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Biopersistence and potential adverse health impacts of fibrous nanomaterials: what have we learned from asbestos?

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Abstract

Human diseases associated with exposure to asbestos fibers include pleural fibrosis and plaques, pulmonary fibrosis (asbestosis), lung cancer, and diffuse malignant mesothelioma. The critical determinants of fiber bioactivity and toxicity include not only fiber dimensions, but also shape, surface reactivity, crystallinity, chemical composition, and presence of transition metals. Depending on their size and dimensions, inhaled fibers can penetrate the respiratory tract to the distal airways and into the alveolar spaces. Fibers can be cleared by several mechanisms, including the mucociliary escalator, engulfment, and removal by macrophages, or through splitting and chemical modification. Biopersistence of long asbestos fibers can lead to inflammation, granuloma formation, fibrosis, and cancer. Exposure to synthetic carbon nanomaterials, including carbon nanofibers and carbon nanotubes (CNTs), is considered a potential health hazard because of their physical similarities with asbestos fibers. Respiratory exposure to CNTs can produce an inflammatory response, diffuse interstitial fibrosis, and formation of fibrotic granulomas similar to that observed in asbestos-exposed animals and humans. Given the known cytotoxic and carcinogenic properties of asbestos fibers, toxicity of fibrous nanomaterials is a topic of intense study. The mechanisms of nanomaterial toxicity remain to be fully elucidated, but recent evidence suggests points of similarity with asbestos fibers, including a role for generation of reactive oxygen species, oxidative stress, and genotoxicity. Considering the rapid increase in production and use of fibrous nanomaterials, it is imperative to gain a thorough understanding of their biologic activity to avoid the human health catastrophe that has resulted from widespread use of asbestos fibers.

The manufacturing of engineered nanomaterials for both consumer and industrial applications is undergoing exponential growth. Among the many nanomaterials produced since the discovery of fullerenes in 1985,¹ carbon nanofibers (CNFs) and carbon nanotubes (CNTs) are exceptionally attractive, because of their high strength-to-weight ratio, high surface area, thermal stability, and resistance to chemicals.² Their unique physicochemical characteristics are expected to be useful in a wide range of applications, including reinforcement of biomaterials, optical devices, drug delivery, biosensors, as well as conducting and reinforcing fillers in polymer composites.

Inhalation exposure in occupational environments can occur during the synthesis, collection, purification, handling, and packing of nanomaterials. Because of their shape and dimensions, in particular their high aspect ratio, graphenic CNTs may have similar pathogenic potential as naturally occurring asbestiform fibers. Their structural resemblance to asbestos fibers³ and potential biopersistence make fibrous carbon nanomaterials a potential human health hazard.

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⁴ The aim of this review is to address the similarities between asbestos fibers and high aspect ratio nanoparticles with respect to their physicochemical and toxicologic properties.

FIBROUS MATERIALS

Naturally Occurring

Asbestos fibers are naturally occurring silicates, with the following properties: fibrous shape, high tensile strength and flexibility, low thermal and electrical conductivity, high absorbency, high mechanical and thermal stability, and resistance to acids and bases.⁵ They can be divided into two groups: serpentines and amphiboles (Figure 1). Chrysotile is the only member of the serpentine group. Actinolite, amosite, anthophyllite, crocidolite, and tremolite belong to the amphibole group. Chrysotile is comprised of an octahedral magnesium hydroxide layer intercalated between silicate tetrahedral layers which form tightly rolled sheets ranging in diameter from 25 to 100 nm.⁶ As the brucite layer of chrysotile is acid-sensitive, fibers that reach the lung can undergo focal fiber disintegration, either in the acidic (pH ~4.5) phagolysosomes of macrophages, or if cleared from the lung and swallowed, by hydrochloric acid in the lumen of the stomach.

Amphiboles exhibit prismatic cleavage and diameters in the range of 100–200 nm and their chemical composition is complex (Table 1). Amphibole fibers can split longitudinally into thinner fibers that are extremely biopersistent.⁷

Fiber toxicity is related to the physicochemical characteristics of the particular fiber and sample, including geometry, surface reactivity, crystallinity, and chemical composition, particularly the presence of redox-active transition metals. Other naturally occurring asbestiform fibers include, but are not limited to, wollastonite and erionite, the latter being a highly carcinogenic fibrous zeolite. In contrast to asbestos and other asbestiform fibers, wollastonite is highly-soluble and has minimal toxicity.⁸

Synthetic Fibers—CNFs and CNTs

Classification—Although synthetic fibers encompass a wide variety of products with variable chemistries,⁷ for the purpose of this review we will focus on CNFs and CNTs. CNF is a generic term used to describe filaments comprised of graphene layers stacked at an angle to the fiber axis, where the angle of the graphene layers determines the fiber type⁹ (Figure 2).

CNFs are used commercially as polymer additives, gas storage materials, catalyst supports, and in biomedical devices.¹⁰ CNTs are either single walled (SWCNTs) or multi-walled (MWCNTs). SWCNTs are comprised of a single cylindrical graphene sheet with diameters ranging from 0.4 to 3 nm and are often closed at one end. MWCNTs contain several concentric, coaxial graphene cylinders with diameters ranging from 2 to 200 nm,^{11,12} and bear a structural resemblance to chrysotile asbestos (*vide infra*). CNTs can coalesce to form ropes or bundles up to 500 nm or 3 μ m in diameter, for SWCNT and MWCNT¹³ respectively.¹⁴

Synthesis—Two components are needed for carbon nanomaterial synthesis, a carbon source and an energy source. Depending on the material and method of synthesis, a metal nanoparticle catalyst may be used to increase yield and sample homogeneity, and to reduce the synthesis temperature.²¹ Carbon nanomaterials can be synthesized by several distinct methods, most commonly used are chemical vapor deposition (CVD), arc discharge, laser ablation, and nanochannel templating methods (Table 2).

The diameter of the fibers depends on the dimensions of the metal nanoparticle used as a catalyst, and the orientation of the graphene layers can be steered by the growth temperature and/or the nature of the metal. Iron catalysts preferentially generate parallel fibers, whereas

nickel generates fishbone-like fibers. The fiber strength depends on the graphene layer arrangement and the rate of growth. Fast growth of thin fibers generates weak products, while slow growth of thick fibers leads to strong products. The mechanical strength at the macroscopic scale is also strongly influenced by the structure of CNF agglomerates.^{20,22}

SWCNTs and MWCNTs exhibit unique characteristics depending on the method and conditions of synthesis. The shape, symmetry, dimensions, growth rate, yield, and crystallinity of the materials are influenced by the selection of the catalyst, carbon source, temperature, and time of the reaction, thereby leading to significant heterogeneity among the final products. Moreover, batch-to-batch differences in the amounts of residual catalyst, (typically partially encapsulated inside the graphenic tube or covered by a carbon shell), disordered graphenic or fullerenic carbon, support materials, and other impurities have been observed.²³ Consequently, post-synthesis treatments are used to increase the purity of the product. The most common purification approach involves selective oxidation of the amorphous carbon and/or carbon shells at a controlled temperature followed by washing or sonicating the sample in acid (HCl, HNO₃, H₂SO₄) or base (NaOH) to partially remove the catalyst and/or support. Breakage can occur during purification, resulting in defective or shorter tubes. In addition, surface functionalization by air^{24,25} or oxidizing acids can occur as a side reaction.²³ Removing imbedded metal without damaging the desired tube structure is difficult, so even purified materials typically have measurable and often significant metal content. As there are many types of purification processes, the purified materials will exhibit differences in the content of trace elements and residual materials.^{26,27}

FIBER CHARACTERISTICS THAT DETERMINE BIOACTIVITY

The toxicity, fibrogenicity, and carcinogenicity of asbestos are related to its physical and chemical properties, some of which are also shared by fibrous carbon nanomaterials. The following parameters are important in governing the lung burden and subsequent development of fibrosis or cancer following fiber inhalation.

