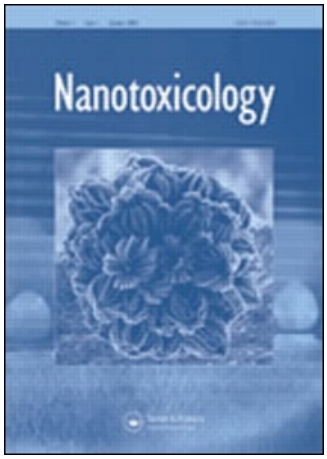

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Carbon nanotubes and their toxicity

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Abstract

The vital need of studying the toxicological profile of carbon nanotubes (CNTs) has emerged from the rapidly enhancing utility of CNTs in the field of nanobiology and drug delivery. This review highlights the vivid aspect of CNTs' toxicity comprising of *in-vitro* to *in-vivo* toxicological profile vis-à-vis the various potential routes of CNTs exposure. The article also underlines the various surface modifications on carbon nanotubes and its role in imparting biocompatibility to the CNTs, further suggesting their utility as a safer delivery module for bioactives.

Keywords: *Carbon nanotubes, toxicity, fullerenes, carbon nanoforms, functionalization, biocompatibility*

Introduction

Nanotechnology, a contemporary discipline, has emerged in the field of cell biology in the form of nanosized particles ranging from nanoliposomes (Dubey et al. 2007) to dendrimers (Bhadra et al. 2005) and quantum dots to carbon-based nanoparticles including nanotubes, nanofibers and fullerenes (CNTs). Nanotechnology is an applicable aspect of a broader area of nanoscience (nano means 10^{-9} m), which is one of the upcoming and highly challenging as well as a rewarding key research area in the modern scientific set-up. It is the science of small particles having unique properties, which change on altering the size of the particles as well as the melting point, electronic and optical properties (Martin & Kohli 2003). In this context, different nanocomposites have been employed or proposed in areas as diverse as from imaging to the tissue engineering (Iijima 1991; Koerner et al. 2004; Lin et al. 2004; Sen et al. 2004), microelectronics, coating and paints, as well as in the field of biotechnology (Martin & Kohli 2003). Further, these nanosized particles could be used as the visual indicators in over-the-counter medical diagnostic kits (Martin & Mitchell 1998) in the form of gold nanoparticles. Other examples of nanosized particles in biomedical sciences and in biotechnological fields include their use as a vehicle for enzyme encapsulation (Chang & Prakash 2001), DNA transfection (Radlar et al. 1997; Koltover et al. 1998; Kneuer et al. 2000), biosensors (Cao et al. 2002; Demers et al. 2002; Park et al. 2002) and drug delivery (Ulrich et al. 1999; Lee et al. 2002)

In this nanotechnological development, CNTs have attracted a great deal of attention due to their unique structural, electrical and mechanical properties. Some of the properties of CNTs for example, superior strength; flexibility, electrical conductivity and easy chemical functionalization (Chen et al. 1995; Hamon et al. 1999; Zhao et al. 2004) have been exploited for the biological application both at the cellular and molecular level. The CNTs have the unique absorption in the near infrared region, and specific properties of fluorescence can be used for biological sensing (O'Connell et al. 2002; Cherukuri et al. 2004; Barone et al. 2005). Furthermore, CNTs have also been employed for the cancer therapy (Kam et al. 2005). A variety of cells can engulf the CNTs, so they can also be employed as an intracellular delivery module for biologically active cargo (Cherukuri et al. 2004; Kam et al. 2004; Kam & Dai 2005; Pantarotto et al. 2004b). Although non-dispersible in any solvent and non-biodegradable in nature, they are inherently cytotoxic, thus imposing limitation on their use in the biological systems (Shvedova et al. 2003; Cui et al. 2005; Jia et al. 2005) and hence posing a challenge to pharmaceutical drug delivery scientists to modulate it as a

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vector for drug delivery. The present review describes the different toxicities of CNTs studied in *in vitro/in vivo* systems, effect of functionalization of carbon nanotubes on their toxicity and curbing the toxicities of carbon nanotubes in order to realize their possible potential.

Nanosized particles and Nanotoxicology

Development of a vast variety of nanomaterials simultaneously imposed a need for the assessment of the potential health and environmental effects. It has become another source of human exposure to the different nanoparticulate matter by different routes, i.e., inhalation (respiratory tract), ingestion (gastrointestinal tract), dermal (skin) and injection (blood circulation) (Oberdörster et al. 2005a). These nanomaterials have been ascribed various terminologies. In general nanosized particles (NSPs) include all the engineered and nanosized special particles <100 nm and engineered (specially engineered in laboratory) nanoparticles defined by their shapes, e.g., nanotubes, nanofibers, nanowires and nanorings and so on. Apart from these the nanomaterials are variably called ultrafine particles (UFPs) by toxicologist (Oberdörster et al. 2005a), air taken mode and nucleation particles by atmospheric scientists (Oberdörster et al. 2005a) and engineered nanostructured materials by material scientist (Oberdörster et al. 2005a).

Due to the extraordinarily high number concentration of NSPs per given mass, they would have toxological significance, when these particles interact with cell and subcellular component. Another toxicologically significant characteristic is that they are having increased surface area per unit of mass. The study of the adverse effects of nanosized particles and fibers and their interaction in the living organism has been termed as *Nanotoxicology.* Taylor and Francis have recently introduced a research journal, *Nanotoxicology*, that offers an exclusive forum encompassing studies that enhance safety during the production, use and disposal of nanomaterials (*Nanotoxicology 2007*).

Specific properties of nanosized particles are due to small size and corresponding enormous surface area. When the ratio of surface to the atom or molecules increases exponentially, surface reactivity also increases leading to the greater biologic activity per given mass as compared with the larger particles. This increased biologic activity can be either positive or desirable (i.e. antioxidant activity, carrier capacities, for therapeutics and penetration of cellular barriers for drug delivery) or can be negative and undesirable (e.g., toxicity, induction of oxidative stress or cellular dysfunction). A nanosized particle could have the potential to cause the toxicity. Certain properties determine the toxicity of nanosized particles:

- The surface area/mass ratio of the particles; if the particle is having the larger surface area, it provides the greater contact with the cellular membrane, and also provides greater capacity for adsorption and transport of toxic substance.
- Retention of particles within a physiological environment; retention time determines the cellular contact and hence causes the greater chances for damage. Retention time also determines its mobility either through clearance or migration to surrounding tissue.
- Inherent toxicity of the contaminants present in nanomaterials.

The basic idea of nanomaterials' toxicity can be revealed by its lung deposition. The lung deposition of a nanosized material depends upon its surface area/mass ratio. A study on the toxicity of the nanosized particles reports the effect of high purity carbon black (14 nm) and carbon black (16 nm) on pulmonary tissue in which the high purity carbon black caused the increased oxidative stress (Smart et al. 2006) in human type II alveolar epithelial cells *in vitro*, increased murine alveolar macrophage migration in fetal-calf serum nearly two folds as compared high purity carbon black (260 nm). On the other hand carbon black (16 nm) causes the development of the pulmonary tumor in rates by subchronic inhalations. Although C_{60} fullerenes do not show significant toxicity, it shows the rapid distribution in rates and deposition in many tissues like brain, liver and spleen.

Carbon-based nanosized particles

With the advancement of nanotechnology, various engineered nanomaterials are rapidly proliferating. These nanomaterials are designed and produced to have structural features with at least one dimension of 100 nm or less. Engineered nanomaterials pose nanostructure-dependent properties, i.e., chemical, mechanical, electrical, optical, magnetic, and biological, which make them to be useful as for commercial and medical application (Oberdörster et al. 2005b). Carbon nanoforms have been a component of natural atmosphere since combustion was discovered and human being have been constantly exposed to it (Kang et al. 2006). Various carbon-based biomaterials or nanostructured materials have been discovered so far. One of them is pyrolytic carbon (Smart et al. 2006) that has been employed for several decades in biomedical implants and coating, particularly in the manufacturing of heart

valve prostheses although it was initially developed for the aerospace and nuclear industries. Two forms of pyrolytic carbon have been found, one of them is anisotropic and the other is isotropic which is developed by a special chemical vapor deposition process. The isotropic form of carbon atoms possesses special biocompatibility properties.

Another carbon-based biomaterial is diamond-like carbon. It is a metastable form of carbon having high hardness, low coefficient of friction, chemical inertness and good corrosion and wear resistance (Smart et al. 2006). Biocompatibility of diamond like carbon is revealed by the application of its coating for cardiovascular and orthopedic application (Smart et al. 2006).

Besides these biomaterials various forms of carbon based nanosized particles like fullerenes, carbon nanofoam, carbon nanohorns and carbon nanotubes are also available. Carbon nanotubes are discussed later in this section.

