WORKING FOR A HEALTHY FUTURE

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An outline scoping study to determine whether high aspect ratio nanoparticles (HARN) should raise the same concerns as do asbestos fibres

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EXECUTIVE SUMMARY

Potential concerns about the potential health effects of high aspect ratio nanoparticles (HARN) are based primarily on toxicology studies of industrial fibres including asbestos. The objectives of this study are:

1. to undertake a scoping study to review the existing literature on industrial fibres and HARN to determine whether high aspect ratio nanoparticles (HARN) should raise the same concerns as do asbestos fibres and
2. to set out a research strategy towards determining whether the health concerns about HARN are well–founded.

Our approach focused on the following activities:
Literature and information collection. Information was collected from the peer-reviewed, grey literature and published conference proceedings of relevance to the stated project objectives.
Critical review. Critical reviews of the identified literature were conducted by the different partners of our consortium. The following areas are integral to the critical reviews:

Asbestos & Industrial Fibre Characterisation
Asbestos & Industrial Fibre Health Effects in Humans
Asbestos & Industrial Fibre Mechanisms of Toxicity
HARN Characterisation
HARN Mechanisms of Toxicity

The development of a research strategy to investigate the potential hazard of HARN based on identified gaps in knowledge
The facilitation of a workshop to disseminate the findings of this study to stakeholders including key members of the toxicology, regulatory and other appropriate communities.

In this study, we have reviewed the state-of-the-art knowledge on the toxicity of asbestos and HARN and we have compared the health effects from exposure to asbestos (as an example of an industrial fibre) with carbon nanotubes as an example of a HARN. As part of the review we have also compiled the current information on the characterisation of the physico-chemical properties of HARN

The research on the toxicity of asbestos and other industrial fibres has lead to the 'fibre paradigm';

1. **Diameter**: fibres must be *thin* enough reach past ciliated airways. The aerodynamic diameter of fibres is not affected much by their length
2. **Length**: essential for the onset of e.g. frustrated phagocytosis and other inflammatory pathways
3. **Biopersistence**: important when considering the setting of e.g. maximum exposure limits to a fibre over time.

This review has identified many similarities between HARN and asbestos with regard to their physico-chemical properties and toxicological effects and has concluded that there is sufficient evidence to suggest that HARN which have the same characteristics (diameter, length and biopersitance) as pathogenic fibres are likely to have similar pathology.

This review has also highlighted the lack of data in key areas of toxicology, exposure and assessment.

- The rationale of this strategy was based on information on the similarities and differences between HARN and asbestos hazard.
- The objective of the strategy was to reduce the knowledge gap in order to better ascertain the HARN hazard.

The results from the critical review were used to formulate a research strategy to investigate the potential hazard of HARN. The main components of this strategy are:

- **Hazard Identification:** The characterisation of the physico-chemical properties of HARN especially the length of the fibres and their biopersistence
- **Dose-Response Assessment:** Acute and chronics adverse effects of HARN; Cellular and molecular mechanisms of HARN toxicity investigated with in vitro and in vivo models
- **Exposure Assessment:** Identification and quantification of the routes (e.g. inhalation, dermal); the pattern and the intensity of exposure
- **The Risk Assessment of HARN:** Combining exposure and Hazard to calculate the health risks from exposure to HARN.

Recommendations on future studies to cover the identified information gaps have been made.

1 INTRODUCTION

Asbestos is a naturally occurring fibrous mineral that occurs in two main varieties, amphibole and serpentine (chrysotile). The amphibole asbestos varieties are all straight needle like fibres. Far and away the major type of asbestos that was, and still is, commercially exploited was chrysotile (white) asbestos but two types of amphibole asbestos have been used commercially: amosite (brown asbestos) and crocidolite (blue asbestos). In addition, a third type of amphibole asbestos, tremolite forms an important part of the research literature on fibres. For regulatory purposes, fibres are defined on the basis of their size and shape (aspect ratio ≥ 3:1, length ≥5 μm, and width ≤3 μm) and unlike other dusts are regulated by number per unit volume of air and not mass concentration per unit air. All of the asbestos types are found with long (>20um) fibres. Both crocidolite and tremolite contain very thin and long fibres, in contrast to the size distribution of amosite. Scientific studies of the health effects of these fibres, in man and in rodents, have provided a great deal of information on the effect of the shape and size of fibres which is relevant to high aspect ratio particles. In fact, fibres are the only particle type that has a well verified 'structure/activity relationship' (SAR) that has been found to be applicable over a number of different types of fibre – crystalline silicates like asbestos, amorphous vitrious fibres such as glass wool and ceramic fibres and at lest one organic fibre p-aramid. Such a robust SAR gives some optimism that HARN may also be included and this will be addressed below. The applicants have been involved in the research that lead to the existing SAR for industrial fibres for over thirty years, and therefore we know and understand the literature on fibres and can explain its relevance to HARN. In the text below, we will summarize the current knowledge on the health effects of asbestos.

Health Effects of Asbestos comprise several types of pleural and parenchymal lung disease associated with inhalation of asbestos fibres. Asbestos fibres have been classified by the International Agency for Research on Cancer (IARC) as carcinogenic for humans. Rodent models have proven to be appropriate surrogates for humans in reproducing the lung diseases associated with asbestos exposure. Respirability (the fraction of inhaled fibres reaching the alveolar region) is an important aspect of fibre pathogenicity. Respirability is determined by the aerodynamic diameter of the fibre, which is a function of diameter, length, and density. Surface charge and hydrophilicity, as well as adsorbed finishes and other physical and chemical factors, determine whether fibres can be easily dispersed or will agglomerate into larger, nonrespirable masses. For respirable fibres tested in rodent bioassays, the dose, dimensions, durability in the lung and in some cases surface reactivity of the fibres have been identified as critical parameters related to adverse health effects. Fibre length is hypothesized to be a major determinant of pathogenicity: Fibres that are too long to be completely phagocytised by macrophages are cleared less efficiently. If fibres are composed of material that resists leaching and breakage in the lungs (i.e. are biopersistent), then the long fibres have the potential to interact with target cells in the lungs or be translocated to the interstitium or the pleura where they may cause disease. In chronic rodent inhalation studies, fibres that persisted in the lungs caused sustained inflammation and fibrosis. These pathologic endpoints were associated in most cases with the development of lung cancer or mesotheliomas in rodents after 1–3 yr. Fibre length, diameter, and chemical composition are major determinants of biopersistence. Additional properties that have been linked to asbestos toxicity include free radical

generation, mobilization of transition metals, acquisition of iron, ferritin, or other proteins in the lungs, and surface hydrophilicity/hydrophobicity.

There is a correlation between the type of asbestos exposure, the intensity and duration of exposure, and the severity of the disease. Pleural plaques and fibrosis (including pleural pseudo tumours or rounded atelectasis) are markers of asbestos exposure but are not causally related to lung cancer or diffuse malignant mesothelioma. Fibrotic scarring of the pleura or lung parenchyma is a nonspecific reaction to chronic inflammation or trauma; however, bilateral, symmetrical pleural plaques usually indicate occupational or environmental exposure to asbestos fibres or erionite (Travis et al., 2002). Asbestosis or diffuse interstitial fibrosis may progress after cessation of exposure and is considered a risk factor for development of lung cancer by some investigators (Churg & Green, 1998) but not by others (Nelson & Kelsey, 2002). The incidence of lung cancer is greatly increased in cigarette smokers or ex-smokers who are also exposed to asbestos fibres. Diffuse malignant mesothelioma arising in the pleura or peritoneum occurs less frequently and after a longer latency period than pleural fibrosis or asbestosis; this cancer can develop in the absence of asbestosis or cigarette smoking and occurs more frequently in people exposed to amphibole asbestos fibres or erionite.

HARN: Carbon Nanotubes, Nanowires and Nanorods

Carbon nanotubes (CNT) are an important new class of technological materials that have numerous novel and useful properties. A review of their properties in relation to pulmonary toxicology and workplace safety has been undertaken recently by the present applicants (Donaldson et al., 2006). Comprised entirely of carbon, the structure of pure CNT can be visualized as a single sheet of graphene rolled to form a seamless cylinder. There are two classes of CNT: single walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT). MWCNT are larger and consist of many single-walled tubes stacked one inside the other. CNT are distinct from carbon fibres, which are not single molecules but strands of layered graphite sheets. CNT are reported to be physically strong and stiff; for example SWCNT can be as much as 10 times as strong as steel and 1.2 times as stiff as diamond (Walters et al., 1999; Yu et al., 2000).

Nanorods are one morphology of nanoscale objects. Each dimension of nanorods ranges from 1–100 nm. They may be synthesized from metals or semi-conducting materials. Standard aspect ratios are 3-5. Nanorods are produced by direct chemical synthesis. A combination of ligands acts as shape control agents and bond to different facets of the nanorod with different strengths. This allows different faces of the nanorod to grow at different rates, producing an elongated object. The applications of nanorods are diverse, ranging from display technologies (the reflectivity of the rods can be changed by changing their orientation with an applied electric field) to micro electromechanical systems.

Nanowires consist of a wire of diameter of the order of a single nanometer (10^{-9} meters). At these scales, quantum mechanical effects are important — hence such wires are also known as "quantum wires". Many different types of nanowires exist, including metallic (e.g., Ni, Pt, Au), semiconducting (e.g., Si, InP, GaN, etc.), and insulating (e.g., SiO_2, TiO_2). Molecular nanowires are composed of repeating molecular units either organic (e.g. DNA) or inorganic (e.g. $Mo_6S_{9-x}I_x$).

Health effects of HARN. Currently, information on the toxicity of HARN exist mainly for carbon nanotubes. A review of the fibre paradigm (see later), as it relates to CNT, has been conducted recently (Donaldson et al. 2006) and some further information on the toxicity of nanowires and rods is available in conference proceedings (refs). Several existing studies address the potential pulmonary and cellular toxicity of nanotubes, and yet, none have addressed whether the particle or fibre paradigm is dominant. Of the *in vivo* studies, none have used inhalation, which is much needed. All use instillation where the high dose and dose rate raise questions about physiological relevance; no study has addressed the role of length, in the sense that none has compared long (greater than ~ 20 μm) to short (< 10 μm) nanotubes. Although there are several *in vitro* studies in various models and end points, no biopersistence study has been carried out.

It is clear that CNT can cause some of the adverse effects observed in asbestos. However, a close comparison of the exposure-dose-response relationship between asbestos and CNT (also nanorods and wires) needs to be implemented in order to ascertain the similarities and differences in the induced health effects between asbestos and HARN and therefore to determine whether the health concerns about HARN are well–founded. Creative approaches are to be emphasised and cell work e.g. with mesothelial cells (Heintz et al. 1993) and exposure of mesothelial surfaces that promote fibre/mesothelial interaction (e.g. Donaldson et al. 1994) may be useful approaches.

2 OBJECTIVES

The objective is to undertake a scoping study to review the existing literature and set out a research strategy towards determining whether the health concerns about HARN are well–founded.

3 METHODS AND PLAN OF WORK

3.1 Literature information collection process

Information was collected from the peer-reviewed, grey literature and published conference proceedings of relevance to the stated project objectives. Using hosts such as Dialog-Datastar and STN, access was gained to numerous commercial databases covering chemistry, medicine toxicology, and the life sciences. In addition, extensive use wias made of Web-based sources, including the US National Library of Medicine's PubMed, TOXNET and TOXSEEK meta-search and clustering engine, and Web of Science. Original papers and books identified in the searches were obtained from existing library resources and current journal subscriptions amongst the partners. A reference database was maintained by the project coordinator using 'RefWorks' software enabling the reference sources to be visible to all partners in real time via the internet. The findings from the component scoping activities were collated into an overall review document by IOM.

3.2 The critical review process

The critical reviews were conducted by the different partners of our consortium with each main Task lead by an expert in the field. The following Tasks are integral to the critical reviews:

Task	Topic	Lead Partner
3.1.1	Asbestos & Industrial Fibre Characterisation	IOM
3.1.2	Asbestos & Industrial Fibre Health Effects in Humans	IOM
3.1.3	Asbestos & Industrial Fibre Mechanisms of Toxicity	KD
3.1.4	HARN Characterisation	NPL
3.1.5	HARN Mechanisms of Toxicity	Napier

Table 1 Tasks for the critical review

For each Task, our approach for the critical review is:

- To organise and collate the information obtained in 3.1 into each Task in Table 1
- To review, extract the important data and summarise the findings for each Task.
- To synthesise the findings across all the Tasks for the purpose of comparison. Table 2 illustrates the theoretical framework constructed for the comparison between asbestos and HARN.
- To summarise the findings of the comparison as input for the planned Research Strategy (Section 3.3).

The critical reviews have delivered information on the state-of-the-art of the potential hazard of HARN in comparison to asbestos hazard and the knowledge gap in HARN potential hazard.

	Asbestos	CNT	Nanorod	Nanowire
Characterisation	✓	✓	✓	✓
Health effects in Humans	✓	?	?	?
Mechanisms of Toxicity	✓	✓	?	✓

Table 2 Scheme for the comparison of Asbestos and HARN potential toxicity based on similarities and differences (tick marks are for illustration purpose only)

3.3 Develop a research strategy to investigate potential hazard of HARN (Lead Partner: IOM)

The results from the critical review are used to formulate a research strategy to investigate the potential hazard of HARN.

- The rationale of this strategy is based on information on the similarities and differences between HARN and asbestos hazard.
- The objective of the strategy is to reduce the knowledge gap in order to better ascertain the HARN hazard. We will design:
- Specific toxicology experiments (*in vitro* and *in vivo*) to address the questions highlighted in the critical reviews. We will also take into account:
    - the necessary scientific issues (e.g. validation of *in vitro* results by *in vivo* experiments);
    - regulatory implications (e.g. safe level of exposure).

The research strategy has layout a plan for *in vitro*, *in vivo*, mathematical modelling studies to reduce the knowledge gap and ascertain whether the concern for the health effects of HARN is justified.

3.3 Workshop (Lead Partner: IOM)

A draft final report was prepared, discussed with Defra/HPA, and used as prior information for the workshop. The project team, jointly with Defra/HPA, has selected the stakeholders to be invited for the workshop. Stakeholders include key members of the toxicology, regulatory and other appropriate communities. We invited a limited number of participants from the US and EU to reflect the international dimension of this activity. The workshop was informed by the state of the arts reviews which was provided to all participants prior to the workshop. The workshop was structured as follows:

- Facilitator introduced the workshop aims, objectives and protocol;
- Overview of the key components of the study;
- Chaired round table discussion, with break out groups as required;
- Consensus on recommendations / comments to finalise the report.

The workshop was held at the Central Science Laboratories (CSL) in York. IOM undertook to record the discussion to inform the final stages of the study. Based on the discussion and outcomes of the workshop, the draft report was updated and finalised prior to submission to Defra/HPA at the conclusion of the project.