Size and Shape

Fiber size and geometry determine the extent of fiber deposition into the lung and are well known to influence carcinogenicity.⁷ The deposition of inhaled fibers in the lung is determined by their length, width, shape, density, and by the anatomy of the respiratory tract. These parameters determine the aerodynamic behavior of fibers in the conducting airways, as well as their probability of deposition and retention in the distal lung (Figure 3). Aerodynamic diameter (AD) impacts the movement and deposition efficiency of inhaled fibers and particulates in the respiratory tract. Most of the mathematical models used to determine the AD of fibers are extrapolated from studies of the AD of particles, which describe a direct correlation between increased particle density and/or diameter (leading to an increase in the AD) and reduced deposition in the respirable region of the lung. Mechanisms of fiber deposition include impaction, sedimentation, and diffusion. For larger particles with an AD > 5 μm, deposition occurs by impaction as a result of an abrupt directional change in the airways, and sedimentation or settling by gravitational forces, while diffusion is the predominant mechanism for deposition of smaller fibers (AD < 5 μm).⁷

Biopersistence

The ability of fibers to persist in the body following inhalation determines the retained dose. The dose of fibers retained in the respiratory tract is expressed as the initial number of deposited fibers minus the number of fibers subject to clearance, which can occur by both physicochemical and physiological processes. Fibers that cannot be cleared by any of these processes are considered to be biopersistent and are predicted to accumulate during chronic

exposure. Physicochemical processes leading to increased clearance include leaching of elements from the fiber matrix, which can alter fiber composition, dissolution in surfactant or physiological fluids, and transverse breakage or splitting into shorter or thinner fibers that are more readily cleared.²⁹ Clearance is believed to occur by the following four major mechanisms.

Clearance via the Mucociliary Escalator in the Nose and Tracheobronchial region—The trachea, bronchi, and larger bronchioles are lined with ciliated epithelial cells, which are covered with a thin layer of mucous. The mucous traps foreign material, which the cilia rhythmically beat and move toward the throat where it can be swallowed or expelled from the body.²⁹

Phagocytosis by Alveolar Macrophages—A limiting factor for complete phagocytosis of fibers is the diameter of the alveolar macrophages. As macrophages range in diameter from ~10 to 20 μm , shorter fibers are more likely to be completely phagocytosed by alveolar macrophages than longer fibers. This leads to incomplete or ‘frustrated’ phagocytosis, which is characterized by prolonged production of reactive oxygen species (ROS).²⁹

Dissolution—Fiber durability is typically assessed by dissolution *in vitro*. Studies using mathematical models in both static and flow-through methods have been described.^{30,31} As the pH of the extracellular lung fluid is 7.2, the pH in the phagolysosome of the rat alveolar macrophage is 4.5–5.0, and the intracellular (cytoplasmic) pH of lung tissue has been measured as 6.5, dissolution studies are performed in buffer solutions resembling the pH and composition of these different milieus.^{29,30,32,33}

Translocation—Fibers can also leave the lung by migration, passing across the alveolar wall and into the lung interstitium, where they can reach the lymphatic system. Chrysotile fibers may split into thinner fibrils over time. Compared to amphiboles, chrysotile asbestos is more effectively cleared, in part because of its brucite layer, which dissolves in the acidic milieu of alveolar macrophages, leading to focal fiber disintegration. Because of the carbonaceous nature of CNFs and CNTs, it has been suggested that they may be as biopersistent as amphiboles. Using an *in vitro* flow-through assay with phagolysosomal simulant fluid at pH 4.5, SWCNTs have been shown to persist for two months.²⁷ It is unknown whether the biopersistence of CNFs and CNTs more closely resembles that of serpentines or amphiboles, and whether uncleared nanomaterials can translocate to the pleura.³⁴

Surface Properties

Surface properties of fibers can affect their biological activity. Unfunctionalized carbon nanomaterials tend to be hydrophobic and are difficult to disperse in physiological solutions. The wide variety of surface functionalization schemes now available for nanotubes, coupled with their high intrinsic surface area, gives these materials the potential to adsorb a wide range of small molecules and macromolecules in biological environments. Characteristics such as pore volume and surface structure can impact the total area over which physical adsorption can occur, while charge and chemical modification can determine the location and degree of adsorption.^{14,35–37} These phenomena may selectively impact interactions with biological systems, thus altering their potential health risk throughout the product life-cycle.

Asbestos fibers can adsorb xenobiotics from the environment, including polycyclic aromatic hydrocarbons and iron.^{38–40} Upon contact with the lining fluid and alveolar macrophages in the lung, asbestos fibers adsorb endogenous proteins including ferritin,^{38,41} immunoglobulin G^{42,43} and vitronectin.^{44,45} Formation of ferruginous or asbestos bodies is the consequence of endogenous iron, protein, and mucopolysaccharide deposition on biopersistent fibers in the lungs.⁴⁶

Carbon nanomaterials can also adsorb biomolecules. Post-synthesis treatments of CNFs and nanotubes can modify their surface, leading to alterations in the selective adhesion of biomolecules, opsonization by complement,⁴⁷ and adsorption of small solutes, such as folic acid or riboflavin, from biological fluids.³⁶ Unmodified graphene surfaces are hydrophobic, but many types of surface modifications commonly performed on CNTs and CNFs can increase hydrophilicity and improve their dispersion in medium or biological fluids.³⁷ As hydrophilic materials interact more readily with cell receptors, surface modifications that enhance hydrophilicity can modify target cell interactions and subsequent biological responses.³⁷

Surface Reactivity: Role of Transition Metals

Redox-active transition metals are an important determinant of fiber toxicity and carcinogenicity, and have been proposed to mediate, in part, the genotoxic, mitogenic, and cytotoxic effects of amphibole asbestos fibers.⁷ The ability of asbestos fibers to generate reactive species is associated with the presence of surface redox-active iron, which can be present in both ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms. Among commercially used asbestos fibers, crocidolite and amosite asbestos are particularly iron-rich, containing 20–30% iron by weight.³⁹