Fullerenes

Fullerenes are the molecules of carbon formed into hollow cage like structure. The most prevalent form of fullerene is C_{60} or buckminster fullerene (buckyballs). It is made up of 60 carbon atoms arranged in a ball shaped of hexagonal and pentagonal panels. Fullerenes having different no of carbon atoms (e.g., C_{70}, C_{76}, C_{84}), fullerene derivatives (with other atom inserted within the structure) and endohedral fullerenes also exist as present in the buckyballs with shell around them (Becker et al. 2000).

Carbon nanofoam

Another carbon-based nanomaterial is the fifth allotrope of carbon, i.e., carbon nanoforms. These are the clusters of carbon atoms (with an average diameter of 6–9 nm), randomly interconnected to form a web-like structure. It can act as a semiconductor due to its extremely lightweight and spongy solid characteristics. It also possesses magnetic character in contrast to other carbon nanoforms; due to heptagonal arrangement of carbon atoms and also contains some iron and nickel as trace impurities (Lacerda et al. 2006).

Carbon nanohorns

Carbon nanohorns are a a recently discovered carbon nanostructure in the form of spherical aggregate of graphite tubes. One main form of carbon nanohorns is Single Wall Carbon Nanohorns (SWNHs) having unique physicochemical property like large surface area, suggesting their possible utility as carriers in drug delivery systems (Murata et al. 2002). SWNHs are aggregates of graphitic tubes that have closed ends with cone-shaped caps (horns). Each tube has a diameter of 2–3 nm, which is larger than the 1.4 nm of typical single-wall CNTs, while the aggregates are 80–100 nm in diameter and have 'dahlia-like' or 'bud-like' spherical structures (Murata et al. 2001) Surface area of these nanomaterials can be further enlarged by oxidizing the SWNH walls, creating the nanowindows which facilitate the entry of small molecules [e.g., N_2, Ar, and fullerene (C60)] (Murata et al. 2002).

Nanotubes

The emerging field of research is related to the conception and fabrication of small-scale system for drug delivery (Lacerda et al. 2006). These include micro and spherical nanoparticles, liposomes, micelles, dendrimer and different polymers (Kostarelos 2003). Micro and nanotube structure are alternative to the spherical nanoparticles. The spherical C60 molecule was discovered in 1985 by Harry Kroto and Richard Smally through experiments with laser vaporization of graphite. Carbon nanotubes were later observed through efforts in fullerene research and were discovered by Sumio Iijima in 1991. The Kroto and Smally group detected the predominant generation of C60 and hypothesized that it may exist as a stable closed assembly. Later work by Wolfgang Krätschmer and Donald Huffman produced larger quantities of fullerenes through an arc-discharge process and confirmed the proposed spherical structure of C60. Through transmission electron microscopy (TEM), Iijima scrutinized carbon soot produced by the Krätschmer and Huffman method where he found CNTs. The initial discovery was of Multiwalled carbon nanotubes (MWCNTs) the smallest being double-walled, and later discoveries in 1993 independently reported on the first SWCNTs (Iijima 1991).

Nanotube technology is a field of research in full expansion in the current scenario. Nanotubes offer some special advantages over spherical nanoparicles (Martin & Kohli 2003).

(a) Nanotubes have longer inner volume (relative to diameter of nanotube), which can be filled with desired chemical or biochemical species, ranging in size from small molecules to proteins (Mitchell et al. 2002);
(b) Nanotubes have distinct inner and outer surface which can be differentially modified for chemical or biochemical functionalization (Mitchell et al. 2002).This makes the possibility of loading a moiety on inside of

nanotube, and at the same time imparting chemical features to the outer surface that render it biocompatible;
(c) Nanotubes have open end, which makes the inner surface accessible and subsequent incorporation of species within nanotube is particularly easy.

The different types of nanotubes so far discovered are:

(a) Organo silicone nanotube (Linksy et al. 1971);
(b) Self-assembling lipid microtubules (Martin & Kohli 2003);
(c) Fullerenes carbon nanotubes (Iijima 1991);
(d) Template synthesized nanotubes (Martin 1994);
(e) Peptide nanotubes (Ghadiri et al. 1994).

Among the different types of nanotubes, carbon nanotubes (CNTs) are very promising and are gaining a lot of interest.

Carbon nanotubes

Carbon nanotubes are cylindrical molecules composed solely of carbon atoms. This nanocomposite belongs to the family of fullerenes, the third allotropic form of carbon (others are graphite and diamond) (Lacerda et al. 2006). They can be thought of as a seamless cylinder formed from a graphitic sheet with a hexagonal lattice structure but end capped with pentagons (Iijima et al. 1992). The CNT ends resemble hemispherical buckyballs connected by a graphene cylinder. The properties of individual CNTs vary depending on their atomic structure. The CNTs can be obtained as single wall carbon nanotubes (SWCNTs) characterized by the presence of one layer of cylindrical graphene or multiwall nanotubes (MWCNTs), made up of concentric graphene sheets. Mostly they have a diameter (SWCNTs: 0.4–3.0 nm and MWCNTSs: 1.4–100 nm) and length (SWCNTs: 20–1000 nm and MWCNTSs: 1–50 μm) in nanometric range. Multi-walled carbon nanotubes (MWCNTs) has been produced by arc discharge laser ablation and chemical vapor deposition or gas phase catalytic process (HiPco) methods (Park et al. 2002). The physical structure of carbon nanotube (CNTs) confers extreme strength and electrical conductivity.

Characteristics of carbon nanotubes

Three common forms of CNT raw materials have been proposed so far (Figure 1) (Kang et al. 2006). These are Single Wall Carbon Nanotubes (SWCNTs), Multi-Wall Carbon Nanotubes (MWCNTs) and Carbon Nanofibers (CNF). Various combinations of these forms have been used to produce smart materials. These nanotube materials are commercially available (Carbon Nanotechnologies) and can also be synthesized using commercial Chemical Vapor Deposition (CVD) systems. Computer controlled CVD nanofurnace (Peng & Cho 2000; Hata et al. 2004) to grow SWCNTs and MWCNTs is also available. Electronic property of nanotubes depends upon its atomic structure, mechanical deformation and chemical doping. Alteration of these properties can induce strong changes in electrical conductance of the nanotube (Peng & Cho 2000). The electrical impedance of CNTs was shown to be very sensitive to chemical exposure (Collins et al. 2000; An et al. 2004) and mechanical deformation (Tombler et al. 2000). The properties of carbon nanotubes vary with the types of nanotube (Carbon Nanotechnologies). The various properties of nanotubes that make the nanotubes as a smart fibers material are summarized in Table I.

Toxicity of carbon nanotubes

Engineered nanomaterials may have various shapes like spheres, fibers, tubes, rings and planes. Toxicological studies of spherical and fibrous particles have been well established, i.e., natural asbestos and man-made, e.g., biopersistance vitterous fibers are associated with increased risk of pulmonary fibrosis and cancer of the prolonged exposure (Oberdörster et al. 2005a). Since the carbon nanotubes possess nanosized dimension and fibrous structure, they are prone to be toxic. In general, fibers are defined as elongated structure with a diameter to length ratio (aspect ratio) 1:3 or greater and with a length of >5 μm and diameter <3 μm (World Health Organization [WHO] 1985). Carbon nanotubes have aspect ratio of up to >100, and length can exceed 5 μm with a diameter ranging from 0.7–1.5 nm for single wall nanotubes and 2–50 nm for multiwall nanotubes. Single walled nanotubes possess strong Vander walls forces and self-organize in the rope like structure that can range in length up to several microns. The carbon nanotubes as nanosized particles affect the physiological system, depending upon various portals. However potential health hazard of inhaled carbon nanotubes have not been sufficiently evaluated but several results of *in-vitro* and non-aerosol associated *in-vivo* studies suggests that exposure of these material could lead to significant hazard.

