shown different types of pulmonary lesions (Shvedova et al., 2005). Long fibres of CNT may induce frustrated phagocycytosis, but if the fibres entangle during and/or after dosing, the toxicity effects appear to be different. Therefore, studies indicating different toxicity effects, even for the CNTs with similar lengths, should be considered carefully as their differing behaviours during and after administration and resulting health effects may be affected by test conditions.

Impurities

Different impurities, their concentrations and various methods of surface modifications (functionalisation) and surface oxidation (Panessa-Warren et al., 2009) may affect the toxic properties of CNTs.

The two studies that have shown mesothelioma formation in mice and rats used the same test material with 0.35% Fe impurities (Takagi et al., 2008 and Sakamoto et al., 2009). The test materials investigated by Muller et al. (2009) for carcinogenicity had Fe < 0.01% (without defects) or 0.49% (with defects), Al 0.37% and Co < 0.01% (without defects) or 0.48% (with defects). Considering the variability of test materials used in these studies, it is not possible to correlate impurity levels with toxicity effects reported.

Kagan et al. (2006) showed SWCNTs with low iron content were significantly less cytotoxic than iron contaminated SWCNTs. In addition treatment of raw SWCNT with a chelator to remove contaminating iron has been shown to significantly decrease both oxidant generation and cytotoxicity (cited from review by Shvedova et al., 2009). Although some researchers have found a possible link between metal catalysts and impurities (disordered carbon, polycyclic aromatic hydrocarbons) with cytotoxicity (Koyama et al., 2009), there have also been reports where no such correlation has been demonstrated (Simon-Deckers et al., 2008). However in the latter study it was reported that the iron was encapsulated in the core of the CNT and was not bioavailable. Purified and unpurified MWCNTs have shown similar toxicity when Fe content in unpurified test material was mainly internalised (Cheng et al., 2009). However, dose dependent cytotoxicity has also been reported with purified MWCNTs (Hirano et al., 2008 and Walker et al., 2009). The bioavailability of metal impurities should be considered when designing experiments to investigate the toxic effects of CNTs.

Surface properties

From all the publications cited in this report, there were only four studies that used functionalised CNTs. Two of these investigated the excretion rate of SWCNTs after injection in mice. Ammonium functionalised SWCNTs (diam. 1 nm, length 300 – 1000 nm) were rapidly excreted through the renal route (Singh et al., 2006), whilst polyethylene glycol functionalised SWCNTs (dimensions not reported) persisted for 4 months within the liver and

spleen in mice (Schipper et al., 2008). Considering that the chemical used in functionalisation is not the only variable between the two test materials, it is difficult to conclude that the rate of excretion was affected by functionalisation. The other two studies used functionalised CNTs to investigate different toxicity properties: acid functionalised SWCNTs showed increased cardiopulmonary toxicity in mice (Tong et al., 2009) and carboxylic functionalised MWCNTs did not affect the immune responses in human dendritic cells *in vitro* (Wang et al., 2009). Based on the limited number of studies designed to achieve various objectives, it is not possible to draw conclusions on the effect of functionalisation on persistence or toxicity of CNTs.

From the limited studies available on each physicochemical property summarised above and due to many variables in the test material and test conditions used, it is not possible to draw conclusions on how these parameters will impact on overall toxicity of CNTs.

5. Human health hazard assessment and classification

5.1 Hazard classification status

The hazard classification provided below was determined using the studies available for each health end point according to the *Approved criteria for classifying hazardous substances* (NOHSC, 2004) and the Globally Harmonised System of Classification and Labelling of Chemicals (GHS) (United Nations, 2009) referred to from here on as the Approved Criteria and GHS, respectively.

Based on the available data, NICNAS used the following approach to classify (or not classify) CNTs for specific health end points where:

1. OECD test guideline studies and/or other suitable scientific data acceptable for regulatory decision making are available for SWCNTs/SWCNHs and/or MWCNTs however the data do not meet the criteria specified in the Approved Criteria or the GHS – not classified as a hazardous substance for the specific health end point*;

2. No guideline studies or other suitable scientific data acceptable for regulatory decision making (i.e. administration route relevant for human exposure) are available for SWCNTs/SWCNHs or MWCNTs, or the available data are not sufficient to make a classification decision – cannot be classified according to the Approved Criteria or the GHS for specific endpoint;

3. Results of guideline studies or other suitable study outcomes meet the criteria described in the Approved Criteria or the GHS for SWCNTs/SWCNHs and/or MWCNTs – classified as hazardous for the specific health end point.

5.1.1 Acute oral toxicity

According to the Approved Criteria (NOHSC, 2004), substances with oral LD50 >2000 mg/kg bw are not classified as hazardous. Under the GHS (UN, 2009), substances with oral LD50 up to 5000 mg/kg bw are classified as hazardous (Category 5) with a signal word 'Warning'. However, Australia will classify chemicals for acute oral toxicity up to Category 4 (oral LD50 up to 2000 mg/kg bw) for classification (Safe Work Australia, 2011b).

Based on the oral LD50 of > 5000 mg/kg bw for MWCNTs established in the study conducted according to the OECD TG 423, MWCNTs are not classified as hazardous substances according to the Approved Criteria or the GHS with regards to acute oral toxicity.

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* Note: The impact of the wide range of variables on the hazard classifications is not clear, and therefore the read-across of data from one negative test to all CNTs (and thus the outcome "not classified as hazardous") may need to be revisited if data for any given endpoint indicates that the toxicity for this endpoint covers a wider range than is implicit in the read-across assumption.

The LD50 determined to be >1000 mg/kg bw for SWCNTs does not provide a conclusive value to make a classification decision on the acute oral toxicity of SWCNTs as the dose tested falls within the classifiable range to assign the risk phrase 'Harmful if swallowed' (200 < LD50 ≤ 2000 mg/kg bw), according to the Approved Criteria (NOHSC, 2004) and, within the Category 4 classification range of GHS (1000 < LD50 ≥2000 mg/kg bw). However, the LD50 for SWCNHs (considered a reasonable analogue for SWCNTs) was > 2000 mg/kg bw. Although it is possible to classify substances with oral LD50 up to 5000 mg/kg bw under Category 5 of GHS, Australia will classify chemicals for acute oral toxicity up to Category 4 (oral LD50 up to 2000 mg/kg bw) (Safe Work Australia, 2011b). Therefore given the results of these two studies, SWCNTs are not classified as hazardous based on their acute oral toxicity according to the Approved Criteria (NOHSC, 2004) or the GHS (UN, 2009).

5.1.2 Acute dermal toxicity

According to the Approved Criteria (NOHSC, 2004), substances with dermal LD50 > 2000 mg/kg bw are not classified as hazardous. Under the GHS (UN, 2009), substances with dermal LD50 up to 5000 mg/kg bw are classified as hazardous (Category 5) with a signal word 'Warning'. However, Australia will classify substances for acute dermal toxicity up to Category 4 (dermal LD50 up to 2000 mg/kg bw) (Safe Work Australia, 2011b).

MWCNTs (mean diameter 10-15 nm; mean length ~200-1000 nm; Co 0.53%) were reported to be of low acute dermal toxicity with LD50 > 2000 mg/kg bw in a study conducted in rats according to the OECD TG 402 (Pauluhn et al., 2010b). There were no published data on acute dermal toxicity of SWCNTs.

Based on the dermal LD50 of > 2000 mg/kg bw, MWCNTs are not classified as hazardous according to the Approved Criteria or the GHS (UN, 2009) with regards to acute dermal toxicity.

There is no evidence to indicate that SWCNTs should be classified as hazardous based on acute dermal toxicity.

5.1.3 Acute inhalation toxicity

According to the Approved Criteria (NOHSC, 2004), substances with inhalation LC50 > 5 mg/L/4hr are not classified as hazardous substances with regard to acute inhalation toxicity (Section 4.14 of the Approved Criteria).

According to the GHS (UN, 2009), substances with inhalation LC50 > 5 mg/L can be classified as hazardous under Category 5, if under certain circumstances the substance may present a danger to vulnerable populations (Section 3.1.2 Note (g)). However, Australia will classify chemicals for acute inhalation toxicity up to Category 4 (LC50 up to 5 mg/L for inhalation of dusts) (Safe Work Australia, 2011b).

There are three acute inhalation toxicity studies for MWCNTs conducted in rats and mice (see Table 2 Section 4.2.2 for details). Although the LC50s have not been reported in these studies, there were no deaths after 6 h nose-only exposure to MWCNTs at doses up to 241 mg/m^3 concentration, indicating LC50 > 0.241 mg/L. However, this value is not definitive to make a classification decision on the acute inhalation toxicity of MWCNTs as this value falls within the classifiable range to assign the risk phrase 'Very toxic by inhalation' (i.e. LC50 ≤ 0.25 mg/L/4hr), according to the Approved Criteria.
Therefore further studies using higher doses are required in order to determine the LC50 and classification status of CNTs for acute inhalation toxicity according to the Approved Criteria or the GHS.

There are some studies conducted using intratracheal and pharyngeal aspiration techniques to investigate pulmonary toxicity of CNTs. As these routes of exposure are not relevant for human exposure and may substantially contribute to pulmonary response through overloading the lung clearance, the results of these studies were not used in the hazard classification of CNTs. The Approved Criteria and the GHS do not provide guidance on alternative exposure routes such as intratracheal instillation or pharyngeal aspiration in the hazard classification for inhalation toxicity.

Based on the limited data available, CNTs cannot be classified according to the Approved Criteria (NOHSC, 2004) or the GHS (UN, 2009) with regards to acute inhalation toxicity.

5.1.4 Non-lethal irreversible effects after a single exposure (Approved Criteria) / Specific target organ toxicity – Single exposure (GHS)

According to the Approved Criteria (NOHSC, 2004), if there is strong evidence that irreversible damage other than carcinogenicity, mutagenicity and reproductive effects, is likely to be caused by a single exposure by an appropriate route, the substances can be assigned the risk phrase, 'Danger of very serious irreversible effects (R39)'.

According to the GHS (UN, 2009), substances can be classified under Category 1 or 2 as specific target organ toxicants following single exposure (GHS Chapter 3.8). All significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed should be considered for classification. The classification should be determined based on expert judgement on the basis of the weight of all evidence available, including the use of recommended guidance values provided (GHS Section 3.8.2.1.9). Substances should be classified under Category 1 if there is significant toxicity in humans or in experimental animals following single exposure at low doses (guidance values: oral, rat ≤300 mg/kg bw; dermal, rat or rabbit ≤1000 mg/kg bw; inhalation (dust), rat ≤1 mg/L/4 h). Substances should be classified under Category 2 if there is evidence from studies in experimental animals that

can be presumed to have harmful health effects in humans following single exposure to generally moderate concentrations of the substance (guidance values: oral, rat >300 - ≤2000 mg/kg bw; dermal, rat or rabbit >1000 - ≤2000 mg/kg bw; inhalation (dust), rat >1 - ≤5 mg/L/4 h).

In a mouse study, increases in sub-pleural fibrosis due to embedded nanotubes in the sub-pleural wall and macrophages was observed after 2 and 6 weeks following inhalation exposure to MWCNTs at 30 mg/m^3 (0.03 mg/L) for 6 h. Although some of the inhaled nanotubes remained in the sub-pleural wall after 14 weeks, most appeared to be cleared by this time and the incidence of fibrosis also diminished (Ryman-Rasmussen et al., 2009b). In a rat study (Ellinger-Ziegelbauer and Pauluhn, 2009), the pulmonary inflammation associated with 6-h exposure to MWCNTs at 2.41 mg/L subsided with time in these studies over a period of 3 months.

Based on the transient nature of the pulmonary effects reported, CNTs are not classified as hazardous according to Approved Criteria in terms of irreversible effects after a single exposure via inhalation. Although there were pulmonary effects observed in rats and mice at doses within the classifiable range under the GHS, considering that there was no functional impairment and the effects subsided following exposure, CNTs were not classified as hazardous according to the GHS in terms of specific target organ toxicity following a single exposure. Cardiovascular effects implicated were not considered for classification purposes due to the testing methods used (instillation and *in vitro*) to derive these effects (Legramante et al., 2009; Radomski et al., 2005; Bihari et al., 2010) and limited supportive data on translocation of CNTs into the systemic circulation.

It should be noted that the effects that may progress to carcinogenicity/mesothelioma were not considered under this classification as such effects are considered under classification for carcinogenicity.

According to the Approved Criteria (NOHSC, 2004), substances which cause serious irritation to respiratory system based on observations in humans or results of animal tests can be assigned the risk phrase ‘Irritating to respiratory system (R37)’(Section 4.30 of Approved Criteria).

GHS Category 3 (transient target organ effects) can be assigned if there are narcotic effects or respiratory tract irritation from exposure to substances (GHS Figure 3.8.1). This special classification would occur only when more severe organ effects in the respiratory system are not observed (Section 3.8.2.2.1 of GHS).

The available single dose inhalation toxicity studies in animals or human exposure to dust containing CNTs do not report respiratory irritation from exposure. Therefore, CNTs are not

classified as hazardous according to Approved Criteria or the GHS in terms of irritation to respiratory system.

5.1.5 Skin irritation

According to the Approved Criteria (NOHSC, 2004), a substance is determined to be a skin irritant if it causes significant inflammation of the skin that persists for at least 24 hr after an exposure period of up to 4 hr (Section 4.27 of Approved Criteria).

According to the GHS (UN, 2009), substances that produce reversible damage to the skin following application for up to 4 hr are classified as skin irritants (Sections 3.2.1 and 3.2.2.5 of GHS).

MWCNTs (mean diam. 10-15 nm; mean length ~200-1000 nm; Co 0.53%) have shown no evidence of skin irritation in a study conducted on rabbits according to the OECD TG 404 (Pauluhn et al, 2010b). Based on this study, MWCNTs are not classified as hazardous according to the Approved Criteria (NOHSC, 2004), or the GHS (UN, 2009) with regards to skin irritation.

No skin irritation studies are available on SWCNTs. SWCNHs (considered a reasonable analogue for SWCNTs) also showed no skin irritation in rabbits exposed to SWCNHs (0.15 g) applied as an occlusive patch for 24 hours on abraded and unabraded skin (Miyawaki *et al*, 2008). However, the dose used in this study was significantly less than the specified dose (0.5 g) in the OECD TG 404. Based on this study SWCNTs are not classified as hazardous according to the Approved Criteria (NOHSC, 2004), or the GHS (UN, 2009) with regards to skin irritation.

5.1.6 Eye irritation

According to the Approved Criteria (NOHSC, 2004), a substance is determined to be an eye irritant if it causes significant ocular lesions which occur within 72 hr after exposure and which persist for at least 24 hr. Ocular lesions are significant if the mean scores of the eye irritation test meet the scores provided in the Approved Criteria (Section 4.29 of Approved Criteria).

According to the GHS (UN, 2009), substances that produce changes in the eye following application to the anterior surface of the eye, which are fully reversible within 21 days of application are classified as eye irritants (Sections 3.3.1 and 3.3.2 of GHS).

In a study conducted according to the OECD TG 405, MWCNTs (mean diam. 10-15 nm; mean length ~200-1000 nm; Co 0.53%) were reported to produce only a very mild eye irritation in rabbits (Pauluhn et al, 2010b). No study details or eye irritation scores are available to determine the degree of eye irritation caused by MWCNTs. Based on this study

MWCNTs are not classified as hazardous according to the Approved Criteria (NOHSC, 2004), or the GHS (UN, 2009) with regards to eye irritation.

No eye irritation studies are available for SWCNTs. An eye irritation study conducted in rabbits with SWCNHs (considered a suitable analogue for SWCNTs), reported no eye irritation (Miyawaki *et al*, 2008). However, the dose used in this study (0.02 g) was five times less than the specified dose (0.1g) in the OECD TG 405. Based on this study SWCNTs are not classified as hazardous according to the Approved Criteria (NOHSC, 2004), or the GHS (UN, 2009) with regards to eye irritation.

5.1.7 Skin sensitisation

According to the Approved Criteria (NOHSC, 2004), substances are classified as skin sensitisers if practical experience shows sensitisation by skin contact in a substantial number of persons, or where there are positive results from an appropriate animal test (Section 4.42 of Approved Criteria).

According to the GHS (UN, 2009), substances are classified as skin sensitisers if there is evidence in humans that the substance can lead to sensitisation by skin contact in a substantial number of persons, or if there are positive results from an appropriate animal test (Sections 3.4.1 and 3.4.2.2 of GHS).

MWCNTs (mean diam. 10-15 nm; mean length ~200-1000 nm; Co 0.53%) did not produce skin sensitisation in guinea pigs in a modified maximisation test conducted according to the OECD TG 406 (Pauluhn et al, 2010b). Based on this study, MWCNTs are not classified as hazardous according to the Approved Criteria (NOHSC, 2004), or the GHS (UN, 2009) with regards to skin sensitisation.

No skin sensitisation studies are available for SWCNTs. In a Magnusson-Kligman maximisation test conducted in guinea pigs SWCNHs (considered a suitable analogue for SWCNTs) showed no skin sensitisation potential (Miyawaki et al., 2008). No signs of irritation were observed during the induction phase. Based on this study SWCNTs are not classified as hazardous according to the Approved Criteria (NOHSC, 2004), or the GHS (UN, 2009) with regards to skin sensitisation.

5.1.8 Respiratory sensitisation

According to the Approved Criteria (NOHSC, 2004), if there is evidence that a substance can induce specific respiratory hypersensitivity in animals (may include Immunoglobulin E (IgE) measurements in mice or specific pulmonary responses in guinea pigs), the risk phrase ‘May cause sensitisation by inhalation (R42)’ can be assigned.

According to the GHS (UN, 2009), if there is evidence in humans that the substance can lead to specific respiratory hypersensitivity and/or if there are positive results from an appropriate animal test (may include measurements of IgE and other specific immunological parameters in mice or specific pulmonary responses in guinea pigs), a substance is classified as a respiratory sensitiser.

Some studies have reported that CNTs may promote allergic immune responses or suppress immunity in mice. Nygaard et al. (2009) reported increased IgE levels in mice after subcutaneous or intranasal administration of SWCNTs and MWCNTs with the allergen ovalbumin. MWCNTs have shown adjuvant activity for allergen-specific IgG1 and IgE in mice when instilled intratracheally for 6 weeks (Inoue et al., 2009). Although both these mice studies indicated altered IgE levels after administering the test material internally (compared to topical exposure indicated for mice IgE tests) the responses were stronger with added allergens, indicating potential concerns for asthmatic individuals.

Based on the limited data available demonstrating that an endotoxin, allergen booster or pre-existing inflammation is required prior to CNT exposure to promote allergic response, CNTs cannot be classified according to the Approved Criteria (NOHSC, 2004) or the GHS (UN, 2009) with regards to respiratory sensitisation.