Reactive species can be generated in the presence of transition metals, whether these metals are present at the fiber surface in a poor coordination state,³⁷ or as a result of mobilization from the crystalline lattice.⁴⁸ In cell-free systems, iron can be mobilized from asbestos fibers using chelators such as ferrozine, or reductants such as ascorbate or citrate.³⁹ Iron mobilization and redox cycling lead to production of hydroxyl radicals, DNA breaks, and formation of premutagenic DNA adducts such as 8-OHdG.³⁹ Redox-active iron has been proposed to contribute to the development of human cancer.⁴⁹ Iron saccharate induces sarcomas in rats at the site of injection,⁵⁰ and iron nitriloacetic acid (Fe-NTA) induces renal tumors and mesotheliomas when injected intraperitoneally.⁵¹

Adsorption of endogenous iron onto the fiber surface or into pores may also contribute to the toxic and carcinogenic effects of fibers. This mechanism has been suggested for erionite, a fibrous zeolite that does not contain iron within its molecular structure. The surface area of erionite is 10–100 times greater than that of asbestos and erionite has been shown to accumulate iron in its porous structure.^{39,40} Erionite is highly carcinogenic in rodents, inducing mesotheliomas more efficiently than crocidolite or amosite asbestos,⁵² and has been linked to a mesothelioma epidemic in central Turkey, where the incidence exceeds 50% in select villages.⁵³

Metal catalysts are used in the synthesis of CNTs, primarily iron, nickel, yttrium, cobalt, and molybdenum, which are known to raise toxicity concerns. Of primary concern is whether the metal catalysts present in synthetic nanomaterials are bioavailable, especially considering their apparent encapsulation by carbon observed by TEM. Using buffers that model physiological fluids (e.g., phagolysosomal simulant fluid) or biologically relevant fluids, mobilization of iron and nickel from CNTs has been demonstrated.^{26,54} Although purification methods can be devised to remove the majority of contaminating metals, no purification process is 100% efficient; therefore, even purified samples are not completely metal-free. Recent work has shown that soluble iron and nickel can be mobilized even from vendor-purified CNT samples.^{26,54} Importantly, the amount of contaminating, and presumably bioavailable, metal varies widely among commercially produced CNTs, which may confound interpretation of toxicological studies.

In addition to iron, contaminating nickel catalysts may also have toxic and carcinogenic potential in the respiratory tract. Inhalation exposure to nickel induces acute respiratory symptoms in nickel workers, and increases respiratory tract cancers in chronically exposed

workers.^{55,56} Nickel induces acute lung injury and tumorigenesis in rodent models, including mesotheliomas in rats when injected intrapleurally or intraperitoneally with metallic nickel powder.^{57,58} Carcinogenic nickel compounds, which are generally non-mutagenic, are hypothesized to transform target cells via epigenetic mechanisms.⁵⁹ Altered DNA methylation within regulatory regions of genes may result in transcriptional silencing of tumor suppressor genes. Epigenetic control of gene expression can also be mediated through altered modifications of histone proteins that are responsible for the degree of DNA condensation. Deacetylation or methylation of histones can induce gene silencing by turning transcriptionally active euchromatin into transcriptionally repressive heterochromatin.⁶⁰ Both water-soluble and water-insoluble nickel compounds have been shown to induce transgene silencing in the absence of mutation, but in association with increased DNA methylation, decreased histone acetylation, and increased histone methylation.^{61,62}

Tendency to Agglomerate

The degree of fiber dispersion influences physiological responses, tissue distribution, clearance, and translocation. In this review, we use the term agglomerates to describe clusters of high-aspect ratio nanomaterials held together by non-covalent interactions.⁶³ Agglomerates of microscale diameter conserve most of the surface properties of the constituent nanoscale materials.¹⁴ Agglomeration introduces an additional level of complexity to their behavior in the lungs. *In vivo* and *in vitro* studies correlating the degree of agglomeration with the toxicological outcome are still controversial. Several groups have shown that micron-sized CNT agglomerates are more toxic than well-dispersed nanotubes,⁶⁴ and in some cases are as toxic as asbestos.^{65,66} It is important to note the high degree of sample heterogeneity in these studies and to avoid premature conclusions regarding the primary cause for the observed toxicity. To prevent agglomeration, functionalization via covalent and non-covalent methods is used to introduce positive or negative charges onto nanomaterial surfaces. Functionalization is also useful for the attachment of biomolecules or drugs, making carbon nanomaterials attractive as carrier systems for therapeutic agents.⁶⁷ In addition to improving dispersion, the nature of the functional group and the degree of functionalization can fundamentally change the interaction of fibrous materials with target cells, which itself can lead to different physiological or toxicological outcomes.

BIOLOGICAL RESPONSES TO FIBROUS NANOMATERIALS

There is concern that exposure to fibrous carbon nanomaterials will produce pathological reactions similar to that of asbestos fibers. The most probable route of exposure to fibrous carbon nanomaterials, because of their very light weight, is inhalation.²⁸ With a global production capacity of CNTs in excess of 300 tons per year,⁶⁸ inhalation exposure may be occurring in workers providing the impetus to assess the potential pathogenicity of these materials.

Studies examining the biological reactions to fibrous carbon nanomaterials have encountered problems with routes of exposure. Rodent models of asbestos-induced disease administer fibers by either inhalation or direct intratracheal instillation.⁶⁹ Delivery of fibrous carbon nanomaterials using similar techniques has been complicated by their tendency to agglomerate. Inhalation studies have been hindered by electrostatic interaction of nanomaterials which stimulates the formation of large, nonrespirable particles.⁶⁶ Intratracheal instillation is widely used, but it delivers a bolus of nanomaterials that clump together, potentially limiting distribution to the distal lung parenchyma and translocation to the interstitial space.^{7,66,70}

Acute and Chronic Reactions to Fibrous Nanomaterials

Macrophages attempt to phagocytize fibers that are not removed by mucociliary clearance. Biopersistent fibers stimulate macrophage activation characterized by production of cytokines and ROS.⁷¹ This inflammatory environment produces injury and proliferation of lung epithelial cells.⁷¹

The acute inflammatory response to fibrous carbon nanomaterials depends on surface properties, fiber length, and chemical composition of the fibers.^{72,73} Following intratracheal instillation or intraperitoneal injection, CNTs have been shown to produce an inflammatory response characterized by the recruitment of neutrophils and macrophages, and formation of multinucleated giant cells.^{66,73,74} In contrast, exposure to CNFs produces an inflammatory response consisting predominantly of macrophages.^{75,76} The rougher, prismatic edges and lower metal content of CNFs have been proposed to facilitate uptake by macrophages, preventing the development of frustrated phagocytosis, and eliciting a different inflammatory response than that of CNTs.^{7,77,78}

Long, biopersistent asbestos fibers trigger persistent inflammation characterized by activated macrophages and multinucleated giant cells, leading to fibrosis of the lungs and pleura.⁷⁹ Similarly, mice instilled intratracheally with CNTs develop granulomas in the bronchioles, surrounded by hyperplastic epithelial cells and containing multinucleated giant cells, macrophages, and other inflammatory cells.⁶⁶ The reaction to biopersistent CNTs in rodents parallels the response to asbestos showing recruitment of macrophages, fibroblasts, lymphocytes, neutrophils, and eosinophils,^{70,74,80} as well as an active fibrotic response.^{66,80} The pulmonary response may be influenced by degree of CNT agglomeration, as well-dispersed SWCNTs evoke a more potent interstitial fibrotic reaction, in the absence of granuloma formation.⁸¹ Asbestos fibers^{82,83} and CNTs^{66,70,84} induce the formation of mature granulomas, composed of macrophages and multinucleated giant cells in a more acellular and fibrotic microenvironment. The proximity between the persistent inflammatory microenvironment within granulomas and the hyperplastic surface epithelial or mesothelial cells suggests that chronic macrophage activation could stimulate persistent cell proliferation, possibly facilitating malignant transformation.^{80,85}