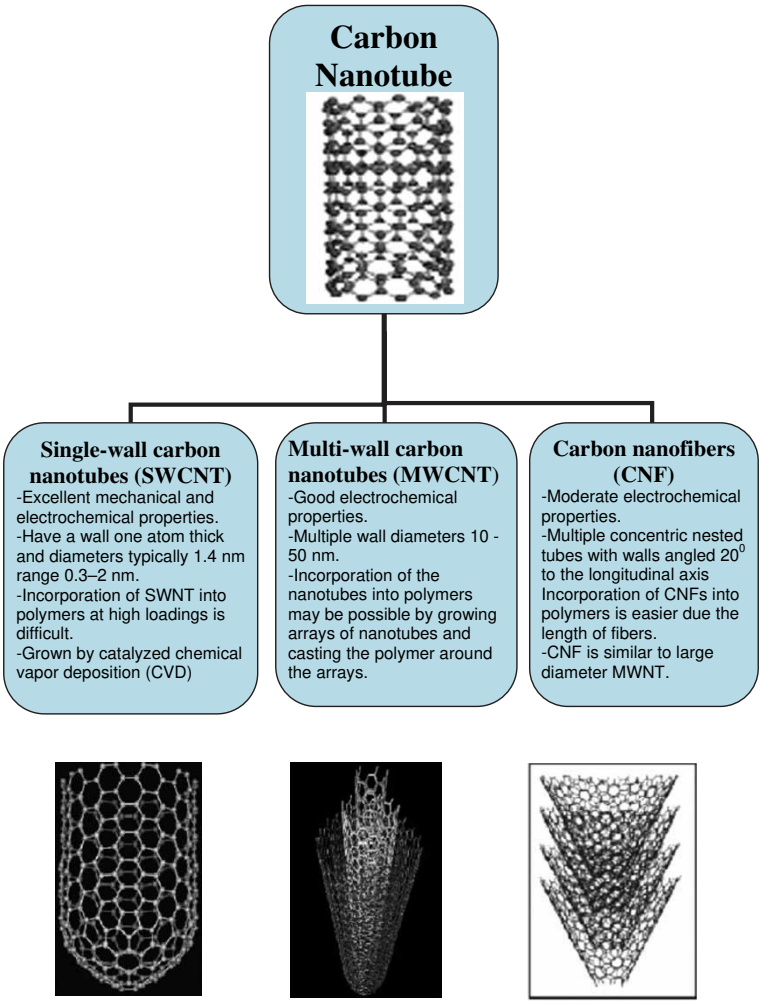


Figure 1. Various forms of carbon nanotubes and their general properties (Kang et al. 2006).

Possible routes of exposure of carbon nanotubes and their biokinetics

Carbon nanotubes are highly mobile in both humans and environment and thus could enter the body through several portals including skin, lungs and ocular routes. Carbon nanotubes or various carbon nanoparticles can enter the body through several portals. They include mainly skin, lungs and eyes. Afterwards the translocation can occur via the blood stream leading to an accumulation in many tissue including kidney, muscles, skin, spleen and femur (Singh et al. 2006). However carbon black particles are thought to interfere with cell signaling (Brown et al. 2004). It was observed when DNA was used for the size separation of nanotubes (the DNA wraps itself around the nanotubes if the tube diameter is right) (Zheng et al. 2003) but this observation equally raises concerns over the effect of carbon nanotubes on the human body.

Skin as a portal of entry

Human skin (*ca*. 1.5 m^2 in area in an adult human) normally functions as strict barrier (Argyle & Robinson 2006). The epidermis of the skin forms a very tight protective layer for the underlying dermis. The dermis has a rich supply of blood and tissue macrophages, lymph vessels, dendritic cells (Langerhans, also in stratum spinosum of epidermis), and five different types of sensory nerve endings. The CNTs may enter through dermis into the blood circulation followed by uptake into the lymphatic system and regional lymph nodes. The CNTs may provoke the immune response after transporting into the lymph nodes (skin macrophages and dendritic: Langerhans cells). Chen et al. (1998) were able to raise antibodies in mice specific for C60 after intraperitoneal injections of C60 conjugated to thyroglobulin and serum albumin (Chen et al. 1998). Another study reported

Table I. General properties of nanotubes.

1	Elastic and thermal properties (Arepalli et al. 2001)	• Stiffer known fiber. (Ball 1999) • Young's constant 1–1.4 tera Pa. (Vigolo et al. 2000; Yu et al. 2000) • Tensile strength >50 Pa. • Thermal conductivity in the direction of nanotubes – 1700–5800 W/mk (Hone 2001; Cumings & Zettl 2000). • Strength to weight ratio of nanotubes in the axial direction is 4 times greater than carbon reinforcing fibres (Schulz et al. 2005).
2	Electrical conductivity (Ball 2001)	• Metallic or semi conducting, depending on chirality. • Have the capability to conduct current ballistically without dissipating heat. • Conductance is independent of diameter and strength. • Resistance is affected by temperature and magnetic field. • High current density 109 A/cm^2. • Resistivity 10^{-4} Ω cm at 300 K (Kang et al. 2006).
3	Magnetoresistance (Mehrez et al. 2000)	• It is a spin dependent transport properties
4	Piezoresistance (Kang et al. 2006)	• It depends upon chirality of nanotubes. • It is a promising property for sensing.
5	Electrokinetic of nanotubes (Hughes 2000)	• Nanotubes have different electrical properties like conductivity and dielectric properties.
6	The piezoelectric properties (Lebedev et al. 2001)	• The piezoelectric effect of nanotube is very small.
7	Electrochemical effect (Kang et al. 2006)	• The electrochemical properties can generally large strain/force using low voltages. • The electrochemical property of CNTs is considered provision for actuation.
8	Telescoping nanotubes (Cumings & Zettl 2000)	• MWCNTs have been considered as rotational and translational bearings. • Employed as nut and screw for building nanomedicines, due to spiral chirality.
9	Power generation (Kang et al. 2006)	• Due to ionic flow over the nanotubes surface. • Property is promising for medical application and flow sensing.

(Monteiro-Rivierea et al. 2005) the exposure of CNT (grown using a microwave plasma enhanced chemical vapor deposition system) to the human epidermal keratinocytes (HEK). The results of the study discussed in detail in later section, revealed that MWCNTs, not derivatized or optimized for biological applications, are capable of both localizing within and initiating an irritation response in a target epithelial cell that composes a primary route of occupational exposure for manufactured nanotubes.

Lungs as portal of entry

Airways are a relatively robust barrier, in the gas exchange area (300×10^6 alveoli), the barrier between the alveolar wall and the capillaries is very thin merely 0.5 μ away from the blood flow (Argyle & Robinson 2006). Since various nanomaterials and devices including the carbon nanotubes, carbon nanoparticles etc. are formed from, or use, aerosols and colloidal suspensions so that exposure is most likely to occur through lung inhalation (Maynard et al. 2002). Spherical solid material can be inhaled when its aerodynamic diameter is <10 μm and so nanoparticles travel deeper into the lungs and deposit in the alveoli via Brownian motion. If inhaled concentrations are low, then the retention time is about 70 days (Argyle & Robinson 2006). Main mechanism of inhaled NSPs is diffusion when they collide with the air molecule; electrostatic precipitation may also occur for the nanosized particles, which carry the significant charge. Depending upon size and physical structure of nanosized particles, they are deposited in the different regions of the respiratory tract (Oberdörster et al. 2005a). After deposition, the nanosized particles are translocated to the extrapulmonary site and reach the target organ site by different transfer route and mechanism. The nanomaterials access the blood circulation probably by transcytosis across the respiratory tract into the interstitium. Clearance of the deposited particles in the respiratory tract is basically by two mechanisms: (a) Physical translocation of particles by different mechanisms, and (b) chemical clearance process. Chemical dissolution is directed at the biosoluble particles or the components of the particles, which are either the lipid soluble or soluble in the intracellular or extracellular fluids. After that the soluble component of the particles undergoes absorption and diffusion or binding to proteins and other subcellular components (Oberdörster et al. 2005a). A possible pathway leading to Inflammation and longer-term effects as a result of sustained inhalation exposure to Carbon Nanotubes (CNTs) is given in Figure 2. A very recent study regarding interaction of carbon nanotubes and lung proteins is discussed later (Morales et al. 2007).