5.1.9 Severe effects after repeated or prolonged exposure (Approved Criteria) / Specific target organ toxicity – Repeated exposure (GHS)

According to the Approved Criteria (NOHSC, 2004), if serious damage is likely to be caused by repeated or prolonged exposure by an appropriate route, the risk phrase 'Danger of serious damage to health by prolonged exposure (R48)' can be assigned.

The serious damage to health includes death, clear functional disturbances or morphological changes that are toxicologically significant. It is particularly important when these changes are irreversible. Generalised changes of a less severe nature involving several organs, or severe changes in general health status should also be considered (Section 4.15 of Approved Criteria).

Substances should be classified 'at least harmful (Xn)' when the effects are observed at the following doses: Inhalation, rat $\leq$ 0.25 mg/L, 6 h/day; Oral, rat $\leq$ 50 mg/kg bw/day and Dermal, rat or rabbit $\leq$ 100 mg/kg bw/day.

The above values apply directly when lesions have been observed in 90-day toxicity studies. If results of studies of more than one duration are available, those from the longest study should normally be used for classification.

R48 should be assigned if major functional changes occur in organ systems (for example the lung); if any consistent changes in clinical biochemistry, haematology, or urinalysis

parameters, which indicate severe organ dysfunction; severe organ damage noted on microscopic examination following autopsy indicates widespread or severe necrosis, fibrosis or granuloma formation in vital organs with regenerative capacity; severe morphological changes that are potentially reversible but are clear evidence of marked organ dysfunction or evidence of appreciable cell death in vital organs incapable of regeneration is reported (Section 4.17 of Approved Criteria).

According to Approved Criteria (Section 4.19), R48 risk phrase should not be applied for adaptive responses (eg. macrophage migration in the lung, liver hypertrophy and enzyme induction, hyperplastic responses to irritants) and for local effects on the skin by repeated dermal application.

According to the GHS (UN, 2009), substances can be classified under Category 1 or 2 as specific target organ toxicants following repeated exposure (GHS Chapter 3.9). All significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed should be considered for classification. Specific target organ toxicity can occur by any route that is relevant for humans, i.e. principally oral, dermal or inhalation.

Non-lethal toxic effects observed after a single exposure, other toxic effects such as acute toxicity, eye and skin irritation, respiratory or skin sensitisation, carcinogenicity, mutagenicity, reproductive toxicity and aspiration toxicity are not considered for specific target organ toxicity classification following repeated exposure. These can, however, be used to consider the weight of evidence for the relevance of effects seen in repeat dose studies.

The classification should be decided based on expert judgement on the basis of the weight of all evidence available, including the use of recommended guidance values which take into account the duration of exposure and the dose/concentration which produced the effect/s (GHS Section 3.9.2.9). Examples of relevant toxic effects in humans and/or animals include: multifocal or diffuse necrosis, fibrosis or granuloma formation in vital organs with regenerative capacity; morphological changes that are potentially reversible but provide clear evidence of marked organ dysfunction (eg. severe fatty changes in the liver); evidence of appreciable cell death in vital organs incapable of regeneration (GHS Section 3.9.2.7.3).

For Category 1 classification, significant toxic effects should be observed in a 90-day repeated dose inhalation study in rats at doses <0.02 mg/L/6-h/day (GHS Table 3.9.1). For Category 2 classification, significant toxic effects should be observed in a 90-day repeated-dose study in rats at concentrations between 0.02 and 0.2 mg/L/6-h/day (GHS Table 3.9.2).

Effects not considered to support this classification include small changes in clinical biochemistry, haematology or urinalysis parameters and/or transient effects, when such

changes or effects are of doubtful or minimal toxicological importance and adaptive responses that are not considered toxicologically relevant (GHS Section 3.9.2.8).

No repeat dose oral toxicity studies are available for CNTs.

In the only repeat dose dermal toxicity study available, unpurified SWCNTs (diam. 1-4 nm, 80% dispersed particles and 20% aggregates, Fe 30%) induced localised inflammation around or within the hair follicles in mice after 5 days of dermal exposure at 40, 80 or 160 μg (Murray et al., 2009). Skin irritation effects (local effect) reported after repeated exposure are not considered under Approved Criteria or the GHS under this classification.

There are no reported repeat dose inhalation toxicity studies conducted with SWCNTs, but there are five repeat dose inhalation toxicity studies conducted with MWCNTs in rats or mice (see Table 4 in Section 4.3.2 for details). Out of these, two mouse studies examined effects after up to 14 or 15 days of exposure and one mouse study examined effects after 30 or 60 days of exposure. One of these short-term mouse studies did not show any significant lung damage up to 5 mg/m^3 dose, but did show suppression of the immune system (Mitchell et al., 2007). The test sample was later confirmed as a mixture of MWCNTs and MW carbon nanofibres (MWCNF).

Four out of the five studies reported some kind of pulmonary effect/response in mice or rats after MWCNT exposure (see Table 4 - proliferation and thickening of alveolar walls, inflammation and granuloma formation in the lung and associated lymph nodes, increased interstitial collagen staining).

The two 90-day repeat dose inhalation toxicity studies in rats were conducted using MWCNTs with the same dimensions (D: 5-15 nm, L: 0.1-10 μm) but at doses of 0.1 to 2.5 mg/m^3 (0.0001 to 0.0025 mg/L) (Ma Hock et al., 2009) and 0.1 to 6 mg/m^3 (0.0001 to 0.006 mg/L) (Pauluhn, 2010a).

Pauluhn (2010a) reported no treatment related effects at 0.1 mg/m^3, but exposure related lesions in the upper and lower respiratory tract were reported at 0.4 mg/m^3 and above due to inflammatory responses at the site of initial deposition and retention of MWCNT structures. Increased septal collagen staining was depicted as equal to interstitial fibrosis in this study and focally increased collagen staining was observed adjacent to deposited particles along with particle-laden macrophages and inflammatory infiltrates. Pauluhn (2010a) reported that the pathological changes observed in the 90-day study are consistent with overload-related phenomena and the etiopathological sequence of inflammatory events caused by this type of MWCNT appears to be related to the high displacement volume of the low-density MWCNT assemblage structure rather than to any yet ill-defined intrinsic toxic property. The author also emphasised that the results obtained with this particular MWCNT (Baytubes) may not

necessarily apply to nanotubes with differing physico-chemical properties. However the pulmonary effects observed at such a low dose (0.0025 mg/L) cannot be disregarded as non-adverse for classification purposes.

Ma Hock et al. (2009) reported dose dependent granulomas in the lung and associated lymph nodes from the lowest dose of 0.1 mg/m^3. Given the MWCNTs used in the Ma Hock et al. (2009) study contained ~10% metal oxide impurities (9.6% Al_2O_3) it is not possible to definitively conclude that dose dependent granuloma formation was solely due to the MWCNT exposure. However, as also indicated by the study authors there is no evidence to suggest that Al_2O_3 can induce progressive granulomatous inflammation in the lung after inhalation at such low doses (used in this study), hence it is considered unlikely that the Al_2O_3 impurity contributed to the observed lung lesions. Ma-Hock et al. (2009) reported particle clumps (agglomerates with MMAD of 0.7 to 2 µm) in the test atmosphere and estimated 46.8 µg to 1170 µg MWCNT per lung after 90 days exposure, based on 0.2 L/min respiratory rate and 10% pulmonary deposition. According to this estimation, Ma-Hock et al. (2009) reported that only the high dose (2.5 mg/m^3) was in the 'overload' range. This study supports classification of MWCNTs for severe effects based on the effects reported in rats at such low doses (ie. granuloma formation in the lung and associated lymph nodes at doses above 0.0001 mg/L).

No clinical signs of toxicity or systemic effects were observed in rats in both 90-day studies to indicate any impaired function.

Four out of five repeat dose inhalation toxicity studies did not contain a post exposure recovery period. The 90-day study that had a 6-month post exposure observation period (Pauluhn, 2010a) reported only a slight reduction of inflammatory effects during the 6-month post exposure period, and increased septal collagen staining was seen at the end of this period. As the overload-related inflammation was reported to occur at much lower doses with this particular type of CNT, and also considering the retention kinetics at high exposure, the length of the post exposure observation period used in this study was reported too short to determine whether the effects were completely reversible. The other 90-day study did not contain a post exposure recovery period to compare the transient or irreversible nature of the granulomas observed at very low doses.

Although not a relevant route of exposure for humans, the instillation studies in mice and rats were also considered for weight of evidence (Table 3). Rapid progressive fibrosis was reported with SWCNTs of 1-4 nm diameters with distinct morphologies specific to aggregates and dispersed CNTs (Shvedova et al., 2005). Some studies reported fibrotic reactions in mice and rats with exposure to SW or MWCNTs (Mercer et al., 2008; Porter et al., 2010;

Muller et al., 2005). Other instillation studies indicated effects due to agglomeration, inflammatory responses or apoptosis of alveolar macrophages. However, these studies do not consider dust deposition behaviour and possible effects on the upper respiratory tract (Ma-Hock et al., 2009). It is possible to argue that if single instillations up to 80 µg/mouse or 5 mg/rat caused pulmonary fibrosis, long-term exposure to lower doses may also cause severe pulmonary effects. However, some of these studies reported that fibrogenic effects could be the result of aggregates formed during instillation and that a respirable cloud may not produce the same effect.

By administering MWCNTs (D: 20-50 nm, L: 0.5-2 um) to rats via repeated inhalation up to 15 days or with a single intratracheal instillation, Li et al. (2007) reported that inhaled particles are delivered more slowly and regularly into the airways and alveoli over a long period of time where small aggregates can be phagocytised and cleared before clumps can form, compared to larger clumps being formed when MWCNTs were administered by intratracheal instillation. The pathological lesions induced by the two administration methods were reported to be different due to their aggregation size and distribution differences. Due to possible bolus effect from instillation methods leading to lung overloading, it is prudent not to use the results of these instillation studies to classify CNTs for severe effects. However, these studies show that SWCNTs and MWCNTs may behave the same way and cause pulmonary effects (including fibrosis) in rodents exposed to low doses (Shvedova et al., 2005; Porter et al., 2010).

The study authors postulated that the adverse effects in the Pauluhn and Ma-Hock study for MWCNTs were likely to be due to overloading. However, lung effects resulting from overload mechanisms are not specifically excluded under the Approved Criteria and GHS. There are cut off values for applying both the Approved Criteria and GHS, and these would generally exclude effects based on overload of otherwise inert dusts. The doses at which these effects were observed for MWCNTs are significantly below the classification cut-off under both the Approved Criteria and GHS, and therefore can be considered to be an intrinsic property of these particles. Hence, it is considered that inhaling MWCNTs repeatedly even at very low doses could be at least harmful to humans and as such it is considered that they should be provisionally classified as hazardous with the following risk phrases in accordance with:

the Approved Criteria (NOHSC, 2004)

Xn; R48/20 Harmful: Danger of serious damage to health by prolonged exposure through inhalation;

and

the GHS (UN, 2009)

Specific target organ toxicity following repeated exposure Category 2

Warning: May cause damage to lungs/respiratory system through prolonged or repeated inhalation exposure

Based on the concentration cut-offs given in the Approved Criteria, a higher classification of 'Toxic: Danger of serious damage to health by prolonged exposure through inhalation (T; R48/23)' could be considered; however it is considered that the uncertainty about the severity of effects, such as collagen staining, at the low doses used in these studies is too great to warrant this classification.

Despite there being no positive data on SWCNTs, given the adverse effects have been postulated to be mainly due to overloading, SWCNTs are not expected to behave differently; particularly as the applicability of the pathogenic fibre hypothesis to granuloma and fibrosis induction in not clear. Therefore it is prudent to consider the above classification for SWCNTs.

5.1.10 Genotoxicity/Mutagenicity

According to the Approved Criteria (NOHSC, 2004), substances are classified as mutagens with specific reference to inherited genetic damage under 3 categories, depending on the level of evidence available. However, substances showing positive results only in one or more *in vitro* mutagenicity assays should normally not be classified (Section 5.34 of Approved Criteria).

According to the GHS (UN, 2009), substances known to induce heritable mutations or to be regarded as if they induce heritable mutations in the germ cells of humans should be classified as Category 1 mutagens. The substances which cause concern for humans owing to the possibility that they may induce heritable mutations in the germ cells of humans should be classified under Category 2, if positive evidence obtained from experiments in mammals and/or in some *in vitro* experiments (mammalian chromosome aberration test, mammalian cell gene mutation test or bacterial reverse mutation test) (GHS Sections 3.5.2.7 to 3.5.2.9).

Both SWCNTs and MWCNTs have shown some potential to cause DNA damage or genotoxicity under certain *in vitro* circumstances (see Table 5 in Section 4.4).

Two studies reported dose dependant DNA damage by SWCNTs in the Comet assay using human epithelial BEAS 2B cells and Chinese hamster lung fibroblast V79 cells respectively (Kisin et al., 2007 and Lindberg et al., 2009). Some DNA damage or genotoxic potential by SWCNTs was reported in the chromosomal aberration test (in Chinese hamster lung fibroblast V79 cells) and in the micronucleus assay. However, SWCNHs were negative in the

chromosomal aberration test using the same cell line used to test SWCNTs (Miyawaki et al., 2008).

Mouse embryonic stem cells have shown DNA damage and mutations when exposed to purified MWCNTs, but the study was of limited value due to only one dose being tested and also the lack of positive controls (Zhu et al., 2007).

Based on the limited positive *in vitro* data available and the lack of sufficient *in vivo* data, CNTs cannot be classified according to the Approved Criteria (NOHSC, 2004), or the GHS (UN, 2009) with regards to their genotoxicity/ mutagenicity potential.

5.1.11 Carcinogenicity

According to the Approved Criteria (NOHSC, 2004), 'Substances that cause concern for man owing to possible carcinogenic effects but in respect of which the available information is not adequate for making a satisfactory assessment' (Section 5.7 of Approved Criteria) can be classified as Category 3 carcinogenic substances with the risk phrase, 'Limited evidence of a carcinogenic effect (R40)'.

Category 3 comprises 2 sub categories: (i) substances which are well investigated but evidence of a tumour inducing effect is insufficient for classification, (ii) substances that are insufficiently investigated (Section 5.14 of Approved Criteria).

Category 3 applies even though tumours have been induced at the site of application, in very sensitive test systems (eg. intraperitoneal), if the particular target is not relevant to man (5.15 of Approved Criteria).

According to the GHS (UN, 2009), known or presumed human carcinogens are classified under Category 1 and suspected human carcinogens are classified under Category 2 (GHS Chapter 3.6).

Long-term carcinogenicity studies conducted according to the OECD TG 451 are not available for CNTs. The available studies to date have all focused on assessing the potential of CNTs to cause asbestos-like carcinogenesis i.e. mesothelioma after a single intraperitoneal, intrapleural or intrascrotal injection, with post-exposure observation periods varying from 7 days to 24 months. There are seven studies that have assessed the potential for CNTs to cause mesothelioma.

SWCNTs with lengths of ~20-80 nm (ultrashort) and 1-2 µm showed granuloma formation in mice in a dose dependent manner after being exposed to 50-1000 mg/kg bw by intraperitoneal injection with a post-exposure observation period up to 150 days (Kolosnjaj-Tabi et al., 2010). However, in a short-term study, no inflammatory response was observed after intraperitoneal administration of longer SWCNTs (4±2 µm) in mice with a post-exposure

observation period of 7 days (Safe Work Australia, 2011a). In the latter study, an inflammatory response was observed for asbestos fibres and MWCNTs of pathogenic fibre dimensions.

Of particular note in the Kolosnjaj-Tabi et al. (2010) study was the finding that the SWCNTs coalesced to form fibre-like structures inside the body, inducing granuloma formation when they exceeded lengths of 10 µm through a postulated frustrated phagocytosis mechanism. The granulomas for the longer SWCNTs (1-2 µm) were located on the surface of the organs whereas the ultrashort SWCNTs (~20-80 nm) given their ability to form compact bundles were able to penetrate the organs and cause the formation of granulomas both on the surface of and inside the organs.

For MWCNTs, a study using intraperitoneal injection in mice (3 mg/mouse) and another using intrascrotal injection in rats (1 mg/kg bw) produced mesotheliomas after 25 and 52 weeks, respectively (Takagi et al., 2008 and Sakamoto et al., 2009). Single injection of MWCNTs into the pleural cavity, the more relevant site for mesothelioma, in mice (5 µg/mice) did not produce mesothelioma in the 24 week post-exposure period but did induce formation of fibrotic lesions along the parietal pleura and proliferation in the mesothelial layers that increased with time (Murphy et al., 2011). Based on these studies, it is possible to conclude that there is potential for MWCNTs to induce mesothelioma formation.

Furthermore, there are studies that have demonstrated a correlation between length and the induction of a pathogenic response that may lead to mesothelioma formation. After direct intraperitoneal or intrapleural injection of MWCNTs in mice, granuloma formation or fibrosis occurred with long straight MWCNTs (length > 10 µm) whereas a negligible response was observed with short (< 5 µm) MWCNTs (Poland et al., 2008; Murphy et al., 2011; Safe Work Australia, 2011a). The pathogenicity of long MWCNTs was also found to decrease after incubation in Gambles solution for 10 weeks had reduced the proportion of fibres > 15 µm and > 20 µm by ~50% (Safe Work Australia, 2011a).

Based on the limited studies available it is not possible to definitively conclude the minimum length at which CNTs may cause a pathogenic response that may lead to mesothelioma. However, the evidence to date have indicated that only CNTs (or structures of CNTs) having lengths of pathogenic fibre dimensions as defined by the WHO (i.e. length > 5 µm) have induced, or elicited a pathogenic response that may lead to, mesothelioma. Hence, a minimum length of 5 µm is considered a reasonable conservative estimate for the minimum length of CNTs to cause mesothelioma formation. This is supported by both studies where mesothelioma was observed with CNTs exceeding this length (Takagi et al., 2008; Sakamoto

et al., 2009), and the absence of mesothelioma formation in a two year study using shorter fibres of 0.7 µm (Muller et al., 2009).

There is also evidence to suggest that as well as length, rigidity of the wall structure of carbon nanotubes (based on their diameter) may also be a factor in mesothelioma formation. Diameters less than a certain limit may not create rigid straight fibres that cause toxicity effects similar to asbestos. Less rigid, fragile long fibres may break into smaller strands or get entangled to form agglomerates that can be engulfed by macrophages, without leading to mesothelioma formation. For example long, thin CNTs (length 5-20 µm; diameter ~ 10 nm) reported as tangled aggregates of respirable size (< 5 µm) did not elicit a pathogenic response in short-term intraperitoneal and intrapleural studies (Poland et al., 2008; Murphy et al., 2011).

Given the limited data available, it is not possible to definitively determine the minimum diameter at which CNTs cause mesothelioma, however a minimum diameter of 20 nm is hypothesised (see Section 4.5 for details).