Lung Diseases as a Result of Chronic Exposure to Fibers

The most common benign pathologic response to asbestos exposure is development of pleural plaques, which appear on the parietal pleura as raised white calcified lesions or nodules with a smooth surface covered by mesothelium. Pleural plaques occur in approximately 50% of individuals with a history of heavy and prolonged exposure to asbestos, and are considered to be a clinical marker of asbestos exposure whose extent correlates with the degree of asbestos exposure.⁸⁶ However, the presence of pleural plaques is not associated with an increased risk of malignancy,⁸⁷ nor are plaques considered to be a precursor lesion in the development of mesothelioma,⁸⁸ although pleural plaques and mesothelioma may be present simultaneously. Plaques are believed to develop as a result of collagen deposition by submesothelial fibroblasts, which are stimulated by macrophages that have phagocytosed asbestos fibers and are translocated, via the lymphatics, to the pleura.⁸⁹

Asbestosis refers to the progressive fibrosis of the lung parenchyma as a result of asbestos exposure,⁹⁰ involving epithelial cell hyperplasia, infiltration of inflammatory cells, and excessive deposition of collagen by fibroblasts. Individuals with asbestosis may remain asymptomatic for 10–30 years, although cigarette smoke and asbestos fibers may act additively to accelerate its progression.⁹¹ It is unclear at present whether asbestosis increases the risk for lung cancer, or whether asbestosis is a prerequisite for developing asbestos-related lung cancer

or mesothelioma. It is similarly unclear whether exposure to carbon nanomaterials will induce pulmonary fibrosis in humans.

The mechanisms of asbestos carcinogenicity remain unclear, although fibrosis, chronic inflammation, production of reactive species, and interference with the mitotic spindle may each have a role.⁷ In contrast to asbestos-induced lung disease, graphite-containing dusts accumulate in the lungs of workers but produce minimal fibrosis and no increased risk of cancer.⁹² In rodent models, all carcinogenic mineral fibers induce fibrosis⁷; therefore, there is concern that some CNTs that induce lung fibrosis may also have the potential to induce lung cancer.

TOXICOLOGIC ASSESSMENT OF CARBON NANOMATERIALS

Given the similarities in high aspect ratio between manufactured carbon nanomaterials and asbestos fibers and their projected widespread use, considerable effort is being devoted to identify the physical and chemical parameters responsible for their potential toxicity. Although there is an increasing body of evidence concerning toxicity of fibrous nanomaterials (Table 3), results are sometimes conflicting and inconclusive. *In vitro* studies have reported toxicity of CNTs in a variety of relevant cell types, including macrophages, lung epithelial cell lines, and normal and malignant mesothelial cell lines,^{65,66,93–97} while other studies have found CNTs to be nontoxic.^{98–101} It has been reported that CNF and CNT, like asbestos, are capable of inducing ROS and oxidative stress,^{23,78} while another study indicates that highly purified MWCNT can act as radical scavengers in a cell-free system.¹⁰² The apparently conflicting results obtained from current studies is likely because of inherent sample variation among nanomaterials, including structure (SWCNTs vs. MWCNTs), dimensions, extent of purification, degree of agglomeration, and accessibility of metal catalyst residues. Nevertheless, *in vivo* data have demonstrated the potential for CNTs to produce acute lung injury, inflammation, and fibrosis (Table 4).

When evaluating the cytotoxic and genotoxic potential of fibrous nanomaterials *in vitro*, several biological and technical caveats must be taken into consideration. First, it is critical to evaluate the uptake of nanomaterials by the target cell population; nanomaterials that are poorly engulfed by cells will have limited opportunity to interact with DNA or to generate intracellular ROS, which could lead to an underestimation of fiber toxicity. For example, SWCNTs that were not readily engulfed by a macrophage cell line *in vitro* did not generate an intracellular oxidative burst or NO production, or induce macrophage apoptosis.⁸⁰ In addition, it is important to note that *in vitro* assays evaluate the oxidative potential of nanomaterials themselves, and do not provide information on ROS generated indirectly by target cells involved in an inflammatory response.

A significant technical caveat in assessing the cytotoxic potential of nanomaterials lies in the use of colorimetric or fluorescence-based viability/cytotoxicity assays. Nanomaterials are highly adsorptive, and interactions have been described between nanomaterials and commonly used viability dyes, including WST-1, MTT, Neutral Red, and Alamar Blue.^{35,98,104,105} It is necessary to demonstrate that the nanomaterials being tested do not interfere with the viability indicator, otherwise the apparent reduction in viability may be erroneously attributed to fiber cytotoxicity and not to quenching of the fluorescent or colorimetric dyes. A recent study used a clonogenic assay as an alternative endpoint of SWCNT toxicity in human lung cell lines.¹⁰⁶ By measuring both the number of colonies and surface area of the colonies, it was possible to determine the effects of SWCNT on cell viability and proliferation simultaneously.

MECHANISMS OF FIBER CARCINOGENICITY

The causal association between exposure to asbestos fibers and development of lung cancer and mesothelioma is well documented. The mechanisms by which asbestos fibers induce carcinogenesis are complex, involving pathways related to cell proliferation, genomic integrity, survival, and apoptosis. On the basis of studies using asbestos fibers, several hypotheses⁶ have been proposed regarding mechanisms of fiber carcinogenicity (Tables 5 and 6).

Free Radical Generation

Asbestos fibers are thought to promote carcinogenicity, in part, through iron-dependent generation of reactive metabolites, including superoxide, hydrogen peroxide, hydroxyl radical, and nitric oxide.¹²⁷ Superoxide anion is formed by reduction of molecular oxygen (reaction 1), which is then dismutated via the action of superoxide dismutases to hydrogen peroxide (reaction 2). Highly reactive hydroxyl radicals may then be generated from superoxide anion and hydrogen peroxide via the iron-catalyzed Haber-Weiss reaction (reaction 3a), or via the Fenton reaction (reaction 3b).¹²⁷



Antioxidants, including glutathione and N-acetylcysteine, and iron chelators ameliorate many but not all of the effects of asbestos fibers *in vitro*, suggesting a role for iron-dependent ROS generation in asbestos toxicity and carcinogenesis.¹²⁸ ROS produced by asbestos can damage cellular macromolecules via lipid peroxidation and oxidative DNA damage.^{127,129}

Alternatively, ROS may act as second messengers to alter signal transduction pathways and gene expression.⁷¹ A role for reactive nitrogen species, asbestos-induced inflammation, and pleural injury has also been suggested, including nitric oxide (NO⁻) and peroxynitrite (ONOO⁻). Asbestos-treated macrophages and mesothelial cells show increased expression and activation of inducible nitric oxide synthase, and enhanced immunoreactivity for nitrotyrosine, a marker for peroxynitrite formation, which has been observed in the lungs of rats exposed to chrysotile or crocidolite asbestos.^{130–132} Importantly, peroxynitrite has been shown to activate numerous components of the extracellular-regulated kinase (ERK) signaling pathway,¹³³ whose activity is associated with proliferation of asbestos-exposed mesothelial and lung epithelial cells.