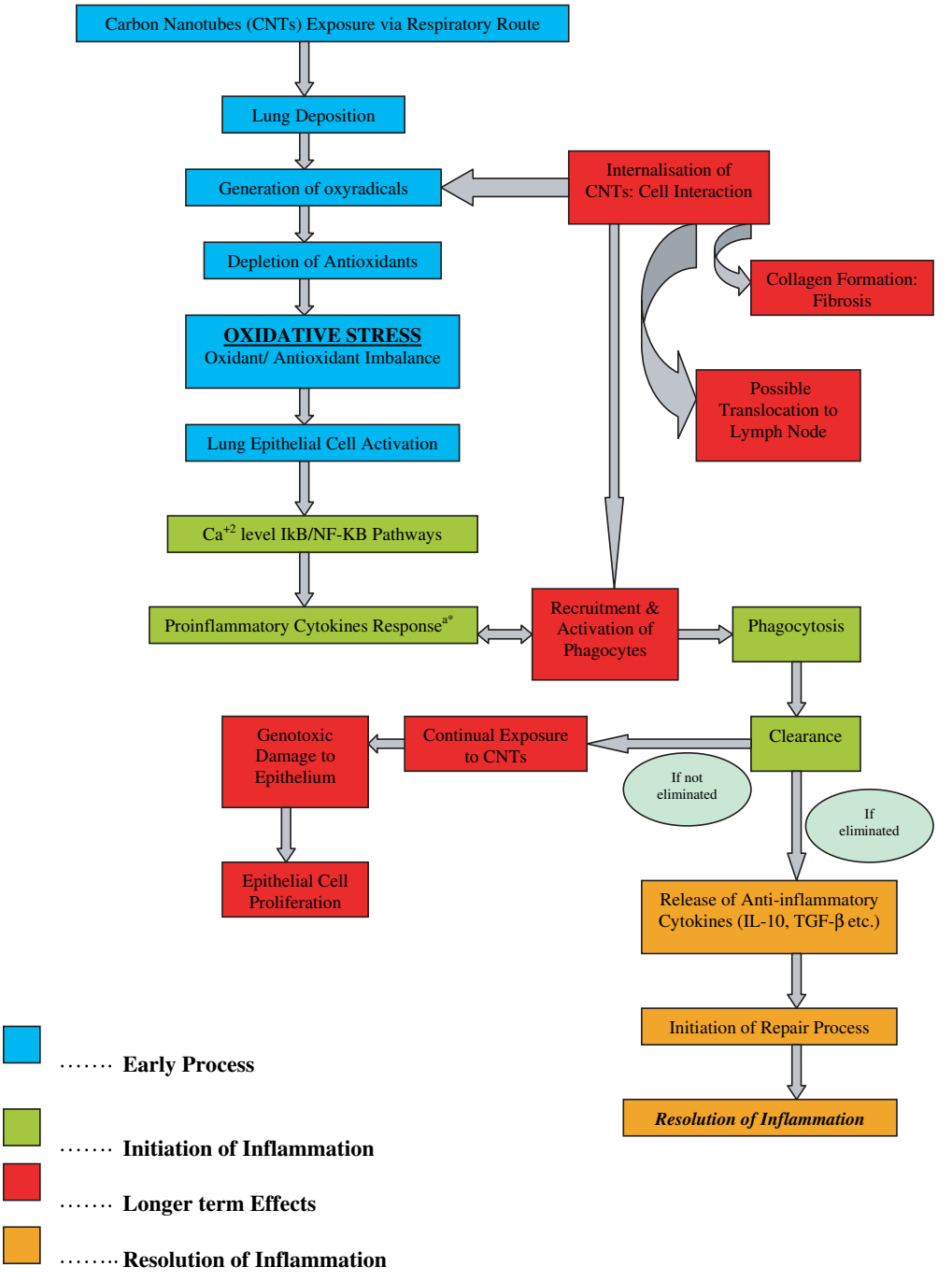


Figure 2. Possible pathways after the sustained inhalation to carbon nanotubes (CNTs). (Faux et al. 2003). a*(Muller et al. 2005).

Once the nanosized particles have reached pulmonary interstitial sites, uptake into the blood circulation, in addition to lymphatic pathways, can occur; again, this pathway is dependent on particle size. Berry et al. (1997) were the first to describe translocation of NSPs across the alveolar epithelium using intratracheal instillations of 30-nm gold particles in rats. It has been suggested by Oberdörster (2004a), that 35 nm carbon particles are mainly translocated to liver. However Cagle et al. (1999) showed that metallo-fullerene having size <220 nm accumulated mainly in bone marrow after liver; and continuously increased in bone marrow but decreased in liver (mouse). Another group of researchers (Oberdörster et al. 2005a) also reported the neuronal translocation of carbon particles ^{13}C particles (CMD ~ 36 nm) in olfactory bulb after whole-body inhalation exposure in rats.

On the contrary, the non-biodegradable fibers show a different toxicity paradigm, as far as the lung toxicity is concerned. The various nonbiodegardable fibers are exemplified by asbestos, synthetic

vitreous fibers (SVF), refractory ceramic fibers, synthetic organic fibers, nanotubes and nanowires (Donaldson & Tran 2004). These fibers pose health risk mainly by respiratory routes, i.e., by inhalation. These respiratory fibers have the ability to cause lung disease such as fibrosis, lung cancer and mesothelima. It is a dimension of the fiber that affects the respirability and clearance of fibers. Long fibers cannot be adequately phagocytosed by the lung macrophages and result into the frustrated phagocytosis or multiple phagocytosis of one fiber by several macrophages. This leads to the stimulation of cells to release inflammatory mediators (Ye et al. 1999) and ultimately leads to fibrosis. The short fibers are easily phagocytosed and are mechanically cleared by macrophages.

Biodistribution of carbon nanotubes and possible biokinetics:

Because of nanoscale dimension and carbon backbone of carbon nanotubes, carbon nanotubes could also exert a number of harmful effects. They may arise from the capability to readily enter the respiratory tract (portal of entry), depositing in the lung tissue, redistributing from the site of deposition, escaping from the normal phagocytic defense mechanism and modifying the structure of proteins. When the nanotubes are administered by, i.v., route, they could be translocated via the blood circulation to the various organs, accumulate there and exert the pharmacological effects. A possible biokinetics of carbon nanotubes through various portal of entry is given in Figure 3.

Singh et al. (2006) determined the different critical pharmacological parameters such as blood circulation and clearance half-life, organ biodistribution and tissue accumulation after i.v. administration of functionalized carbon nanotubes (Singh et al. 2006). They employed the two types of radiolabelled functionalized single walled carbon nanotubes (*f*-SWCNTs). Ammonium functionalized SWCNTs have been functionalized again with the chelating molecules of diethylenetriaminepentaacetic (DTPA) dianhydride and labeled with indium (^{111}In) for imaging purpose. They compared the tissue biodistribution and affinity over time after i.v. injection of (^{111}In) DTPA-SWCNTs which possess no free amino groups, with that of (^{111}In) DTPA-SWCNTs that posses 40% free amino groups (different surface

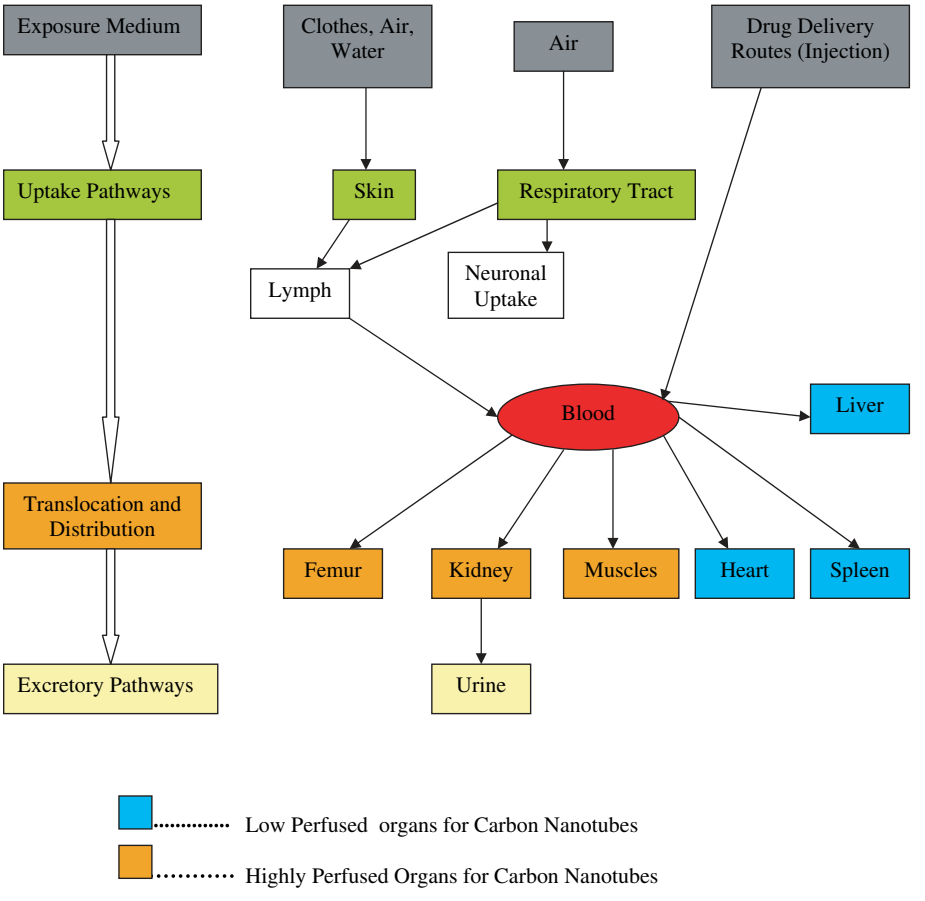


Figure 3. Possible biodistribution of carbon nanotubes. (Singh et al. 2006)