CNTs have a strong tendency to bundle together in ropes (nanoropes) as a consequence of attractive van der Waals forces (Donaldson et al., 2006). It has been shown that short, thin CNTs (length < 2 µm; diameter ~ 1 nm) can form pathogenic fibres through aggregation within the body that were able to diffuse into the organs causing the formation of granulomas (Kolosnjaj-Tabi et al., 2010). Hence the possibility that CNTs as produced form aligned bundles of pathogenic fibre dimensions held together by van der Waals forces also cannot be discounted. Therefore determination for the potential to cause mesothelioma should not solely be based on the length and diameter of the individual CNT fibres. Rather the determination for the potential to cause mesothelioma should be based on the ability for the specific CNT to present as a rigid, straight fibre with pathogenic dimensions as defined by WHO (i.e. length > 5 µm), either as individual fibres or through aggregation.

Although the Sakamoto et al. (2009) and Takagi et al. (2008) studies alone demonstrate that MWCNTs can induce mesothelioma if they come in contact with the mesothelial cells lining the lung, recent studies have provided evidence that CNTs can rapidly translocate to the intrapleural space and be persistent after pulmonary exposure, depending on the dose administered (Mercer et al., 2010). MWCNTs have also been shown in mice to reach the sub-pleural region after inhalation exposure (Ryman-Rasmussen et al., 2009b) (see Section 4.1).

Based on the two studies that produced mesotheliomas following intraperitoneal injection of MWCNTs in mice (Takagi et al., 2008) and following intrascrotal injection of MWCNTs in rats (Sakamoto et al., 2009) combined with studies demonstrating that MWCNTs have the

potential to migrate to the pleural space (Mercer et al., 2010; Ryman-Rasmussen et al., 2009b), and evidence showing a correlation with length and rigidity and a pathogenic response, MWCNTs can be suspected to be a human carcinogen where they can present (either as individual fibres or through aggregation) as a rigid, straight fibre with pathogenic dimensions as defined by WHO (i.e. length > 5 µm).

However, given CNTs have been shown to form pathogenic fibres through aggregation within the body (Kolosnjaj-Tabi et al., 2010), combined with batch to batch variation, and technical difficulties in measuring CNTs (e.g. measurements may vary in different environments), determining whether or not a specific CNT can present as a pathogenic fibre is not straightforward.

Therefore, given the difficulty in conclusively determining whether a specific CNT can present as a fibre of pathogenic dimensions, it is prudent to consider all MWCNTs as hazardous with respect to carcinogenicity.

Hence, MWCNTs should be classified for carcinogenicity as follows in accordance with:

the Approved Criteria (NOHSC, 2004)

Xn; R40	Harmful: Limited evidence of a carcinogenic effect;

and

the GHS (UN, 2009)

Carcinogen Category 2	Warning: Suspected of causing cancer

There are no studies demonstrating that SWCNTs cause mesothelioma. Neither is there evidence to suggest that SWCNTs will behave any differently with respect to the potential to form granulomas or mesotheliomas given they have been shown to be durable and have shown to elicit a fibre pathogenic response through the ability to form rigid fibre-like structures through aggregation inside the body. Hence it is prudent to consider the above classification according to the Approved Criteria or GHS as also being applicable to SWCNTs.

5.1.12 Reproductive and developmental toxicity

According to the Approved Criteria (NOHSC, 2004), classification of chemicals as toxic to reproduction is intended to be used for chemicals that have an intrinsic or specific property to produce such toxic effects. Substances are classified under Category 1, 2 or 3 based on the evidence available on impaired fertility or developmental toxicity (Section 5.35 to 5.45 of Approved Criteria).

According to the GHS (UN, 2009), reproductive toxicity includes adverse effects on sexual function and fertility in adult males and females, as well as developmental toxicity in the offspring. Substances are classified under one of two categories depending on the evidence available in humans or/and animals (Section 3.7.2 of GHS).

There is only one study which investigated reproductive and development effects of functionalised MWCNTs after five repeated intravenous injections over 13 days into tail vein of mice (Bai et al., 2010). In this study, water-soluble MWCNTs have caused reversible testicular damage in mice without affecting mating and fertility.

Due to the limited data available (a study that does not meet the criteria for classification), CNTs cannot be classified for reproductive/development effects according to the Approved Criteria (NOHSC, 2004) or the GHS (UN, 2009).

5.2 Effect of intrinsic properties, impurities and structural defects of CNTs on hazard classification

In order to draw conclusions on how the intrinsic properties (CNT type, wall numbers, diameter, length, status eg. agglomeration/aggregation, functionalisation), impurities and structural defects of CNTs can influence health effects and therefore classifications, the full range of available studies was examined (see Section 4.10).

Overall, from the limited studies available and many variables in the test material and test conditions, it is not possible to draw definitive conclusions on how the intrinsic parameters, impurities and structural defects will impact on overall toxicity of CNTs. Therefore, the classification recommended above should be used when health hazard information specific to a particular CNT is not available.

6. Recommendations

The health hazard classification provided below should be used when specific case-by-case toxicity data are not available. Since it is possible to manufacture CNTs with different physical and chemical properties that alter the toxicity profile, if material specific data relating to specific health end-points are available, those data should be considered in determining the hazard classification for that specific nanomaterial.

6.1 Classification against the Approved Criteria

Based on the information available and in accordance with the Approved Criteria (NOHSC, 2004), SWCNTs and MWCNTs are determined to be hazardous and should be classified as:

Xn; R48/20 Harmful: Danger of serious damage to health by prolonged exposure through inhalation;

and

Xn; R40 Harmful: Limited evidence of a carcinogenic effect

According to the Approved Criteria (NOHSC, 2004) the Xn; R48/20 classification should be applicable for all mixtures containing SWCNTs or MWCNTs at concentrations of ≥ 10% (w/w), and

the R40 classification should be applicable for all mixtures containing SWCNTs or MWCNTs at concentrations of ≥ 1% (w/w).

6.2 Classification against the Globally Harmonised System (GHS) of Classification and Labelling of Chemicals

Comparable GHS classification (United Nations, 2009) for CNTs is provided below.

Health effect	Health Hazard	Classification
Specific target organ toxicity following repeated exposure	Category 2	Signal word: Warning Hazard statement: May cause damage to lungs/respiratory system through prolonged or repeated inhalation exposure
Carcinogenicity	Category 2	Signal word: Warning Hazard statement: Suspected of causing cancer

According to the implementation of the GHS in Australia under the National model Work Health and Safety Regulations (Safe Work Australia, 2011b), the classification for specific target organ toxicity should apply for mixtures containing SWCNTs or MWCNTs at $\geq$ 10%, and the classification for carcinogenicity should apply for mixtures containing SWCNTs or MWCNTs at $\geq$ 1%.

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Appendix 1: Details of toxicity studies

A1-1 Toxicokinetics

Cherukuri et al. (2006) studied the pharmacokinetics of SWCNTs (diam. ~1 nm; length ~300 nm) administered intravenously as an aqueous dispersion in 1% Pluronic F108 (an non-ionic poloaxamer surfactant) to New Zealand rabbits (20 µg/kg bw), using near-infrared fluorescence over a 24 hour period. The study showed that the SWCNTs had a half-life of 1.0±0.1 hrs and that after 24 hours significant amounts were only found in the liver.

Yang et al. (2007) studied the biodistribution of ^{13}C-enriched pristine SWCNTs (bundles of 10-30 nm diameter and 2-3 µm long and treated to remove metal catalysts and carbonaceous impurities) *via* a single tail vein injection of an aqueous suspension of CNTs in 1% Tween 80 to male KM mice at a dose of 200 µg using isotope ratio mass spectroscopy. The results indicated distribution in the entire body with major accumulations in the liver, lungs and spleen. Little excretion was evident *via* urine and faeces. The level in the liver was found to be relatively constant, whereas, there was a gradual decrease in the lungs (from 15% to 9.4% of injected dose per gram of organ base) in 28 days. The authors propose that a possible pathway for lung clearance is transfer through the lymph nodes and into the spleen (consistent with the observed gradual increase in uptake by the spleen over the same period). The SWCNTs were also found in many other tissues, including the brain (up to ~3% of injected dose per gram of tissue after 7 days that decreased after 28 days), which suggests that the nanotubes could overcome the blood brain barrier to enter into the brain.

No animal exhibited any signs of acute toxicity responses and no animals died during the 4-week observation period. The clearance for most organs was generally slow (estimated average total clearance rate < 1 µg of SWCNTs/day).

In a follow-up study, Yang et al. (2008) examined further the toxicity of SWCNTs in the liver, lung and spleen due to the high accumulation observed in these organs in previous studies. Male CD-ICR mice were dosed with an aqueous suspension of CNTs in 1% Tween 80 *via* a single tail vein injection with SWCNT (bundles of CNTs with 10-30 nm diameter and 2-3 µm length; containing impurities (wt%): Fe 0.4, Ni 3.0 and Y 1.3) at 40 µg, 200 µg or 1.0 mg per mouse. The mice were sacrificed at 90 days post-exposure and examined. The study showed long-term accumulation of the SWCNTs in the liver, spleen and especially the lungs. The mice showed no abnormal symptoms during the 90-day post-exposure. Reduced glutathione (GSH) levels found in the liver and lungs of all exposed groups (with the 1.0 mg/mouse group showing the most serious oxidative damage) and increase in malondialdehyde (MDA) levels in the liver and lung in the 1.0 mg/mouse group indicate SWCNTs induce oxidative damage.

In a study by Elgrabli et al. (2008b) intratracheal instillation of 10 or 100 µg MWCNT (20-50 nm diameter, 0.5-2 µm length) dispersed in bovine serum albumin (more than 80% of MWCNT agglomerate less than 10 µm) in male Sprague-Dawley rats demonstrated that the CNTs are taken up by alveolar macrophages and removed from the lung to the lymph nodes over a period of months. At 10 µg, CNTs were undetectable in lung tissue after 90 days, but at 100 µg were detectable up to 180 days post exposure. For the 100 µg exposure group only 37% of the MWCNT remained within the lungs after 3 months and only 16% remained in the lung after 6 months. Other than the lungs, the MWCNTs were not detected in any other systemic organs. The MWCNT were shown to be chemically modified (presence of alcohol, carbonyl and nitrogen function assessed by infrared spectroscopy) and cleaved in the lung.

Although there are studies conducted on biodistribution of CNTs after respiratory exposure using different labelling techniques, none supported the hypothesis for CNTs to translocate from the lung into the systemic circulation or central nervous system and induce direct effects (Simeonova, 2009).

Ryman-Rasmussen et al. (2009b) reported that MWCNTs reached the sub-pleural tissue of the lung when male C57BL/6 mice were exposed to an aerosol of MWCNTs (average diameter = 30-50 nm; length = 0.3-50 µm; > 94% pure; Ni 0.34%; La 0.03%) at 30 mg/m^3 for 6 hours in nose-only inhalation chambers. Lung tissue examination was conducted at 1 day, 2 weeks, 6 weeks or 14 weeks post-inhalation exposure. The aerosolised MWCNTs comprised a mixture of agglomerated and individual nanotubes with lengths from less than 100 nm to more than 10 µm with a mass median aerodynamic diameter (MMAD) of 183 nm. After 14 weeks although some of the inhaled nanotubes remained in the sub-pleural wall, most appeared to be cleared by this time. There was no evidence in this study that the MWCNTs reached the mesothelial lining

Porter et al (2010) also demonstrated that MWCNTs reached the pleura after C57BL/6 mice were exposed to MWCNTs (dimensions in dispersion medium: median length 3.86 µm, mean width 49 ± 13.4 nm; Fe 0.32%, Na 0.41%) dispersed in dispersion medium calcium and magnesium free phosphate-buffered saline, (pH 7.4, supplemented with 5.5 mM D-glucose, 0.5 mg/ml mouse serum albumin, and 0.01 mg/mL 1,2-dipalymitoyl-sn-glycerol-3-phosphocholine) by pharyngeal aspiration at a single dose of 80 µg. The study showed that the MWCNTs were rapidly incorporated in the alveolar cells and walls within one hour post-exposure and were later present in the alveolar interstitium and/or interstitial cells. Pleural penetration by the MWCNTs was observed in two of the four mice at 56 days post-exposure and in one of these mice, the MWCNT protruded through the pleura.

In a follow-up to the Porter et al (2010) study, Mercer et al (2010) investigated the level and rate of migration of the same MWCNTs to the subpleural tissue and intrapleural space at different lung burdens. C57BL/6J mice were exposed by pharyngeal aspiration to 10, 20, 40 and 80 µg MWCNT dispersed in the same dispersion medium and the distribution of MWCNT penetrations determined at 1, 7, 28 and 56 days after exposure. The study demonstrated a rapid and direct transport of the MWCNTs to the visceral pleura (subpleural tissue and intrapleural space). At one day post-exposure for the 80 µg dose, 18%, 81.6% and 0.6% of the MWCNTs was in the airway, the alveolar and the visceral pleura regions, respectively, with the alveolar macrophages receiving 62% of the total dose administered. At day 56, there were significant numbers of sub-pleural and intrapleural penetrations at 20, 40 and 80 µg doses per lung in a dose-response relationship. However, there were essentially none observed at the lowest dose of 10 µg.

Genaidy et al. (2009) conducted a critical appraisal on published articles on CNTs using meta analysis/statistical techniques and concluded that it is 'somewhat possible' for the CNTs to penetrate the human cells in target organs and to cause cellular damage, although the weight of evidence is not sufficient.

Singh et al. (2006) studied the biodistribution and blood clearance rates of water soluble ammonium-functionalised SWCNTs (diam. 1 nm and length 300-1000 nm for unfunctionalised SWCNTs (manufacturers specifications); accurate length determination of ammonium-functionalised SWCNTs could not be achieved as dispersed tubes organised themselves into ropes) intravenously administered (60 µg) into the tail vein of mice. The study showed that the functionalised SWCNTs although distributed throughout the circulatory system within 30 minutes with affinity for kidneys, muscle, skin, bone and blood, rapidly cleared mainly through the renal route. A maximum blood circulation half-life was determined as 3.5 h. No signs of acute toxicity were observed in these experiments. In addition, urine excretion studies revealed that both ammonium-functionalised SWCNTs and MWCNTs were excreted as intact nanotubes.

Water soluble hydroxylated-SWCNTs were found to be distributed quickly throughout the whole body, when administered to mice regardless of the administration route (intraperitoneal, subcutaneous, oral (by stomach intubation) and intravenous), and cleared via the renal route (Lacerda et al., 2006). The preferred organs for accumulation were the stomach, kidneys and bone. No tissue damage or distress was reported.

Schipper et al. (2008) reported that polyethylene glycol functionalised SWCNTs (dimensions not reported) injected into the blood stream of nude mice persisted within liver and spleen macrophages for 4 months without apparent toxicity effects.

A1-2 Acute toxicity

A1-2.1 Acute oral and dermal toxicity

Acute oral toxicity studies have been conducted in rats and mice with SWCNTs, SWCNHs and MWCNTs. Acute dermal toxicity studies have only been conducted with MWCNTs.

Three types of SWCNTs [ultrashort (diam. ~ 1 nm, length ~20-80 nm, Fe < 1.5%), raw (diam. ~ 1 nm, length ~1-2 µm, Fe 25%) and purified (diam. ~ 1 nm, length ~1-2 µm, Fe < 4%)] did not show toxic effects up to 1000 mg/kg bw when administered by gavage to Swiss mice in purified water (Kolosnjaj-Tabi et al., 2010).

SWCNHs were determined to be of low acute oral toxicity (LD50 > 2000 mg/kg bw) when administered by gavage to male Wistar rats in DMSO/water (3:7) (Miyawaki et al., 2008).

MWCNTs (Baytubes®; mean diameter 10-15 nm; mean length ~200-1000 nm; Co 0.53%; surface area 257 m^2/g) were reported to be of low oral toxicity (LD50 > 5000 mg/kg bw) in a study conducted in rats according to the OECD TG 423 (Pauluhn et al., 2010b) (study details not reported).

There were no published acute dermal toxicity data on SWCNTs.

MWCNTs (Baytubes®; mean diameter 10-15 nm; mean length ~200-1000 nm; Co 0.53%; surface area 257 m^2/g) were reported to be of low acute dermal toxicity (LD50 > 2000 mg/kg bw) in a study conducted in rats according to the OECD TG 402 (Pauluhn et al., 2010b) (study details not reported).

A1-2.2 Acute inhalation toxicity and pulmonary effects

There are a number of acute inhalation toxicity studies in different species reported for MWCNTs. However, there are no acute inhalation toxicity studies reported for SWCNTs.

Studies in rats

Ellinger-Ziegelbauer and Pauluhn (2009) compared the pulmonary toxicity of crystalline quartz (MMAD 2.3 µm) with MWCNTs (diam. 10-16 nm; MMAD 2.2 µm; Co 0.53%) following a single 6 h nose-only exposure in male Wistar rats at 241 mg/m^3 MWCNT or 248 mg/m^3 quartz and a 3-month post-exposure observation period. The study also included a comparison of MWCNTs with different levels of cobalt. The pulmonary inflammation associated with MWCNTs and quartz was different. Inflammatory response increased with time following exposure to quartz, but the response subsided with time following exposure to MWCNTs. A comparative study with MWCNTs containing different levels of cobalt [0.12% (MMAD 2.5 µm) and 0.53% (MMAD 2.9 µm)] at 11 mg/m^3 indicated that the time-course of pulmonary inflammation was independent of the concentration of residual cobalt. No rat deaths were reported.

Studies in mice

Ryman-Rasmussen et al. (2009a) examined the effects of inhalation exposure to MWCNT on allergic airway inflammation *in vivo*. Normal and ovalbumin-sensitised C57BL/6 mice (40 males) were exposed to an aerosol of MWCNT (average diameter = 30-50 nm; length = 0.3 - 50 µm; aerosol MMAD 714 ± 328 nm; > 94% pure; Ni 0.34%; La 0.03%) at 100 mg/m^3 or saline aerosol for 6 hours in nose-only inhalation chambers. Animals were studied at 1 and 14 days after inhalation. The inhaled MWCNTs were observed to be evenly distributed in the lung and engulfed by macrophages without causing an increase in LDH release (a measure of cytotoxicity) or total lung protein. Quantitative morphometry showed significant airway fibrosis (accompanied by significantly increased PDGF-AA and TGF-β1 – two profibrogenic growth factors) at 14 days in mice that received a combination of ovalbumin and MWCNT, but not in mice that received ovalbumin or MWCNT only. Combined ovalbumin sensitisation and MWCNT inhalation also synergistically increased IL-5 mRNA levels by 6-fold, which could further contribute to airway fibrosis. These data indicate that inhaled MWCNT require pre-existing inflammation to cause airway fibrosis. However, the study did not perform pulmonary function tests and therefore it is unknown whether the increased airway fibrosis in ovalbumin-challenged mice that inhaled MWCNT correlates with decreased lung function.