The workshop informed an audience of relevant toxicologists, regulators on the findings of the review process and invited their comments which were incorporated in the final report.

4 REVIEW OF THE EVIDENCE

4.1 THE NATURE OF ASBESTOS RISK – EVIDENCE IN HUMANS

4.1.1 The asbestos industry

Asbestos refers to a group of crystalline silicate mineral characterized by a fibrous habit and a tendency, when worked, to break into microscopic fibres that become airborne. They are divided into two classes as shown in Figure 1. The serpentine group contains only chrysotile, the most commercially utilised form of asbestos. The amphibole class, contains 5 members, two of which, amosite and crocodile, were commercially important on a global scale.

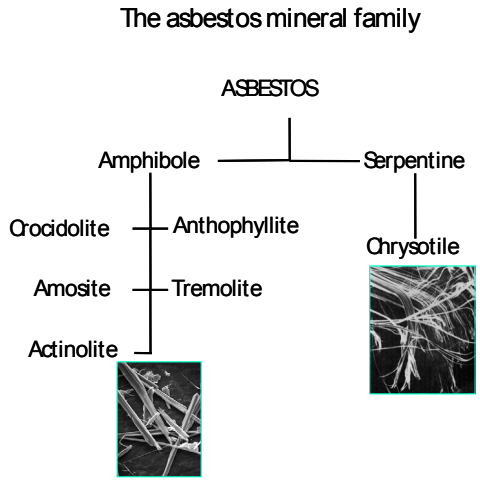


Figure 1 Classification of asbestos minerals

Although sporadic reports in the historical record have described its miraculous fire-resisting properties since 500 B.C., the breakthrough in asbestos production occurred in the late 1800s in Canada with 50 tonnes being mined in 1876 (Selikoff & Lee, 1978). Industry quickly recognized the useful qualities of this cheap bulk fibrous material and chrysotile was incorporated into many different products with the result that by the 1950s chrysotile production was approaching one million tones per year. The USA, China, South Africa and Australia contributed smaller amounts of crocidolite and amosite and world –wide production soon rose to nearly 1000 tonnes with concomitant workplace exposure of a huge number of people in the mining and user industries (Selikoff & Lee, 1978).

4.1.2 Health effects of high aspect ratio thin particles from the evidence on asbestos and industrial fibres in general

The evidence on health effects of asbestos and industrial fibres has been gathered over a century, with the extent and nature of the effects only gradually becoming fully apparent. The role of fibre characteristics were explored over the latter half of the twentieth century, and it was only towards the end of the twentieth century that the significant health risks to populations with relatively low exposure became clear.

By the end of the 1960s, asbestos fibre concentrations were being measured in terms of numbers of fibres/ml, with samples evaluated by phase contrast optical microscopy. Prior to that, asbestos concentrations were measured in terms of millions of particles per cubic foot (mppcf) or as particles per cubic cm. Studies were undertaken to produce conversion factors (see for example the review by Doll and Peto (1990)), and these conversion factors indicate that the occupational exposure limit in the UK in the mid 1960s (5 mppcf) was equivalent to about 30 fibres/ml. In 1970, the control limit for asbestos in the UK was reduced to 2 fibres/ml for chrysotile and amosite asbestos and, because of the evidence that crocidolite produced mesothelioma, to a lower limit of 0.2 fibres/ml for crocidolite (HSE, Asbestos Regulations 1969). In 2006, the UK occupational exposure limit for all types of asbestos became 0.1 fibres/ml (in the 2006 Control of Asbestos Regulations).

It is striking that the reduction in exposure limit in 1970 did not prevent a continuing increase of mesothelioma mortality into the twenty first century. In 1995, Peto *et al* reported on an analysis of mesothelioma mortality since 1968. They found that mesothelioma mortality was increasing and would continue to increase until 2010, perhaps 2020, with the largest occupational group at risk being workers in the construction industry due to the extensive presence of asbestos containing materials in buildings that had been constructed in the 1950s, 1960s and 1970s. The 1969 Asbestos Regulations had been aimed at the workers in the asbestos manufacturing industry, but had neglected the potential exposure of the much larger numbers of workers in the construction industries that installed asbestos containing materials or subsequently disturbed those materials during refurbishment, maintenance, modification or demolition of buildings.

4.1.3 Characterisation of asbestos and industrial fibres in relation to hazard

During the century over which human exposure to asbestos has occurred, the technology for the characterisation of fibres has advanced greatly. The change from measuring airborne asbestos concentrations in terms of numbers of particles to numbers of fibres was a major step forward in the 1960s. However, the chosen size of fibre to be counted (e.g. fibres longer than 5 μm) was not necessarily the most relevant definition for the assessment of risk to human health. Nevertheless, most of the human epidemiology studies assess risk in relation to fibre concentrations measured in terms of fibre concentrations measured by that index of exposure. Consequently, epidemiology demonstrates that there is a relationship between an index of asbestos exposure (in numbers of fibres longer than 5 μm); it does not establish the best or most relevant definition of the size of hazardous fibres. However, where the epidemiological evidence points to a greater hazard from crocidolite asbestos compared to amosite asbestos then it may be inferred that the difference in potency could be due to the presence of more very thin (e.g. less than 200 nm diameter fibres) in the crocidolite asbestos than in the amosite asbestos.

Information on the size distributions of the different asbestos types has been obtained by electron microscopes. Transmission electron microscopy (TEM) provides the highest magnification, but the image obtained is a shadow image of the profile of fibres, and the field of view is limited to being typically less than 100 μm by 100 μm which can restrict the ability to measure length where fibres have lengths that make overlapping the frame likely. Electron diffraction patterns obtained from TEM can help to positively identify the type of asbestos fibres.

Scanning electron microscopy provides an image which has the perspective of three dimensions, does not have the restricted area of a TEM image but does not have a resolution quite as powerful as the TEM. However, modern SEMs provide a very high resolution and the difference between fibre dimensions observable by TEM and modern SEM are probably only significant for fibres with diameters less than some tens of nano meters, but can still be significant for detecting fibrils of chrysotile.

One important study of the relationship between fibre dimensions and incidence of lung tumours in rodent experiments was reported in 1995 by Berman et al. Their study was based on TEM characterisation of airborne fibres size distributions for re-generated samples from a series of inhalation experiments that had been conducted at the IOM over more than two decades. Their findings pointed towards the longer fibres (longer than about 15 µm) being the most important factor determining the risk of lung cancer from inhaled asbestos.

4.1.4 Exposure to asbestos and industrial fibres and human health effects

Asbestosis Fibrosis of the lung was a commonplace industrial disease in the first quarter of the twentieth century, and the main cause was exposure to silica dust. The published account of a case of fibrosis of the lung being attributed to asbestos exposure was published by Dr Montague Murray in 1907 (Hunter 1957). However, it was not until 1930 that there was confirmation of the association between exposure to asbestos and the occurrence of fibrosis of the lung. In 1930, Merewether and Price, from the Factory Inspectorate, produced a report on the health of workers in the British asbestos textile industry where there was no exposure to crystalline silica. They found that a significant number of those examined had fibrosis and that the disease was more prevalent in those with long exposure to high dust concentrations.

Lung Cancer In 1954 Richard Doll of the Medical Research Council carried out a study at the invitation of Turner and Newall Ltd (at the TBA factory in Rochdale) with the help of Dr JF Knox, TBA's Chief Medical Officer. That study collected and analysed the data from 105 necropsies and Doll concluded that "*all the cases of lung cancer were confirmed histologically and all were associated with the presence of asbestosis*". Following that study, there was considerable debate as to whether it was necessary for a worker to have asbestosis before there was a risk of lung cancer. That uncertainty continued into the 1960s.

The studies that demonstrated that exposure to asbestos produces a risk of lung cancer in the absence of asbestosis were published later, typically in the late 1970s and early 1980. For example, the review by Doll and Peto lists several studies from those decades that show lung cancer related to asbestos exposure.

Recently, Hodgson and Darnton (2000) undertook a meta analysis of published epidemiological studies to estimate the cancer risks arising from exposure to asbestos. For exposure between 10 and 100 fibre/ml.years, they estimated the increase in risk of lung cancer at about 4.8% for each fibre/ml.year of exposure to amphibole asbestos (crocidolite or amosite). So 25 fibre/ml.years of exposure to amphibole would increase the risk by 120% i.e. approximately double the risk. That estimate of the asbestos exposure that would double the risk of lung cancer was consistent with a prevoious estimate known as the "Helsinki criterion" (Tossavainen 1997).
For exposure to mixed asbestos, i.e. exposure largely to chrysotile, Hodgson and Darnton (2000) estimated that the risk would increase by about 0.5% for each fibre/ml.year of exposure. So according to that estimation, 25 fibre/ml years of

exposure to chrysotile asbestos would increase the risk by about 12.5%. However, their best estimate of the relationship between exposure and risk for mixed (chrysotile mainly) exposure was non-linear and gives risk of about 2% for 25 years of exposure to chrysotile. There were uncertainties in the basis for the chrysotile model, and therefore in addition to a best-fit model they also reported a "cautious, steepest model" and that gives about 8% increased risk of lung cancer for 25 fibre/ml.years of exposure to chrysotile.

Mesothelioma In 1960 Wagner, Sleggs and Marchand published a paper in the British Journal of Industrial Medicine. They reported thirty-three cases of diffuse pleural mesothelioma in an area of South Africa and that all but one had a probable exposure to crocidolite, i.e. blue asbestos.

In 1965 Newhouse and Thomson published the first important scientific paper dealing with mesothelioma in the UK. They reviewed a series of 83 patients (41 men and 42 women) from the London area who had been diagnosed with mesothelioma (7). Fifty six of the cases had tumours of the pleura and 27 of the peritoneum. Investigations conducted into their exposure history showed that about half had either an occupational or domestic exposure to asbestos.

During the 1980s and 1990's, several epidemiological studies examined the relationships between exposure and incidence of mesothelioma for workers in asbestos mining or manufacturing. These consistently indicated that the risk of mesothelioma, relative to measured exposure, was higher for crocidolite asbestos than for amosite asbestos, and amosite posed greater risk than chrysotile asbestos. A meta-analysis (i.e. an analysis combining the evidence from many separate epidemiological studies) by Hodgson and Darnton (2000) estimated the relative risk as being 500 for crocidolite, 100 for amosite, and 1 for chrysotile. Whether the differences are really as large as that is still debated, but the risks from chrysotile are significantly less than for similar exposure to amphiboles asbestos.

The epidemiological studies have produced models of the relationship between exposure and risk of mesothelioma that include a linear dependence on the exposure concentration and a stronger dependence on the time from first exposure, e.g. risk proportional to time raised to the power n where *n* is of the order of 3 or 4 (HEI,). The incidence rate *I* of mesothelioma at time *t* is given by the following equation where *c* is the exposure concentration, t_1 is the time from start of exposure, and t_2 is the time from the end of the exposure. The proportionality coefficient K_m expresses the risk of mesothelioma per unit of exposure, where exposure is concentration multiplied by time raised to the exponent *n*. Where there are repeated exposures, the effect of each increment of exposure is additive.

$$I_m(t) = K_m \cdot c \cdot \left[(t - t_1)^n - (t - t_2)^n\right]$$

This equation has been used with an assumption of a ten year lag between exposure and any incidence, and with the 10 year lag incorporated then the exponent *n* becomes *n*=3.

$$I_m(t) = K_m \cdot c \cdot \left[(t - t_1 - 10)^3 - (t - t_2 - 10)^3\right]$$

If a relationship of this form applies to high aspect ratio nano particles, it implies that the consequences for people becoming exposed may not be evident until more than a

decade after the exposure, and that the highest levels of risk may not become apparent until 20 to 30 years after the exposure.
The increasing risk is eventually curtailed by the competing effects of natural mortality from other causes. There has been some evidence (Macdonald et al, 2007) that the risks may not be as high as predicted from the model towards the end of the human lifespan e.g. at ages above about 70.

In general, the epidemiological information points to relationships between an index of exposure (in terms of numbers of fibres as measured by the phase contrast optical microscope method) and the risk of disease. Hodgson and Darnton (2000) from their meta-analysis concluded that the risks of mesothelioma from chrysotile asbestos, amosite asbestos, and crocidolite asbestos, differed greatly relative to this index of exposure. Amosite was 100 times more potent than chrysotile, and crocidolite 500 times more potent than chrysotile. The amosite and crocidolite are both much more biopersistent in the human lung than chrysotile and that may be part of the explanation for the hundred fold difference. Crocidolite has finer diameter fibres (i.e. fibres with diameter less than 200 nano metres) and that may be part of the explanation for the difference between crocidolite and amosite.

Timbrell (1989) reviewed the evidence on occurrence of mesothelioma in areas where amphibole asbestos was mined. His findings suggested that the areas where mesothelioma occurred were characterised by having a pattern of airborne fibres with diameters less than 200 nanometres. By contrast, where mesothelioma was apparently absent, the fibre distributions apparently were characterised by fibre diameters generally greater than 200 nanometres.

Animal studies to investigate risks of mesothelioma have generally employed injection of fibres into the pleural or peritoneal cavities. Injection studies with glass fibres of different size distribution have demonstrated that fibres longer than about 8 µm produce much greater risk of mesothelioma than shorter fibres, e.g. the much quoted studies of Stanton and Wrench. However, the main caveat on these studies is that they rely on a non-physiological delivery (i.e. injection) of fibres into an internal body cavity. The diameter of fibres may be critical in determining whether inhaled fibres would reach the pleura or peritoneum.
Where man made mineral fibres have diameters greater than 200 nano metres, there does not appear to be a risk of mesothelioma by inhalation.

Pleural plaques are a benign condition associated with exposure to asbestos. They comprise fibres of collagen lying parallel to the surface of the pleura. Calcification may occur in the centre of the plaques. According to HSE guidance "*Pleural plaques are discrete fibrous or partially calcified thickened areas, which arise from the surface of the parietal pleura and can be detected in chest X-ray or Computer Tomogram (CT) examination. Pleural plaques do not become malignant and do not normally cause impaired lung function."* However, the detection of pleural plaques in an individual is a sign of past asbestos exposure.