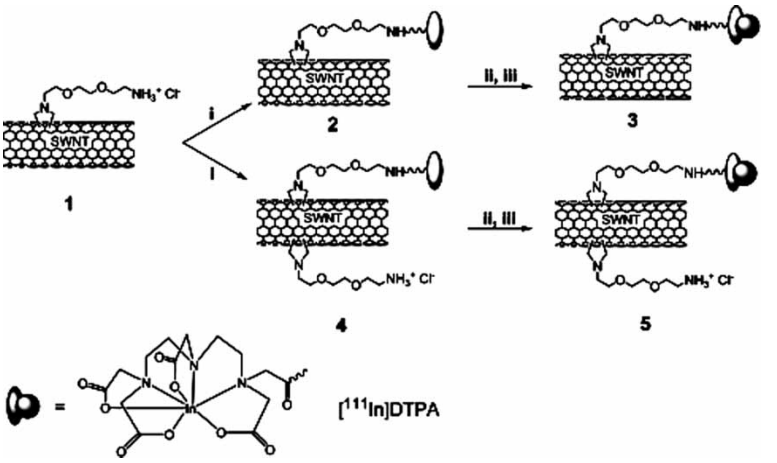


Figure 4. Steps reveal the functionalization of single-walled nanotubes with ^{111}In-DTPA; two types of nanotubes, i.e., having no free amino groups (sample 3) and having 40% amino groups. Synthesis of ^{111}In-labeled CNT. (i) DTPA dianhydride and diisopropylethylamine (DIEA) in DMSO; (ii) Sodium citrate in H_2O; (iii) $^{111}InCl_3$ in H2O. Compound 3: No free amino group & compound 5: 40% free amino group. Compound 2 & 4 represents the DTPA functionalized Single Walled Nanotubes (SWCNTs). Reprinted by the permission of PNAS. Copyright (2006) National Academy of Sciences, USA.

charge distribution). A detailed pathway adopted by researchers is given in Figure 4.

Various organs are examined after 30 min of i.v. administration. Tissue biodistribution studies resulted into the higher level of radioactivity in muscles, skin, kidney and blood for both types of nanotubes. Although it appeared that after 30 min, the (^{111}In) DTPA-SWCNTs (having 40% free amino groups) could be found in kidney, muscles, skin and lungs at a slightly higher concentration than (^{111}In) DTPA-SWCNTs (having no free amino groups). Although both types of nanotubes are cleared rapidly from all the tissues, the researchers found that most of the nanotubes are eliminated through the renal excretion routes, because of high lavel of ^{111}In found in kidney after 30 min and rapid decline in radioactivity. Another study performed by researchers, was the *in-vivo* excretion study to investigate the presence of *f*-SWCNTs and *f*-MWCNTs in the excreted urine. 400 μg of DTPA-SWCNTs and DTPA-MWCNTs were administered i.v. and urine was collected within 18 h period after administration. Both *f*-SWCNTs and *f*-MWCNTs were found to be present in urine sample as confirmed by TEM analysis of urine suggesting that they were cleared from the systemic blood circulation through renal excretion route. They also found the blood circulation half-life of sample having no free amino groups to be 3.5 h and then of sample having 40% amino groups to be 3 h. All the parameters observed were not altered by the degree of functionalization, i.e., surface charge density difference of functionalized, water soluble and (^{111}In) DTPA-SWCNTs. Finally, the authors concluded that the biodistribution study by a radiotracer technique might help in further pharmacological investigation of different types of nanotubes to determine the limitations and offer the various opportunities for CNTs based drug delivery systems. A biodistribution pattern of both types of nanotubes i.e. having no free amino groups, and 40% free amino groups is given in Figure 5.

Ecotoxicological effects

Being nanosized particles, carbon nanotubes and related structures exert certain ecotoxicological effects. A study performed with uncoated, water-soluble, colloidal fullerenes (nC60) revealed that after using standard U.S. EPA protocols (U.S. EPA 1994), the 48 h LC50 (median lethal concentration) in *Daphnia magna* was 800 ppb.[78] Although no mortality was seen in largemouth bass (*Micropterus salmoides*), lipid peroxidation in the brain and glutathione depletion in the gill was observed after exposure to 0.5 ppm nC60 for 48 h (Oberdörster 2004b). The possible mechanism for the lipid damage in the brain includes direct redox activity by fullerenes reaching the brain via circulation or axonal translocation and dissolving into the lipid-rich brain tissue; oxyradical production by microglia; or reactive fullerene metabolites may be produced by cytochrome P450 metabolism. Recently the ectoxicological effects of CNTs have been described by Smith et al. (2007). They first reported the toxicity of SWCNTs to rainbow trout by using the body system approach (Smith et al. 2007). The researchers' main aim was to measure a range of endpoints including the behaviors, gill ventilation rates, hematology and plasma ions trace elemental profile in major organs. They also performed the some biochemical measurements relating to the

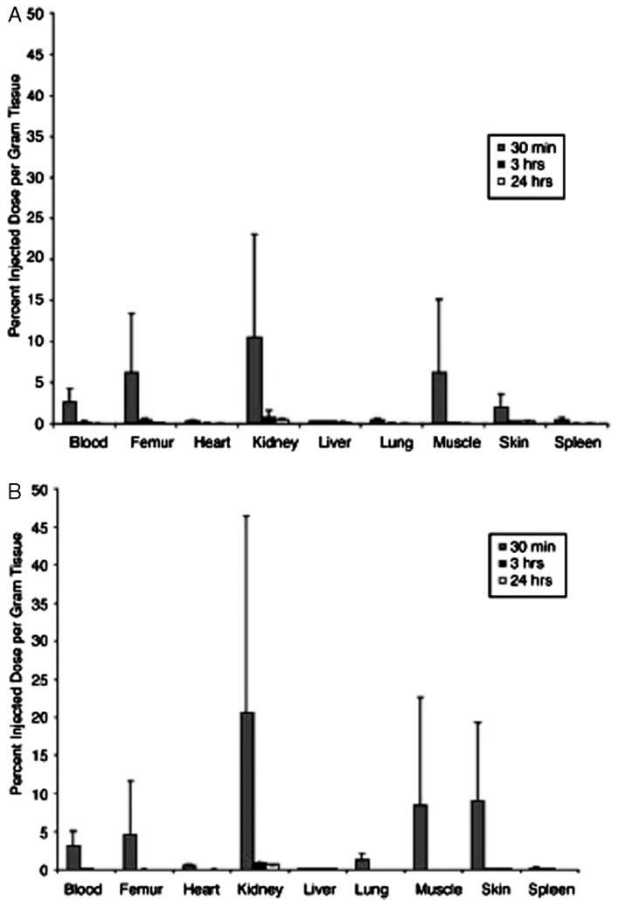


Figure 5. Biodistribution pattern of various ^{111}In-DTPA functionalized Single Walled Nanotubes (SWNTs) samples (i.e., with no free amino group & with 40% free amino group) after i.v. administration. Biodistribution of various samples of Functionalized Nanotubes after i.v. administration (collected per gram of tissue) (A) [^{111}In] DTPA–SWCNT (having no free amino group) (B) [^{111}In] DTPA–SWCNT (having 40 % amino group). Reprinted by the permission of PNAS. Copyright (2006) National Academy of Sciences, USA.

physiological functions (e.g., Na^+K^+-ATPase activity) or oxidative stress (Thiobarbituric Acid Reactive Substance test). The authors concluded that SWCNTs were respiratory toxicant in trout. The fish were able to manage oxidative stress and osmoregulatory disturbances, but other cellular pathologies raised the concerns about cell cycle defects, neurotoxicity, and there were some unidentified blood borne factors that possibly mediate systemic pathologies. Engineered nanomaterials used as antimicrobials may shift microbial communities if they are released into the environment via effluents. Aqueous fullerenes and coated SWCNTs are stable in salt solutions (Cheng et al. 2004; Warheit et al. 2004) cell culture media (Lu et al. 2004; Sayes et al. 2004) reconstituted hard water, and MilliQ water (Dieckmann et al. 2003). Nanosized particles (NSPs) will tend to sorb onto sediment and soil particles and be immobilized because of their high surface area: mass ratio (Lecoanet & Wiesner 2004). Biologic transport would occur from ingested sediments, and one would expect movement of nanomaterials through the food chain. To make engineered nanomaterials more biocompatible, both surface coatings and covalent surface modifications have been incorporated. Some studies have shown that both the surface coating and the covalent modifications can be weathered either by exposure to the oxygen in air or by ultraviolet (UV) irradiation for 1–4 h (Rancan et al. 2002; Derfus et al. 2004). Therefore, although coatings and surface modifications may be critically important in drug-delivery devices, the likelihood of weathering under environmental conditions makes it important to study toxicity under UV and air exposure conditions. Even coatings used in drug delivery of NPs may not be biopersistent or could be metabolized to expose the core NP material.