Accumulating evidence suggests that ROS generation can occur upon exposure to manufactured nanomaterials. This can occur directly, in the presence of redox-active transition metals, or indirectly, as a result of ROS production by target cells. Unpurified SWCNTs containing 30% iron have been shown to induce ROS production in human keratinocytes and bronchial epithelial cells.^{134–136} Exposure to asbestos fibers induces DNA damage, including

oxidized bases (8-OHdG), DNA single-strand breaks (SSB) and double-strand breaks (DSB), mutations, and numerical and structural chromosomal aberrations.^{109,137} Preliminary evidence suggests that fibrous carbon nanomaterials may also be genotoxic. In a cell-free system, mobilization of redox-active iron from CNTs induced SSB in plasmid DNA,⁵⁴ while *in vitro* cellular assays suggest that CNTs can induce DNA damage as assessed by COMET assay and micronucleus formation.^{138,139} Embryonic stem cells exposed to MWCNTs also upregulate DNA damage response proteins.¹⁴⁰ Mice deficient in the *p53* tumor suppressor gene, a critical regulator of DNA damage responses, have been used as an *in vivo* model to investigate the role of genotoxicity in fiber carcinogenesis. The *p53* deficiency increases susceptibility to asbestos-induced mesotheliomas, and the majority of mesotheliomas that form in *p53* heterozygous mice exhibit loss of the remaining wild-type allele.¹⁴¹ Recent work suggests that *p53* heterozygous mice are a sensitive model for assessing the carcinogenic properties of fibrous carbon nanomaterials, as peritoneal tumors form following injection of MWCNTs.¹⁴²

It has been hypothesized that polymorphisms in genes related to xenobiotic metabolism, oxidative stress, or DNA repair could also influence susceptibility to asbestos-related diseases.¹⁴³ Although no susceptibility gene has been conclusively identified, a recent study of genetic polymorphisms in DNA repair genes among individuals exposed to asbestos indicated an association between polymorphisms in the X-ray repair cross-complementing group 1 (XRCC1) gene and malignant mesothelioma.¹⁴³ Although no human 'nanodiseases' have yet been identified, it is important to consider the possibility that genetic modifiers may contribute to fiber-induced diseases.

Physical Interference with Mitosis

It is hypothesized that long, rigid, biopersistent fibers, may induce carcinogenicity through physical interaction with the mitotic spindle, leading to chromosomal breakage or loss, or improper chromosomal segregation during mitosis. This may contribute to genetic instability, in the form of micronuclei and chromosomal imbalances, or aneuploidy, in daughter cells. Asbestos fibers are known to have aneuploidogenic effects in mesothelial cells and lung epithelial cells *in vitro*.¹⁴⁴

Physical interference with mitosis requires interaction between fibers and mitotic chromosomes or penetration of the nucleus during interphase. While some *in vitro* studies have shown that asbestos fibers interact with the nucleus or chromosomes,¹⁴⁵ other studies have shown that intracellular asbestos fibers can be surrounded by a phagolysosomal membrane, which could sequester the fibers in the cytoplasm.¹⁴⁶ It is possible that longer fibers, which cannot be completely engulfed by cellular membranes, are more likely to pierce the nucleus and interact with chromosomes. Internalization of long fibers has been associated with lagging mitotic chromosomes,¹⁴⁷ and exposure to chrysotile or crocidolite asbestos fibers *in vitro* increases the percentage of binucleated cells in rat pleural mesothelial cells,¹⁴⁸ and in primary and immortalized human mesothelial cells.¹⁴⁹ Together with *in vivo* studies showing formation of micronuclei following asbestos exposure,¹⁴¹ these results suggest that asbestos may interfere with both chromosome segregation and cytokinesis. Although limited data are available on the ability of carbon nanomaterials to interfere with mitosis, two recent studies have suggested that carbon nanomaterials may have the potential to induce aneuploidy. Exposure to SWCNTs containing 0.23% iron induced a slight increase in micronucleus formation in Chinese hamster lung fibroblasts at the highest dose tested,¹³⁸ although this study did not distinguish between clastogenic and aneugenic micronuclei. Ground, purified MWCNTs containing less than 2% iron produced a dose-dependent increase in micronuclei in rat type II pneumocytes as well as in clastogenic and aneugenic micronuclei in a human lung epithelial cell line.¹³⁹

Stimulation of Target Cell Proliferation

In order for fiber-induced mutations or chromosomal aberrations to be transmitted to daughter cells, exposed cells must be stimulated to proliferate. Exposure to asbestos fibers *in vivo* induces proliferation of mesothelial cells and bronchiolar epithelial cells.⁷ Mesothelial cell proliferation is associated with fiber biopersistence, as crocidolite asbestos induces a more extended proliferative response than wollastonite, which is susceptible to dissolution and clearance *in vivo*.¹⁵⁰ Biopersistent asbestos fibers may induce chronic cellular proliferation directly through upregulated expression of growth factors and activation of growth factor receptors. Asbestos fibers have been shown to bind and activate the epidermal growth factor receptor, and downstream mitogen-activated protein kinases (MAPKs): ERK1 and ERK2.¹⁵¹ ERK1/2 activity leads to expression of c-fos and c-jun,^{151–153} which are components of the redox-sensitive activator-protein 1 (AP-1) transcription factor. As transcription of AP-1 target genes influences cell proliferation, survival, and apoptosis, persistent activation of MAPKs and AP-1 may drive proliferation and survival of asbestos-exposed cells.¹⁵³ Recent *in vivo* studies suggest a critical role for the ERK signaling pathway in asbestos-induced cell proliferation.¹⁵⁴ Alternatively, asbestos may induce target cell proliferation indirectly. Release of cytokines and growth factors, including tumor necrosis factor (TNF)- α and transforming growth factor (TGF)- α , potent mitogens for mesothelial and lung epithelial cells, may stimulate proliferation of nearby mesothelial and epithelial cells.¹⁵⁵ In addition, asbestos-induced cellular injury can induce compensatory proliferation, as has been shown for mesothelial cells *in vivo*.⁸² It has recently been shown that carbon nanomaterials, like asbestos fibers, may activate some of the same stress-response pathways; for example, SWCNT-induced oxidative stress is associated with NF- κ B activation via MAPK signaling in human keratinocytes as well as in normal and malignant mesothelial cells.^{96,135} In human fibroblasts, exposure to MWCNTs induces transcriptional activation of numerous genes involved in the p38/MAPK stress-signaling cascade.¹⁵⁶