Other Cancers There have been studies to assess whether ingestion of asbestos fibres leads to cancers of the gastrointestinal system. Most studies have not demonstrated a significant effect. However, one study (Andersen *et al*, 1993) in Norwegian light housekeepers who drank water collected from asbestos cement tile roofs which contained a high level of asbestos fibres in suspension (1,760 to 71,350 million fibres/litre of water) showed a significant excess (e.g. 2.4 fold) of stomach

cancers. However, Gamble (2007) in reviewing the evidence on occupational exposure to asbestos and lung cancer commented on this study that "*The cause of the stomach excess is not certain because of possible confounders such as diet (many of the lighthouse keepers were retired seamen). Exposure is not well defined, as it is not known when the* (asbestos cement) *tiles began to deteriorate and the size of the cohort is small*." These comments indicate the difficulty of establishing causation of an excess of gastrointestinal cancer. Nevertheless, Anderson and associates (Kjærheim 2005) in a further report on the cohort concluded that "*The results support the hypothesis of an association between ingested asbestos and gastrointestinal cancer risk in general and stomach cancer risk specifically*". Gamble (2007) concluded from his extensive review of the published studies that "*The epidemiological evidence detracts from the hypothesis that occupational asbestos exposure increases the risk of stomach, colorectal, colon, and rectal cancer.*" Therefore, the evidence from asbestos epidemiology suggests that suggests that gastrointestinal cancers from inhaled or ingested high aspect ratio nano particles may be unlikely but, if they did occur would be difficult to confirm (or disprove) as being exposure-related.

The main conclusions drawn from this brief review of the evidence of health effects of fibres are:

- The key characteristics of fibres that cause high risk of mesothelioma appear to be small diameter (e.g. less than 200 nanometres), length and durability;
- The length that creates the greatest risk of mesothelioma may depend on the cell penetration properties of the fibre or high aspect ratio particle;
- Mesothelioma or lung cancer are likely to occur many years after exposure, for asbestos normally after more than ten years and typically 20 to 3 years later, and the same would be expected if other high aspect ratio particles cause these diseases;
- The history of diseases caused by occupational exposure to asbestos illustrates the difficulties in recognising and proving the causation of the diseases, and demonstrates the importance of recognising the hazards without waiting for epidemiological evidence;
- The exposure response relationships estimated for mesothelioma and asbestos-related lung cancer show no threshold below which there is absolutely no risk. Predicted risk diminishes as exposure becomes lower, but it has not been possible to establish if there is a threshold below which the risk is zero.
- Measuring concentrations in air of high aspect ratio nano particles with techniques that have adequate resolution capabilities (to detect the thinnest of fibres) will be important.

The similarities in shape and durability between HARN and asbestos suggest that exposure to HARN may cause similar adverse health effects. HARN deposited in the lung, because of its length, may be able to translocate to the pleura and cause mesothelioma like asbestos. The lag time after exposure and the absence of the threshold level, as the hallmark of mesothelioma, would be also expected with HARN.

4.2 THE CELLULAR AND MOLECULAR MECHANISMS OF ASBESTOS TOXICITY

4.2.1 Synthetic Vitrous Fibre (SVF) and an emerging fibre pathogenicity paradigm

By the last few decades of the 20th century asbestos had become the most epidemiologically studied of all industrial materials (McDonald 2000) and was also the focus of a huge amount of toxicological research. This in turn led to an emerging understanding that not all asbestos types, nor size categories within any type were equally harmful. Whilst the epidemiological studies were often complex and difficult to interpret in view of the complexities of exposure (different fibre types, different sizes, combined exposures etc) the general conclusion could be drawn that amphibole asbestos was more carcinogenic than chrysotile and that in cohorts exposed to chrysotile alone mesothelioma was very rare (McDonald 2000). Where carcinogenic effects had been seen in chrysotile-exposed population this was considered to result from a amphibole fibres, most often tremolite, that were present along with the chrysotile (McDonald & McDonald, 1997). A full toxicological understanding, however, was not fully gained until there was a focus on SVF, occasioned by the banning or virtual banning, by tight workplace regulation, of asbestos. This led directly to growth in replacement 'man-made vitreous fibres' eventually named SVF, many of which had superior resistive properties compared to asbestos. Importantly for fibre toxicology, this research eventually placed all fibres, including asbestos and the SVF, within one single paradigm, exemplified by the classification in Figure 2.

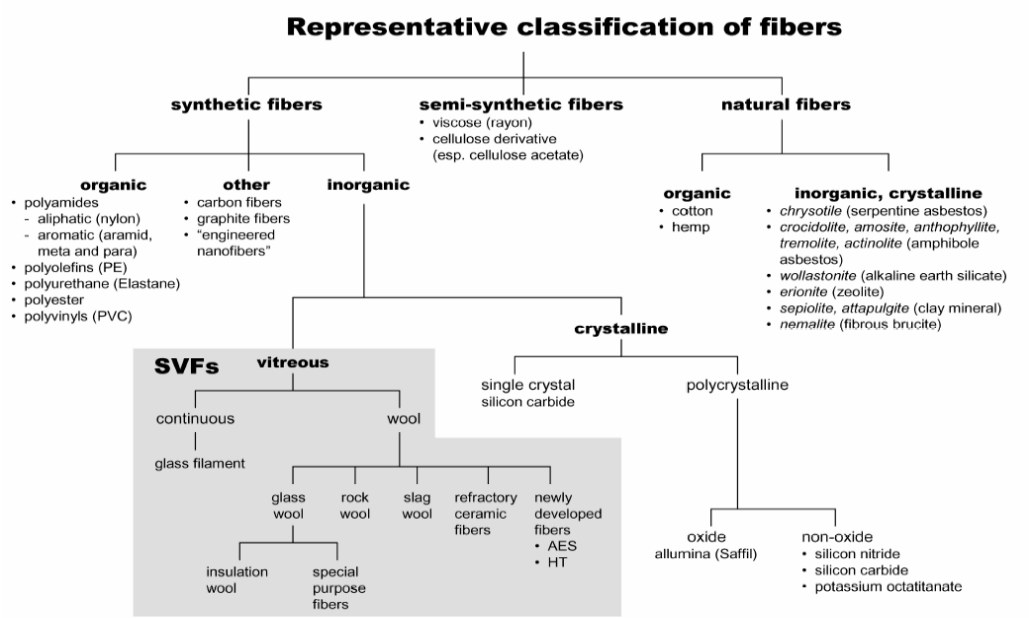
Table 1 Asbestos/ fibre-related diseases and their incidence in various populations and in experimental rodent studies

Disease	*Description*	*Seen in asbestos-exposed humans?**	*Seen in asbestos-exposed rats?***	*Seen in SVF-exposed humans?****	*Seen in SVF-exposed rats?***
Asbestosis/ interstitial fibrosis	Interstitial fibrosis of the lung parenchyma, also called honeycomb lung when severe	yes	yes	no	yes
Bronchogenic carcinoma	Malignant tumour of the epithelial cells of the airways	yes	yes	no	yes
Mesothelioma	Malignant tumour of the mesothelial lining of the pleural and peritoneal cavities	yes	yes	no	yes
Pleural plaques	Benign flattened fibrous lesions on the chest wall	yes	no	Yes ****	no

* Very high exposures were encountered in the past in the older studies
** Very high lifetime exposures often used in rat studies

*** Exposure in these newer, cleaner industries (compared to asbestos) were generally below the levels at which asbestos would have been expected to cause disease (Boffetta et al, 2000)
**** low levels of pleural plaques seen in the ceramic industry (Boffetta et al, 2000)

This development of this over-arching 'fibre toxicology paradigm' is fundamentally important since it identifies the possession of a high aspect ratio (length to width ratio) as the paramount characteristic and not the chemical make-up nor any other classification. e.g. asbestos. There are two caveats to the primacy of shape over composition for fibres, however, in that chemistry is seen as important in determining fibre bioperstistence and may be important for the extra carcinogenicity of a small number of highly pathogenic fibres (see below). The paradigm is centrally important in the present review since carbon nanotubes fulfilled the criterion of high aspect ratio and so they can be legitimately considered as to whether they exemplify the paradigm or not.



4.2.2 The current fibre pathogenicity paradigm

There are boundaries as to what we classify as fibres that are to be considered an inhalation hazard. The WHO define a fibre as a particle longer than 5□m, less than 3 μm in width and having an aspect ratio of >3:1 (WHO/EURO Technical Committee for Monitoring and Evaluating MMMF 1985, WHO 1997). This is not a health-based criterion but one based on a practical definition of a respirable fibre. The fibre toxicology paradigm can be seen as an extension of this definition, in that length, thinness and aspect ratio are all important. However the research into the characteristic of fibres that imbues them with pathogenicity has refined the definitions of length and included bioperstistence.

Width Width or diameter as it pertains to fibres is important because of the central role that fibre diameter plays in controlling aerodynamic diameter (D_{ae}) and the dependence of pulmonary deposition on D_{ae}. The proportion of fibres that deposits beyond the ciliated airways is the fraction that is the slowest clearing. This is because clearance from beyond the ciliated airways is dominated by macrophage-mediated clearance (Gehr, Brand & Heyder 2000), requiring macrophages to load up then migrate to the foot of the muco-cilary escalator (MCE) prior to MCE clearance. Therefore the fibres

that deposit beyond the ciliated airways have the potential to contribute most to the build-up of dose as they are most slowly cleared by macrophages. Length impacts surprisingly little on D_{ae} for thin fibres as shown in Table 2. In Table 2 the D_{ae} of all the fibres is 2 and the density also remains constant; although the length increases from 1 to 30 m the actual diameter has only to decrease by one third to maintain the D_{ae}.

Table 2 Impact of length on the D_{ae} of fibres

Aerodynamic diameter	Actual diameter	Actual length	Density
2	0.9	3	2
2	0.7	10	2
2	0.6	30	2

Length The evidence showing that length is a key factor in pathogenicity of fibres comes from a number of sources but the best data is from experimental studies. Human epidemiological studies of exposure to fibres always represent exposure to a very mixed population of long and short fibres and often to different fibre types. In animal and cell studies it is possible to isolate length categories and assess their effects. This has been done in studies using a number of approaches. Davis et al (1986) used a long amosite sample and a short amosite sample obtained from it by ball-milling and exposed rats to the long and the short amosite at the same airborne mass concentration. After lifetime exposure there was substantial tumour and fibrosis response in those rats exposed to the long amosite and very little effect at all in rats exposed to the short amosite. Using the peritoneal cavity as a means to exposing the mesothelium to a controlled dose of fibres in mesothelioma studies, length was found to be pivotal. A given mass dose of the short amosite caused only one mesothelioma in 36 rats whilst the long sample produced mesothelioma in 95% of rats. Adamson et al (1987) used long and short crocidolite and following deposition in mouse lungs found fibrosis and proliferative responses (Adamson et al. 1993), including proliferation at the pleura with the long, but not the short. The peritoneal cavity has been used as a model of direct mesothelial exposure and much greater toxic (Goodglick & Kane 1990), inflammatory (Donaldson et al. 1989) and granuloma-generating (Moalli et al. 1987) responses were evident in mice that were exposed to the long than the shorter fibres.

In vitro systems have also been used to compare the potency of long and short fibres. Macrophages in culture react more extravagantly in terms of TNFα (Dogra & Donaldson 1995) and oxidative burst (Hill et al. 1995) on exposure to long fibres of amosite than to short fibres. In support of the general importance of the fibre paradigm, long glass fibres were also more effective in stimulating TNFα release *in vitro* than short fibres of the same type (Ye et al. 1999); the long fibres also activated the inflammatory transcription factor NF-κB to a greater extent than was seen with the short fibres (Ye et al. 1999). Length also impacts on genotoxic endpoints and Code 100/475 glass fibres, which contained a proportion of long fibres (>20μm) were found to cause dose-dependent transformation of SHE cells. However, when these were milled to shortness the dose response was abolished (Hesterberg et al. 1983). The long and short amosite fibres were also used in a study that showed greater chromosomal abnormalities and aneuploidy in epithelial cells treated with longer fibres (Donaldson & Golyasnya 1995). Long fibres were also observed to interfere with mitosis in cultures of dividing cells by preventing cell separation result in diploidy (Jensen & Watson 1999).

Biopersistence In addition to long fibres being the hazardous fibres or effective dose for stimulating pro-pathogenic effects in a range of cells there is also evidence that long fibres are more slowly cleared. The lungs are well evolved to get rid of deposited particles via 'clearance'. Clearance is a consequence of two factors:

1) the potential of the fibres/particles to dissolve away, in whole or part – this can result in breakage of long fibres into shorter fibres or complete solution
2) mechanical clearance in the macrophages or on the mucociliary escalator

These two combine to clear particles/fibres from the lungs. The time that it takes for half of the fibres to be cleared is called the clearance or retention half time and can be looked at from the point of view of different fibre sizes. The retention half-time (T1/2) of a compact inert respirable tracer particle in the respiratory tract of a rat is ~60 days (Muhle, Bellmann & Creutzenberg 1994). In the case of fibres, size-related clearance is assessed by counting the fibres in the lungs in different size compartments and then assessing numbers of fibres in the different size classes over time post exposure. T he long fibres (>20 μm) are normally slowly cleared in the case of an insoluble fibre whilst the short fibres are usually cleared as though they were particles, because they can be enclosed by macrophages, like particles. This is evident in Figure 3, where, one year after instillation into the lungs the longer size categories are inefficiently cleared whilst the short ones are cleared. Thus long fibres are more likely to accumulate in the lungs due to failure to clear, providing a vicious circle.

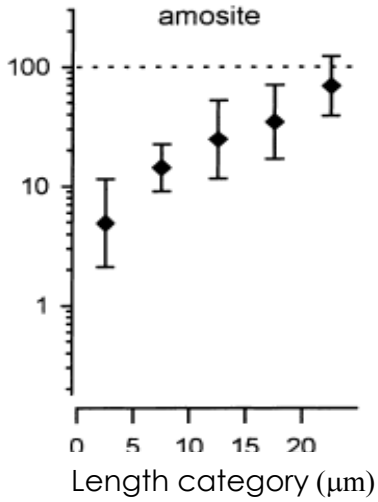


Figure 3 Length dependent clearance of biopersistent amosite asbestos fibres from the lungs of rats (Searl et al. 1999). Figure shows percentage of the fibres of any length category present in the lung 1 year after instillation. Note that short fibres are cleared rapidly but the longer sizes clear more slowly.

However there is a factor that can render a long >20□m fibre low in hazard. This is the potential of that fibre to undergo weakening, and breakage in the lungs. If such a fibre is leached in the presence of lung fluids to lose structural elements they may become weaker and break and will then join the short pool and be cleared from the lungs. Such a fibre has been called non-biopersistent compared to a biopersistent fibre like amphibole asbestos. This is shown in Figure 4 for MMVF10, a non-biopersistent fibre. It has been studied in the same protocol as the amosite in Figure 3 and it is illuminating to compare Figure 3 and Figure 4. As non-biopersistent fibres dissolve and break, they may clear rapidly whilst, at the same time the production of short fibres by breakage can add to the short fibre pool giving the illusion that it is not being cleared (Figure 4).