Given no airway fibrosis was observed in this study in normal lung tissue (i.e. in mice that were not sensitised with ovalbumin), the NOAEL is estimated to be 100 mg/m^3. The results of this study suggest that individuals with asthma may be more susceptible to the profibrotic effects of nanoparticles. No mice deaths were reported.

In a further study, Ryman-Rasmussen et al. (2009b) investigated if the MWCNTs used in the previous study reached the outer mesothelial lining of the lung. Male C57BL/6 mice (10 per group) were exposed once to an aerosol of MWCNT (average diameter = 30-50 nm; length = 0.3-50 µm; > 94% pure; Ni 0.34%; La 0.03%) at 1 or 30 mg/m^3 or saline aerosol for 6 hours in nose-only inhalation chambers. Lung tissue examination was conducted at 1 day, 2 weeks, 6 weeks or 14 weeks post-inhalation exposure.

The aerosolised MWCNTs comprised a mixture of agglomerated and individual nanotubes with lengths from less than 100 nm to more than 10 µm. The MMAD was 164 and 183 nm for the 1 and 30 mg/m^3 MWCNT dose respectively. The calculated dose based on 10% estimated deposition was 0.2 mg/kg bw (1 mg/m^3) or 4 mg/kg bw (30 mg/m^3) in mice.

The study showed that the nanotubes were embedded in the sub-pleural wall and within sub-pleural macrophages. For the high dose group only, increases in sub-pleural fibrosis was observed after 2 and 6 weeks following inhalation. After 14 weeks although some of the inhaled nanotubes remained in the sub-pleural wall, most appeared to be cleared by this

time. The incidence of fibrosis also diminished with time. There was no evidence in this study that the MWCNTs reached the mesothelial lining.

Human exposure

Wu et al. (2010) investigated seven previously healthy individuals who were exposed to World Trade Centre (WTC) dust and later developed severe respiratory impairment. SWCNTs of various lengths were identified in lung tissues of three patients with interstitial disease. One other patient with CNT had mild chronic bronchiolitis and occasional peribronchiolar and submucosal fibrosis. CNTs were also identified in four of the seven WTC dust samples. However, lung tissue analysis showed as well as CNTs of various sizes, variable amounts of aluminium and magnesium silicates, chrysotile asbestos, calcium phosphate and calcium sulfate, and shards of glass. Hence it remains unclear whether the CNTs caused the lung pathology.

Pharyngeal and intratracheal administration

Researchers have used other administration techniques such as intratracheal instillation and pharyngeal aspiration to assess the pulmonary toxicity of CNTs. Intratracheal instillation involves injection of the particles in a saline solution through a catheter inserted in the trachea of the animal. Pharyngeal aspiration involves placing the particles in a saline solution onto the base of a restrained extended tongue of an anaesthetised animal that remains in place until the particle suspension is aspirated into the lung. The greatest benefit from these methods of administration is that the actual delivered dose is known. The methods also circumvent the need for specialised equipment and expertise that is usually required for inhalation studies. However one of the major disadvantages of these methods is that the route of exposure is not relevant for human exposure and may substantially contribute to the pulmonary response through overloading the lung clearance mechanisms. A further disadvantage of the intratracheal instillation is that it bypasses the defenses of the upper respiratory tract. Comparing the two methods of administration, pharyngeal administration provides a better dispersion of the CNT throughout the lung than intratracheal instillation (Safe Work Australia, 2009).

Inhalation exposure resembles more closely the human exposure patterns and minimises artifacts occurring following bolus administration protocols, thus the results from intratracheal instillation and pharyngeal aspiration studies must be interpreted with the limitation of these techniques in mind.

Pharyngeal aspiration

A number of pharyngeal aspiration studies conducted in mice have been reported for both SWCNTs and MWCNTs.

SWCNT

Studies in mice

A study was conducted to assess various markers of inflammatory and fibrogenic pulmonary responses and oxidative stress responses to SWCNTs (Shvedova et al., 2005). Female C57BL/6 mice (7-8 weeks old) were treated with either SWCNTs (1-4 nm diameter, 1040 m^2/g surface area, 99.7% purity with 0.23% iron) at 0, 10, 20, 40 µg/mouse, or two reference materials, ultrafine carbon black (14.3 nm diameter, 254 m^2/g surface area) or SiO_2 (2.14 µm diameter and 4.95 m^2/g surface area) at 40 µg/mouse as a suspension in PBS. Necroscopy examination was conducted 1, 3, 7, 28, and 60 days following pharyngeal aspiration.

The study demonstrated that pharyngeal aspiration of SWCNT elicited unusual pulmonary effects in mice that combined an early onset of a robust acute inflammation with progressive fibrosis and granulomas. A dose-dependent increase in the protein, lactate dehydrogenase (LDH), and γ-glutamyl transferase activities in bronchoalveolar lavage (BAL) were found along with accumulation of 4-hydroxy-2-nonenal (oxidative biomarker) and depletion of glutathione in lungs.

The rapid progressive fibrosis found in mice exhibited two distinct morphologies: 1). the formation of granulomas apparent at 7 days mainly associated with hypertrophied epithelial cells surrounding SWCNT aggregates; and 2). diffuse interstitial fibrosis and alveolar wall thickening observed after 28 days likely to be associated with dispersed SWCNT.

The study also demonstrated functional respiratory deficiencies and decreased bacterial clearance in mice treated with SWCNT. Equal doses of ultrafine carbon black particles or fine crystalline silica (SiO_2) did not induce granulomas or alveolar wall thickening and caused a significantly weaker pulmonary inflammation and damage.

Mercer et al. (2008) investigated whether exposure to more dispersed SWCNTs (i.e. smaller sized agglomerates) would alter pulmonary distribution and response in mice. SWCNTs (agglomerate mean diameter 15.2 µm) or a dispersed preparation of SWCNTs (agglomerate mean diameter 0.69 µm, < 2% unspecified contaminants) was given by pharyngeal aspiration to C57BL/6 male mice (10 µg/mouse) and lung responses were studied at 1 h, 1 d, 7 d, and 1 month post aspiration.

Lung sections and lavage cells demonstrated an early, transient neutrophilic and inflammatory phase for both the SWCNTs and dispersed SWCNTs that rapidly resolved and was similar to that previously observed by Shvedova et al. (2005) with a less dispersed SWCNT preparation. The study revealed that the dispersed SWCNTs were more highly distributed throughout the lung and rapidly incorporated into the alveolar interstitium producing an accelerated increase in collagen deposition without granuloma formation. For

the less dispersed SWCNT preparation, ~80% deposited in the lung as large agglomerates that resulted in rapidly forming granulomatous lesions in the alveolar region immediately proximal to the small airways and ~20% were small enough to enter the alveolar walls and stimulate interstitial collagen accumulation.

The study authors concluded that the improved dispersion of SWCNTs altered the distribution from agglomerates in the proximal alveoli to dispersed smaller sized agglomerates that are rapidly incorporated into the alveolar interstitium, and altered pulmonary responses from granulomatous to an interstitial fibrotic reaction in the absence of granuloma formation.

Shvedova et al. (2008) examined the effects of SWCNTs on bacterial clearance, cytokine/chemokine production, and collagen deposition in mice subsequently infected with *Listeria monocytogenes*. C57BL/6 female mice were treated with SWCNT (99.7% carbon; Fe 0.23%, length 1-3 µm; diam. 1-4 nm; surface area 1040 m^2/g) at 0, 10 or 40 µg/mouse in phosphate buffered saline (PBS) by pharyngeal aspiration and 3 days later were exposed to *Listeria monocytogenes* (10^3 bacteria/mouse). Analyses were performed on days 3, 6, 8 and 10. The study showed that SWCNT significantly decreased the pulmonary clearance of LM and enhanced the inflammatory response. The amounts of inflammatory cells in BAL, collagen deposition, and cytokine responses were all markedly enhanced with SWCNT/LM-exposed mice compared to the respective responses to SWCNT and LM alone.

Female CD mice exposed to a single dose of 40 µg of saline-suspended acid-functionalised SWCNT by oropharyngeal aspiration had increased percentage of pulmonary neutrophils, patches of cellular infiltration and edema in small airways and in the interstitium after 24 h compared to mice exposed to non acid-functionalised SWCNTs. In addition, the hearts of mice exposed to the acid-functionalised SWCNTs had significantly lower cardiac functional recovery, greater infarct size and higher coronary flow rate than mice exposed to non acid-functionalised SWCNTs, and also exhibited signs of focal cardiac myofiber degeneration. However, no SWCNTs were detected in the heart tissue. The study concluded that acid functionalisation of SWCNTs increases cardiopulmonary toxicity (Tong et al., 2009).

MWCNT

Studies in mice

Porter et al (2010) investigated the pulmonary toxicity of MWCNTs in C57BL/6 mice with observations at 1, 7, 28 and 56 days post-exposure. MWCNTs (dimensions for MWCNTs in dispersion medium: median length 3.86 µm, mean width 49 ± 13.4 nm; no. of walls 20-50; Fe 0.32%, Na 0.41%) dispersed in calcium and magnesium free phosphate-buffered saline, (pH 7.4, supplemented with 5.5 mM D-glucose, 0.5 mg/ml mouse serum albumin, and 0.01

mg/mL 1,2-dipalymitoyl-sn-glycerol-3-phosphocholine) were administered by pharyngeal aspiration at a single dose of 10, 20, 40 or 80 µg/animal. The transmission electron microscopy images showed that the dispersion medium used was better than PBS to disperse the MWCNTs. The study showed that the MWCNTs were rapidly incorporated in the alveolar cells and walls (within one hour post-exposure) and were later present in the alveolar interstitium and/or interstitial cells. Alveolar macrophages were also observed to be loaded with MWCNTs and many of the CNTs protruded from the macrophage for days and weeks after aspiration. Pulmonary inflammation (assessed by determining BAL polymorphonuclear leukocytes) and damage (assessed by measuring acellular BAL fluid lactate dehydrogenase activities as a measure of cytotoxicity and albumin concentrations as a measure of the integrity of the alveolar-blood barrier) were found to be dose-dependent and peaked at 7 days post-exposure. By 56 days post-exposure, pulmonary inflammation and damage markers returned to control levels, except for the 40 µg dose (highest dose used for BAL studies), which was still significantly higher than the vehicle control. Histopathological studies showed that MWCNT exposure caused rapid development of pulmonary fibrosis by 7 days post-exposure even at the lowest dose tested of 10 µg that persisted throughout the 56-day post-exposure period for the 20 and 80 µg exposure group. Pleural penetration by the MWCNTs was also observed in two of the four mice exposed to 80 µg at 56 days post-exposure and in one of these mice, MWCNTs protruded through the pleura.

Han et al. (2010) investigated the acute pulmonary response of female C57BL mice to MWCNTs with observations at 1 and 7 days after a single exposure. MWCNTs (diam. 31±23 nm, length 20±10 µm; Fe ~3.5% (encapsulated in core)) suspended in PBS (high degree of agglomeration with average aggregate diameter of 98 nm) were introduced by pharyngeal aspiration at a single dose of either 20 or 40 µg. The MWCNTs were purified by refluxing in boiling nitric acid for 3 h, resulting in surface functionalisation with carboxylic and hydroxyl groups (the degree of functionalisation was not reported). The acute pulmonary response was assessed by measuring various cytotoxic and inflammatory markers in the bronchoalveolar lavage fluid. The results showed that a single exposure to MWCNTs induced a cytotoxic and inflammatory response in the lungs of mice after 1 day post exposure with some degree of resolution after 7 days. The study also investigated localised oxidative stress by examining three isoforms of superoxide dismutase in the lung tissue. Although some systemic oxidative stress was observed, indicators of localised oxidative stress were not significantly altered.

Han et al. (2008) investigated the toxicological interactions between MWCNTs and the environmental gaseous pollutant ozone which is known to induce acute pulmonary toxicity

and enhancement of chronic respiratory disease. Female C57BL/6 mice (12/dose) were treated with single dose of 0 or 20 µg MWCNTs (20-30 nm diameter and up to 50 µm long; >95% purity; impurities primarily composed of encapsulated iron catalyst in the nanotube core; pyrolytic carbon was on the surface of the tubes) in phosphate-buffered saline (PBS) per mouse by pharyngeal aspiration followed by nose-only exposure 12 hours later to ozone (0.5 ppm for 3 h). A total of 4 groups were compared: PBS/air-control, PBS/O_3, CNT/air and CNT/O_3. The study showed that mice treated with MWCNTs elicited significant pulmonary responses, reflected by changes in indicators of injury and inflammation. There was a significant increase in total bronchoalveolar lavage (BAL) cells and polymorphonuclear (PMN) leukocytes in the MWCNT exposed animals. Additionally, protein, LDH, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and mucin levels in bronchoalveolar lavage fluid (BALF) at 5 and 24 h were higher in MWCNT-exposed animals compared to air-exposed control animals. A subsequent exposure to an ozone concentration that has produced modest but significant effects in rats not only failed to exacerbate the effects of MWCNTs, but in many cases attenuated the MWCNT effect. The results of this study demonstrate that pulmonary injury effects produced by MWCNTs may be influenced through subsequent exposure to other environmental pollutants such as ozone. The study authors conclude that the study may represent the development of "cross-tolerance" that has been reported by others for some sequentially administered pollutants.

Intratracheal instillation

A number of intratracheal instillation studies conducted in mice and rats have been reported for SWCNTs, SWCNHs and MWCNTs.

SWCNT

Studies in mice

Jacobsen et al. (2009) assessed toxicity following a single intratracheal instillation of 54 µg SWCNT/mouse using a sensitive strain of mice (apolipoprotein E knockout mice – $ApoE^{-/-}$). The study also compared the effects of instillation against inhalation using carbon black. The SWCNTs were stated to be delivered as agglomerates and the primary particle size declared by the supplier was 0.9-1.7 nm as diameter and ≤ 1 µm as length.

There were significant increases of Mip-2, Mcp-1, and Il-6 mRNAs in response to SWCNT. DNA damage (measured by tail length) in BAL cells, the fraction of neutrophils in BAL cells, and protein in BAL fluid increased statistically significantly. Based on a further study which instilled carbon black at 18 or 54 µg compared to an inhaled dose of 60 mg/m^3 (for 30 min or 90 min) in $ApoE^{-/-}$ mice, it was concluded that instillation can produce stronger effects than inhalation.

Studies in rats

Miyawaki et al. (2008) investigated the effects of intratracheally instilled SWCNTs (diam. ~1.0 nm; length several hundred nanometres to several hundred micrometers, mostly aggregated into bundles; > 90% purity with 5-10% Fe) and finely ground quartz (> 97% finer than 5 µm) in male Wistar rats at a single dose of 17.3 mg/kg bw over a 7 and 90 day observation period. The study showed limited evidence of pulmonary damage including foamy macrophages and foreign body granulomas. The foreign body granulomas were only observed in one of the four animals in the 7 day observation group and one of the five animals in the 90 day observation group. Given there was not much difference in the degree of lesions between both observation periods, the long-term outcome of the lesions cannot be determined. Quartz on the other hand gave a more serious toxicological response including sarcoido-like granulomatous inflammation and fibrosis.

SWCNH

Studies in rats

Miyawaki et al. (2008) investigated the effects of intratracheally instilled SWCNH (dimensions not reported; no metal contaminants) in male Wistar rats at a dose of 17.3 mg/kg bw over a 7 and 90 day study period. The study showed limited evidence of pulmonary damage including foamy macrophages and anthracosis. The result was similar to that observed with the SWCNTs described previously except that foreign body granulomas were observed with the SWCNTs, albeit in only one of the four animals in the 7 day group and one of the five animals in the 90 day group. Quartz on the other hand gave a more serious toxicological response including sarcoido-like granulomatous inflammation and fibrosis.

MWCNT

Studies in mice

Li et al. (2007) compared the pathological lesions induced by MWCNTs in bronchi and alveoli of Kunming mice after a single intratracheal instillation or a repeated dose inhalation exposure. MWCNTs used in the experiment had a mean diameter of 50 nm, length of 10 µm and a surface area of 280 m^2/g (purity > 95%; impurities of La and Ni ash < 0.2% and amorphous carbon < 3%).

For intratracheal instillation, MWCNTs were suspended and sonicated in sterile 0.9% saline containing 1% of Tween-80, to have a concentration of 0.5 mg/mL. A 0.1 mL of this suspension containing 0.05 mg MWCNTs was instilled into lungs of mice. Pathological lesions were examined after 8, 16 or 24 days (5 females per group) in comparison to vehicle control groups. See Appendix 1 Repeat dose inhalation toxicity (Li et al, 2007) on administration by inhalation.

Mice examined after 8 or 16 days of intratracheal exposure had clumps of MWCNTs on the lining wall of bronchi without obvious inflammatory cells around them. After 24 days, the clumps led to inflammation of the lining wall of bronchi and severe destruction to alveolar netted structure around them.

After inhalation, only moderate pathological lesions were observed with formation of aggregates rather than clumps of MWCNTs that resulted in induced proliferation and thickening of the alveolar walls without altering the general alveolar structure and bronchial inflammation.

The study authors conclude that the pathological lesions induced by MWCNTs administered by intratracheal instillation and inhalation are different, and may be due to their different aggregation sizes and the distribution of aggregates in lung.

Studies in rats

The pulmonary toxicity and persistence (by measuring lung cobalt content) of MWCNT in rats exposed by intratracheal instillation to unground (5.9 µm long; C > 98%, Co 0.95%) or ground MWCNT (0.7 µm long; C > 98%, Co 0.95%) at 0.5, 2 or 5 mg/animal was studied by Muller et al. (2005). The MWCNT were ground in an oscillatory agate ball mill and only the length of the CNTs was affected by milling. The study showed that both MWCNT samples caused pulmonary inflammation at 3 and 15 days post-exposure, and induced pulmonary fibrosis at 60 days post-exposure. The unground MWCNTs induced the formation of collagen rich granulomas developing around focal aggregates of CNTs in the bronchi which partially or completely blocked the bronchial lumen. In contrast, the ground MWCNTs were better dispersed throughout the lung and induced granulomas in the interstitial tissue. These granulomas were localised in the alveolar spaces or the interstitium. The fact that the pulmonary lesions induced by the unground MWCNTs were mainly localised to the airways in contrast to the ground MWCNTs that are better dispersed is suggested by the study authors to indicate that the unground MWCNTs formed agglomerates that limited their dispersion in the lungs. The ground MWCNTs cleared more rapidly, but a significant fraction of the administered dose (36%) still remained in the lung after 60 days.