Persistent Chronic Inflammation

Persistent release of ROS and inflammatory mediators can contribute to establishment of a chronic inflammatory environment, which has been linked to carcinogenesis.¹⁵⁷ Activation of macrophages is accompanied by production and release of ROS, cytokines, and inflammatory mediators.¹⁵⁸ Cell proliferation in the context of chronic inflammation may result in accumulation of DNA damage and mutations that promote carcinogenesis. Recent work has suggested a direct link between asbestos-induced inflammation and mesothelial cell transformation. Asbestos-induced TNF- α release is associated with upregulation of TNF- α receptor on mesothelial cells, as well as NF- κ B activation, suggesting that activation of pro-survival pathways by inflammatory cytokines may prolong the exposure of mesothelial cells to the genotoxic effects of asbestos fibers.¹²⁶ Chronic inflammation accompanied by oxidant generation has also been associated with epigenetic gene silencing by hypermethylation.¹⁵⁹

Exposure to SWCNT in rodent models produces pulmonary inflammation and fibrosis. Fibrous nanomaterials can induce release of TNF- α and O₂[•] from human peripheral blood mononuclear cells, as well as frustrated phagocytosis and impaired macrophage function, although there is considerable variability among commercial CNT and CNF samples.⁷⁸ The importance of oxidative stress in physiological responses to fibrous nanomaterials is highlighted by recent work showing that mice fed diets deficient in Vitamin E, a potent antioxidant, showed an enhanced inflammatory and fibrotic response to SWCNTs.¹⁶⁰ These responses were accompanied by increased recruitment of inflammatory cells and enhanced production of pro-inflammatory (TNF- α and interleukin-6) and profibrotic (TGF- β) cytokines.¹⁶⁰

REMIEDIATION OF ASBESTOS FIBERS/GREEN NANOTECHNOLOGY

Although its use has been banned or restricted in many countries, asbestos remains a human health hazard throughout the world. Despite the established link between asbestos exposure and human disease, asbestos use continues in developing countries. Even in countries where asbestos use has been discontinued, fibers are still present in homes and schools in the form of insulation, ceiling, and roofing tiles or in the environment as naturally-occurring deposits. Liberation of asbestos fibers during demolition of buildings or disturbance of previously unidentified asbestos deposits could lead to new exposure scenarios. As iron mobilization from asbestos fibers is associated with increased free radical generation and DNA damage,^{39,128} selective removal of iron has been proposed as a method of asbestos remediation. Exciting recent work has shown that soil fungi¹⁶¹ and lichens¹⁶² are capable of detoxifying crocidolite and chrysotile asbestos, respectively, through chelation and removal of redox-active iron.

Unlike naturally-occurring asbestos fibers, fibrous carbon nanomaterials are manufactured and therefore more amenable to modification before use. Removal of redox-active metals through purification may reduce fiber toxicity prior to their use in various downstream applications. However, residual metal catalysts may persist because of incomplete purification or redeposition of mobilized metal onto the surface of the nanotube.²⁷ Acid-based purification may inadvertently result in increased metal bioavailability by damaging the protective carbon shells surrounding the catalysts in cases where the subsequent acid dissolution of the uncovered metal is incomplete. Evidence for all three mechanisms is provided by a recent study of commercial SWCNT, which addresses the source of bioavailable metal in purified SWCNTs and proposes metal removal as a potential detoxification strategy.²⁷ Additionally, tocopheryl polyethylene glycol succinate, a synthetic vitamin E analogue linked to polyethylene glycol, when used in carbon nanomaterial processing, has the potential for simultaneously acting as a surfactant to promote nanomaterial dispersion, and as an antioxidant, to ameliorate oxidative stress.¹⁶³

SUMMARY

The epidemic of asbestos-related diseases has been an unfortunate consequence of wide-scale use of fibers for industrial and consumer applications in the absence of the recognition and acceptance of their toxicologic and carcinogenic properties. Although an asbestos ban may go a long way toward preventing occupationally-related diseases, it cannot completely eliminate environmental exposures and continuing exposure to asbestos-in-place. Enormous effort has been devoted to identifying the properties of asbestos that determine its bioactivity. Currently, the toxicologic and carcinogenic properties of high aspect ratio nanoparticles await complete investigation, and it is unclear whether they will be equally, more, or less hazardous than asbestos fibers. Given the relative novelty of manufactured carbon nanomaterials, we are now presented with a unique opportunity to evaluate nanomaterial bioactivity prior to wide-scale use, and the inevitable scenarios of human exposure. The goal of nanotoxicology should not be to restrict production and use of nanomaterials, as has been done with asbestos, but to recognize that their use in industrial and consumer applications must be selective and balanced against their potentially harmful inherent properties. On the basis of the current understanding of asbestos pathogenicity, we can use this knowledge as a framework to guide the design and post-processing of carbon nanomaterials in a pre-emptive attempt to reduce the potential for exposure-related 'nanodiseases'.

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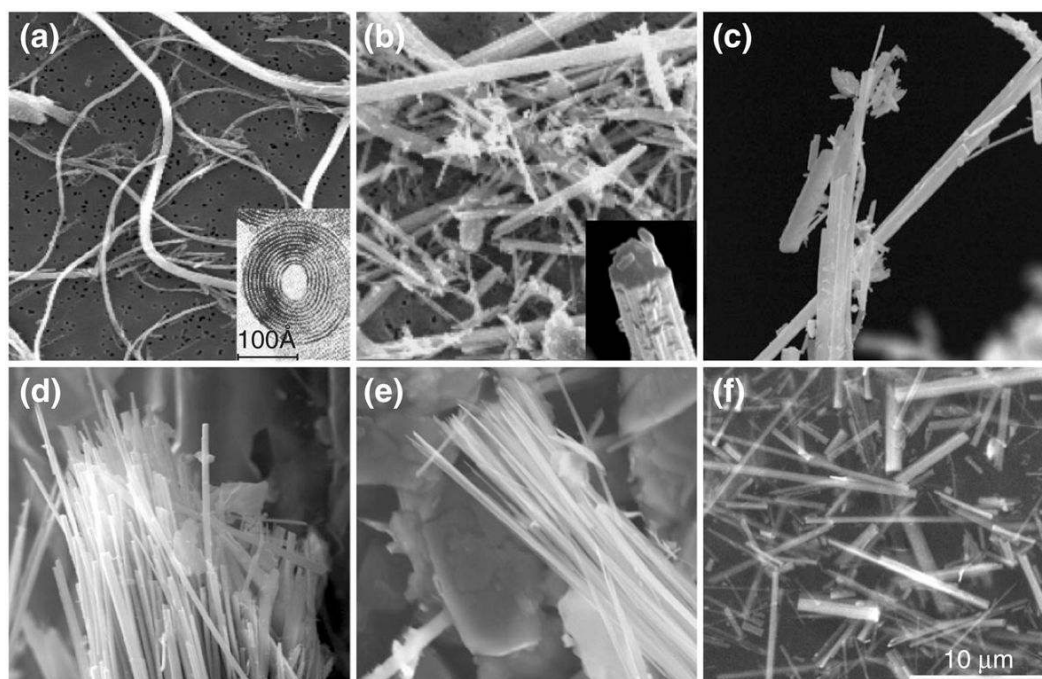
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**FIGURE 1.**

Structure of asbestos fibers by transmission electron microscopy (TEM): (a) serpentine and (b–f) amphiboles. (a) International Union Against Cancer (IICC) asbestos chrysotile ‘A’ standard, (b) IICC asbestos crocidolite standard, Death Valley, California, (c) IICC asbestos anthophyllite standard, (d) winchite-richterite asbestos, Libby, Montana, (e) tremolite asbestos and (f) IICC asbestos amosite standard. Chrysotile is the only member of the serpentine group. Because of the mismatch in the spacing between the magnesium ions and the silica ions, chrysotile curls into a thin-rolled, flexible sheet while amphibole fibers are more rigid. Scale bar = 10 μm . (Reprinted with permission from Denver Microbeam Laboratory at the U.S. Geological Survey).