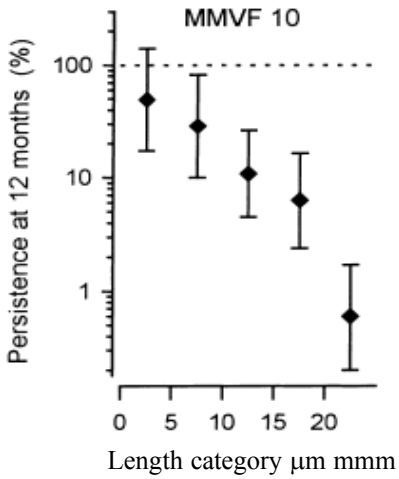


Figure 4 Clearance of non-biopersistent glass fibres from the lungs of rats (Searl et al 1999). Figure shows percentage of the fibres of any length category present in the lung 1 year after instillation. Note that long fibre dissolution and breakage causes it to decrease but that the expected production of short fibres, as the long fibre break up, means that the short size classes do not clear rapidly.

The contribution of large-scale animal studies During the nineties two research programmes contributed greatly to an understanding of what constitutes a pathogenic fibre. The RCC studies were run in RCC Consulting in Switzerland, on behalf of North American SVF manufacturers. On a smaller scale the Colt Fibre Programme in the UK carried out program of pathology and allied studies. Between them these studies examined more than 10 types of fibre including asbestos and various SVF. The strong conclusion from both of the studies was that long (>20 μm), biopersistent fibres were the effective dose for lung cancer, fibrosis and mesothelioma (Hesterberg et al. 1994, Hesterberg et al. 1998, Miller et al. 1999a, Miller et al. 1999b).

Biopersistence as studied by durability *in vitro* Biopersistence potential of a fibre can be studied *in vitro* using models of lung fluid and observing loss of structural molecules or mass, expressed as K_{diss} the dissolution constant (Eastes, Potter & Hadley 2000). This is measurement of the ability of fibres to remain intact in simulated lung fluid (Gambles fluid) is termed fibre durability. It is distinct from biopersistence, which occurs *in vivo* and is the sum of leaching, breakage and macrophage then mucociliary escalator clearance. There is a relationship between K_{diss} and bioperstistence in rat lungs (Figure 5 left) and there is also relationship between K_{diss} and pathogenicity (Figure 5 right).

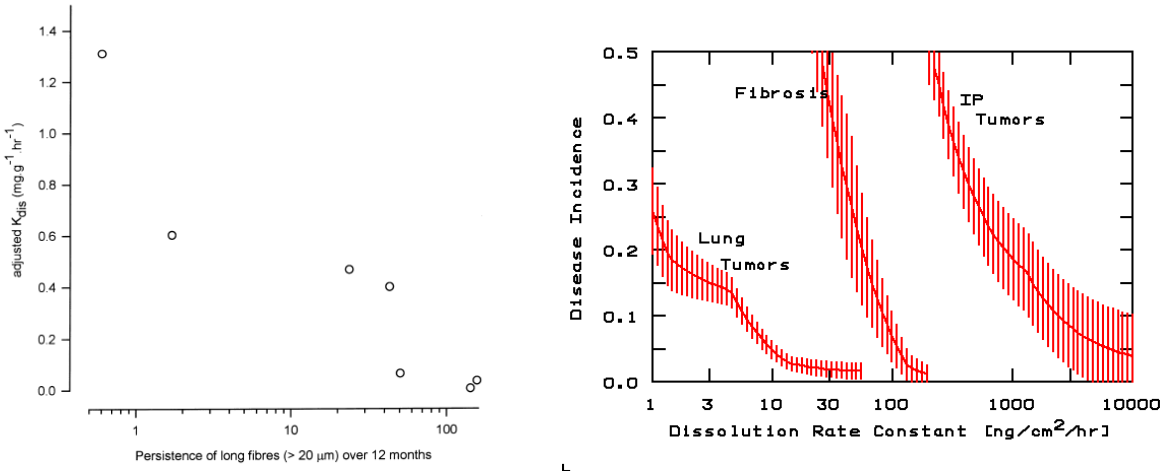


Figure 5 Left Relationship between K_{diss} and bioperstistence in rat lungs for a range of SVF in the Colt Fibre Study (Searl et al. 1999); Right Relationship between K_{diss} and pathology in rats after various lengths of exposure to various fibre types in the RCC studies (Eastes & Hadley 1996). Note that higher K_{diss}, indicating less biopersistence, is associated with less risk for lung tumours, fibrosis and mesothelioma.

Fibre surface reactivity One important question is whether all long biopersistent fibres have equal pathogenicity. There is evidence that some fibres have specially enhanced pathogenicity and this may be a consequence of their surface reactivity e.g. erionite fibres (Wagner et al. 1985) and Silicon carbide fibres (Miller et al. 1999a). The nature of the specially reactive surface for these fibres is unknown but asbestos has been extensively studied and found to deliver oxidative stress to cells via iron-mediated redox chemistry (Gilmour et al. 1995) and oxidative stress is a central hypothetical mechanism in the pro-inflammatory (Donaldson & Tran 2002), fibrotic and carcinogenic (Kane 1996) effects of fibres. An overall model for asbestos and fibre pathogenicity is shown in Figure 6.

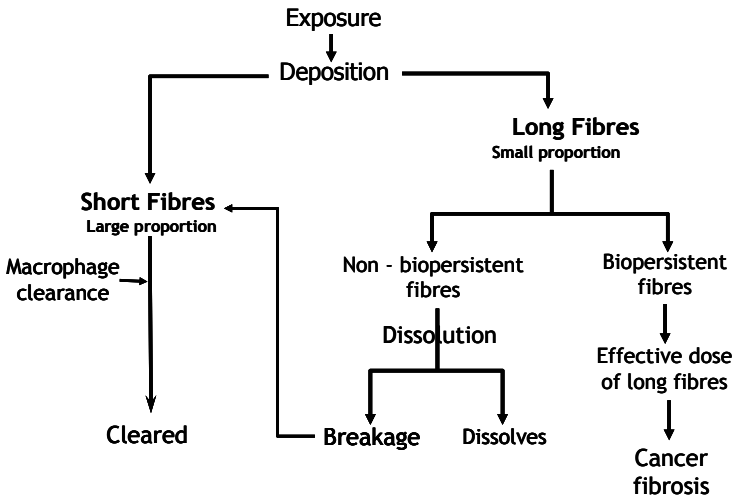


Figure 6 A model for the role of length and biopersistence in the fibre paradigm.

4.3 HARN PHYSICO-CHEMICAL CHARACTERISATION

4.3.1 Classification of HARN

HARN can be classed as part of the 'one dimensional' nanoscale building blocks; other one-dimensional particles include nanobelts and nanoribbons; these are nanostructures with high surface area and high aspect ration (of greater than 10 to 1, length-to-diameter). In this report, we refer to HARN as being: carbon nanotubes, nanowires and nanorods. These three types of nanostructures are of particular interest as they have become the most relevant in the latest focus of intensive research.

Carbon nanotubes *(CNT)* These are considered to be one of the most important species of HARN, as evident by strong commercial applications. They are cylindrical tubes of graphitic carbon 6-member rings, whose structure can be obtained by rolling single layers of graphene sheets into tubes. Depending on how graphene sheets are cut before being rolled up, CNT can be divided into three symmetry groups: armchair, zig-zag and helical nanotubes. More commonly however, nanotubes are divided into two general types: single-walled and multi-walled (this including double-walled CNT) i.e. SWCNT and MWCNT respectively. SWCNT consist of graphene sheets rolled up into singly cylindrical tubes whereas MWCNT is a stack of single-walled carbon nanotubes nested inside one another to make concentric cylinders. Within a given type of nanotube, different radii in the nanometre range can be found, with SWCNT having a typical diameter of ~ 1 nm (the smallest reported to date being 0.4 nm) (Gao et al. 1998) and MWCNT having diameters in the range of 2 – 100 nm with a layer spacing of 0.3 – 0.4 nm. CNT can be many microns in length, with some being 'super long' i.e. up to several tens of microns (Thostenson et al. 2001). Many of the unique properties offered by nanotubes are mainly determined by their diameter and chiral angle; the tube's diameter will determine the tube's band gap (hence, its electronic and optical properties) whereas chirality (a measure of how the graphene sheet rolls up) will determine whether the tubes are metallic or semiconducting (Hu et al. 2006). Figure 7 shows an image of multi-walled carbon nanotubes, taken using Scanning Electron Microscope (SEM) (image obtained from http://groups.google.com/group/Carbon-nanotube-club). Figure 8 shows an image of single walled carbon nanotubes scattered on a substrate, taken using SEM and Atomic Force Microscope (image obtained from http://www-drecam.cea.fr/en/Phocea/Vie_des_labos/Ast/ast_sstheme.php?id_ast=500).

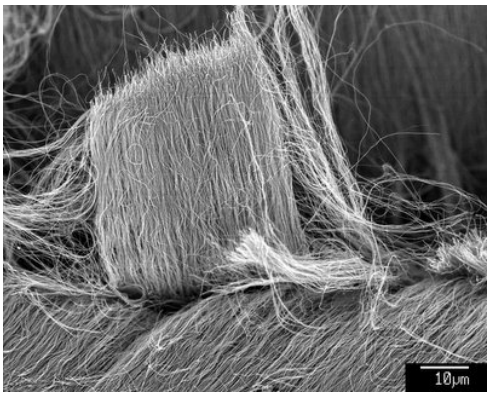


Figure 7 Multi-Walled Carbon Nanotubes produced by Chemical Vapour Deposition (CVD) Method.

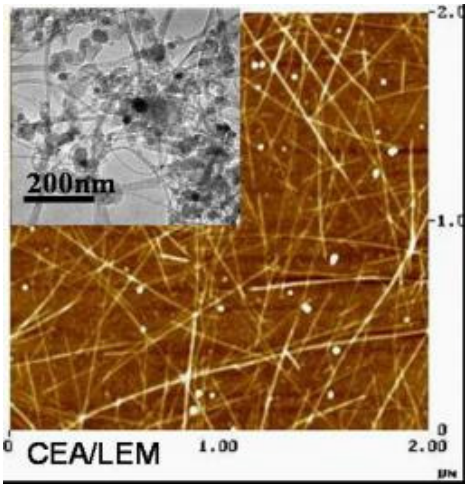


Figure 8 AFM picture of purified SWCNT scattered on a substrate. Upper left, SEM picture of the SWCNT raw material showing catalyst metal particles.

Nanowires Nanowires can be prepared form a variety of materials. A nanowire can be grown from metal (Ag, Au), elemental semiconductors (e.g., Si, and Ge), III-V semiconductors (e.g., GaAs, GaN, GaP, InAs, and InP), II-VI semiconductors (e.g., CdS, CdSe, ZnS, and ZnSe) or oxides (e.g., SiO_2 and ZnO). As with carbon nanotubes, they can be synthesised with various diameters and length, depending on the synthesis technique employed and or/desired application needs. In addition, they also possess unique electrical, electronic and optical properties, different from that of their parent counterpart. Although no real commercial product yet exists, we expect that there will be an increase in future demands in nanowires, as their potential use in different applications is vast. Of particular importance is in the development of better photovoltaic cells, which will lead to better/cheaper solar cells than we currently have (Rao et al. 2003). Figure 9 shows an image of ZnO nanowires grown on a substrate, taken using SEM (image obtained from http://nanotechnologytoday.blogspot.com/2007/04/why-nanowires-make-great-photodetectors.html).

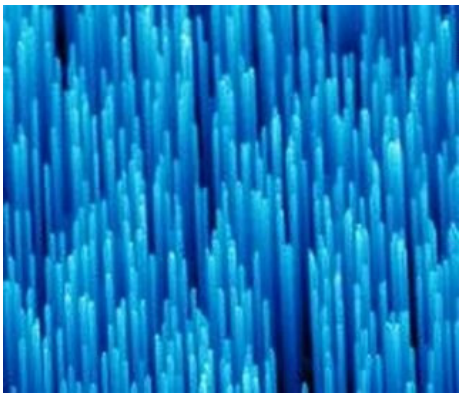


Figure 9 Zinc oxide (ZnO) nanowires grown in the Deli Wang lab at UCSD.

Nanorods Nanorods can be synthesised by direct chemical synthesis and the appropriate choice of ligands (that act as 'shape control agents') will ultimately allow

different faces of the nanorod to grow at different rates, producing a 'rod shaped nanostructure'. Again, like nanowires, no real commercial products yet exist however there are many potential application. The latest discovery is in the use of titanium oxide to fill nanocavities, which have been shown to be more efficient at absorbing UVA and UVB solar radiation than titanium oxide without the nanocavities and subsequently be useful in sunscreen ingredients (Murphy et al. 2008; Liu et al. 2006). Figure 10 shows an image of ZnO nanorods arrays of different shapes, taken using SEM (image obtained from **http://ren.che.ufl.edu/ZnO.html**).

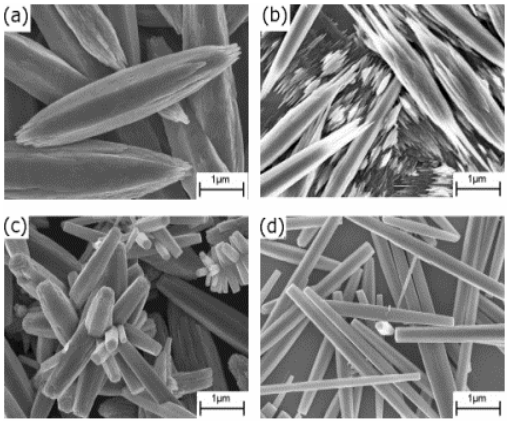


Figure 10 Field emission SEM images of ZnO nanorod arrays. (a) rice (b) needle (c) spherulitic prism, (d) rod shape. (ratio of Zn(NO3)2·6H2O to C6H12N4, from (a) to (d), 1:1, 1:0.8, 1:0.7, 1:0.6).