Reactive oxygen species mechanism of CNTs toxicity

Due to the nanodimensional structures, the carbon nanotubes and other related structures are shown to create *reactive oxygen species* (*ROS*). ROS production has been found in NPs as diverse as C60 fullerenes, SWCNTs, quantum dots, and UFPs, especially under concomitant exposure to light, UV, or transition metals (Brown et al. 2000, 2001; Rancan et al. 2002; Wilson et al. 2002; Li et al. 2003; Yamakoshi et al. 2003; Derfus et al. 2004; Joo et al. 2004; Lu et al. 2004; Nagaveni et al. 2004; Shvedova et al. 2004). The preferential mobilization of NSPs of various sizes and various chemical compositions to the mitochondria (mitochondria are redox active organelles) (Gopinath et al. 1978; Rodoslav et al. 2003; Shvedova et al. 2004) leads to the alteration in ROS production and thereby overloading or interfering with antioxidant defense systems. The C60 fullerene is shown as a model NP producing superoxide (Yamakoshi et al. 2003). The possible suggested mechanism by which each of these diverse NPs generate ROS include: (a) Photo excitation of fullerenes and SWCNTs, causing intersystem crossing to create free electrons; (b) metabolism of NPs to create redox active intermediates, especially if metabolism is via cytochrome P450s; and (c) inflammation responses *in vivo* that may cause oxyradical release by macrophages.

In vitro *toxicity studies*

Carbon nanotubes are high-profile nanoparticles that are under consideration for dozens of applications in materials science, electronics and medical imaging. For medical applications, it is reassuring to

see that the cytotoxicity of nanotubes is low and can be further reduced with simple chemical changes. In their native state, carbon nanotubes are insoluble, meaning they are incompatible with the water-based environment of living systems. Solubility is a key issue for medical applications, and attempts have been made to render nanotubes soluble. In particular, scientists are keen to exploit the fluorescent properties of carbon nanotubes for medical diagnostics. In previous studies with buckyballs, scientists found that even minor surface modifications could dramatically reduce cytotoxicity. The nanotube study also depicted similar results. In both cases, the researchers have identified specific alterations that reduce toxicity (Singh et al. 2006).

Cytotoxicity refers to toxic effects on individual cells. In cytotoxicological studies, identical cell cultures are exposed to various forms and concentrations of toxins. In order to compare the toxicity of different compounds, scientists look for the concentration typically measured in parts per million or parts per billion of materials that lead to the death of 50% of the cells in a culture within 48 h.

The earliest study indicating the CNT cytotoxicity was performed by Shvedova et al. (2003). They investigated the effects of unrefined SWCNTs on immortalized human epidermal keratinocytes (HaCaT). HaCaT cells were incubated for up to 18 h in media containing unrefined SWCNTs (0.06–0.24 mg/ml). When they were exposed to these cells, SWCNTs resulted in increased free radical and peroxide generation and depletion of total antioxidant reserves that was assigned an 'accelerated oxidative stress', loss in cell viability and morphological alterations to cellular structure. It was concluded that these effects were due to high levels (approximately 30%) of iron catalyst present in the unrefined SWCNTs. A possibility was also given regarding the dermal toxicity in handling unrefined CNT, but the authors stressed upon the role of SWCNTs particle size and structure (Shvedova & Castranova 2003).

Another dermal cytotoxicity study was performed in 2005, which found that MWCNTs initiated an irritation response in human epidermal keratinocyte (HEK) cells (Monteiro-Rivierea et al. 2005). Purified MWCNTs (synthesized via CVD) were incubated (at doses of 0.1–0.4 mg/ml) with HEK cells for 48 h and was allowed to localize within the cells. The result showed the production of the pro-inflammatory cytokine (IL-8) and decreased cell viability in a time- and dose-dependent manner. Since these MWCNTs were purified (absence of catalyst particles), this led the authors to conclude that CNTs itself have potential dermatological toxicity, urging a full toxicological assessment before widespread public exposure.

In another study, Muller et al. (2005) incubated peritoneal macrophages (from Sprague-Dawley rats) for up to 24 h in media containing purified MWCNTs and purified 'ground' MWCNTs (in an oscillatory ball mill) at concentrations of 20, 50 and 100 μg/ml . The sign of cytotoxicity was indicated by the release of lactate dehydrogenase (LDH) at 24 h of incubation whilst inflammatory potential was assessed by measuring mRNA expression of TNF-α (a pro-inflammatory cytokine) at 6 h of incubation. It was found that 'ground' MWCNTs had a similar capacity for inducing dose-dependent cytotoxicity and up-regulating TNF-α expression as asbestos and carbon black. On the other side cytotoxicity and TNF-α expression in the 'unground' MWCNTs sample were significantly lower than the 'ground' sample, as an increased agglomeration observed in the 'unground' sample lead to a decrease in MWCNTS availability to the cells, accounting for the less cytotoxic and proinflammatory response (Muller et al. 2005).

Another group of scientists (Jia et al. 2005) investigated the effect of different carbonaceous nano-materials on the cytotoxicity of alveolar macrophages. They exposed alveolar macrophages to SWCNTs (1.4 nm diameter, synthesized by electric arc-discharge and purified to 90%), MWCNTs (10–20 nm diameter, synthesized via CVD and purified to >95%) and C60 fullerenes (synthesized via electric arc-discharge and purified to 99.9%) for 6 h. Since the CNT materials are having the tendency to aggregate, so they attempted to overcome the effect of particle size by utilizing a modified dosing regimen (1.41–226 μg/cm^2 for SWCNT and C60, and 1.41–22.60 μg/cm^2 for MWCNTs). However, they failed to clarify whether an increased dose corresponded to an increase in mass or a decrease in surface area. They also failed to specify how the surface area of the particles, suspended in media, was confirmed. Despite these failures the study revealed that SWCNT exhibited the most cytotoxic response, although both SWCNTs and MWCNTs demonstrated decreased cell viability and impaired phagocytic function. Again, the authors did not provide enough information to determine whether this dose-dependent increase in cytotoxicity is a result of an increase in CNT particle size or an increase in the total mass of CNT, to which the cells were exposed. Also, SWCNTs and MWCNTs variants were found to have a greater negative impact on cell viability than the positive control, quartz.

In another study by Cui et al. (2005) investigated SWCNTs cytotoxicity and showed that SWCNTs inhibited human embryonic kidney (HEK 293) cells

by inducing apoptosis and decreasing cellular adhesion ability. These researchers cultured HEK293 cells in media containing concentrations of SWCNTs ranging from 0.78–200 μg/ml. Cells were tested for a variety of functions, including adhesion ability and protein secretion. Biochip analysis also provided information about genetic expression of cells cultured with SWCNT. Both cell proliferation and adhesion ability decreased in a dose- and time-dependent manner. Genes involved in apoptosis were up regulated, while genes associated with the G1 phase of the cell cycle (the major period of cell growth) were down regulated along with the genes associated with adhesion. Unfortunately, neither the degree of dispersion nor the SWCNTs handling methods were disclosed in this publication (Cui et al. 2005).

Tamura et al. (2004) conducted a brief investigation on the cytotoxic effect of purified CNT on neutrophils isolated from human blood. Purified CNT significantly increased super-oxide anion and TNF-α production after contact with the cells for 1 h as compared to controls, while cell viability was clearly decreased. Unfortunately, no details of CNT structure, synthesis or handling methods were provided, reducing the significance of this publication (Tamura et al. 2004).

Another study by Mustellin and co-workers (2006a) compared the toxicity of pristine and oxidized multiwalled carbon nanotubes on human T cells (diameter 0.4 nm and 100 nm, respectively) (Bottini et al. 2006a). Study was performed with Jurkut T leukemia cells, using RPMI-1640 cell culture medium supplemented with 10% fetal bovine serum albumin (FBS). Cells were incubated with 0.5 ml of nanomaterials (Carbon Black or CNTs both pristine and oxidized) dispersion in water or 0.5 ml water alone, to 4.5 ml of cell suspension containing 2×10^5 cells at 37°C in 5% CO_2 atmosphere. Cells were evaluated for cell viability and proliferation assay by tryphan blue exclusion test. Cell death was measured as a