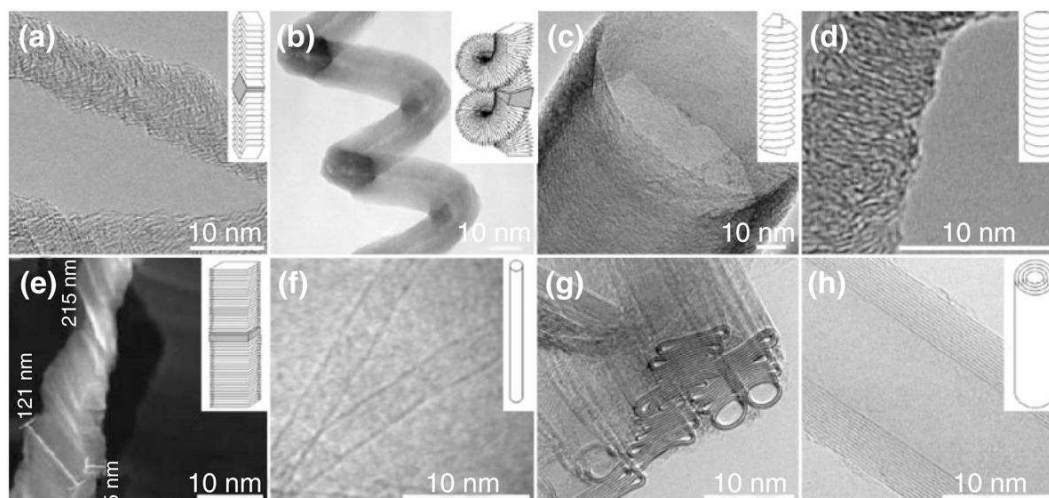


FIGURE 2.

Structure of some carbon fibrous nanomaterials by TEM: (a–e) carbon nanofibers (CNFs) and (f–h) Carbon nanotubes (CNTs); (a) Fishbone solid CNFs, (b) Platelet spiral, (c) Stacked-cup, (d) Platelet-symmetry, (e) Platelet, (f) single-wall CNTs, (g) double-walled CNTs, and (h) multiwalled CNTs. Scale bar= 10 nm. TEM sources: (a) Chen, 2005,¹⁵ (b) Bandaru, 2007,¹⁶ (c) Vera-Agullo, 2007,¹⁷ (d) Jian, 2006,¹⁸ (e) Zheng, 2006,¹⁹ (f) www.rsc.org, (g) www.msm.cam.ac.uk, and (h) www.nccr-nano.org. Inset sources: (a–c, e, f, and h) Martin-Gullon, 2006²⁰ and (d) Jian, 2006.¹⁸

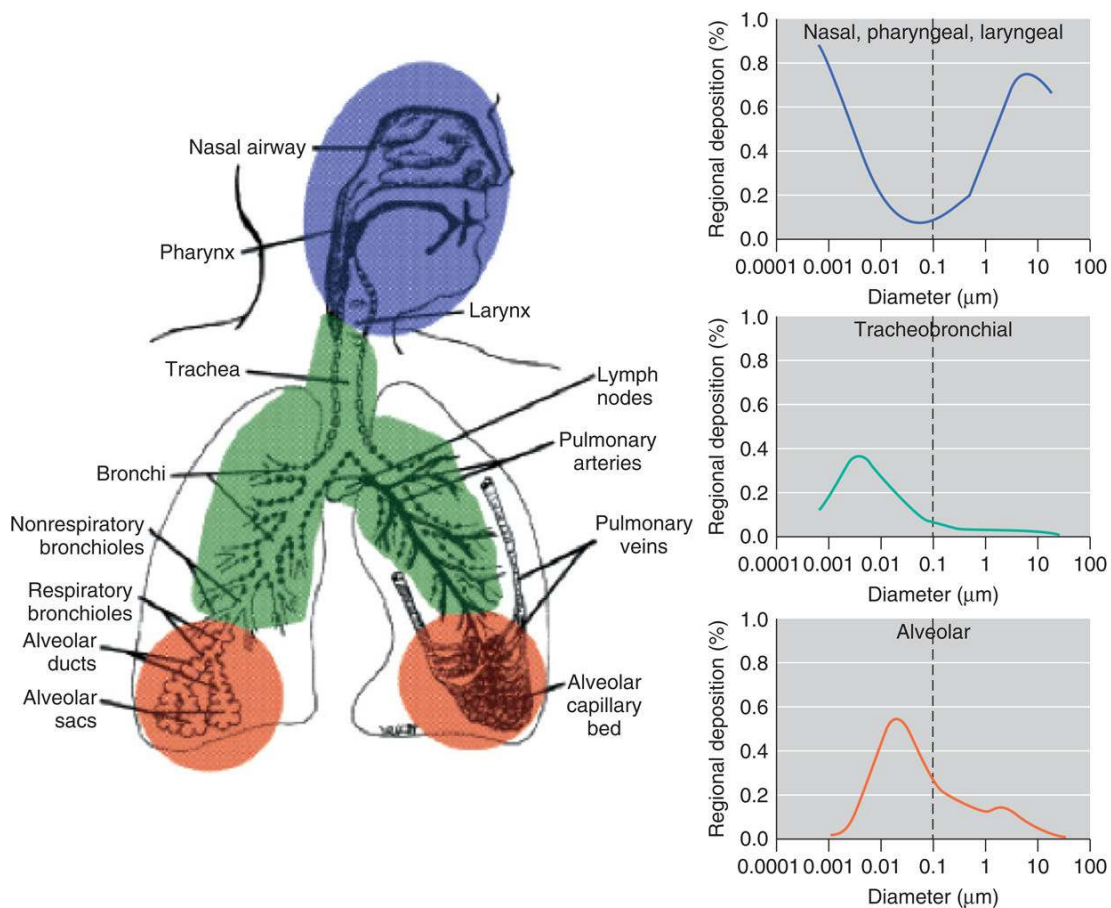


FIGURE 3. Relationship between aerodynamic diameters of particles and lung deposition. (Reprinted with permission from Ref ²⁸. Copyright 2005 National Institute of Environmental Health Sciences).