4.3.2 Challenges to the Characterisation of HARN

In general, challenges that exist with the characterisation of nanoparticles also exist with HARN, which will ultimately have a big impact on the success of the analysis and the end results obtained. It is reasonable to assume that such challenges can be magnified when it comes to HARN because of the higher aspect ratio property compared to other nanoparticles. The high aspect ratio property implies a much increase surface area, which will mean increase in surface interaction and activity, compared to other nanoparticles. These challenges will be discussed in details below:

a) **Sample preparation.** This is one of the most critical steps towards successful characterisation of nanoparticles, in which there are many variables to consider when designing a method for preparation. The first step to consider in sample preparation is the need to have "reliable" sampling, such that "sample collected from bulk represents the physical and chemical characteristics of the entire sample" (NIST 960-1. 2001). Most nanoparticles are received in the powder form or in liquid form i.e. the form of a stable suspension. Powder sampling is more difficult, as there is a natural tendency for nanoparticles to aggregate and unlike in solution phase, it is more difficult to control surface charges on particles; some general guidelines on powder sampling can be found in Allen 2004a/b. The next steps in the sample preparation will be governed by the requirements of individual methods, which may require specialised conditions for measurement; each will represent their own unique challenges. It is not the intention of this report to go into some of the specifics, nonetheless, all of them share a few common challenges, namely the problems regarding the issues of purity and aggregation. Ideally, samples for analysis should be free from: the inherent aggregation problems associated with nanoparticles and other contaminants not associated with the said

nanoparticles. However, to achieve such goals are not trivial. For example, to successfully disperse nanoparticles in a liquid media, researchers often employ the use of sonication methods. However, in the carbon nanotubes, it was observed that this sonication step has the potential to change size distribution of the nanotubes and introduce defects (Islam et al. 2001). Furthermore, questions have been raised concerning the dispersion stability over time (Vaisman et al. 2006). Another thing to establish is the 'state' of the sample required for analysis i.e. whether the nanoparticles should be: fixed on to a solid substrate, suspended in liquid media or aerosolised (solid or liquid aerosols). Lastly, some surface techniques [such as electron microscopy, BET (Brunauer, Emmert and Teller) and XPS (X-ray Photonelectron Spectroscopy)] are carried out under various high vacuum conditions, which implies that such techniques may not be easily applied to liquid samples. If the starting solution in which the nanoparticles are dispersed in contain salts, then such dissolved species may dry into fine crystals, which will eventually interfere with the results.

b) **Identifying Suitable Characterisation tools**. Characterisation of nanomaterials for toxicological study can be complex, as there are many different material attributes that needs to be considered. Ideally, we should only be concerned with those properties known or expected to influence toxicity. According to Powers and co-workers (2006) the relationship between material attributes and the effects of toxicological activity is not clear and so, it may be required to characterise as completely and as practically possible. The need to characterise nanoparticles for toxicological evaluation is made even more complicated by the need to analyse as close as possible to the "as-dosed" form i.e. to what is required under the toxicological investigation. This is not easy as: a) quantities used in the analysis are normally smaller b) state of particles will have likely to change under the conditions of the analysis. Overall, it is the experimental conditions used in toxicological studies, which will eventually determine the choice of techniques used for characterisation. For example, an inhalation type study may require the need to characterise the nanoparticles in dry powder aerosol form. This report cannot does justice to the vast number of techniques that have been proposed in the literature for HARN characterisation. The main challenge here therefore is to identify those techniques deemed to be most 'suitable' for characterisation (in relation to nanotoxicological activities). Our findings will therefore be focused on those techniques that meet certain criteria, namely: well-established/commercially available analytical techniques and those techniques that yield chemical/physical property information that have been linked/hypothesised in some way to toxicological activities. The techniques presented in this report fall into two categories: imaging (high resolution, with the capability of probing individual HARN) and non-imaging techniques (often involves the measurement of a collection/ensemble of HARN).

4.3.3 Characterisation techniques: Imaging at individual HARN level

This group of techniques is extremely advantageous, in that they have potential to yield highly resolved images, which allows direct visualisation of nanoparticles and thus will yield information on size and shape. It is a general consensus amongst nanotoxicologists that properties related to the three-dimensional structure of nanoparticles have a profound influence on their toxicity and the small size has the potential to enter cells and ultimately cause cell death (Porter et al. 2007), as shown in

Figure 11. This figure shows a TEM image of single walled carbon nanotubes that has migrated into a human cell, ultimately causing cell death.
The image in Figure 11 was obtained from http://www.physorg.com/news114348754.html. Hence, the ability to provide images on the nanoscale, with sensitivity that reaches the individual nanoparticle level, is of utmost importance. All of the techniques in this section will be based either on the use of electron or scanning probe microscopes. In the past, such techniques have been mainly used for investigating structure and morphology of nanoparticles. The amount of detail present in the image will ultimately be dependent on the instrument's spatial resolution; a high-resolution microscope for example will results in images that can reveal very small defects or anomalies. Although these tools are extremely useful, they all share the same disadvantages, in that they: do not have a wide field of view, are relatively expensive techniques (to purchase and maintain), require the need of specially trained analysts and have no potential for automation. A pre-requisite requirement for successful imaging is the need to have the nanoparticle well adhered on a solid support and as well as other specifics that will be dependent on the individual technique. Sample preparation can be done either through a simple deposition procedure or may need to have more complicated fixing protocols. Background information on the different imaging technique is detailed as follows:

Electron-Based Microscopies These tools are able to produce highly magnified, resolved images of objects (with a much greater depth of field in comparison to conventional optical microscopes) because electrons (with much shorter wavelength than light waves) are used (Egerton 2005). SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy) are the two most common techniques for nanoparticle characterisation. SEM creates an image by scanning a tightly focused electron beam over the sample and detecting the secondary electrons from the sample on to screen; each point on screen will then correspond to a pixel (picture element). TEM on the other hand forms an image using a system of lenses; unlike SEM, the electron beam passes entirely through the sample and subsequently collected to appear on a screen, generating a 'transmission' electron image (Reimer 1993). There are several advantages and disadvantages associated with both techniques. TEM for example has a far better spatial resolution than SEM but suffers from lengthy, time-consuming sample preparation step; one difficulty is getting the specimen sample thin enough for analysis under TEM. Another disadvantage with TEM is the need for very intense electron beam (with energy in the range ~ 200 – 300 keV) (Kiang et al. 1996) compared to SEM (~20 keV); this will pose some concern as to the (structural and thermal) stability of nanoparticles during analysis when under the influence of high energy electron irradiation.

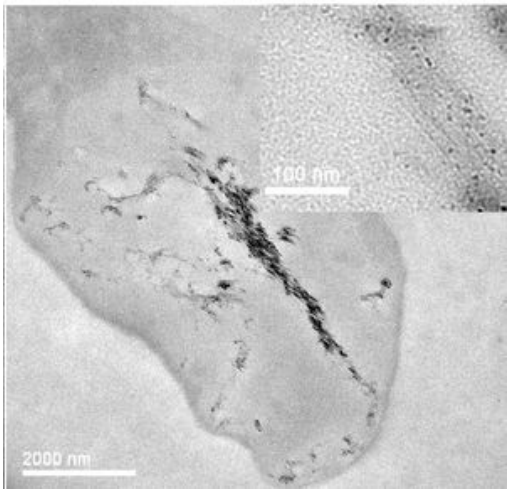


Figure 11 Transmission electron microscope image shows carbon nanotubes (dark areas) within a cell nucleus.

Scanning Probe Microscopies (SPM) These are techniques that acquire an image by raster scanning a sharp, microscopic 'sensor' probe capable of 'sensing' height changes as small as 0.1 Angstrom (10 picometers) (Sakurai 2000). In this report, we will only concentrate on two most widely used high-resolution SPM techniques: AFM (atomic force microscopy) and STM (scanning tunnelling microscopy). In an AFM, it is the cantilever deflection signal versus probe base position that results in the image, whereas in an STM, it is the variation in current as the probe passes over the surface that are translated into an image. Unlike electron microscopes, which have the potential to destroy or modify sample structure in the process due to the use of electron beam, SPM techniques are non-invasive as they are able to create highly resolved three-dimensional images (providing both in-plane as well as height features) without the need for an electron source. Again, sample preparation is critical, in which every single step in the procedure can make a difference. Particularly, it is important for the nanoparticles under analysis to have a greater affinity to the flat substrate surface than the sensor probe tip. If there is weak adhesion between the nanoparticle and substrate, then the image acquired either show a reduced resolution or contains 'artefacts' (e.g. streaking) (Bonnell 2001).

4.3.4 Characterisation of HARN: Non-imaging based techniques

This is a group of techniques, which are "non-imaging" in nature, and most have much lower detection sensitivity than the 'imaging' based techniques described above. Under this group, a myriad of chemical/physical properties of the nanoparticle, related to its nanotoxicological activities, can be extracted:

a) **Size/ "state of aggregation" information**. For nanotoxicologists, the main properties of interest is in relation to size of the nanoparticles under analysis, as the link associated with diminishing particle size and toxicity is well founded (Oberdorster et al. 2005). The "state of aggregation" is a difficult parameter to quantify but is potentially significant for nanotoxicological evaluation. This parameter, used to describe the degree to which particles agglomerated (held together in groups or clusters by attractive inter-particle forces, the most fundamental being Van der Waals forces); particle agglomerate size may play a crucial role in the uptake of such particles inside the body by macrophages (Rudt, S. and R. H. Muller (1992)).

b) ‘Surface related’ information. In addition to size, the concept of surface area is becoming of increasing importance in relation to their toxicological behaviour. In industry, surface area characterisation of nanoparticles are needed as they can be correlated to ‘rate-related’ phenomena such as catalyst activity, electrostatic properties, shelf life and many others, which can influence the processing and behaviour of nanoparticles. The significance of surface area as a parameter can easily extended to its toxicological activities of nanoparticles. Furthermore, the diverse array of surface properties apart from area that will be important from a nanotoxicological point of view (as they may provide mechanistic details in the uptake, persistence and biological activity of HARN inside living cells) include: surface charge (zeta potential measurements) and surface chemistry (Gill et al. 2007). Techniques in this section will be divided into two groups i.e. those techniques that describe chemical properties and those that characterise the physical properties.

Non-Imaging Techniques : Chemical Property Information

These techniques are mainly spectroscopic in nature. Spectroscopic techniques measure the interaction between a probe and matter, yielding a ‘spectrum’ i.e. a response plot as a function of wavelength. In the past, such techniques have been very useful in identifying the ‘chemical class’ of various components of an analyte under study.

Vibrational Spectroscopy This is a tool used to probe the ‘vibrational states’ of a molecule and hence, for the determination of molecular structure. This can be achieved in several ways. In infrared (IR) spectroscopy, the molecule will be exposed to a frequency range of infrared light and ultimately the measure of wavelength-intensity of IR absorption by the molecule; for a vibrational motion to be IR active, the dipole moment of the molecule must change. Raman on the other hand is a measurement of wavelength-intensity of inelastically scattered light from molecules; for a molecule to be Raman active, there must be a change in the polarisability of that molecule. A Raman spectrum of a molecule gives complementary information to its corresponding IR spectrum and both techniques are powerful tools for non-destructive characterisation of nanoparticles and are well suited for routine analysis i.e. can be operated by technicians, quick analysis time and have well-established frequency standards. As a tool, they are relatively much cheaper to buy/maintain in comparison to the high-resolution microscopy techniques so far described (Laserna 1996; Stuart 2004).

XPS This is a surface analytical technique, requiring ultra-high vacuum conditions, that bombards the sample with mono-energetic soft x-rays (~1-2 keV), causing electrons to be ejected. The number and kinetic energy of the ejected ‘photoelectrons’ (from the top 1 to 10 nm of the material to be analysed) are then simultaneously measured. The tool yields ‘information rich’ spectra, being capable of measuring: elemental composition, empirical formula, chemical state and electronic state of the elements. Some advantages of XPS are: the ability to analyse non-conducting materials with minimum charging effects (unlike techniques such as SEM), excellent inter-element resolution (high information content) and speed of analysis. Some of disadvantages include: poor lateral resolution, a relatively weak signal and possible non-uniqueness of its chemical shift information (Watts 2003).

Non-Imaging Techniques: Physical Property Information

Surface Area Analysers, BET technique BET (Brunauer, Emmett and Teller) technique is a traditional method for the measurement of surface area and other characteristics such as pore size and pore distribution of nanoparticles. The techniques are based on the addition of a known volume of gas (the adsorbate) and the subsequent gas adsorption on to the solid material (at cryogenic temperatures), resulting in a direct relationship between the pressure and the volume of gas in the sample vessel. By measuring the reduced pressure due to adsorption, the ideal gas law can then be used to determine the volume of gas adsorbed by the sample and subsequently, the surface area of the sample, which is reported as the specific surface area (i.e. surface area per unit mass, usually m^2/g) (Lowell et al. 2004).

Zeta-potential measurements This is a method to probe 'surface charge' information of nanoparticles in a liquid suspension. Theoretically, zeta-potential is the electric potential in the interfacial double layer i.e. layer at the location of the 'slipping plane' versus a point in the bulk fluid away from the interface. It has been recognised that the zeta potential is a very good index of the magnitude of the interaction between nanoparticles and the measurements of zeta potential are commonly used to assess the stability of colloidal systems. One way to determine zeta potential measurement is to obtain the electrophoretic mobility of the particle and this can be done through the combination of laser Doppler velocimetry and phase analysis light scattering of the sample, under the influence of an applied electric field (Hunter 1981).

Photon Correlation Spectroscopy The most common method used to probe size distribution of nanoparticles information in the sub-micron range is based on Photon Correlation Spectroscopy (more commonly called dynamic light scattering). This non-destructive method determines particle size by measuring the rate of fluctuations in laser light intensity scattered by particles as they diffuse through a fluid. However, "size information" obtained from PCS will be the average diffusion coefficient of the particles and the size is correlated to the equivalent "sphere diameter". Hence, PCS is more likely to be successful in measuring size if the nanoparticle under investigation is close to being spherical. In the case of HARN, size information will be limited and the information obtained will neither be the length nor the width of the particle). Nonetheless, it is possible to probe the "state of dispersion" i.e. evidence of agglomeration, taking place in the suspension (relative to the nanoparticles if in the dispersed state i.e. as close to the 'primary' particle size distribution as possible). Hence, in relation to HARN, PCS will be more suitable for measuring "state of aggregation"; it has been used by Lee and co-worders for measuring polydispersity and the stability of single walled carbon nanotubes (Lee, J. Y., J. S. Kim, et al. 2005).

Scanning Mobility Particle Sizer
If the 'aerosolised' form is of interest, then a different technique is needed. As an example, the Scanning Mobility Particle Sizer spectrometer is able to give particle size distribution of aerosolised as-produced material, again with sub-micron sensitivity; here the particles will be classified with an electrostatic classifier (that is used to deliver singly charged, monodisperse calibration aerosols of known size and composition) and their concentration measured with a condensation particle counter (that measures the number of particles by growing the particles through a condensing process) or an electrometer, yielding particle size distribution information (Berne & Pecora 2000). Again, the interpretation of the results for non-spherical/ aggregated nanoparticles is not straightforward (Van Gulijk et al. 2004).

For physical property related investigations, our primary interests include: size/ ”state of aggregation”, zeta-potential (charge measurements) and surface area. For HARN, Photon Correlation Spectroscopy will be suitable to probe “state of aggregation” rather than primary size distribution information of nanoparticles dispersed in solution. If the size information is required from an aerosolised form of the sample, then other methods such as a scanning particle mobility sizer may be required. However, no research in relation to HARN characterisation has been done with this technique and again its suitability is questionable as the nanoparticle under investigation moves away from the ideal spherical model. Overall, for size information, imaging techniques seems to be a good alternative. Zeta-potential measurements, a measure of dispersibility and stability, are useful for the characterisation of HARN in liquid suspension. Lastly, the BET method and apparatus is appropriate for determining surface area of HARN.