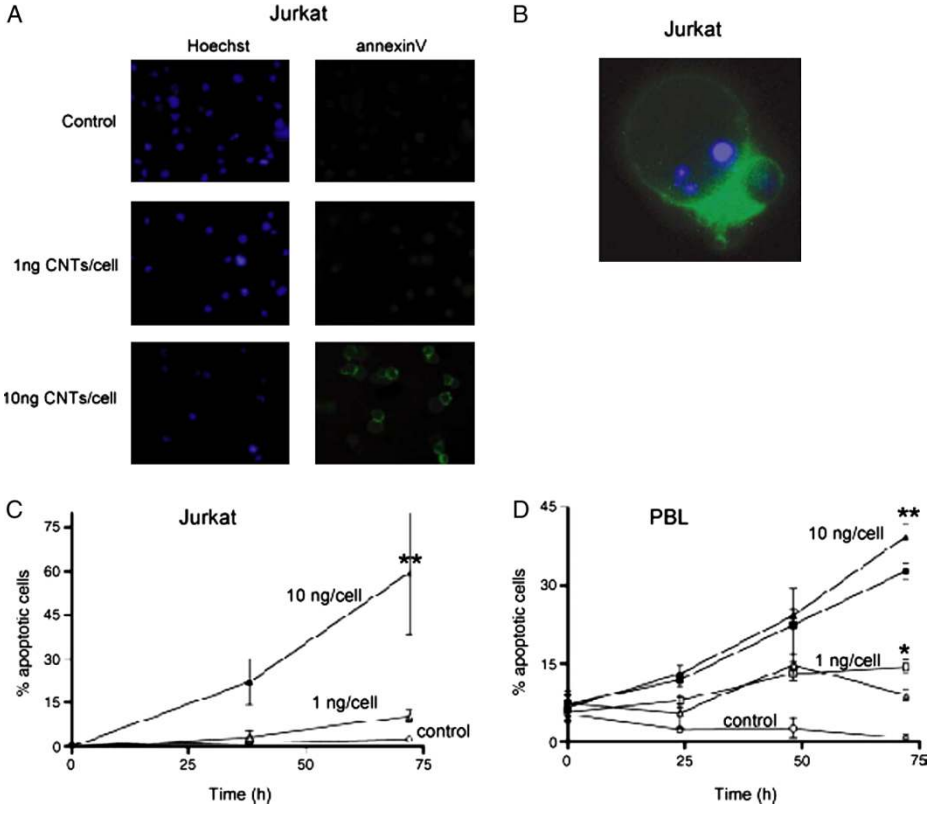


Figure 6. CNTs induce apoptosis of human T cells. (a) Immunofluroscence images of untreated Jurkat cells (upper panels) and of Jurkat cells treated for 24 h with 1 ng/cell (middle panels) or 10 ng/cell (lower panels) of oxidized CNTs. Right panels show cells stained with annexin V-FITC, left panels shows nuclear staining of same cells with Hoechst 33342; (b) At higher magnification annexin V positive Jurkat cells show pyknotic nuclear DNA condensation and membrane blebbing, two typical features of apoptotic cell death; (c) Time course of Jurkat cell apoptosis after treatment with oxidized CNTs. Graph shows percentage of annexin V positive cells after incubation with 1 ng/cell (open triangles) or 10 ng/cell (filled triangles) of oxidized CNTs. Open circles show percentage of apoptosis of control cells; (d) Time course of human peripheral blood lymphocytes (PBL) apoptosis after treatment with oxidized CNTs. Graph shows percentage of annexin V positive cells at three time points after incubation with 1 ng/cell (open symbols) or 10 ng/cell (filled symbols) of pristine (squares) or oxidized (triangles) CNTs. Open circles show percentage of apoptosis of control untreated PBL; Statistical significance $*p<0.05$ and $**p<0.001$. Reprinted with permission from (Bottini et al. 2006). Copyright (2007) Elsevier.

percentage of apoptotic Jurkut cells or peripheral blood lymphocytes (PBL), determined by using annexin-V-FITC (Figure 6).

The authors concluded that the physical form of carbon had a major impact on the toxicity, i.e. CNTs are more toxic than the similar chemical amount of carbon in the amorphous form of carbon (amorphous carbon is non-toxic) even at the highest tested concentration; 400 μg/ ml). They also concluded that the molecular structure and topology are essential for the toxicity of carbon nanomaterials. According to them, the oxidized carbon nanotubes are more toxic as compared to the hydrophobic pristine carbon nanotubes. The results revealed that triggering the T-cell antigen receptor leads to the complex biochemical signaling cascade (recruitment of tyrosine kinase and increased tyrosine phosphorylation of many key signaling proteins), that leads to expression of many genes, increased metabolism, cytoskeleton rearrangement, activation of effector function of T- cells and initiation of immune response. By experimentation (immunobloting of cells extract with mAb against phosphorylation) the concentration of CNTs that has no detrimental effect on the receptor induced T-cell activation was established to be 40 μg/ml (Figure 7).

The author also advised that this limiting concentration should be maintained in all new forms of CNTs and CNTs containing nanodevices.

The *in-vitro* cytotoxic response of SWCNTs (Cui et al. 2005; Bottini et al. 2006a) typically involves their dispersion with in the cell culture medium followed by their subsequent addition to a cell line of interest in the medium in which they have been dispersed. Various studies have been performed for the interaction of SWCNTs with the cell culture medium components and various molecular species including small organic molecules (Hedderman et al. 2004; Valentini et al. 2006), organic polymers (Dalton et al. 2000; Keogh et al. 2004), polysaccharide (Bandyopadhyaya et al. 2002; Chambers et al. 2003; Casey et al. 2005), proteins (Salvador et al. 2006) and DNA (Moulton et al. 2005). They have the tendency to solubilize the SWCNTs. The various carbon-based nanomaterials including SWCNTs interact with cytotoxic assay, further interfering with absorption/fluorescence data used to evaluate the cytotoxicity (Monteiro-Riviere & Inman 2006; Wörle-Knirsch et al. 2006).

Casey et al. (2007) explored the interaction of SWCNTs with the cell growth and its constituent components. UV/visible absorption and fluorescence were employed to study the interaction with the cell culture medium. Cell culture medium and its components were prepared both with and without foetal bovine serum. A noticeable colour change was

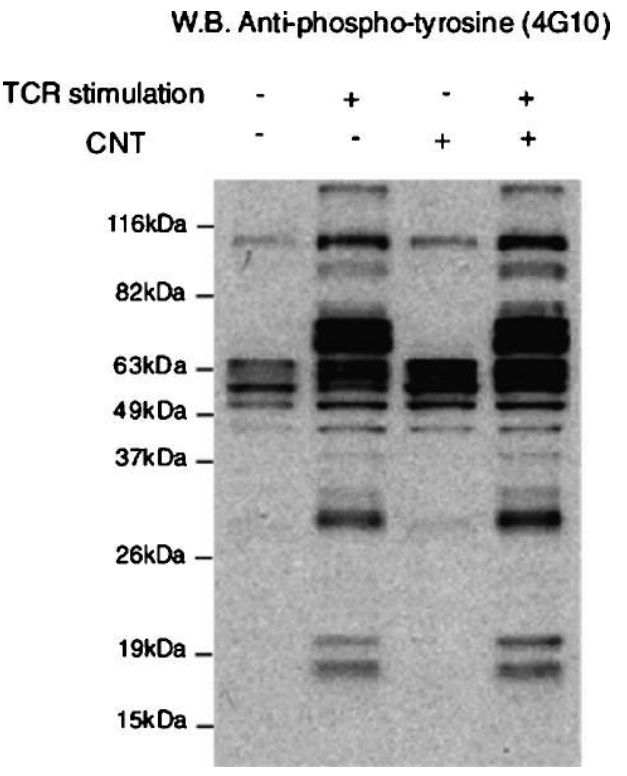


Figure 7. Effects of CNTs on T-cell antigen receptor signaling. Figure shows an anti-phosphotyrosine Western blot of equal amount of total proteins from untreated Jurkat cells (lane 1 and 2) and Jurkat cells treated with 40 μg/ml of oxidized CNT for 24 h. Lanes 1 and 3 show resting T cells, lane 2 and 4 show cells stimulated with anti-CD3ξ plusanti-CD28 mAbs for 5 min. Reprinted with permission from (Bottini et al. 2006). Copyright (2007) Elsevier.

observed when the raw SWCNTs were added to the medium. UV/Visible absorption spectroscopy revealed a dramatic reduction in the absorption attributable to the phenol red, a pH indicator within the medium, without an associated change in pH. These reductions were also observed in absorbance features, which were attributed to various components of the medium indicating an interaction with the SWCNTs. An additional support to the interaction was also provided by fluorescence spectroscopy that reflects reductions in emission features associated with the components of the medium. Concentration dependent studies of the fluorescent emission of the various components of the media were modeled to show a differing degree of interaction between the SWCNTs and the various components. Finally, notable differences were observed between the behavior with and without serum. Raman spectroscopy gave no indication of differences between raw SWCNTs and those deposited from the medium suspension indicating that no debundling of the SWCNTs occurred.

A group of scientists (Wick et al. 2007) also revealed the *in-vitro* cytotoxicity of carbon nanotubes

that was affected by its degree and kind of agglomeration. The *in-vitro* cytotoxic experimentation was carried out with MSTO-211H cells using four samples of CNTs. The first sample represented the starting material termed as CNTs raw material or CNTs-rm, second CNTs-rm that was additionally purified by acid/oxidation means further called as CNTs-agglomerates. Samples third and fourth were prepared by alternately performing the ultrasonication assisted dispersion of raw material by the addition of nonionic and biocompatible surfactants, i.e., polyoxyethylene sorbitan mono oleate (PS 80). This step was followed by ultrasonication and centrifugation steps. The centrifugation step resulted in the two forms: a sediment fraction termed as CNT-pellet and a stable dispersed gray supernatant termed as CNT-bundles. The asbestos was employed in the present study as control material. The researchers measured the two metal ratios (Ni/Y) in all the materials including the reference. They found that ratio remained fairly constant, that was determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICPOES). Later it was suggested that the carbon entrapped Ni and Y had no significant effect on the cells. The mesothelioma cell line (MSTO-211H) was tested for cell activity, proliferation and cell-CNTs interaction (Figures 8 and 9).