TABLE 1

Classification and Chemical Composition of Asbestos Fibers

Mineral group	Mineral species	Asbestiform variety	Ideal chemical composition
Serpentine	Clinochrysotile	Chrysotile	$Mg_3Si_2O_5(OH)_4$
Serpentine	Orthochrysotile	Chrysotile	$Mg_3Si_2O_5(OH)_4$
Serpentine	Parachrysotile	Chrysotile	$Mg_3Si_2O_5(OH)_4$
Amphibole	Riebeckite	Crocidolite	$Na_2Fe_3Si_8O_{22}(OH)_2$
Amphibole	Grunerite	Amosite	$(FeMg)_7Si_8O_{22}(OH)_2$
Amphibole	Cummingtonite	Amosite	$(MgFe)_7Si_8O_{22}(OH)_2$
Amphibole	Gedrite	Amosite	$(MgFe)_5Al_2(Si_6Al_2)O_{22}(OH)_2$
Amphibole	Anthophyllite	Asbestiform anthophyllite	$(MgFe)_7(Si)_8O_{22}(OH)_2$
Amphibole	Tremolite	Asbestiform tremolite	$Ca_2 Mg_5Si_8O_{22}(OH)_2$
Amphibole	Actinolite	Asbestiform actinolite	$Ca_2(MgFe)_5Si_8O_{22}(OH)_2$
Amphibole	Richterite	Asbestiform actinolite	$Na_2Ca(MgFe)_5Si_8O_{22}(OH)_2$
Amphibole	(Alumino) winchite	Asbestiform winchite	$CaNa (MgFe)_4AlSi_8O_{22}(OH)_2$
Amphibole	Ferriwinchite	Asbestiform winchite	$CaNa (MgFe)_4Fe^{3+} Si_8O_{22}(OH)_2$

Source: IOM, 2006.⁶

TABLE 2

Common Methods for Carbon Nanomaterial Synthesis

Synthesis method	Carbon precursor	Catalyst	Energy source	CNFs Symmetry	CNTs
CVD/PECVD HipCO	Gas-phase precursors (methane, carbon monoxide, synthesis gas (H ₂ /CO), ethyne, and ethane)	Fe, Co, Ni Cr, V, and Mo (alone or in combination, as powder or supported)	Heat/radio frequency, direct current glow discharge, inductively coupled plasma or microwave (500 to 1000 °C)	Platelet (iron powder) Tubular (iron supported on silica) Stacked-cup	Single wall/Multiwall
Nanochannel templating	Gas-phase CCVD precursors (as above; discolic liquid crystals	CVD catalysts as above, or non-catalytic	Heat (from 300 to 1000 °C)	Platelet Carbon/carbon composite Nanoribbons	Multiwall
Electric arc discharge	Graphite rod electrodes in inert gas (Ar or He)	Ni, Y	Electric discharge (2000 to 3000 °C)		Single wall (metal-doped electrodes)/ Multi wall (pure graphite electrodes)
Laser ablation (vaporization)	Graphite in Ar flow	Co, Ni	High intensity laser (Nd:YAG) pulses in a high temperature atmosphere (1200 °C)		Single wall (metal-doped electrodes)/ Multi wall (pure graphite electrodes)

PECVD, plasma-enhanced chemical vapor deposition; CCVD, catalytic chemical vapor deposition; Nd:YAG, neodymium-doped yttrium aluminum garnet

TABLE 3

Selected Studies of *In Vitro* Toxicity of Synthetic Fibrous Nanomaterials

Material	Metal content (%)	Cell type	Toxicity assay	Results	Reference
SWCNT/MWCNT	Fe, Y, Ni (trace) Ni (0.6)	Guinea pig alveolar macrophages	MTT	SWCNT were more toxic than MWCNT at an early timepoint (6hr)	(93)
SWCNT/MWCNT	Fe (≤ 0.009), Ni ($< 0.005-1.86$), Co (≤ 2.8), Mo (≤ 4.2)	Rat alveolar macrophages	MTT WST-1 LDH release PI/Annexin V	Neither raw material nor highly purified SWCNT and MWCNT were toxic after 24hr	(101)
MWCNT/CNF	N.D.	Human lung epithelial (H596)	MTT	MWCNT and CNF showed dose-dependent toxicity CNF>MWCNT	(94)
SWCNT	Ni (1.2), Co (1.3)	Human lung epithelial (A549) Rat alveolar macrophages	MTT	SWCNT were nontoxic up to 96hr	(98)
SWCNT	Highly purified	Human monocyte-derived macrophages	PI/nuclear morphology	Highly purified SWCNT devoid of contaminating metal were nontoxic	(99)
SWCNT	Fe (< 0.013)	Human lung epithelial (A549) Human mesothelioma (MSTO-211H)	MTT Hoechst	Unpurified material showed greater toxicity than purified bundles SWCNT were more toxic to MSTO-211H than A549	(95)
SWCNT	N.D.	Human lung epithelial (A549)	MTT Alamar Blue Coomassie Blue	SWCNT showed low acute toxicity following 24hr exposure	(100)
SWCNT	Fe (< 0.013), Ni (2.4-13.8), Y (0.5-1.6)	Human mesothelioma (MSTO-211H)	MTT Hoechst	SWCNT nanoropes >crocidolite >dispersed SWCNT	(65)
MWCNT	N.D.	Murine macrophages Human macrophages Human lung epithelial (A549)	MTT	MWCNT samples were toxic to all cell lines, with EC50 (SWCNT) \leq EC50 (chrysotile)	(97)
MWCNT	Fe (0.47), Co (0.95), Al (0.05)	Rat peritoneal macrophage	LDH release	Ground, but not intact MWCNT, induced dose-dependent toxicity	(66)

N.D., not determined

TABLE 4

In Vivo Toxicity of Fibrous Nanomaterials

Material	Metal content (%)	Dose	Route of exposure	Results	Reference
SWCNT			i.t. (mouse)	Induction of granulomas, fibrotic lesions	(70)
SWCNT	Ni (5), Co (5)	1 mg/kg, 5 mg/kg	i.t. (rat)	Induction of multifocal granulomas	(84)
SWCNT		0.5mg	i.t. (mouse)	Induction of chronic inflammatory response, with formation of pulmonary granulomas and activation of NF- κ B, AP-1	(74)
SWCNT	Fe (0.23)		Pharyngeal aspiration (mouse)	Dispersed SWCNT produced diffuse interstitial fibrosis, while agglomerated SWCNT induced granulomas	(80)
MWCNT		12.5mg	i.t. (guinea pig)	Induction of granulomatous response	(106)
MWCNT	Fe (~ 1) Co (0.95)	0.5–5mg	i.t. (rat)	Intact material showed greater biopersistence; ground MWCNT were cleared more readily	(66)

i.t.: intratracheal

TABLE 5

Direct Mechanisms of Fiber Carcinogenicity

Mechanism	Endpoints	References
<i>Genotoxic</i>		
	Oxidized bases	107,108
	DNA breaks	109
	Aneuploidy	109,110
	Mutations	111
	Deletions	112
<i>Nongenotoxic</i>		
Mitogenic	Target cell proliferation	113,114
	Binding, activation of growth factor receptors	44,115
	Growth factor expression	116,117
	Activation of signaling pathways	85,118
Cytotoxic	Apoptosis	114,119,120
	Necrosis	121

TABLE 6

Indirect Mechanisms of Fiber Carcinogenicity

Mechanism	References
Cofactor with tobacco smoke	121–123
Epigenetic gene silencing	124
Persistent inflammation with secondary genotoxicity	125
Persistent inflammation with release of cytokines, growth factors	117,126