Elgrabli et al. (2008a) investigated the effects of intratracheally instilled MWCNT (with 20-50 nm diameter and 0.5 – 2 µm in length) dispersed with bovine serum albumin (80% MWCNT agglomerate < 10 µm and 98% < 30 µm) in male Sragues-Dawley rats at doses of 0, 1, 10 or 100 µg per rat. The particles were observed inside alveolar macrophages up to 180 days after instillation, but there were no histopathological lesions, fibrosis or synthesis of inflammation markers into the lung. As apoptosis was observed in alveolar macrophages after 30, 90 and 180 days with 100 µg of CNT and after 30 and 90 days with 10 µg CNT, the

study suggests further research is required to investigate whether cell death leads to pathological effect or whether phagocytosis and apoptosis are normal steps in the physiological clearance of MWCNT by the lung.

A1-2.3 Skin and eye irritation

Skin irritation studies conducted to OECD guidelines have been reported with MWCNTs and SWCNHs. No skin irritation studies on SWCNTs are reported.

No irritation or clinical signs of toxicity were observed when six rabbits were exposed to SWCNH (0.15 g) at abraded and unabraded sites via an occlusive patch for 24 hours (Miyawaki et al., 2008). However, the dose used (0.15 g), based on the maximum quantity that remained fully in contact with the skin and did not overflow from the patch, was significantly less than the specified dose (0.5 g) in the OECD TG 404.

MWCNTs (Baytubes®; mean diameter 10-15 nm; mean length ~200-1000 nm; Co 0.53%; surface area 257 m^2/g) were not reported to cause skin irritation (details not provided) in a study conducted on rabbits according to the OECD TG 404 (Pauluhn et al., 2010b) (study details not reported).

Eye irritation studies have been reported with SWCNHs and MWCNTs conducted according to the OECD TG 405. No eye irritation studies have been reported with SWCNTs.

No irritation was observed after the eyes of six rabbits were treated with “as grown” SWCNH (Miyawaki et al., 2008). However, the dose used (0.02 g), based on the maximum quantity that did not overflow when administered into the conjunctival sac, was five times less than the specified dose (0.1g) in the OECD TG 405.

MWCNTs (Baytubes®; mean diameter 10-15 nm; mean length ~200-1000 nm; Co 0.53%; surface area 257 m^2/g) were reported to have only a very mild eye irritation potential based on a study conducted on rabbits according to the OECD TG 405 (Pauluhn et al., 2010b) (study details not reported).

A1-2.4 Sensitisation

Dermal

Skin sensitisation studies have been reported with SWCNHs and MWCNTs. No skin sensitisation studies have been reported with SWCNTs.

A modified Magnusson-Kligman maximisation method was used to evaluate the skin sensitisation potential of SWCNHs (Miyawaki et al., 2008). In this study, guinea pigs were induced on day 0, 1 and 2 with a topical application of SWCNHs (0.02 g) using an occlusive patch for 24 hours on abraded sites in combination with intradermal Freund’s Complete Adjuvant (FCA). On day 7, 24 hours after treatment with 10% sodium lauryl sulfate, an

occlusive patch with SWCNHs (0.01 g) was applied for 48 hours at the original site. After 3 weeks, the guinea pigs were challenged with occlusive patches of SWCNHs (0.01 g) for 24 hours at untreated sites. At 24- or 48-hours post challenge, the SWCNHs with FCA did not induce skin sensitisation reactions. No signs of irritation were observed during the induction phase.

MWCNTs (Baytubes®; mean diameter 10-15 nm; mean length ~200-1000 nm; Co 0.53%; surface area 257 m^2/g) were stated not to show skin sensitisation in guinea pigs in the modified maximisation test conducted according to the OECD TG 406 (Pauluhn et al., 2010b) (study details not reported).

Respiratory

A study was conducted to investigate whether SWCNTs and MWCNTs promote allergic immune responses (Nygaard et al., 2009). In the study, BALB/cAnNCrl mice were exposed via intranasal administration to three doses (low, medium, high) of SWCNTs, MWCNTs and ultrafine carbon black particles together with 10 µg ovalbumin (OVA) on three consecutive days. On days 21, 22 and 23, all mice were given an intranasal OVA booster. The following table gives the physical characteristics of the CNTs used in this study.

	SWCNT	**MWCNT**
Description	aggregated particles, ~50% graphitic material of arbitrary shape, ~25% SWCNTs and ~25% MWCNTs	aggregated particles, > 90%, containing many defects
Diameter	4.05 ± 0.23 nm	15.04 ± 0.47 nm
Length	0.5-100 µm	0.5-200 µm
Surface area	542.9 m^2/g	139.7 m^2/g
Impurities	> 95% carbon; Co (1-20 nm)*; Fe (50-500 nm)*	> 95% carbon; Ni-(Fe) (5-40 nm)*; Fe-(Ni) (several hundred nanometers)*
Endotoxin	0.0079 ng/mg	0.0066 ng/mg

* Scanning transmission electron microscopy was used to estimate size of metal impurities

Five days after giving the first allergen booster ovalbumin (OVA), allergen specific antibody levels (IgE, IgG1 and IgG2a) in serum and the numbers of inflammatory cells and cytokine levels in bronchoalveolar lavage fluid (BALF) were determined. The acute inflammatory response 24 h following a single intranasal application of the particles at all three doses without the allergen was also investigated.

Large nanoparticle agglomerates which have less relevance for airway exposure were removed by sedimentation from the test samples and mice were exposed to the particle suspension supernatant for both mouse models. However, removal of the particle agglomerates meant that an accurate determination of dose could not be achieved.

The study demonstrated that CNTs promote allergic responses in mice. Both SWCNTs and MWCNTs together with OVA increased IgE, the numbers of eosinophils in BALF and the secretion of Th2-associated cytokines in the mediastinal lymph node. However, only MWCNTs with OVA increased IgG2a levels, neutrophil numbers and tumour necrosis factor-alpha and monocyte chemoattractant protein-1 levels in BALF, as well as acute influx of neutrophils 24 h after single exposure to the particles alone.

Inoue et al. (2009) investigated pulmonary effects after exposure to intratracheally instilled MWCNTs (25 or 50 μg/animal weekly for 6 weeks), with and without an allergen (ovalbumin, 1 μg/animal) in mice. MWCNTs aggravated allergen induced airway inflammation characterised by the infiltration of eosinophils, neutrophils and monocytes in the lung and increased goblet cells in the bronchial epithelium. MWCNTs with an allergen, amplified lung protein levels of Th cytokines and chemokines compared to allergen alone. MWCNTs exhibited adjuvant activity for allergen-specific IgG1 and IgE. Allergen specific IgG1 and IgE levels were significantly greater in the allergen and MWCNT or allergen groups, compared to MWCNT or vehicle control groups. The study concluded that MWCNT can exacerbate murine allergic airway inflammation, at least partly, via the promotion of a Th-dominant milieu and suggests that inhalable MWCNTs may become an important environmental risk factor for allergic asthma.

Inhalation exposure to MWCNTs has indicated that pre-existing inflammation is required to cause airway fibrosis raising concerns for more susceptible individuals (Ryman-Rasmussen et al., 2009a) (see Appendix A1-2 for study details).

In-vitro

Wang et al. (2009) investigated the influence of carboxylic MWCNTs (c-MWCNTS) on the immune activities of dendritic cells *in vitro*. Dendritic cells (DC) are the most important antigen-presenting cells due to their unique ability to activate native T-lymphocytes, which initiate immune responses. Human monocytes were isolated from standard peripheral blood leukocyte preparations from healthy blood donors and immature dendritic cells were generated from these monocytes. c-MWCNTs were prepared from raw MWCNTs (series of diameters: 10-20 nm, 20-40 nm, 40-60 nm, 60-100 nm; purity >95%). The process removed most of the metal contaminants and *ca.* 4.5% of surface C atoms were oxidised to carboxyl groups. Following functionalisation with carboxylic acid the average lengths of the c-MWCNTs (and impurities) were: 530 ± 190 nm (Fe: 0.11%, Co: 0.02%, Ni: 0.26%, Mo:0.004%), 380 ± 170 nm (Fe: 0.025%, Co: 0.001%, Ni: 0.16%, Mo:0.001%), 660 ± 330 nm (Fe: 0.06%, Co: 0.006%, Ni: 0.22%, Mo:0.0007%), 690 ± 300 nm (Fe: 0.018%, Co: 0.0011%, Ni: 0.36%). The materials were dispersed in water and then diluted by cell culture medium to have individual CNTs without aggregation in the suspension.

Immature DCs were seeded in plates and then treated with a medium containing cytokines and stimulators [c-MWCNTs: four different diameter preparations with three concentrations of 10, 50, 100 µg/mL] and incubated for 48 h. c-MWCNTs had negligible cytotoxicity. The study found that the number of cells phagocytosing c-MWCNT increases with increasing c-MWCNT concentration and the level of phagocytosis varied with the diameter of the c-MWCNTs. Treatment with c-MWCNTs did not interfere with normal DC activation induced by stimulus.

A1-3 Repeated dose toxicity

A1-3.1 Oral and dermal toxicity

No repeat dose oral toxicity studies have been reported to date on CNTs.

Murray et al. (2009) investigated the dermal toxicity of unpurified SWCNT (30 wt% Fe; diam. 1-4 nm, dispersed particles (80%) and aggregated particles (20%)) in a repeated exposure study (daily application of 40, 80 or 160 µg for 5 days) on immune-competent hairless SKH-1 mice. The unpurified SWCNT induced inflammation in the skin as observed by increased skin thickness, accumulation of polymorphonuclear leukocytes and mast cells, release of myeloperoxidase (a marker of neutrophil influx into tissue) and pro-inflammatory cytokines. Inflammation was reported to be localised around or within the hair follicles, however the study did not evaluate if the SWCNT penetrated and deposited within the skin compartments. In another study, increased epidermal thickness and release of pro-inflammatory cytokines were also observed when bioengineered skin (EpidermFT, MatTek Corporation) was exposed to the unpurified SWCNT (75 µg in 150 µl Dulbecco's Modified Eagle's Medium; 18 hours) (Murray et al., 2009).

No repeat dose dermal toxicity studies have been reported with MWCNTs.

A1-3.2 Inhalation toxicity

MWCNT

Studies in rats

Ma Hock et al. (2009) conducted a 90-day inhalation toxicity study with MWCNT [(diameter 5-15 nm, length 0.1-10 µm, purity 90% C and 10% metal oxide (9.6% aluminium oxide with traces of iron and cobalt)] undertaken according to the OECD test guideline 413 under GLP conditions. Male and female Wistar rats were head-nose exposed to the MWCNT aerosol for 90 days (6 hours per day, 5 days per week, for 13 weeks; a total of 65 exposures) at concentrations of 0 (control), 0.1, 0.5 or 2.5 mg/m^3. The MWCNT aerosol was generated using a proprietary brush generator that reportedly caused no damage to the structure of the tubes and there was no increase in the level of reactive oxygen species on the particle surface despite it being a high energy level process. Microscopic examination of the dust

particles in the inhalation chambers revealed agglomerates of a few hundred nanometers to a few micrometers diameter, with a “hairy” surface consisting of numerous ends of MWCNT. The MMAD as measured by a cascade impactor was in the range 0.7 and 2.0 µm.

No clinical signs of toxicity (i.e. food consumption, body weight, motor activity) or systemic effects were observed during the study. However, at concentrations of 0.5 and 2.5 mg/m^3, grey lung discoloration and concentration-dependent lesions in the lung and lymph nodes (primarily inflammation and multifocal granuloma formation) were observed. Even at the lowest concentration of 0.1 mg/m^3 single granulomas were observed. Diffuse pulmonary fibrosis was not observed, however in the mid and high dose groups alveoli contained multifocal eosinophilic, granular material representative of alveolar lipoproteinosis. The lowest observed adverse effect concentration (LOAEC) for MWCNTs established in this study is therefore 0.1 mg/m^3 based on effects observed, albeit minimal, at the lowest concentration tested. The study authors estimated the pulmonary deposition to be 46.8 µg, 243 µg and 1170 µg MWCNT per lung after 90 days exposure to 0.1, 0.5 and 2.5 mg/m^3 MWCNT, respectively, based on 0.2 L/min respiratory rate and 10% pulmonary deposition. The study authors claim that although the MWCNTs contain 9.6% aluminium oxide as impurity, there has been no indication that this impurity is capable of progressive granulomatous inflammation in the lung after inhalation exposure at concentrations as low as tested in the study, implying that the lesions are induced by the MWCNTs. According to this estimation, only the high dose (2.5 mg/m^3) was in the ‘overload’ range. Interestingly, dustiness measurements of the MWCNT prior to the dust generation procedure detected no nanoscale particles above ambient levels and only a small number of microscale agglomerates were detected indicating its dust forming potential to be relatively low.

Pauluhn (2010a) conducted a 90-day inhalation toxicity study with MWCNTs (diameter 5-15 nm, length 0.1-10 µm, Co ~0.5%). Male and female Wistar rats were nose-only exposed to the MWCNT aerosol for 90 days (6 hours per day, 5 days per week) at concentrations of 0 (control), 0.1, 0.4, 1.5 or 6.0 mg/m^3. Pulmonary toxicity was examined by histopathology and bronchoalveolar lavage (BAL) during week 8 and 13 (end of exposure period) and weeks 17, 26 and 39 during post-exposure (up to 6 months). Translocation of tracer Co to the lung-associated lymph nodes was also examined in 6 males per group per time point. To increase dustiness, the MWCNT was micronised using a Resch centrifugal ball mill that reportedly caused no damage to the structure of the tubes or physical characteristics. Microscopic examinations of the dust particles in the inhalation chambers revealed agglomerates of coiled, tangled assemblages. The mass median aerodynamic diameters of agglomerates as measured by critical orifice cascade impactor was in the range of 2.7 and 3.4 µm. Examinations of the test substance before and after the dust generation procedure confirmed

that there was no damage caused to the structure of the tubes by this method and no effect on the metal catalyst content.

No clinical signs of toxicity (i.e. food consumption, body weight, motor activity, etc) or systemic effects were observed during the study. Histopathology revealed principal exposure-related lesions at 0.4 mg/m^3 and above in the upper respiratory (goblet cell hyper- and/or metaplasia, eosinophilic globules, and focal turbinate remodelling) and the lower respiratory tract (inflammatory changes in the bronchioloalveolar region and increased interstitial collagen staining). Granulomatous changes and a time-dependent increase of bronchoalveolar hyperplasia occurred at 6 mg/m^3. The no observed adverse effect concentration (NOAEC) was therefore considered by the study authors to be 0.1 mg/m^3 for the MWCNTs tested based on no treatment related adverse effects at this dose. It is worth noting that no differences were observed in the MWCNT structures between the starting material, the dispersed MWCNT into inhalation chamber atmospheres, and MWCNT deposited and retained in lung cells.

Increased cumulative lung burden was proportional to the exposure level during the treatment period. An increase of Co into the hilus lymph nodes occurred at 1.5 and 6 mg/m^3 during the post-exposure observation period. Reduction in the severity of the inflammatory response probed by BAL was seen at 0.4 mg/m^3 and to a lesser extent at 1.5 and 6 mg/m^3 where levels still remained elevated during the post-exposure observation period. The author concluded that a post-exposure observation period as short as 6 months was unlikely to reveal any appreciable reversibility of the findings.

Studies in mice

In a short-term study, Mitchell et al. (2007) studied pulmonary and systemic response to inhaled MWCNTs in mice. C57BL/6 male mice were exposed (whole-body) to control air or 0.3, 1 or 5 mg/m^3 respirable aggregates (i.e. < 3 µm) of MWCNTs (diam. 10-20 nm; length 5-15 µm; > 98% C; 0.5% Ni; 0.5% Fe) for 7 or 14 days for 6 h/day. It was noted by the study authors that the MWCNTs did not appear rigid, but instead they were flexible and the majority were coiled into agglomerates that were less than 1 µm. The MWCNT aerosols were a mixture of material in varying states of agglomeration (MMAD of agglomerates up to 1.8 µm) including some free tubes. The agglomeration increased with increasing aerosol concentration. The exposure system was developed to produce CNT aerosols that simulate resuspended CNT powders that may exist in the workplace. As a consequence there were no attempts to intentionally produce free, nonagglomerated MWCNTs. There were no indications of fibrosis, increased cellularity or granuloma formation at any dose level. However, mice exposed for 14 consecutive days to all MWCNT concentrations demonstrated a suppressed T-cell-dependent antigen response. NK cell-mediated lysis of Yac-1 target

cells (a mouse lymphoma cell line sensitive to NK cells), a measure of innate immune response, was suppressed by MWCNT inhalation only at the 1 mg/m^3 concentration. The study authors concluded that the MWCNTs did not cause any significant lung damage but did suppress the immune system. Most of the nanoparticles were taken up by macrophages.

As the results of this study contradicted the results of some previous investigations that reported marked inflammatory and fibrotic lung responses, Lison and Muller (2008) examined the physicochemical characteristics of the test material used in this study and determined that the test material used was carbon nanofibres, and not nanotubes. The test material had a herringbone structure comprised of graphite layers arranged at a variable angle to the axis of the filament, forming a stacked and discontinuous arrangement of cones. Carbon nanofibres have different properties to CNTs, mainly based on their structure (eg: mechanical strength of carbon nanofibres are several orders of magnitude lower than that of CNTs). In response to the comments by Lison and Muller (2008), Mitchell states that the material used in the study was a mixture of nanofibres and nanotubes, with the majority as nanotubes (McDonald and Mitchell, 2008).

Eighteen female Kunming mice were exposed (whole-body) to an aerosol of 32.61 mg/m^3 MWCNTs (diam. 50 nm, length 10 µm; purity > 95%; impurities of La and Ni ash < 0.2% and amorphous carbon < 3%) for 6 h/day in an inhalation chamber using a dust generator over a period of 8, 16 or 24 days (actual exposure was for 5, 10 or 15 days) (Li et al., 2007). Based on the fractional deposition ratio of aerosolised CNTs in the lung (~4%, assuming that a 30 g mouse breathes 30 mL air per minute), the lung deposition doses were calculated to be 0.07, 0.14, and 0.21 mg for 8, 16 and 24 days exposure groups, respectively. The aerosol in the inhalation chambers were reported to consist of small size clusters of MWCNTs almost all in the respirable range.

The aggregation of MWCNTs was observed on the lining wall of bronchi, but there were no inflammatory cells around or covering them. Most of the aggregations of MWCNTs in the alveoli were smaller than that in bronchi, and they induced proliferation and thickening of alveolar walls without altering the general alveolar structure. The larger size aggregations of MWCNTs distributed in bronchi and smaller size ones distributed in alveoli, and did not further aggregate into larger clumps as observed with administration by intratracheal instillation. It was considered by the study authors that this result is likely due to the inhaled MWCNTs being delivered more slowly and regularly into the airways and alveoli over a long period of time such that the smaller size aggregates can be phagocytised and cleared by alveolar macrophages before clumps can form.