The molecular composition and structure of the surface of nanoparticles will ultimately define its chemistry. There are various methods to characterise these and can be divided into two groups: surface sensitive and bulk techniques. X-ray photoelectron spectroscopy and secondary ion mass spectroscopy (Lee, H. C., P. S. Alegaonkar, et al. 2007), in particular have been extensively used for characterising nanoparticles. These are very powerful surface techniques and has been used to give information rich spectra detailing the ‘chemical state’ at the surface; XPS in particular, has the advantage of being quite oxidation specific but this is dependant on the particular element which is analysed. Past workers have used XPS to probe information relating to structural modification due to chemical interaction with organic compounds or gases adsorption and sidewall functionalisation of HARN. A recent review by Powers and co-workers indicated that the technique is applicable to correlating biomaterial surface properties to physiological endpoints (Powers, K. V., M. Palazuelos, et al. (2007)).
For bulk techniques, the chemical composition of nanoparticles requires the concurrent application of several spectroscopic techniques such as inductively coupled plasma (ICP), nuclear magnetic resonance (NMR), UV-vis and fluorescence, which are widely used to measure the atomic structure and composition of pure nanoparticles (Powers, K. V., M. Palazuelos, et al. 2007). Techniques such as Raman/Infra-red spectroscopy has been popular choices for the characterisation of HARN to determine impurities, surface functionalisation/chemistry and catalytic properties. Raman spectroscopy, in particular has been widely used for the characterisation of carbon nanotubes, to obtain a variety of information to include: structural, chirality, surface modification/ functionalisation and in the determination of their vibration and electronic energies. Of particular interest has been in the characterisation of single-walled carbon nanotubes as there is a vibrational mode (i.e. the so called ‘breathing mode’) in the spectrum that is unique to the tube diameter of SWCNT (when illuminated and ‘in resonance’ to a particular wavelength of light).

4.4 CURRENT EVIDENCE ON THE POTENTIAL TOXICITY OF SOME HARN

4.4.1 HARN aerosol generation and exposure

Inhalation is a particularly relevant route of exposure when considering HARN hazard if comparing HARN to other pathogenic materials such as asbestos. However, there is much discussion among researchers regarding the ability to generate airborne carbon nanotubes in an industrial or consumer setting. This scepticism is driven by the observation that nanotubes exhibit a high propensity to aggregate. In order to address this question, Maynard et al. (2004) investigated unrefined single walled CNT agitated in a controlled laboratory setting, and found that such agitation resulted in fine particle

release into air. A subsequent study of four production facilities found airborne concentrations in the vicinity of single walled CNT production equipment to be relatively low (less than 53 µg/m^3). While this is a low concentration when compared to the occupational exposure limits for other nuisance particles such as TiO_2, it is relatively high compared to ambient air pollution (associated with morbidity and mortality). Furthermore, in comparison to fibres, exposure limits are set according the number of fibres per cc of air. The relationship between ug/m^3 and number of CNT per cc is currently unknown, but is likely to vary between different CNT types and sources. We were not able to identify any published information regarding aerosol exposure to multiwalled CNT in a laboratory, experimental or industrial setting.
It is important to note that it is difficult to produce inhalable or respirable aerosols of CNT because of the tendency of these materials to form aggregates. However, efforts are being made to develop more efficient methods for aerosolising CNT (NIOSH paper, 2008).
The properties and quality of SWCNT and MWCNT vary greatly between sources, and even between batches from the same source. In addition, composition, length, wall number and catalyst can all be manipulated through the manufacture process. This means that it is still too early to disregard inhalation as a feasible route of exposure since some HARN may be more easily airborne than others.

4.4.2 HARN Respiratory Toxicology

The pulmonary toxicology of SWCNT has been investigated in mice by Shvedova et al. (2005). This study delivered the SWCNT by pharyngeal aspiration at a dose of up to 40 µg/mouse. The SWCNT generated an acute inflammatory response that included increased neutrophils in the lung 1 day after exposure, and a subsequent increase in macrophages at day 7. Granuloma formation, diffuse fibrosis and alveolar wall thickening were all identified by histological analysis of the lung tissue within 28 days of exposure to doses of 20 or 40 µg/mouse. The granuloma formation was mainly associated with hypertrophied epithelial cells that surrounded aggregates of the SWCNT. Biochemical markers of bronchoalveolar lavage fluid also indicated lung damage. Induction of oxidative stress was indicated by increased 4-hydroxynonenal production and glutathione depletion of lung tissue.

In a different study, Muller et al. (2005) treated rats with either MWCNT or ground MWCNT via instillation (0.5, 2 or 5 mg/animal). At a dose of 2mg/rat, both the intact and ground MWCNT induced inflammation and fibrosis. The particles persisted in the lung tissue for 60 days, and by 2 months a collagen rich granuloma had formed. The ground (and presumably shorter) MWCNT were found to disperse more effectively within the lung tissue, but were still associated with inflammation and fibrosis. The study by Muller et al. 2005 also compared the MWCNT with carbon black nanoparticles, and found the MWCNT to be more pathogenic. Other studies comparing SWCNT with nanoparticle carbon black have made similar observations (Warheit et al. 2004; Lam et al. 2004). The reason for the enhanced potency of SWCNT and MWCNT relative to nanoparticle carbon black is not clear. A potential explanation by Warheit et al. (2004) suggests that chemical differences in the graphitic surface of the particles or the physical size of the aggregates produced in the studies could determine toxicity.
In a study by Lam et al. (2004) the SWCNT were compared with quartz. Respirable α-quartz is known to induce fibrosis and cancer in the lung following chronic exposure. Lam et al., 2004 found that at the same mass dose SWCNT were more potent than quartz at inducing pathological markers such as granulomas and fibrotic lesions. This data suggests that the SWCNT investigated in this study have the potential to be highly pathogenic.

However, it is important to note that all of the studies outlined above use different CNT samples that vary in source, metal contamination and particle dimensions. At this time the relationship between physicochemical characteristics and toxicity remains to be determined, especially in relation to fibre length and durability, the factors known to determine asbestos toxicity. In fact, dimension data is missing from most of the studies described above. It is also worth noting that all of the studies described above have used a protocol that delivers a bolus dose of aggregated or agglomerated HARN or nanoparticles.

The lung uses a number of defence mechanisms to clear particles from the lung surface including clearance via macrophages. Macrophages are immune cells that migrate from blood into the lung airspaces to identify and ingest foreign particles by a process known as phagocytosis. Once ingested, the cell will attempt to degrade the foreign particles. For inorganic materials such as asbestos degradation is not possible, and so the macrophage physically carries the particle away from the lung surface by either moving out of the airways with mucus, or by migrating to the lymphatic system. In order for macrophages to carry out this function the cell must be able to completely engulf the particle, an act which is not possible for pathogenic fibres, resulting in frustrated phagocytosis, prolonged inflammation, tissue damage and potentially disease. In a study by Kagan et al. (2006), different SWCNT, varying in their iron content (26 wt. % versus 0.23 wt. % of iron) were compared in a cell free system. There appeared to be a relationship between iron content and free radical generation in that the SWCNT, with a higher iron content, generated more detectable free radicals. Kagan et al., 2006 also treated the RAW 264.7 macrophage cell line with the SWCNT and found that the iron-rich particles induced oxidative stress such as indicated by glutathione depletion and lipid peroxidation. These results suggest that iron content is important in determining redox dependent effects in macrophages. The bioavailability of iron in a variety of commercially available SWCNT was investigated by Guo et al. (2007). The amount of iron that could be mobilised was not associated with the total iron content present in the SWCNT, suggesting that there are differences in the amount trapped within the nanotube structure.

The research of Brown et al., 2007 has found that the physical structure of MWCNT influences the way in which they interact with macrophages *in vitro*. Long (50 μm) rigid MWCNT appear to form fibre-like aggregates or structures that are too long to be phagocytosed by the macrophage cells. This results in reactive oxygen species (ROS) production, frustrated phagocytosis and production of the pro-inflammatory cytokine TNFα (Brown et al. 2007). In the same study, entangled MWCNT formed aggregates that were easily ingested by the macrophages resulting in little ROS or TNFα production.

Li et al. (2007) exposed mice to SWCNT at a dose of 10 and 40 μg/mouse. They identified markers of oxidative markers in various tissues including the lung, aorta and heart. Respiratory exposure to particulate air pollution (PM_{10}) has been linked via epidemiology studies to cardiovascular effects, although the final mechanism remains unclear (Donaldson et al. 2001; Mills et al. 2005). One hypothesis relating air pollution to cardiovascular effects, is that the pollution particles can accelerate atherosclerotic plaque formation (Suwa et al. 2002). Lie et al. (2007) found that the SWCNT accelerated formation of atherosclerotic plaques in a mouse model of cardiovascular disease ($ApoE^{-/-}$ mice fed an atherogenic diet). This obviously requires much more investigation in order to identify whether HARN have the potential to increase the risk of cardiovascular disease.

4.4.3 Dermal effects of HARN

Dermal toxicity of HARN has been investigated using either *in vitro* cell line models, or implantation of particles into the skin of rodents. The dermal toxicity of CNT has been tested due to the previous observations that graphite and other carbon materials (e.g. carbon fibres) are able to induce dermatitis.

SWCNT containing 30% iron have been found to induce oxidative stress and cytotoxicity in a human keratinocyte cell line (0.6 mg/ml for 18 hours) (Shvedova et al. 2003). MWCNT have also been added to human epidermal keratinocytes at relatively high concentrations of 100, 200 and 400 μg/ml. These studies resulted in detection by TEM of the CNT within cytoplasmic vacuoles as early as 1 hour after exposure (Monteiro-Riviere et al. 2005). In this study all concentrations resulted in IL8 production at 8 hours suggesting a pro-inflammatory response, and cytotoxicity at 24 hours.

Zhang et al. (2007) treated human epidermal keratinocytes in vitro with 6-aminohexanoic acid-derivatized SWCNT, developed for drug delivery. Pro-inflammatory responses were observed in the form of IL6 and IL8 protein production on exposure to particle concentrations of 50 μg/ml for up to 48 hours.

Koyama et al. (2006) implanted SWCNT and MWCNT into the subcutaneous tissue of mice for a period of up to 3 months. The authors noted that both types of CNT induced detectable subcutaneous toxicity, but the extent of toxicity was low compared to asbestos. The toxic effects included activation of antigen/antibody response systems. Specific responses included activation of major histocompatibility complex (MHC) class 1 of CD4+/DC8+ T-cells one week after the SWCNT implantation. After 2 weeks all CNT samples activated MHC class II markers in CD4+/DC8+ cells and in CD4+ cells. Granulomatous tissue was found to encapsulate agglomerates of SWCNT and MWCNT. A study by Yokoyama et al. (2005) used hat-stacked carbon nanofibres implanted into rats. The inflammatory response detected was described by the authors as mild, in that it did not generate widespread necrosis, but it did induce the appearance of granuloma like structures.

Currently there is no study designed specifically for assessing the potential health effects (e.g. biokinetics or dose-response) of HARN on the gastrointestinal tract. Since ingestion is a likely route of exposure, this is an area of research which merits further attention.

4.4.4 Biomedical applications of HARN

Many studies have been published which investigate the use of CNT as nanomedicines, including drug delivery (Wu et al. 2007), gene delivery (McKnight et al. 2003) and nanocomposites for implantation (Price et al. 2004). A full review of these applications is beyond the scope of this study and the reader is referred elsewhere for this information (Lacerda et al. 2006). Instead, this review will focus on studies that provide toxicologically relevant information.

In a study by Singh et al. (2006), mice were injected with radio labelled functionalised SWCNT into mice in order to follow their biodistribution and subsequent clearance. The purpose of the functionalisation of the SWCNT was to make them more water soluble and therefore more useful in biomedical applications. Intravenous administration of the functionalised SWCNT resulted in rapid excretion via the kidney

with a half-life in blood of 3 hours. The presence of SWCNT in urine was verified by EM. This study suggested that functionalisation of the SWCNT prevented their uptake by the reticulo-endothelial system, including the liver and spleen. Such functionalisation is obviously useful in preventing accumulation in the body, facilitating clearance and thereby reducing the potential for toxicity. However, rapid clearance is also a disadvantage for drug delivery purposes. Another study injected intravenously unmodified SWCNT into rabbits and identified their biodistribution using near-infrared fluorescence. Again the nanotubes were found to clear from the blood, this time with a half life of one hour. The nanotubes were found accumulated in the liver 24 hours after administration, the consequences of which remain unknown.

4.5 ASBESTOS AND HARN: STUDIES DIRECTLY COMPARING THEM

The superficial similarities between CNT and asbestos was amongst the first things that led to perceptions of the potential risk of HARN (Royal Society Acd Eng report 2004) The similarities between chrysotile asbestos fibrils and nanotubes has re-invigorated an interest in chrysotile asbestos as a nano material. This has taken the form of comparisons and the inevitable renaming of chrysotile fibrils as 'chrysotile nanotubes' (see Soto et al 2004). The perceived uses of more controlled chrysotile structure, coapred to the variation that occur geological samples of chrysotile, has led to synthesis of 'chrysotile nanotubes' (Foresti et al 2006) and chrysotile nanotubes have been synthesis with wire inside them (Ivanova et al 1995) . It is ironic that the greatest similarity is with chrysotile asbestos since chrysotile is the least harmful of the asbestos types as a result of its lesser biopersistence than the amphiboles. CNT on the other hand are likely to be biopersistent on account of the chemical resistance of graphene.