Li et al. (2009) examined the pulmonary toxicity of aerosolised MWCNT in mice after 30 days or 60 days repeat dose inhalation exposure. Female mice (n=9) were exposed to an aerosol of MWCNTs (diam. 50 nm; mean length 10 µm, purity > 95%; La, Ni < 0.2 wt.%; amorphous carbon < 3%) at a mean concentration of 32.61 mg/m^3 for 6 h/day once every two days for each exposure period. The lung deposition dose in mice in the 30-day and 60-day group were reported to be roughly 0.21 mg and 0.42 mg respectively based on a deposition of 4% of the aerosolised particles. Aerosolised MWCNTs were reportedly to be predominantly in respirable sizes (no further details reported).

The study showed that, based on changes in biochemical indices (elevation of total protein, alkaline phosphatase (ALP), acid phosphatase (ACP) and lactate dehydrogenase (LDH) in BALF) and pathological lesions (aggregations of MWCNTs on bronchial and alveoli walls and consequent obvious thickening of the alveoli wall), the MWCNTs induced severe pulmonary toxicity in the 60-day exposure group but not in the 30-day exposure group. The 30-day exposure group showed only a slight increase in the biochemical indices compared to the control group, formed smaller aggregates on bronchial and alveoli walls and only slight alveolar wall thickening. It was hypothesised by the study authors that the higher deposition of MWCNTs in the 60-day exposure group exceeded the threshold for lung clearance.

A1-4 Genotoxicity

In vitro studies

SWCNT

Kisin et al., (2007) examined the genotoxic effects of SWCNT (diam. 0.4-1.2 nm; > 99% pure; Fe 0.23%) *in vitro* using three different test systems: the Comet assay, the micronucleus test in Chinese hamster lung fibroblast (V79) cell line, and the bacterial reverse mutation assay (Ames test) with two strains of *Salmonella typhimurium* (YG1024 and YG1029) without metabolic activation only. The Ames test gave negative results in the two strains of *Salmonella typhimurium* tested. The *in vitro* micronucleus assay indicated limited but not statistically significant induction of DNA damage at all concentrations tested (12, 24, 48 or 96 µg/cm^2). In the Comet assay after exposure to SWCNT at concentrations of 0, 24, 48 or 96 µg/cm^2 for 3 h an induction of DNA damage was seen at the highest dose only. However, after 24 h exposure dose dependent induction of DNA damage was observed at all concentrations tested.

Yang et al. (2009) studied the inter-relationship between particle size, shape, chemical composition and toxicological effects between nanomaterials (carbon black, SWCNTs, silicon dioxide and zinc oxide) with comparable properties in relation to cytotoxicity (measured using MTT assay, the WST-1 assay and lactate dehydrogenase (LDH) assay), genotoxicity

(evaluated by DNA damage through comet assay) and oxidative effects (determined through measurement intracellular levels of reactive oxygen species, glutathione, superoxide dismutase activity and malondialdehyde) on primary mouse embryo fibroblast cells. The SWCNTs used in this study were rod-shaped with lengths < 5 µm and diameters ~8 nm (C > 99.9%). The particle state during treatment with the cells was not elucidated. The authors concluded that engineered nanoparticles may induce cytotoxicity through an oxidative stress mechanism; however, the study authors suggest that the DNA damage caused by SWCNTs were more likely from mechanical injury from penetration of the cell nucleus than to an oxidative effect. Particle surface properties determined by chemical composition played a critical role in the generation of the reactive oxygen species. However, the potential genotoxicity of nanoparticles were mostly attributed to particle shape.

Lindberg et al. (2009) examined the potential genotoxicity of fibrous carbon nanomaterials *in vitro*. The CNTs used in this study contained > 50% SWCNTS and ~40% other CNTs (not specified) with 1.1 nm diameter and 0.5-100 µm length. The impurities were at < 5% and included cobalt and molybdenum. Genotoxicity was assessed using the Comet assay and micronucleus assay (cytokinesis-block method) in human bronchial epithelial BEAS 2B cells cultured for 24, 48 or 72 h with doses of 1, 5, 10, 20, 40, 60, 80 and 100 µg/cm^2 (3.8, 19, 38, 76, 114, 228, 304 and 380 µg/mL) of nanomaterial. The Comet assay was performed in alkaline conditions (pH > 13) and was used to study DNA strand breaks and alkaline labile sites in BEAS 2B cells. The CNTs are reported to have induced a dose-dependent increase in DNA damage in the Comet assay at all treatment times, with a statistically significant effect from the lowest dose tested. The micronucleus assay was used to study chromosomal damage in BEAS 2B cells. No increase in the frequency of micronucleated cells (at any dose) was observed after the 24 and 72 h treatments. However, a significant increase was seen after 48 h treatment at 10, 60 and 100 µg/cm^2 but a clear dose-response was not seen. The study authors concluded that the result may be due to increasing size of agglomerates formed at higher dose levels. The study concluded that CNTs are genotoxic in human bronchial epithelial BEAS 2B cells and speculated that the activity may be due to the fibrous nature of the nanomaterials used. However, the contribution of catalyst metals to genotoxicity cannot be ruled out.

SWCNH

In a bacterial reverse mutation assay (Ames test), SWCNH did not increase the number of mutant colonies when tested in strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and *Escherichia coli* (WP2uvrA) with or without metabolic activation at the test concentration range of 78-1250 µg per plate (Miyawaki et al., 2008). However, precipitation of the SWCNH was observed at all doses above the lowest dose tested (78 µg per plate).

Cytotoxicity was not observed at any test dose. The SWCNH also did not significantly increase the frequency of chromosome aberrations observed in a mammalian chromosome aberration test using Chinese hamster lung fibroblast cells (< 2% for all dose levels tested as against < 1% for vehicle and control). However, precipitation of the SWCNH was observed at all tested doses above 20 µg/mL.

MWCNT

Di Sotto et al. (2009) found MWCNT (diam. 110-170 nm; length 5-9 µm; > 90% pure with metal impurities < 0.1%) not to be mutagenic in an Ames test when tested with *Salmonella typhimurium* (TA98 and TA100) and *Escherichia coli* (WP2uvrA) with and without metabolic activation at the test concentration range of 0.01-9.0 µg per plate. The upper concentration was chosen based on precipitation at concentrations > 9.0 µg/plate in a preliminary study.

Zhu et al. (2007) found that purified MWCNTs (free of metal catalysts, no other details specified) can accumulate and induce apoptosis in mouse embryonic stem cells and activate the tumour suppressor protein p53 within 2 hours of exposure, an indicator of DNA damage. Expression of two key double strand break repair proteins, Rad 51 and XRCC4 provided further evidence of DNA damage induced by the MWCNTs. The study also showed using an endogenous molecular marker, adenine phosphoribosyltransferase, that MWCNTs increased the mutation frequency two-fold compared with the spontaneous mutation frequency in mouse ES cells. However, given the test was only conducted at one concentration of the MWCNTs so a dose response relationship cannot be determined and no positive controls were used to show the expected response of the test, it cannot be concluded that the study demonstrates a mutational effect.

In both a pre-incubation and plate incorporation bacterial reverse mutation assay (Ames test) conducted according to the OECD TG 471, MWCNT (Baytubes®, macro-sized agglomerates of 0.1-0.3 mm diameter; Co < 0.1%) did not increase the number of mutant colonies when tested in strains of *Salmonella typhimurium* (TA1535, TA1537, TA98, TA100, TA102) with or without metabolic activation at the test concentration range of 50-5000 µg per plate (Wirnitzer et al., 2009). However, precipitation was reported at and above 158 µg per plate. The agglomerates did not release free nanotubes during the tests. The MWCNTs were also found to be negative in a chromosomal aberration assay using Chinese hamster lung fibroblast V79 cells conducted according to the OECD TG 473 (Wirnitzer et al., 2009). The test was conducted at concentrations up to 10 µg/mL given that precipitation was observed at this concentration and above in a preliminary continuous exposure study.

Belluci et al. (2009) studied the effects of MWCNT buckypaper (BP) on cell proliferation of 5 different types of human cell cultures *in vitro* [2 solid cancer cell lines (Caco-2, human

colorectal cancer cell line; MCF-7, human breast adenocarcinoma cell line), a leukemic cell line (HL-60, human promyelocytic cell line) and 2 normal primary cells (HA-SMCs, human arterial smooth muscle cells; HFs, human dermal fibroblasts)]. Buckypaper is an innovative material made from MWCNTs that bears a structural resemblance to woven asbestos. In addition to carbon (95.93 wt%), the BP contains iron (1.65 wt%) and other elements in trace amounts (< 1 wt%). The sterilisation procedure (details not stated) reportedly did not modify the properties of BP used in the test. Cells were seeded in 12-well culture plates (2 x 10^4 to 4 x 10^4 cells/well). BP fragments (0.1 cm^2 squares) were placed on the bottom wells at zero time (T_0) or after 24 h (T_{24}) of cell culture. Plates were incubated for 72 and 120 h at 37ºC in a 5% CO_2 atm in air, before being prepared for particle counting and size analysis. Two replicate wells were used for each data point and all experiments were performed three times. Proliferation of cells of solid cancer lines was significantly reduced by the presence of BP and effects were more prominent for MCF-7 cell line when compared to Caco-2 (reported to be consistent with previous findings). Complete inhibition of cell-growth was observed in HL-60 cell line at T_0. In this experiment viable cells made up only 30% of total cells after 72 and 120 h of culture. For T_{24} a statistically significant reduction in cell proliferation was seen at 72 h and complete inhibition of cell growth occurred. The cytotoxic effect of BP on HL-60 is suggested to not be due to oxidative stress induced by BP metallic impurities. No modification of cell proliferation pattern and viability was observed in the normal primary cells. Thus, the effect of BP is thought to be specific to transformed cells.

In vivo studies

No *in vivo* genotoxicity studies have been reported to date on CNTs, however there is an *in vivo* study reported with MWCNT buckypaper (BP) (Belluci et al., 2009). In this study, a patch of BP (2 x 2 cm^2) was placed into a pocket created between the muscular fascia and lumbar niusele within 25 Sprague Dawley male rats. The same operation was performed on control mice implanted with a polypropylene mesh and the tissue effects were compared. A moderate inflammatory reaction with fibroblast deposition around the foreign body granuloma that wraps the BP sample was observed. After implantation, the animals showed an inflammatory reaction followed 2 weeks later by a cicatrization reaction with the organisation and fibrosis of the scar. Therefore, the authors suggest that BP does not appear to produce any toxic effects in a healthy animal. Tissue modifications were studied following BP implantation for 5 weeks after the operation and no mutagenic effects were observed.

A1-5 Carcinogenicity

SWCNT

Kolosnjaj-Tabi et al. (2010) examined the effects of SWCNT length, surface chemistry and metal impurities after a single intraperitoneal administration in Swiss mice at doses up to 1000 mg/kg bw with post-exposure observation periods of 14 and 150 days. Three types of SWCNTs were tested: ultrashort (< 80 nm in length; Fe < 1.5%), raw (length ~1-2 µm; Fe 25%) and purified (length ~1-2 µm; Fe < 4%) SWCNTs. All SWCNTs tested induced the formation of granulomas in a dose-dependent manner. The granulomas were composed of phagocytic cells and foreign body giant cells (FBGCs) loaded with large CNT aggregates (mostly > 10 µm length). The granulomas formed from the raw and purified SWCNTs were located on the surface of the organs whereas the granulomas formed from the ultrashort SWCNTs were located both on the surface and inside the organs, irrespective of the dose. This was explained by the study authors to be a result of the raw and purified SWCNTs forming large bundles that cannot diffuse inside the organs in contrast to the short, compact bundles of ultrashort SWCNTs.

There was no evidence of mesothelial lesions. However, it is stated by the study authors that it is highly unusual for rodents to develop mesotheliomas after such a short time period. Smaller aggregates (< 2 µm) did not induce granuloma formation but persisted inside cells for up to 5 months after administration. The results demonstrate that SWCNTs irrespective of their length or dose can coalesce inside the body to form fibre-like structures. When structure lengths exceed 10 µm, granuloma formation may be induced through a frustrated phagocytosis mechanism. The study also demonstrated that short (< 300 nm) discrete SWCNTs can be excreted through the kidneys and bile duct. Small aggregates of SWCNTs (< 10 µm) were engulfed by phagocytes without granuloma formation. It is concluded by the study authors that the key physical property of CNTs that may be relevant for potential granuloma formation induction is not only their length but also their ability to form fibre-like structures through aggregation.

SWCNT/MWCNT

Safe Work Australia (2011a) commissioned a study to investigate the durability of SW- and MWCNTs using treatment in a simulated biological fluid. In addition the study also compared the fibre pathogenicity in mice of the CNTs before and after treatment.

For the durability study four types of CNTs (see table below) were tested in comparison to two types of asbestos fibres and one type of glass wool fibres.

Type of CNT	Diameter (nm)	Length (µm)	Soluble metals > 5 µg/g	Description of morphology
SWCNT	5±2	4±2	**Fe 185**, Co 442, Ni 47.4, Mo 144, Mn 15.7, Al 6.2	Bundles of tightly agglomerated SWCNTs in which the presence of individual CNTs could not readily be determined.
$MWCNT_{spin}$	9±3	200-300*	**Fe 50.1**, Sr 48.4	Agglomerated sheets of very long fibres with a hair-like appearance.
$MWCNT_{tang2}$	10.3±5	5-20*	**Fe 606**, Mo 655, Al 41.6, Zn 9.5	Bundles of intermediate length MWCNTs. Often stellate in form with longer fibres protruding from the central tangled agglomerate, a large proportion of which are in respirable size range < 5 µm.
$MWCNT_{long1}$	64±16	12±6	**Fe 15.6**	Dispersed bundles and singlets of long and intermediate-length MWCNTs, many in the range 10-20 µm and longer. Many very short fibres often decorate the long fibres.

*Length as supplied by manufacturer

In the study, samples were incubated in a simulated biological fluid (Gambles solution) for up to 24 weeks, with samples removed, filtered, dried and weighed at defined time points (0, 3, 6, 10 and 24 weeks). SWCNT and $MWCNT_{spin}$ showed no loss of mass and no change in morphology or average fibre length. $MWCNT_{tang2}$ showed ~25% loss of mass after 24 weeks incubation with no change in morphology. This loss of mass was mainly only observed at the 24 week time point. $MWCNT_{long1}$ had only ~70% of the original mass at all time points from week 3 onwards except for week 10 where a mass loss of ~80% was observed, with slightly decreased fibre length and decreased proportion of long fibres (>20 µm fibres decreased from 10% to 8% by week 3, to 4% by week 10; >15 µm fibres decreased from 30% to 18% by week 3 and to 13% by week 10), indicating that they had undergone fibre dissolution and/or breakage. Although the $MWCNT_{tang2}$ showed a mass loss of ~25%, the study authors have considered that this mass loss is not significant as it reflects experimental error (~20%) experienced in the study for recovery of the CNTs and a consistent trend was not evident across all time-points. However, the study authors did not rule out that the $MWCNT_{tang2}$ may have experienced a small mass loss. In contrast, the study authors considered the loss of mass observed for $MWCNT_{long1}$ was consistent and statistically significant across all time-points.

Out of the four CNT types incubated above, only two were used in the second part of the study ($MWCNT_{long1}$ - previously shown to induce an asbestos-like response when injected into peritoneal cavity of mice and SWCNT) to investigate the impact of incubation (for 10 weeks) in Gambles solution on CNT pathogenicity in mice (4 females per test group and 3 control mice) in comparison to asbestos and glass wool fibres. A 50 µg test sample in sterile saline with 0.5% BSA was injected into the peritoneal cavity of the mouse. The mice were sacrificed after 24 h or 7 days, peritoneal cavity washed and lavage fluid collected for a number of *in-vitro* assays to identify an acute inflammatory response. The incubated $MWCNT_{long1}$ showed decreased pathogenicity in mice compared to the strong inflammatory and granuloma response induced by injection of $MWCNT_{long1}$ that had been not incubated. Both incubated and non-incubated SWCNTs formed very tightly agglomerated particle-like bundles and did not elicit an inflammatory response in mice. The study authors concluded that if CNTs are of sufficient length and aspect ratio, they can induce asbestos-like responses in mice, but this may be mitigated if CNTs are of a less durable nature. Out of the 4 CNT types tested, 3 showed no fibre shortening and 2 showed no loss of mass. Therefore, the CNT durability might be sample specific and pathogenicity may not necessarily be consistent across all types of CNTs. The paper concludes that CNTs manufactured with some kind of surface defects which make CNTs vulnerable to chemical attack and biodegradation in biological systems, or manufactured to form clump-like agglomerates may be useful to minimize the potential biological hazards.

MWCNT

Studies in mice

Poland et al. (2008) investigated the effect of length on the fibrogenic and carcinogenic potential of MWCNTs based on the paradigm by Donaldson and Tran (2004) that a hazardous fibre is one that is thinner than 3µm, longer than ~20 µm and is biopersistent in the lung. Four types of MWCNTs were used in the study. Two of the MWCNTs contained a substantial portion of long straight fibres longer than 20 µm and the other two samples consisted of CNTs arranged in low aspect ratio tangled aggregates a large proportion of which are in the respirable range < 5 µm. Nanoparticulate carbon black (NPCB) was used as a non-fibrous graphene control and short-fibre amosite (SFA) and long-fibre amosite (LFA) were used as negative and positive controls, respectively. A single dose of the test materials (50 µg/animal) was injected into the intraperitoneal cavity of mice (C57BL). The inflammatory response was assessed after 24 hours by measuring protein levels and cell populations in the lavageate of the peritoneal cavity. Histopathological evaluation of granulomas was carried out after 7 days. The study showed that the long straight MWCNTs caused an early inflammatory response and the formation of FBGCs and granulomas similar

to that observed with the long-fibre amosite. In contrast, the tangled MWCNTs, NPCB and SFA failed to cause any significant inflammation or giant cell formation with only a small non-significant granuloma response observed in one test animal treated with the tangled MWCNT. The latter is speculated by the authors to be either caused by contamination of the tangled MWCNTs with long fibres that were too low to be detected or arose spontaneously by chance. Given the short, tangled MWCNTs contained similar or indeed higher levels of impurities to the long MWCNTs, the study authors stated that the level of impurities could not explain the differences in the peritoneal response observed. Therefore the study authors concluded that asbestos-like pathogenic behaviour associated with CNTs conforms to a structure-activity relationship based on length.

Takagi et al. (2008) examined the fibrogenic and carcinogenic potential of MWCNTs using p53 heterozygous mice that are reported to be sensitive to asbestos and develop mesotheliomas rapidly. Expression of p53 protein is an early cellular response to DNA damage that activates cell cycle checkpoints and directs a cell with DNA damage to self destruct via apoptosis. Groups of 19 animals were intraperitoneally administered a single dose of MWCNTs (average width 100 nm; length 27.5% > 5 µm (100% < 20µm)) or crocidolite (the positive control) at 3 mg/animal (equivalent to 1×10^{10} and 1×10^{9} particles for MWCNTs and crocidolite, respectively). It was reported that the administered MWCNT suspension in 5% methyl cellulose solution contained aggregates among dispersed rod-shaped or fibrous particles. The study was allowed to run until one of the groups reached 100% mortality. This occurred for the MWCNT-treated group after 25 weeks. Only three crocidolite-treated animals survived the study period of 25 weeks. The study showed that the MWCNT and crocidolite caused large mesothelial tumours that were invasive to several tissues but no metastases were noted. The overall incidence of mesothelioma was greater for the MWCNT treated animals (14/16) than the crocidolite treated animals (14/18).

Murphy et al (2011) assessed the inflammatory responses and role of length of MWCNTs directly instilled into the pleural space of mice. Five types of MWCNTs were used in the study; two long (both containing a substantial portion of long straight fibres longer than 20 µm), one short (length 0.5-2µm) and two short and tangled (composed of aggregates of respirable size i.e. < 5 µm). NPCB was used as a non-fibrous graphene control and SFA and LFA were used as negative and positive controls, respectively. A single dose of the test materials (5 µg/animal) was injected into the intrapleural cavity of mice (C57BL). The inflammatory response was assessed after 24 hours, 7 days, 4 weeks, 12 weeks and 24 weeks by measuring protein levels and cell populations in the lavageate of the pleural cavity. Histopathological evaluation of the parietal pleura was also carried out at the same time points. The study showed that only the long MWCNTs and LFA gave rise to an acute

inflammatory response that was characterised by granulocyte influx, mostly neutrophils, and increased protein concentration in the pleural fluid after 7 days. This inflammatory response declined greatly at 4 weeks but was still above the vehicle controls at the end of the observation period of 24 weeks. Histopathological evaluation indicated that the acute inflammation observed with the long MWCNTs was followed by the progressive development of fibrotic lesions along the parietal pleura and proliferation in the mesothelial layers. In contrast, the short and short and tangled MWCNTs did not elicit a significant inflammatory response or development of fibrotic lesions. The study authors conclude that the results show a clear length-dependent inflammogenicity for CNTs when injected into the pleural space of mice that adheres to the fibre pathogenicity paradigm proposed by Donaldson and Tran (2004). The study also evaluated the likely mechanism by which this length-dependent pathogenicity might occur. The study authors hypothesised that the inflammatory effects of long CNTs and long fibres in general arise as a consequence of retention at stomata whose maximum diameter is 10 µm. Normally, particles in the pleural cavity are cleared by passive removal in the pleural fluid through stomata in the parietal pleura to the underlying lymphatic system and thereby to the mediastinal lymph nodes (LNs). In support of this hypothesis the study authors examined the clearance to the mediastinal LNs using short (~4.3 µm) and long (~24 µm) versions of alternative high-aspect ratio nanowires made of nickel. NiNWs were used as they are more readily visualised histologically than CNTs and can be synthesised with a narrower distribution of sizes. The results showed that significantly more short NiNWs migrated to the LNs from the pleural space than the long NiMWs lending support to the hypothesis that long fibres are retained in the pleural cavity as they cannot negotiate the stomata leading to persistent inflammation and eventually fibrosis.

Studies in rats

Three groups of 50 male Wistar rats were injected intraperitoneally with a single dose of MWCNT with defects (dangling bonds and products of reaction of dangling bonds with air; Al 0.37%, Fe 0.49%, 0.48%) (2 or 20 mg/animal) and without defects (low level of structural defects and depleted metal content; Al 0.37%, Fe < 0.01%, Co < 0.01%) (20 mg/animal) (Muller et al., 2009). Two additional groups of 26 rats were used as positive controls (injected 2 mg crocidolite/animal) and vehicle controls (2 mL phosphate-buffered saline). Both types of MWCNT had a diameter of 11.3 nm and a length of 0.7 µm and formed agglomerates so the size distribution could not be analysed. The proportion of individual nanotubes with a length > 5 µm was estimated to be extremely low.

After 24 months, crocidolite induced a clear carcinogenic response with 34.6% animals with mesothelioma compared to 3.8% in the vehicle control group. MWCNT with or without structural defects did not induce mesothelioma under these experimental conditions.

However, MWCNTs with defects dosed at 20 mg did elicit inflammatory responses similar to crocidolite, but these could not be sufficiently sustained in the peritoneal cavity of the rat to show a carcinogenic activity after 24 months.

More recently, Sakamoto et al. (2009) studied the carcinogenic potential of MWCNTs (diam. 82% between 70-110 nm; length 72.5% between 1-4 µm with a peak at 2 µm and 27.5% of particles with 5-19 µm length with the majority ≤ 10 µm) in rats after administration by a single intrascrotal injection. In rats the scrotal cavity is freely connected with the peritoneal cavity and thus intrascrotal injection is regarded as similar to intraperitoneal administration. The MWCNTs used in this study was reportedly the same as used by Takagi et al. (2008). It was reported that the administered MWCNT suspension in 2% carboxymethyl cellulose solution contained agglomerates and dispersed multi-sized rod-shaped or fibrous particles. Fischer 344 rats were administered with MWCNT (1 mg/kg bw (3.62×10^8 particles/kg bw), 7 animals), crocidolite (2 mg/kg bw (58.6×10^8 particles/kg bw), 10 animals) or vehicle (2% carboxymethyl cellulose, 5 animals) and maintained for 52 weeks. While no mesothelioma was found in vehicle- or crocidolite-treated rats, 6 out of 7 of the MWCNT-treated rats developed mesothelioma and died before the end of the study period. The study authors suggested that the dose of crocidolite used in the study may have been too low to induce mesothelioma based on doses that have induced mesotheliomas in previous studies conducted with asbestos. The dose in this study was chosen in order to have an equivalent dose (on at least weight basis) with that of the MWCNTS. The study authors have calculated the dose used in the present study as 120 times less than that used in the study by Takagi et al. (2008).

A1-6 Reproductive and developmental toxicity

No reproductive and developmental toxicity studies in mammals or male/female reproductive systems have been reported to date on unfunctionalised CNTs.

Bai et al (2010) evaluated the effects of water soluble amine (NH_2-) and carboxylate (COOH)-functionalised MWCNTs on the male reproductive systems in mice. The functionalised MWCNTs (diam. ~20-30 nm, length ~0.5-2 µm, Fe 0.2-0.3%) dispersed in phosphate buffered saline with 0.1% Tween 80 were administered intravenously into the tail vein of male BALB/c mice as five doses over 13 days at 5 mg/kg bw per dose. The study reported that within 24 hours, both types of nanotubes were found in the testis, and accumulation resulted in oxidative stress and tissue damage. However, the damage was reversed after 2 months and no effect on mating and fertility was observed. The study authors caution that although the results showed that water soluble carbon nanotubes have minor effects on the male reproductive system in mice, the oxidative stress and the

alterations in the testis raises concern because it is possible that these materials may accumulate at higher quantities over a longer period and may have adverse effects on male fertility.

A1-7 Neurotoxicity

SWCNT

Belyanskaya et al (2009) analysed the influences of SWCNTs with different degrees of agglomeration on primary mixed neuro-glial cultures derived from chicken embryonic spinal cord of the central nervous system (CNS) or dorsal root ganglia of the peripheral nervous system (PNS). Two distinct SWCNTs with different degrees of agglomeration were used: SWCNT-agglomerates (dense rope-like aggregates with diameter ~100 nm; Ni 2.4%, Y 0.5%) and better dispersed SWCNT-bundles consisting of 10-20 parallel aligned tubes (diam. ~ 20 nm; Ni 5.5% and Y 0.7%). When measured by the reduction in total DNA content both types of SWCNTs at dose concentrations of 30 µg/mL elicited adverse effects in primary cultures derived from both the CNS and PNS of chicken embryos with the level of toxicity being partially dependent on the agglomeration state of the tubes (i.e. at higher concentrations, the SWCNT-agglomerates were found to be more toxic than the SWCNT-bundles at the same concentration). The study authors conclude that if SWCNTs can enter the nervous system at sufficiently high concentrations, they may cause adverse effects on glial cells and neurons.

MWCNT

MWCNTs (97% pure, diameter = 10-30 nm, length = 2 µm, coated with pluronic surfactant PF127 (polyoxyethylene-polyoxypropylene block co-polymer), metal particles 2.94%, bulk density = 0.15 g/cm^3, carbon impurities < 1%) were used in two studies, *in vivo* and *in vitro*, to investigate apoptosis of mouse primary cortical neurons (Bardi et al., 2009).

In the *in vivo* assay, intracerebral injections were made at specific stereotaxic location in the visual cortex by a glass pipette (350 nL at 700 µm and 350 nL at 444 µm below the cortical surface) to allow homogeneous dispersion of CNTs along the cortical depth. After an 18 day recovery period, the injected mice were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (0.1 M). Brains were sectioned on a sliding microtome in 40 µm sections, and cresyl violet staining was performed to take images.

A more detailed study related to molecular mechanisms of MWCNT-induced toxicity was performed *in vitro*. Primary mouse cortical cultures were used in the *in vitro* assay and the cells were incubated with 3.5 µg/mL CNT or without CNT for 24 or 48 h.

PF127 alone produced apoptosis of the neurons, but PF127-coated MWCNTs did not show any cell death, indicating no toxicity to brain cells. MWCNTs did not exhibit cytotoxic effects

in neurons and glial cells *in vitro* or *in vivo.* The study authors conclude that the presence of MWCNTs can reduce PF127 toxicity.

A1-8 Immunotoxicity

MWCNT

Mitchell et al. (2007) demonstrated a suppressed T-cell-dependent antigen response in a short term study in mice exposed for 14 consecutive days at all tested MWCNT (< 3 µm aggregates) concentrations of 0.3, 1 or 5 mg/m^3 (see Section 4.3.2 for details). However, Lison and Muller (2008) examined the physicochemical characteristics of the test material used in this study and determined the material to be carbon nanofibres and not CNTs. It was later confirmed that the test material was a mixture of nanofibres and nanotubes, with the majority being nanotubes (McDonald and Mitchell, 2008)

Mitchell et al. (2009) investigated the mechanistic aspects of how inhaled MWCNTs suppress systemic immune function in mice by perturbing the cyclooxygenase-2 pathway. Male C57BL/6 mice (7/dose) were exposed to 0, 0.3 or 1 mg/m^3 MWCNTs for 6 h/d for 14 days in whole-body inhalation chambers. Based on particle mass, the median particle diameter was 590 nm.

Mice exposed to 1 mg/m^3 presented suppressed immune function up to 30 days post exposure. The study authors reported that the immunosuppressive effects seen *in vivo* are not likely to be due to MWCNTs entering the circulation and acting directly on the spleen cells, but rather due to the cytokine TGFβ released from the lung after inhalation of MWCNTs at low levels activating the cyclooxygenase pathway in the spleen, leading to prostaglandin and IL-10 production and release, causing T-cell dysfunction and altered systemic immunity. Mice deficient in cyclooxygenase-2 gene did not develop lung inflammation after inhaling MWCNTs, indicating the importance of this signaling pathway. A lung-specific TGFβ knockout mouse was not developed to fully elucidate the role for TGFβ signaling in MWCNT-induced systemic immunosuppression.

A1-9 Cardiovascular toxicity

Radomski et al. (2005) investigated the effects of nanoparticles including SW- and MWCNTs on human platelet function *in-vitro* and rat vascular thrombosis *in vivo*. Incubation of platelets with SW and MWCNTs resulted in a concentration dependent increase in platelet aggregation. Infusion of SW and MWCNT (5 µg/mL systemic level) significantly accelerated time and rate of development of carotid artery thrombosis in rats. The paper discussed the mechanisms of platelet aggregation and the impact of it due to the formation of agglomerates, shape of nanoparticle, surface properties and contaminants.

A significant decrease in the number of baroreflex sequences (from 498 ± 1 27.1 at baseline to 287 ± 40.2 after 4 weeks from the first instillation) was reported in SWCNT-instilled rats (two intratracheal instillations at 2 week interval, dose: 1 µg/g bw dispersed in phosphate buffer saline) after 4 weeks compared to rats in the control group, suggesting that SWCNT may alter the arterial baroreflex function, consequently affecting the autonomic cardiovascular control regulation (Legramante et al., 2009).

Bihari et al. (2010) investigated various nanoparticles including SWCNT for their effects on platelet activation *in-vitro* and on macro- and microcirculatory thrombosis formation in mice. Nanoparticles were injected into mice (1 mg/kg) and ferric chloride induced thrombosis formation was measured in small mesenteric arteries using *in vivo* microscopy. In a separate experiment, SWCNT at 0.01-1 mg/kg was injected into mice and light/dye induced thrombosis formation was investigated in the cremasteric microcirculation. The paper reported that SWCNT significantly increased platelet P-selection expression, the number of platelet-granulocyte complexes, and platelet aggregability *in vitro*, and reduced the occlusion time in mesenteric arteries as well as in cremasteric arterioles.

A1-10 Impact of physical and chemical characteristics on potential toxicity

In-vitro

SWCNT

Fiorito et al. (2006) examined the ability of pure SWCNTs (containing no nickel or cobalt, but a very low amount of amorphous carbon) to elicit an inflammatory response in murine (J774 cell line) and human macrophage cells *in vitro* and cytotoxicity against these cells. The results demonstrated that the highly purified SWCNT at doses up to 60 µg/mL did not stimulate the production of nitric oxide in murine cells. Their uptake by human cells was very low, and they do not induce damage and death of human macrophage cells. On the contrary graphite particles were shown in the same study to be effective in eliciting considerable amounts of nitric oxide from murine macrophages and their uptake by human cells was very much higher. In addition graphite particles induced activation of human macrophage cells as well as morphological changes consistent with severe cell damage and death. The authors speculate that differences in inflammatory response between graphite particles and SWCNTs could be due to the much lower amount of chemical reactive sites on SWCNTs because reactive sites on graphite may be capable of generating radicals causing oxidative stress.

Kagan et al. (2006) examined the effect of iron content of SWCNTs on murine macrophages. Murine macrophages (RAW 264.7) were exposed to well-dispersed non-purified SWCNTs containing 26 wt.% Fe and purified SWCNTs (0.12-0.5 mg/mL) containing 0.23 wt.% Fe (mean diameter 1-4 nm; surface area 1040 m^2/g). The results of the study demonstrated that

neither iron-rich (non-purified) nor purified SWCNT were able to induce intracellular production of superoxide or nitric oxide by RAW 264.7 macrophages indicating the failure of macrophages to recognise dispersed SWCNT. It is suggested by the study authors `that the failure of macrophages to recognize dispersed SWCNT *in vivo* may result in their diffusion into systemic circulation, dissemination, and subsequent effects on distant tissues. However, when the cells were stimulated with zymosin in the presence of SWCNTs, the non-purified SWCNTs much more effectively converted extracellular generated superoxide radicals into hydroxyl radicals. Concomitant with the notion that iron rich SWCNTs could enhance extracellular oxidative stress induced by other agents there was a larger decrease in intracellular glutathione and increased lipid hydroperoxides. The authors concluded the presence of iron in SWCNT may be important in determining redox-dependent responses of macrophages.

Pulskamp et al. (2007) investigated the *in vitro* responses of lung macrophages (rat alveolar macrophage cell line NR8383) and epithelial cells (human lung cell line A549) after exposure to four types of CNTs: 1xSWCNT [diam. 1-2 nm; Co (2.8%); Ni (< 0.005%); Cu (0.03%); Mo (4.2%)] and 2xMWCNT [(i): diam. 10-20 nm; no metal catalysts and (ii) diam. 30-50 nm; Ni (1.86%); Fe (0.55%)] and a purified SWCNT [diam. (not specified); (Co (1.3%); Ni (1.2%)] at doses up to 100 µg/mL. The CNTs consisted of large agglomerates forming bundles or ropes, although single nanotubes were also visible. The agglomerates were tightly bound together even when the particle suspensions in water or medium were sonified prior to use. The study demonstrated that CNT agglomerates were taken up by rat alveolar macrophages with large bundles observed in the cytoplasm. None of the CNTs tested induced inflammatory mediators or caused cytotoxicity. However a dose and time-dependent increase of intracellular reactive oxygen species and a decrease of the mitochondrial membrane potential was observed with all the CNTs except for the purified SWCNT that demonstrated none of these effects. The authors conclude that metal traces associated with the commercial nanotubes are responsible for the biological effects. NICNAS notes that according to the analysis certificate of the supplier, one of the commercial MWCNTs producing an effect did not contain any metal traces. However, the study authors have determined that the level of metal content is much higher than that stated by the supplier upon analysis of one MWCNT type used in the study.

Witasp et al. (2009) studied the effect of SWCNTs on chemotaxis and phagocytosis of macrophages. The study showed that purified SWCNTs (length 500 nm to 1-2 µm; mean diameter of 1-4 nm; surface area 1040 m^2/g; Fe content 0.23%) at doses of 0.1 mg/mL impaired engulfment of apoptotic target cells by primary human monocyte-derived macrophages without exerting cytotoxic effects and suppressed chemotaxis.

Panessa-Warren et al. (2009) examined the cytotoxicity to human lung epithelial cells with commercially prepared SWCNT in their “as-prepared” (diam. 1.4-1.5 nm, length 400-1000 nm, nanoropes 20 nm diam, 2-5 µm long; other constituents: Ni 23.2%; Y 4.8%, carbon black, graphite shells, amorphous carbon, non-tubular grapheme, metal catalyst particles) and “acid cleaned” states using two acid treatments; acid/air-oxidised SWCNT (diam 2.1-2.3 nm, length 180-400 nm, nanoropes 0.4-2 µm length; other constituents: Ni 5.9%, Y 0.9%, graphitic shells, carbon black, amorphous carbon, metal catalyst particles) and acid/peroxide SWCNT (diam 1.4 nm with bundles; no Ni and Y present; other constituents: cut nanoropes, little amorphous carbon, few empty carbon shells). CNTs are commonly cleaned industrially by acid treatment as a means to remove metal catalysts and carbon debris.