Soto et al noted the similar ultrastructure of CNT and chrysotile using transmission electron microscopy (see Fig 12). The resemblance and size of chrysotile fibrils and individual MWCNT is striking but the underlying structure is very different. The structure is a graphene lattice in the case of the nanotubes versus rolls of magnesium silicate and brucite layers in the case of chrysotile,

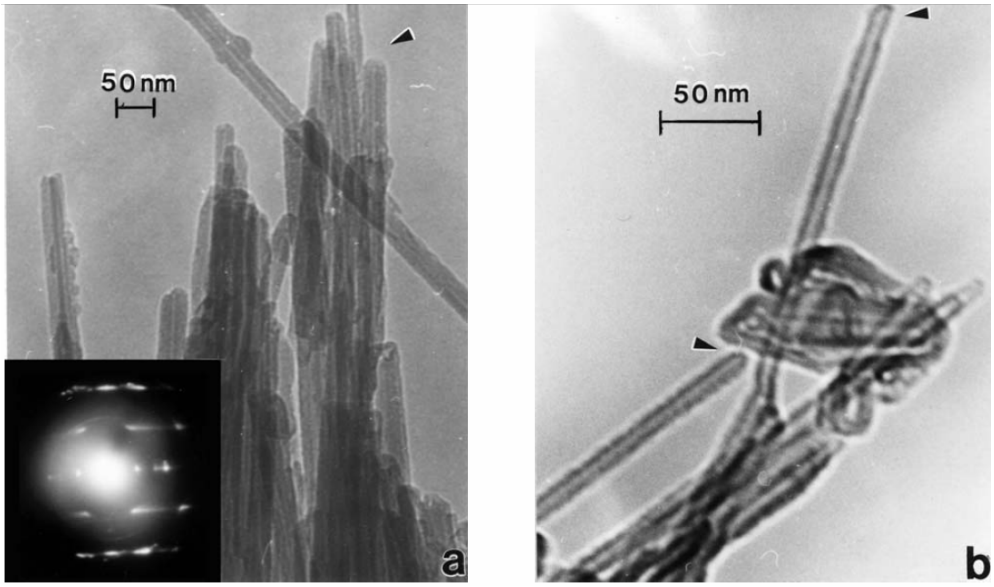


Figure 12 shows for comparison a chrysotile fibril bundle or aggregate (Fig 12(a)) and a carbon nanotube aggregate extracted from a natural gas (-96% CH4) kitchen stove top flame exhaust using thermal precipitation (Figure 12(b)). The arrows in Figure 12 (a) and (b) indicate the closed ends on the corresponding multiwall nanotubes. The multiwall and monoclinic nanocrystal fibril structure for the asbestos nanotubes is illustrated in the selected-area electron diffraction pattern insert in Figure 12(a).

In keeping with the fibre pathogenicity paradigm Brown et al., 2007 have found that the physical structure of MWCNT influences the way in which they interact with macrophages *in vitro*. Long (50 μm) rigid MWCNT appear to form fibre-like aggregates or structures that are too long to be phagocytosed by the macrophage cells. This results in reactive oxygen species (ROS) production, frustrated phagocytosis and production of the pro-inflammatory cytokine TNFα (Brown et al. 2007). In the same study, entangled MWCNT formed aggregates that were easily ingested by the macrophages resulting in little ROS or TNFα production. The phenomenon of

‘frustrated phagocytosis’ is well known in fibre toxicology (Manning et al., 2002). This study has demonstrated that MWCNT length can interfere with the ability of macrophages to phagocytose just like asbestos.

In a study published in Nature Nanotechnology Poland et al., 2008 specifically asked whether carbon nanotubes satisfied the fibre paradigm rule regarding length –in other words were long carbon nanotube fibres harmful whilst short fibres were not. This is the case with asbestos as has been shown in several key studies (e.g. Davis et al 1986). The study focused on the mesothelium, the cells that line the pleura and other body cavities, since asbestos -exposed individuals develop a number of conditions of the pleura, including the tumour mesothelioma; so this seems a special target for harmful fibres.

Nanotubes were chosen that were long and straight (i.e. one dimension longer than about 15 microns) and also nanotubes that were long tangled or short tangled (shorter than about 5 microns in any dimension). The short-term response of the mesothelium in the rat peritoneal cavity was analysed and only the long carbon nanotubes caused damage, inflammation and scarring (granuloma formation). As has been shown for long amphibole asbestos, these endpoints are linked to longer term effects, like mesothelioma. The short carbon nanotubes had no effect. We included a long and a short asbestos control - the long caused damage and inflammation whilst the short had no effect. Nanoparticulate carbon black was included as a control nanoparticle, since they are compact particles composed of graphene in particle, not tubular form -this had no effect on the mesothelium. The conclusions were clear that in terms of the toxicological rules governing fibre pathogenicity multiwalled carbon nanotubes that are long satisfy the criterion of a pathogenic fibre whilst multiwalled carbon nanotubes that are short satisfy the criterion of non-harmful. Asbestos also satisfies the same rules. As for the other rules –carbon nanotubes are very thin and they are durable in preliminary studies that have been carried out.

These studies have suggested that physical characteristics of HARN, such as length and biopersistence control pathogenicity for nanotubes, as is the case with asbestos and so long nanotubes may initiate adverse effects in the lung similar to those caused by asbestos exposure. It is important to note that the current data available are related to CNT only.

4.6 A RESEARCH STRATEGY TO DETERMINE THE POTENTIAL TOXICITY OF HARN

There are accumulating data regarding the potential toxicity of HARN, but as demonstrated in the reviews above, the existing information is mainly related to CNT. This is no doubt a result of the production volume of this material, being substantially greater than any other HARN. Information on other HARN such as nanowires and nanorods is clearly lacking. There are two possible paradigms that HARN could follow to produce toxicity – a particle-based paradigm and a fibre paradigm-based toxic effect. This document concentrates on the fibre-like effects, using asbestos as the example. However, research is need to determine whether HARN as particles/aggregates (<5um in any direction), are harmful to the lungs by mechanisms similar to other nanoparticles. (particles is underlined).

Here we focus on the fibre paradigm and a research strategy to determine the potential toxicity of HARN within the fibre paradigm must have the following *high priority* areas:

- **Hazard Identification:** The characterisation of the physico-chemical properties of HARN especially the length of the fibres and their biopersistence
- **Dose-Response Assessment:** Acute and chronics adverse effects of HARN; Cellular and molecular mechanisms of HARN toxicity investigated with in vitro and in vivo models
- **Exposure Assessment:** Identification and quantification of the routes (e.g. inhalation, dermal); the pattern and the intensity of exposure
- **The Risk Assessment of HARN:** Combining exposure and Hazard to calculate the health risks from exposure to HARN.

For Hazard Identification, the following issues are of important:

- HARN samples to be used in toxicology experiments must be well characterised for their physico-chemical properties by standard metrology protocols. This relates especially to length which should be measured as best as can be attained with difficult materials like CNT that tend to form tangles.
- Models should be used that are optimal for detecting fibre-type effects
- Asbestos fibres should be used as controls.
- The target organ dose of HARN, in animal experiments, must be determined in order to better- assess the dose-response relationship for HARN.
- Assessment of biopersistence or durability of HARN as it relates to accumulation of HARN dose, since long non-biopersistent fibres are cleared as they dissolve, fragment and join the short fibres (clearable) pool. Further work is also required on the links between biopersistence and the shape of HARN
- Full assessment of the length distribution, as far as is possible and assessment of how this relates to the accumulation of dose since long fibres are inefficiently cleared
- If evidence of compliance with the fibre paradigm is sought, then a long sample (a substantial proportion of fibres more than 15-20microns) and a short sample (less than 5 microns and few fibre longer than about 10 microns) should be used with the expectation that the short fibres would have less or no activity compared to the long sample

- Assessment of the role of structural features such as number of walls (CNT only), metal contamination, shape over and above length, crystallinity, surface reactivity etc…

For Dose-Response Assessment, the important issues are:

- Use of key cells postulated to be involved in the pathogenicity of fibres such as macrophages, epithelial cells, mesothelial cells. It should be noted that the pleural/ mesothelial responses are the only ones that are asbestos/ fibre-specific since inflammation and fibrosis occur with other pathogenic particles and so the mesothelial response should receive special attention
- Initial *in vitro* dose-response studies for the following endpoints,

    - Oxidative stress
    - Pro-inflammatory gene expression
    - Genotoxicity
    - Pro-fibrogenic effects

Depending on the specificity of fibre effects being sought then the mesothelium should be considered then epithelial cells and macrophages.

- Generation of aerosol for CNT and other HARN: Studies to develop a method for aerosolising respirable HARN for use in inhalation experiments.

- *in vivo* bio-kinetics studies: Toxicokinetics studies concerning the potential for inhaled or intratracheally delivered nanotubes to translocate to the pleura, mesothelial tissue and other body organs.

- *in vivo* toxicology studies: Acute single and multi-dose *in vivo* exposure studies by inhalation. For the lung, special consideration should be given to intrapleural and intraperitoneal administration, given the fact that both of these provide direct exposure of the mesothelium. Since mesothelioma in animals is inefficient (a few % of exposed rats with even a highly fibrous material) then direct mesothelial exposure has much to commend it

    - Endpoints should include mesothelial changes inflammation, fibrosis and mesothelioma.
    - Chronic and sub-chronic inhalation studies animal studies to investigate the toxicokinetic and inflammogenic, genotoxic and carcinogenic potential of HARN.

- Screening approach – based on physicochemistry: Building purely on the fibre pathogenicity paradigm, a potential screening approach is shown in Figure 13.

- Screening approach – animal based: The mouse peritoneal cavity has been show to be highly response to long fibres and to long nanotubes (Poland et al 2008). This provided a rapid and potential useful screen for HARN. Mesothelial cells and macrophages seem especially sensitive to long fibres and could form the basis of screening *in vitro* tests. Endpoints would be those mentioned above:

    - Oxidative stress
    - Pro-inflammatory gene expression

- Genotoxicity
- Pro-fibrogenic effects

It is important to note that the *in vivo* studies recommended here are clearly required, although if HARN are used in cosmetics, ingredients and formulations cannot be tested on animals from 2009 and 2013, respectively.

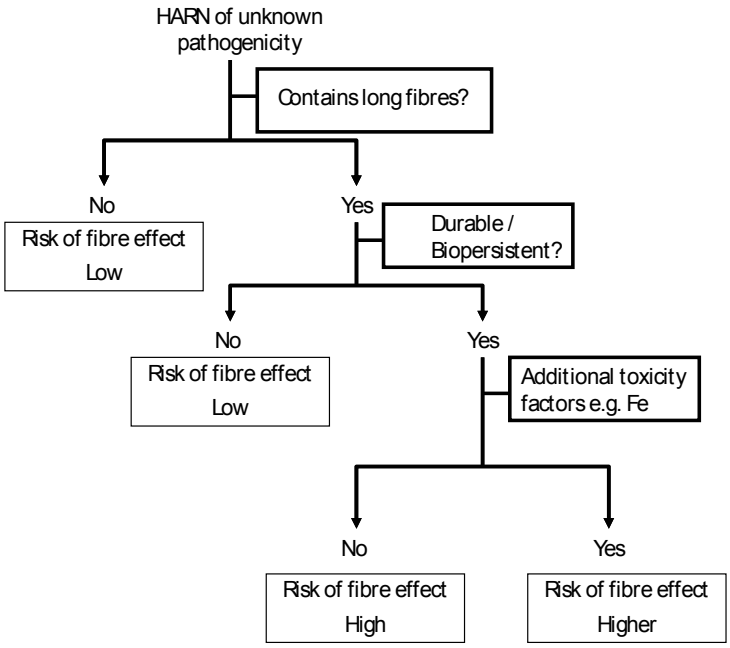


Figure 13. Potential screening approach for HARN

For Exposure Assessment the important issues are:

- Identification of the routes of exposure for HARN in the workplace, environment and consumer products
- Measurement of HARN concentration levels (in the air, soil, etc…)
- Modelling of the exposure scenarios for workers, consumers etc…including population exposure

And for Risk Assessment,

- Use data from the Dose-Response Assessment to calculate the Derived No Effect Level (DNEL)
- Assess risk by comparing the DNEL with exposure levels identified in the Exposure Assessment

WORKSHOP

The HARN workshop, discussing aspects of cellular penetration of nanoparticles in the lung epithelium, was held on Tuesday 21st April 2008, at DEFRA's Central Science

Laboratories in York. Its aim was to bring together a diverse range of stakeholders from the nanotechnology field, to disseminate the primary findings of the scoping study, and then through facilitated breakout discussions establish research priorities for further investigation of the mechanisms of nanoparticle translocation across the respiratory epithelium and the resulting possible toxic effects in and beyond the lung.

Stakeholders from across the UK and wider European nanotechnology community attended the workshop, with representation from the following institutes present:

Table 3: Workshop Attendance List

IonBond Ltd.	Imperial College, London
Joint Research Centre (JRC)	Central Science Laboratory (CSL)
Leeds University	University of Torino, Italy
Napier University	University of Parma, Italy
Institute of Occupational Medicine	Department for Environment, Farming and Rural Affairs (DEFRA)
Manchester University	United Bristol Healthcare NHS Trust
Health and Safety Laboratory	Nanotechnology Industries Association (NIA)
Edinburgh University	Centre for Environment, Fisheries and Aquaculture Science (CEFAS)
Aberdeen University	
Durham University	
Cardiff University	
University of Chester	
Health Protection Agency (HPA)	

Workshop Format

The workshop was divided into three main sessions – scientific presentations throughout the morning, followed by facilitated breakout discussions, and a summary and discussion of findings in the afternoon.

The morning's presentations were given by HARN's authors. The smaller group breakout, and final summary discussions in the afternoon aimed to build on the morning's dissemination and work toward reaching a consensus on future research.

Morning Session: Plenary presentations

The morning's proceedings began with a welcome from Professor Mike Roberts of CSL, and Professor Anthony Seaton of SnIRC. Prof. Roberts welcomed delegates to the workshop & gave a brief summary of CSL's involvement in the nanosafety field, after which Prof. Seaton introduced himself to delegates as workshop chair, and outlined what he hoped could be achieved during the HARN workshop.

The first scientific presentation of the day was from Dr Lang Tran of the IOM. Dr Tran introduced delegates to the HARN project in detail, explaining the brief given be DEFRA, and the importance of developing a research strategy for investigation of HARN given the recent parallels that have been drawn between high aspect ratio nanomaterials and asbestos. Dr Tran and Prof. Seaton then gave a brief outline of physicochemical characteristics of fibres in the asbestos family and its industrial history

from widespread use, through the emergence of a link with cancer and its ultimate banning in the workplace under the Asbestos Regulations in the late 1960s. Prof. Seaton summarised key aspects of the asbestos, and particularly the mesothelioma story for consideration when examining potential harm from HARN. These included emphasising:
1. that fibres holding a high mesothelioma risk were of a small diameter, but were highly durable and long in relation to their diameter
2. there is no threshold of risk for mesothelioma
3. mesothelioma has a latency of 20-40 years before it presents thus making recognising and proving causality difficult.
Dr Tran and Prof. Seaton concluded by stressing to attendees that the lessons of asbestos must be built upon rather than repeated to ensure the safe development of HARN nanomaterials.

The second presentation of the morning was from Professor Ken Donaldson of Edinburgh University, who outlined to delegates relative aspects of asbestos toxicity for application when considering HARN. He explained that due to the widespread nature of disease caused by asbestos exposure, the fibre toxicity paradigm is the most robust structure activity relationship model for any particle, having been built up from both human data, *in vivo* and *in vitro* experimentation over 25 years, and holding true to all fibres to which it is applied.

Prof. Donaldson outlined to attendees three key factors in the fibre paradigm for consideration in relation to HARN;

1. **Diameter**: fibres must be *thin* enough reach past ciliated airways. The aerodynamic diameter of fibres is not affected much by their length
2. **Length**: essential for the onset of e.g. frustrated phagocytosis and other inflammatory pathways
3. **Biopersistence**: important when considering the setting of e.g. maximum exposure limits to a fibre over time.