Human lung epithelial cells were dosed with 0.12 mg/L of the SWCNT for 2 or 3 hours at 37°C. The “as prepared” SWCNT was found to be less cytotoxic to human lung cells than the acid cleaned SWCNTs. These results appear to show that reduction in metal catalyst content (Ni and Y) does not reduce cytotoxicity whereas increased surface oxidation seems to correlate with increased cytotoxicity. The cytotoxicity of the acid cleaned SWCNTs was significantly reduced when evaluated after aging in saline solution for 1-120 days. As salt was found to be strongly adsorbed onto the CNT it was hypothesised that this salt deposition may have formed a physical barrier that limited interactions with the plasma membrane surface. Very small CNT bundles were often seen within the apical cytoplasm, in nuclei or the perinuclear space. Cell death seemed to be initiated by CNT contact with the plasma membranes and by the reactive groups on the surface. A similar reduction in cytotoxicity was observed when the highly toxic acid-peroxide SWCNT were aged in natural organic matter. This is thought to be due to the formation of aggregates between the SWCNT and the components of natural organic matter (fulvic/humic acids).

MWCNT

Hirano et al. (2008) investigated the mechanism of cytotoxicity of MWCNT. Murine macrophages (J774.1 cell line) were exposed to highly purified MWCNT (curly fibrous particles, average diameter 67 nm, surface area 26 m^2/g, Fe content 2000 ppm) and crocidolite at concentrations up to 1000 µg/mL over 32 hours. Dose dependent cytotoxicity, 25 times greater than for crocidolite, was observed for the highly purified MWCNT. The study demonstrated that the highly purified MWCNT exerted their toxicity by binding to specific receptors (i.e. MARCO) on the plasma membrane of the macrophage, causing extension and disruption of the membrane.

Simon-Deckers et al. (2008) compared the cytotoxicity and intracellular accumulation of MWCNT in respect to fibre length and iron content in A549 human pneumocytes. Three types of MWCNT (fibres) were studied: short and long MWCNT containing Fe impurities

(4.24 wt.%) and purified (by annealing) long MWCNT (0.08 wt.%). The length of long MWCNT ranged from 110 nm to 13 µm, with a mean length of 1.5 µm and a median at 875 nm. Their diameter ranged from 8 to 167 nm with a mean diameter of 44 nm and a median of 42 nm. Length did not differ between the purified and non-purified long MWCNT. The length of the short MWCNT ranged from 83 nm to 5 µm with a mean length of 972 nm and a median of 708 nm. The diameter ranged from 8 to 185 nm with a mean diameter of 26 nm and a median of 40 nm. The major difference between long and short nanotubes is the presence of nanotubes longer than 5 µm in the long nanotubes suspension. In exposure media, the MWCNTs were agglomerated.

The cells were exposed to 10 µg/ml for 48 hours for the internalisation study and at doses from 0.25 to 100 µg/mL for up to 72 hours for the cytoxicity study. The study showed that MWCNT with a maximal length of 2-3 µm were internalised in A549 cells localised mostly in vesicles in the cytoplasm. The MWCNT were not agglomerated inside cells but were isolated and were not observed in the cell nuclei. The presence of Fe did not influence cellular internalisation. Morphological modifications of cells were observed, particularly the presence of multiple multi-lamellar bodies. Cytotoxic effects were only observed after 48 h of exposure when using the lactate dehydrogenase (LDH) assay (35-40% cytotoxicity at 100 µg/ml) and XTT cell proliferation assay (10% cytotoxicity at 50 µg/ml). The study showed that cytotoxicity of MWCNT was not correlated to the presence of Fe impurities or length. However, it is noted that Fe was mainly encapsulated in the tested MWCNT.

Cheng et al. (2009) studied the toxicity of purified MWCNT (length 4-65 µm; mean 12 ± 9.9 µm; diameter 68 ± 30 nm; Fe 0.0005%) and unpurified MWCNT (length 2-164 µm; mean 26 ± 22.7 µm; diameter 68 ± 30 nm; Fe 6.2%) on human monocyte-derived macrophages. The purification of MWCNT from metallic impurities was based on high temperature annealing rather than acid treatment in order to minimise formation of highly reactive sites on the surface. The majority of the iron was internalised within the bore of the unpurified MWCNTs and remained as iron nanoparticles, inaccessible to cells. Some iron was found on the outer surface, potentially accessible to cells but oxidised mainly to Fe_2O_3. Human monocyte-derived macrophages were treated at up to 20 µg/ml of the MWCNT for 4 days at 37°C. The cells were also treated with Fe_2O_3 (1 µg/ml; proportionally equivalent concentration) as this is main contaminant accessible to cells.

The NR (Neutral Red; measures the lysosomal accumulation of NR dye in viable cells), MTT (measures mitochondrial activity based on the enzymatic conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan by dehydrogenase enzymes in viable cells) and live-dead viability assays all clearly revealed concentration dependent cytotoxicity for unpurified MWCNTs. Cytotoxicity was only significant at 20 µg/ml

with cell death occurring by necrosis rather than apoptosis. As most of the apoptotic cells were observed as secondary necrosis, which results from unphagocytosed apoptotic cells, the study authors suggest that unpurified MWCNTs can cause rapid cell degeneration or inhibition of macrophages to phagocytose. However, there was a similar percentage of healthy to dead cells containing the unpurified MWCNT suggesting that the cells may be able to survive with the internalised MWCNT. The purified MWCNT were found to exhibit comparable levels of cell death as unpurified whilst proportionally equivalent Fe_2O_3 alone showed no toxicity. This result might be due to the fact that the iron is within the bore of the unpurified MWCNT and is not bioavailable. Imaging showed unpurified MWCNT inserting through the plasma membrane into the cytoplasm and nucleus. The results suggest that MWCNTs may cause incomplete phagocytosis or mechanically pierce through the plasma membrane and result in oxidative stress and cell death.

Tabet et al. (2009) evaluated the adverse effects (cell viability, apoptosis and oxidative stress) of MWCNT (average diam. 12 nm; length 0.1-13 µm; Al 2.4%; Fe 2%; surface area 219.2 m^2/g) in the human lung epithelial cell line A549 and human mesothelial MeT5A cell line. The MWCNT as produced by chemical vapour deposition consists of spherical sets of agglomerates of primary MWCNT particles in the range of several tens to several hundreds of micrometers of diameter. Dipalmitoyl lecithin (DPL) was chosen as the dispersing agent to resemble *in vivo* respiratory exposure. In DPL the presence of primary MWCNT particles in addition to MWCNT agglomerates of micrometer size were observed. The study showed that incubation with 100 µg/mL MWCNT dispersed in dipalmitoyl lecithin (a component of pulmonary surfactant) for up to 72 hours induced a decrease in metabolic activity without changing cell membrane permeability or apoptosis. Neither MWCNT cellular internalisation nor oxidative stress was observed. In contrast, asbestos fibres penetrated into the cells, decreased metabolic activity but not cell permeability, and increased apoptosis, without decreasing cell number. Carbon black was internalised without any adverse effects.

Walker et al. (2009) examined the effects of purified SWCNTs (a specific surface area average 641 m^2/g) and MWCNTs (a specific surface area average 56 m^2/g) on human aortic endothelial cells *in vitro*. CNTs that were in direct contact with endothelial cells triggered a dose-dependant impaired cell function and viability.

In-vivo

Koyama et al. (2009) examined the contribution of impurities including disordered carbon, metallic impurities and polycyclic aromatic hydrocarbons on toxicity. “As grown” (Fe content 12,000 ppm) and two thermally cleaned MWCNT (MWCNT20 and MWCNT80 containing 20 and 80 ppm Fe respectively) (1 mg) were subcutaneously implanted 1 cm in depth on the back skin in mice. The “as grown” nanotubes also contain disordered carbon (small or

undulated graphene layers and in some cases a fraction of functional groups at chemically active edge sites of the graphene layers) and polycyclic aromatic carbons (PAHs) as contaminants. These impurities are removed by the heat treatment. The control group were treated with 1 mL of physiological saline and the animals were sacrificed 4 weeks post-implantation for blood sampling from the heart and skin tissue sampling. All the MWCNT have a length of 10-20 µm and a diameter of 100-150 nm. The Fe content in the “as-grown” nanotubes is not soluble in hydrochloric acid (0.6N) whereas the Fe content is soluble in the clean nanotubes, suggesting Fe is on the outside of the tubes in the clean nanotubes and thus may be bioavailable and contribute to toxicity.

The mice implanted with the “as-grown” MWCNT and MWCNT80 exhibited severe hair loss and thick, hard callus formation. However, the animals treated with MWCNT20 did not experience any skin hair loss. The “as-grown” tubes showed inflammatory cells located around them that were encapsulated tightly by granuloma tissue. These effects were less pronounced for the MWCNT80 group and the MWCNT20 showed only a slight granulomatous reaction. The author considers the toxicity of the “as grown” tubes is due to the presence of disordered carbon and PAHs, whereas the toxicity of MWCNT80 is due to the availability of iron that can act as redox catalysts generating reactive oxygen species. Given the highly pure MWCNT (MWCNT20) did not induce significant toxicity under the conditions of the study, the study authors suggest that toxicity of carbon nanotubes might be directly linked to the presence of impurities and not the nanotubes themselves.

Appendix 2: References reviewed but not considered in the health hazard profile or classification of CNTs

1. Auffan M, Rose J, Bottero J, Lowry G V, Jolivet J and Wisener M R; Review article: Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. Nature Nanotechnology 2009, **4**(10):634-641.

After reviewing size-dependant properties of a variety of inorganic nanoparticles, Auffan et al (2009) reported that particles larger than about 30 nm do not in general show properties that would require scrutiny beyond that required for their bulk counterparts. Nanoparticles have diameters of 30 nm or less may undergo dramatic changes in crystalline structure that enhances their interfacial reactivity.

2. Bello D, Wardle BL, Yamamoto N, deVilloria RG, Garcia EJ, Hart AJ, Ahn K, Ellenbecker MJ, Hallock M; Exposure to nanoscale particles and fibers during machining of hybrid advanced composites containing carbon nanotubes. Journal of Nanoparticle Research 2009; **11**:231-249.

Airborne exposure to nanoscale particles and fibres generated during dry and wet abrasive machining of two, three-phase advanced composite systems containing CNTs (micro-diameter continuous fibres (carbon or alumina) and thermoset polymer matrices) was investigated. Submicron and respirable fibres were generated from dry cutting and exposure to nano particles was significant from all composite systems.

3. Erdely A, Hulderman T, Salmen R, Liston A, Zeidler-Erdely PC, Schwegler-Berry D, Castranova V, Koyama S, Kim Y, Endo M and Simeonova PP; Cross-talk between lung and systemic circulation during carbon nanotube respiratory exposure. Potential biomarkers. Nano Letters 2009, **9**(1):36-43.

SWCNT and MWCNT deposited in the lung induced acute lung and systemic effects, which are more pronounced with MWCNT exposure. MWCNT exposed mice showed a greater number of upregulated genes in the lung with qualitatively higher expression compared to SWCNT exposed group. If the systemic response persists, it may trigger or exacerbate cardiovascular dysfunction and disease such as atherosclerosis (build up of a waxy plaque inside blood vessels).

4. Folkmann JK, Risom L, Jacobsen NR, Wallin H, Loft S, Møller P; Oxidatively damaged DNA in rats exposed by oral gavage to C_{60} Fullerenes and Single-Walled Carbon Nanotubes. Environ. Health Persp. 2009, **11**, **7**:703.

In this study, Female Fisher 344 rats received a single dose of SWCNT (diam. 0.9-1.7 nm; fiber length <1 µm; Fe 2 wt.%) in either saline solution or corn oil by oral gavage (0.064 and 0.64 mg/kg bw). Rats were sacrificed 24 h after administration and the liver, lung and colon tissues were then analysed for 8-oxodG (a biomarker for oxidatively damaged DNA) levels. The results showed increased levels (~20%) of this biomarker in the liver and lung but not the colon indicating that the SWCNT may have a genotoxic potential via the gastrointestinal route.

5. Herzog E, Byrne HJ, Davoren M, Casey A, Duschi A and Oostingh GJ; Dispersion medium modulates oxidative stress response of human lung epithelial cells upon exposure to carbon nanomaterial samples. Toxicol App Pharmacol. 2009; **236**:276-281.

The study investigated the potential of SWCNT and MWCNT to induce ROS in lung epithelial cells *in vitro*, to mimic occupational exposure by inhalation of airborne dust particles from

CNT production. The *in vitro* exposure resulted moderate to low oxidative stress under the exposure conditions employed.

6. Kostarelos K; News and Views - The long and short of carbon nanotube toxicity. Nature biotechnology 2008, **26**(7):774-776.

CNTs are seamless cylindrical structures containing single or multiple concentric graphene sheets. Research to date indicates that long, rigid CNTs should be avoided for *in vivo* applications and that chemical functionalisation should be optimized to ensure adequate dispersibility, individualization and excretion rates sufficient to prevent tissue accumulation. More work is needed to assess the persistence and toxicity of MWCNTs after intravenous, pulmonary and other routes of administration.

7. Liu J and Hopfinger AJ; Identification of possible sources of nanotoxicity from carbon nanotubes inserted into membrane bilayers using membrane interaction Quantitative Structure- Activity Relationship analysis. Chem. Res. Toxicol. 2008, **21**:459-466.

Four possible sources of cellular toxicity from the insertion of a carbon nanotube into dimyristoylphosphatidylcholine (DMPC) membrane bilayer, which served as a model for a cellular membrane, were investigated. The insertion of the carbon nanotube diminished the molecular flexibility of the bilayer and its constituent DMPC molecules, suggesting adverse effects on cellular function. The total diffusion coefficient through the membrane bilayer increased for ethanol, urea and caffeine in the presence of carbon nanotubes.

8. Lucente-Schultz RM, Moore VC, Leonard AD, Price BK, Kosynkin DV, Lu M, Partha R, Conyers JL and Tour JM; Antioxidant single-walled carbon nanotubes. J. Am. Chem. Soc. 2009, **131**(11):3934–3941.

This report showed that a pluronic-wrapped pristine SWCNT (pluronic acid is a triblock copolymer of poly(ethylene glycol)/poly(propylene glycol/poly(ethylene glycol) (PEG/PPG/PEG)) and a PEGlayted SWCNT were not cytotoxic to human renal epithelial and HepG2 liver cells when dosed at 83 mg/L and 28 mg/L for 24 hours. These SWCNT are soluble in biologically relevant salts.

9. Malarkey EB and Parpura V Carbon nanotubes in neuroscience. Acta. Neurochir. Suppl. 2010, **106**:337-341.

Review on the use of CNTs in neuroscience, focusing mainly on their use as scaffolds for neuronal growth and as electrical interfaces with neurons. The review states that given carbon nanotubes do not have obvious adverse effects on mammalian heath, in the near future they could be used in brain-machine interfaces.

10. Park KH, Chhowalla M, Iqbal Z and Sesti F Single-walled carbon nanotubes are a class of ion channel blockers. J. Biol. Chem. 2003, 278(50):50212-50216.

The report demonstrated that as-fabricated (i.e. unpurified) and purified SWCNTs blocked K^+ channel subunits in a dose-dependent manner. Blockage was dependent on the diameter of the SWCNT with smaller diameters (~0.9 nm) showing stronger blocking. MWCNTs having larger diameters (10-15 nm) did not show any blocking. Also shape was shown to be an important factor as the SWCNTs were roughly 3- to 2-fold more efficient at blocking than C_{60} fullerenes (diam. 0.72 nm). The mechanism was postulated to be governed by geometrical factors not by electrochemical interactions as is usually required by conventional blocking agents.

12. Powers K, Palazuelos M, Moudgi B and Roberts S Characterisation of the size, shape and state dispersion of nanoparticles for toxicological studies. Nanotoxicology 2007, **1**(1):42-51.

Although nanomaterial size is assumed to be a factor influencing toxicity, there is little specific information available characterising toxic effects relative to the 1-100 nm size range.

13. Silva GA Nanomedicine: Shorting neurons with nanotubes. Nature Nanotechology 2009, **4**:82-83.

It was reported that neurons continue to grow when placed on CNTs and can still carry electrical signals when stimulated by them. The findings of recent research conducted to understand the mechanisms that underlie the effects of CNTs on neural cells were discussed, which could lead to development of neural devices.

14. Tervonen T, Linkov I, Figueira J, Steevens J, Chappell M and Merad M Risk-based classification system of nanomaterials. J. Nanopart. Res. 2009, **11**:757-766.

This paper presented a systemic multi-criteria approach that enables nanomaterials to be assigned into ordered risk classes (extrinsic properties: agglomeration, reactivity/charge, critical functional groups, contaminant dissociation, particle size; other factors: bioavailability, bioaccumulation potential, toxic potential).

Materials assigned to the highest risk class potentially represent areas of important future toxicological studies, while materials exhibiting low risk may be targets of research aimed at commercial use. This approach prevents decisions being unduly based on one particular criterion (such as size versus surface reactivity relationships), as the material may have other physico-chemical characteristics related to size that exhibit a greater impact on its toxicity.

15. Xu H, Bai J, Meng J, Hao W, Xu H and Cao J-M; Multi-walled carbon nanotubes suppress potassium channel activities in PC12 cells. Nanotechnology 2009, **20**:285102.

Undifferentiated PC12 (rat pheochromocytoma) cells were incubated with 5 µg/mL of Carboxyl-terminated MWCNTs (length 300-800 nm, inner diameter 10-20nm, outer diameter 40-50 nm) for 30 min, 1, 2, 3 and 6 h. Measures of current flow through membrane K^+ channels indicated that incubation with MWCNTs suppress current densities in a time dependant and irreversible manner, without inducing oxidative stress.

16. Yu Y, Zhang Q, Mu Q, Zhang B, and Yan, B; Exploring the Immunotoxicity of Carbon Nanotubes. Nanoscale Research Letters 2008, **3**(8):271-277.

Some studies related to the assessment of the immunotoxicity of carbon nanotubes are reviewed with a view to their application in nanomedicine. While data suggests CNTs enter cells, cause oxidative stress and interact with the immune system, a better understanding of the mechanisms of interaction with the immune system is required for the development of nanomedicine carriers.

17. Zhang Y, Ali SF, Dervishi E, Xu Y, Li Z, Casciano D and Biris AS Cytotoxicity effects of graphene and single-walled carbon nanotubes in neural phaeochromocytoma-derived PC12 cells. ACS Nano 2010, **4**(6):3181-3186.

The article compares the *in vitro* toxicity of graphene with SWCNTs using neuronal PC12 cells in similar conditions. As indicated by the MTT assay, both graphene and SWCNTs induced cytotoxicity, however the results showed that at low concentrations the graphene structures induced a more intense toxic response than the SWCNT but as the concentratin

increased the cytotoxic effects reversed, with grapheme showing a lower activity. In addition, the lactate dehydrogenase (LDH) levels, an indicator of cell damage, were found to be significantly higher for the SWCNTs. The authors conclude that these differences are explained by the differences in the shape of the two nanomaterials. The needle-like shape of SWCNTs promote penetration of membranes uptake by cells and strong interactions with various protein systems. Graphene, on the other hand, is expected to have stronger interaction with the cellular membranes due to their flat shapes, indicating different cellular target sites for the two types of nanomaterials.