In relation to biopersistence, Prof Donaldson stressed the importance of considering asbestos as two separate minerals – explaining that this is mainly because the physicochemical characteristics of chrysotile type asbestos mean it is not biopersistent and thus can be broken down & digested by macrophages even if fibres are long and thin.

In conclusion, Prof. Donaldson outlined the physicochemical characteristics of carbon nanotubes in relation to the fibre toxicity paradigm, emphasising that in addition to being long and thin, CNT have a biopersistence profile similar to that of graphite (which is hugely biopersistent) and thus present a very worrying picture to fibre toxicologists.

The next presentation of the day was from Dr Ratna Tantra of the National Physics Laboratory (NPL), who provided attendees with an overview of HARN characterisation. By way of introducing to the huge challenge HARN characterisation poses, Dr Tantra began by presenting to attendees images depicting a selection of the numerous types of HARN in existence. Then, focussing on CNT, nanowires and nanorods, Dr Tantra outlined the characterisation needs of each in relation to both the manufacturer and the regulatory bodies (in this case the Health & Safety Executive), and the challenges posed by handling, preparation and standardising of samples for characterisation.

Dr Tantra broke the tools for characterisation itself down into three main areas –

1. Imaging tools (electron microscopy e.g. SEM and TEM)
2. Non-imaging tools (spectroscopic techniques e.g. Raman, Infa-red and X-ray spectroscopy), and
3. Other tools (including size distribution via dynamic light scattering, charge via zeta potential or surface area information via BET).

For each of these, she gave a brief introduction to the main techniques used, and its pros and cons, stressing that the most powerful option was to use a combination of techniques.

In relation to the HARN study, Dr Tantra explained that deciding on techniques for characterisation must be related to both the profiling requirements of the HSE, and the physicochemical data that they would need to determine the health hazard HARN may pose, and the three mainstays of the fibre paradigm – length, diameter and biopersistence.

The final presentation of the morning was given by Professor Vicki Stone of Napier University, who gave a summary of studies to date investigating mechanisms of HARN toxicity. Prof. Stone concentrated on carbon nanotubes, providing details of the major pulmonary, cardiovascular, immune system and dermal toxicity studies to date, outlining & comparing their results. To relate the evidence for CNT toxicity to their high aspect ratio, Prof. Stone provided details of two recent studies in which explored this hypothesis. The first study, conducted at Napier University by Brown et. al. (2007) showed clearly frustrated phagocytosis by macrophages occurring on exposure to CNT, with a seemingly more potent action than long fibre amosite. The second study, conducted at Edinburgh University by Craig Poland, Ken Donaldson and colleagues compared the behaviour and biological impact of a variety of CNT types. It found that long fibre-like CNT elicited a similar biological response in the Apo E -/- mouse to long fibre amosite (Poland C.A. *et. al*., 2008). To conclude, Prof. Stone asked that based on the growing bank of evidence for serious concern to be raised about HARN, what was the best plan of action to further elucidate and address HARN toxicity.

Afternoon Breakout sessions

For the afternoon breakout sessions, delegates were split into two randomly generated groups, and asked simply 'should HARN share the same concerns as Absestos?'. Groups were led by Professor Enrico Bergamaschi and Professor Vicki Stone. Rapporteurs were Dr Steve Hankin and Bryony Ross.

Summary of outcomes and discussion

The main conclusions of each breakout group were collated and reported to workshop attendees as a whole for wider discussion. A summary of outcomes and points raised in the subsequent discussion are outlined below.

Cross-cutting themes for HARN Research

The major cross-cutting priority for HARN research recommendations was identification of a suitable panel of HARN materials for use in further studies (including controls). It was recommended that materials used be of varying size, shape, length, thickness and bio-persistence.

Workshop attendees highlighted a number of recent pieces of work toward identification of standardised nanomaterials for experimental use, including the REFNANO report and the ongoing efforts of the OECD Working Party on Manufactured

Nanomaterials. It was agreed that cross-referencing to these would be important to development of a panel of standardised HARN.

In relation to this theme, several additional points were raised. These included:

- Development of labelled nanomaterials that:
  - are easy to trace
  - do not have altered behaviour as a result of being labelled
- Access to nanotubes systematically varied in relation to different physicochemical characteristics. These must be:
  - of good quality
  - available in sufficient quantity
  - well characterised
- The issue of acquiring access to appropriate inhalation facilities for investigation of airborne HARN within a controlled environment
- The need for development of technologies to improve aerosol generation
- The importance of making available targeted funding for further research into HARN

HARN Research Recommendations

Research recommendations for HARN identified from the workshop fell into three broad areas: recommendations for toxicology studies, priorities for HARN measurement & characterisation, and priorities for HARN Exposure/Risk Assessment. These are summarised below.

· Priorities for HARN Toxicology Studies

Physicochemical characteristics of interest

Research recommendations for an approach to the study of HARN toxicity, both *in vivo* and *in vitro* were discussed. Physicochemical characteristics of interest for investigation using both approaches were outlined and are summarised in Table 4.

Table 4: Physicochemical characteristics critical to investigation of HARN toxicity

Physicochemical Characteristics critical to investigation of HARN toxicity
1. Assessment of durability in the lung or any relevant tissue compartment, and relating this durability to *biologically relevant endpoints* 1.1 For in vitro studies, this may be conducted using a specifically designed Gambles solution
2. Assessment of aspect ratio (or shape) of SWCNT and MWCNT 2.1 Ascertainment of whether length can be better defined
3. Relation of aspect ratio to *biologically relevant endpoints* for *in vivo* and *in vitro* models 3.1 Determine whether there is a length-based relationship with HARN akin to asbestos to prove whether HARN conform with the Fibre Paradigm 3.2 Comparison of straight and entangled versions of the same material (and how surfactant affects this) 3.3 Comparison of long and short versions of same material 3.4 Comparison of varying diameters of same material 3.5 Comparison of fibre and spherical versions of same material 3.6 Investigate whether the fibre toxicity paradigm is applicable to HARN *bundles*

4. Assessment of composition and contaminants, and relation to biologically relevant endpoints for *in vivo* and *in vitro* models
    4.1 Comparison of Nanotubes with and without metals
    4.2 Comparison of HARN of different composition
    4.3 Comparison of un-functionalised and functionalised HARN

In vivo studies

For research into of whether HARN may cause mesothelioma, other cancers or fibrosis, the following *biologically relevant endpoints* were identified as being important to *in vivo* studies:

- Pathology (mesothelioma, granuloma, fibrosis, oxidative stress, inflammation, and tissue damage)
- Translocation (of HARN into pleural cavity)
- Biochemical changes (oxidative stress, inflammation, lung damage and fibrosis)
- Molecular mechanisms (signalling pathways)
- Genotoxicity

It was agreed that a variety of *in vivo* models would be necessary to study these endpoints. Suitable study models were considered, and their potential pros and cons are summarised in Table 5.

Table 5: *in vivo* models for HARN research

Study type	Potential Difficulties	
Inhalation study	Creation of a respirable aerosol?	Cross-cutting issue – Dose Selection
Instillation study	Creation of a good dispersion?	
Aspiration study	Prevention of LPS interference?	
Intraperitoneal	Good model, but does not represent the pleura	
Intrapleural	Possible, but technically difficult.	

For all *in vivo* studies, length of exposure to HARN necessary to determine potential toxicity was discussed and three main exposure lengths were identified. These are:

- Life time exposure to assess mesothelioma/cancer induction
- Exposure over months to assess pathological changes leading to mesothelioma/cancer
- Exposure over days to assess inflammatory and oxidative changes leading to mesothelial effects

In Vitro Studies

For research into of whether HARN may cause mesothelioma, other cancers or fibrosis, the following *biologically relevant endpoints* were identified as being critical to *in vitro* studies:

- Genotoxicity
- Cell Proliferation
- Pro-inflammatory signalling

- Oxidative stress
- Frustrated phagocytosis
- Cytotoxicity

For *in vitro* investigation of HARN, investigation of several cell types was identified as critical. These were:

- Mesothelial cells
- Epithelial cells
- Macrophages
- Fibroblasts
- Co-cultures

It was agreed that all *in vitro* work should be related to *in vivo* studies, in order to provide an indicator of potential useful screening techniques.

· Priorities for HARN Measurement & Characterisation

Measurement & Characterisation requirements identified as critical to the study of HARN are summarised in Table 6 below.

Table 6: Measurement and characterisation requirements for HARN

HARN measurement & characterisation requirements	
Requirement	Discussion points
Determination of whether HARN can be measured using existing available sample collection & analysis conditions.	Attendees noted that this could be achieved through comparative studies e.g. PCOM vs SEM/TEM comparison study
Determination of whether there are variations in the distribution of HARN observed by light microscopy, SEM, TEM etc.	
Conduction of studies to establish better geometric characterisation of shape.	
Determination of whether standard asbestos fibre measurement techniques are applicable to HARN.	Including determination of whether the current measurement techniques employed for asbestos applicable to HARN in various forms (e.g. airborne)?
Characterisation and quantification of Length/Diameter; Durability; Chemistry; Surface Composition etc.	Including benchmarking against other fibres and particulates
Development of a characterisation protocol to describe tangles	
Establishment of methods to characterise the sample being used as a whole.	This would ensure it is the fibre that is having the effect rather than any contaminants (applicable to both toxicological and measurement studies).
Investigation of aggregation and disaggregation of tangles in surfactant	
Investigate how to quantitatively measure HARN in biological tissue	
Studies of the aerodynamic behaviour and fate of HARN.	
Development of methods to measure release of nanomaterials from composites	This is especially relevant to the development of exposure/risk assessment protocols for HARN

· Priorities for HARN Exposure/Risk Assessment

Priorities identified as critical for development of exposure and risk assessment protocols for HARN are summarised below:

To enable conduction of a comprehensive exposure assessment:

1. Identification of routes of exposure:
2. Identification of Scenarios for exposure
3. Decide which of these give the greatest cause for concern and direct study accordingly.

5 CONCLUSIONS

The paradigm for the health effects of high aspect ratio nanoparticles (HARN) originates from toxicology studies of industrial fibres including asbestos. In this study, we have reviewed the state-of-the-art knowledge on the toxicity of asbestos and HARN and we have compared the health effects from exposure to asbestos (as an example of an industrial fibre) with carbon nanotubes as an example of a HARN. As part of the review we have also compiled the current information on the characterisation of the physico-chemical properties of HARN.

The lesson we have learned from asbestos is that:

1. fibres holding a high mesothelioma risk were of a small diameter, but were highly durable and long in relation to their diameter
2. there is no threshold of risk for mesothelioma
3. mesothelioma has a latency of 20-40 years before it presents thus making recognising and proving causality difficult.

The research on the toxicity of asbestos and other industrial fibres has lead to the 'fibre paradigm' for consideration in relation to HARN;

1. **Diameter**: fibres must be *thin* enough reach past ciliated airways. The aerodynamic diameter of fibres is not affected much by their length
2. **Length**: essential for the onset of e.g. frustrated phagocytosis and other inflammatory pathways
3. **Biopersistence**: important when considering the setting of e.g. maximum exposure limits to a fibre over time.

To assess the health risk posed by HARN, it is important to identify the physico-chemical characteristics of HARN. The tools for HARN characterisation can be summarised into three main areas:

1. Imaging tools (electron microscopy e.g. SEM and TEM)
2. Non-imaging tools (spectroscopic techniques e.g. Raman, Infa-red and X-ray spectroscopy), and
3. Other tools (including size distribution via dynamic light scattering, charge via zeta potential or surface area information via BET).

The techniques for characterisation must be related to both the profiling requirements of the regulatory process, the physico-chemical data that they would need to determine the health hazard HARN may pose and the three mainstays of the fibre paradigm above.

The studies to date, which investigated the mechanisms of HARN toxicity, concentrated on CNT and have provided data on pulmonary, cardiovascular, immune system and dermal toxicity. To relate the evidence for CNT toxicity to their high aspect ratio, two recent studies, which explored this hypothesis, are highlighted.

The first study, Brown et. al. (2007) showed clearly frustrated phagocytosis by macrophages occurring on exposure to CNT, with a seemingly more potent action than long fibre amosite.

The second study, Poland et al (2008) compared the behaviour and biological impact of a variety of CNT types. It found that long fibre-like CNT elicited a similar biological response in the Apo E -/- mouse to long fibre amosite.

This review has identified many similarities between HARN and asbestos with regard to their physico-chemical properties and toxicological effects and has concluded that there is sufficient evidence to suggest that HARN which have the same characteristics (diameter, length and biopersitence) as pathogenic fibres are likely to have similar pathology.
This review has also highlighted the lack of data in key areas of toxicology, exposure and assessment.
Based on the growing bank of evidence for serious concern to be raised about HARN, a research strategy to further elucidate and address HARN toxicity was constructed. The main components of this strategy are:

- **Hazard Identification:** The characterisation of the physico-chemical properties of HARN especially the length of the fibres and their biopersistence
- **Dose-Response Assessment:** Acute and chronics adverse effects of HARN; Cellular and molecular mechanisms of HARN toxicity investigated with in vitro and in vivo models
- **Exposure Assessment:** Identification and quantification of the routes (e.g. inhalation, dermal); the pattern and the intensity of exposure
- **The Risk Assessment of HARN:** Combining exposure and Hazard to calculate the health risks from exposure to HARN.

This review has identified the important issues concerning each of these components and has prioritised them. Finally, recommendations on future studies to cover the information gap are made.

6 RECOMMENDATIONS

This review has highlighted the potential hazard of HARN. The evidence so far has shown that there are similarities in the mode of action of HARN and asbestos. However, further work is needed to ascertain the relationship between the HARN physico-chemical properties and their toxicity.
We make the following prioritised recommendations for future research:

- For HARN measurement & characterisation requirements
  - Of high priority are the studies designed for
    - Characterisation and quantification of Length/Diameter; Durability; Chemistry; Surface Composition etc…of HARN
    - Investigation on how to measure HARN physico-chemical properties in suspension and biological tissue
- For HARN Toxicology Studies
  - The following in vivo studies are of high priority
    - Bio-kinetic study to investigate the distribution of HARN in the pulmonary system.
    - Chronic and sub-chronic multi-dose inhalation experiments with HARN to investigate a range of endpoints (see in vitro below). Most importantly, there is a need for the creation of respirable aerosols of HARN for the study.
  - And the following in vitro studies
    - To Investigate the potential for oxidative stress, inflammation and genotoxicity of HARN
- For Exposure and Risk Assessment
  - The following studies are of high priority
    - Exposure Assessment: Develop models of occupational, consumer and environmental exposure to HARN
    - Risk Assessment: Develop model of exposure-dose-response for HARN, calculate DNEL and compare with the exposure levels identified in Exposure Assessment

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