Cell activity and proliferation were found to be significantly decreased in a dose-dependent manner that confirmed the various results or study regarding the cytotoxicity of CNTs. They found that CNTs-pellets, which were having the highest carbonaceous material, were most toxic to the cells. The cell-CNTs interaction was confirmed by suspending the CNTs in biocompatible surfactants, i.e., PS-80 (it itself did not provoke any cytotoxic response). The results revealed that the toxicity of CNTs-bundles was less than that of CNTs-agglomerates. On the other hand, both the samples contained the same traces of Y and Ni that again confirmed the nondependence on cytotoxicity by these two metals. Finally the cytotoxic response of the reference materials i.e. asbestos and CNTs-agglomerates was found to be similar. The authors suggested that material characterization should be done prior to toxicological studies. Finally they concluded that the toxicity was affected by the presence of carbonaceous material and the degree of CNTs dispersion but not the content of entrapped Y and Ni.

In vivo *toxicity studies*

As the production and applications (Ajayan & Ebbesen 1997) of CNTs expand, potential human exposures will also increase. CNTs can be produced by deposition of carbon atoms vaporized from graphite by electric arc or by laser on to metal particles. More recently they have been produced by chemical vapor deposition (CVD). High-pressure CO conversion (HiPCo) is a CVD process and is a more advanced method that uses carbon monoxide as carbon source; up to 97% of the carbon in the HiPco product ends up in NTs (Bronikowski et al. 2001). All of the products produced by these methods contain residual catalytic metals; some also contain other non-NT carbon materials. An individual NT molecule is about 1 nm in diameter and several microns long (Baron et al. 2003). Microscopically, individual CNTs fibers aggregate into bundles or ropes, which in turn agglomerate loosely into small clumps.

A study reveals that unprocessed CNTs samples could generate the fine particles of respirable size (Baron et al. 2003; Maynard et al. 2003). Fine particles may pose a health risk by inhalation. NTs are rather unique in physical and chemical properties, hence no other dusts, except perhaps graphite, possess any properties similar to that of CNTs. However, graphite does not possess the electrical properties and fibrous structure of CNTs. It is well known that the geometry and surface chemistry of particulates can play an important role in causing lung toxicity (Lippmann 1994). The geometry and surface chemistry of fibers also plays an important role in its toxicity, especially the lung toxicity. Being a fibrous material asbestos cause a number of dysfunctions like asbesteosis (development of bilateral diffuse interstitial pulmonary fibrosis), pleural plaques (white or yellow smooth surfaced lesions on parietal, visceral and diaphragmatic pleura), bronchogenic carcinoma and mesothelioma (pleural & peritoneum). So far the lung toxicity is concerned it is size and aspect ratio of fiber that play a crucial role in assess and entry of fibrous material into the various region of lung. Serpentines (curly fibers) and amphiboles (straight fibers) (Manning et al. 2002) are two groups of physical forms of asbestos. These two forms are having the different toxicity profile and lung penetration capability based on their chemical and physical compositions, shapes, sizes, durability. Biopersistance and clearance are other parameters that have a crucial role in toxicity, carcinogenicity and pathogenicity of various forms of asbestos. Being a mineral in nature the asbestos fiber's surface contain the different elements like magnesium, iron and various transition metals that affect the toxicity and fibrogenicity of asbestos. The interaction of asbestos to fibroblasts causes the generation of ROS that could be directly correlated to toxicity of asbestos. In general the asbestos fibers which contain more iron and longer fibers exert

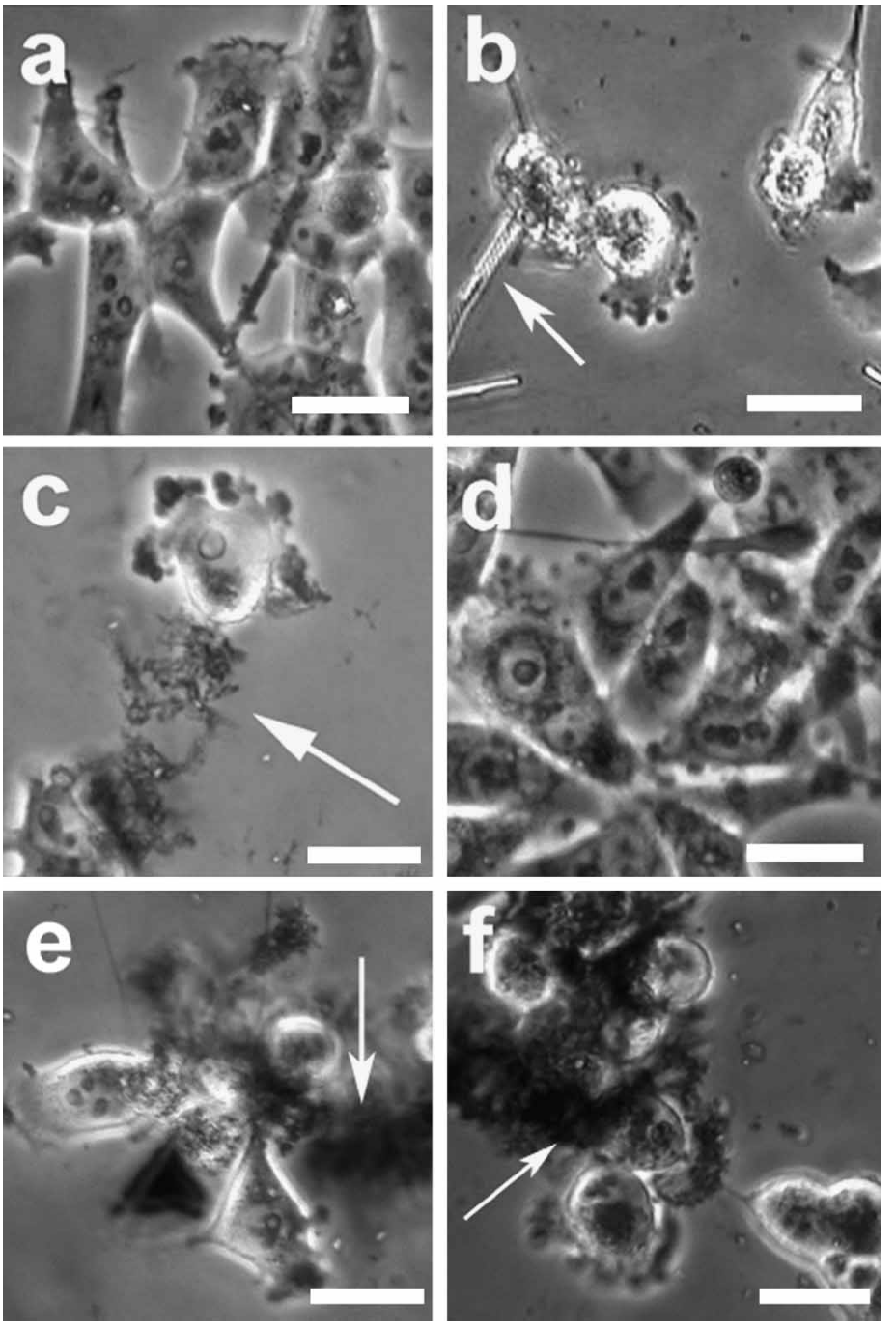


Figure 8. Morphology changes of MSTO-211H cells after 3 days of exposure to 15 μg/ml of different fractions of CNTs and asbestos; (a) Untreated MSTO-211H cell culture; (b) Cell culture exposed to asbestos. Arrows indicate needles of asbestos; (c) Cells treated with CNT-agglomerates were round-shaped and lost their adherence on the cell culture plate. Arrow point to CNT agglomeration; (d) Cells exposed to CNT-bundles showed no visible morphological changes compared to the control cells; (e) Effect of CNT-pellet fraction. Non-tubes material agglomerated during the incubation period to micro-sized structures; (f) Cells incubated with CNT raw material. Arrow indicates agglomerated CNT material: Scale bar 20 μm. Reprinted with permission from (Wick et al. 2007). Copyright (2007) Elsevier.

more toxicity due to more ROS. Briefly, at the cellular level, multiple signaling pathways and transcription factors are activated by asbestos fibers through oxidant-dependent pathways. In general the length of fibers affects the oxidant production after the interaction with phagocytes. If the length of fiber is >5 mm, it causes the 'frustrated phagocytosis' that ultimately leads to the chronic oxidant elaboration from both epithelial cells and macrophages (Manning et al. 2002). Various oxidants are known to interact with macromolecules, such as proteins and DNA, and a significant alteration takes

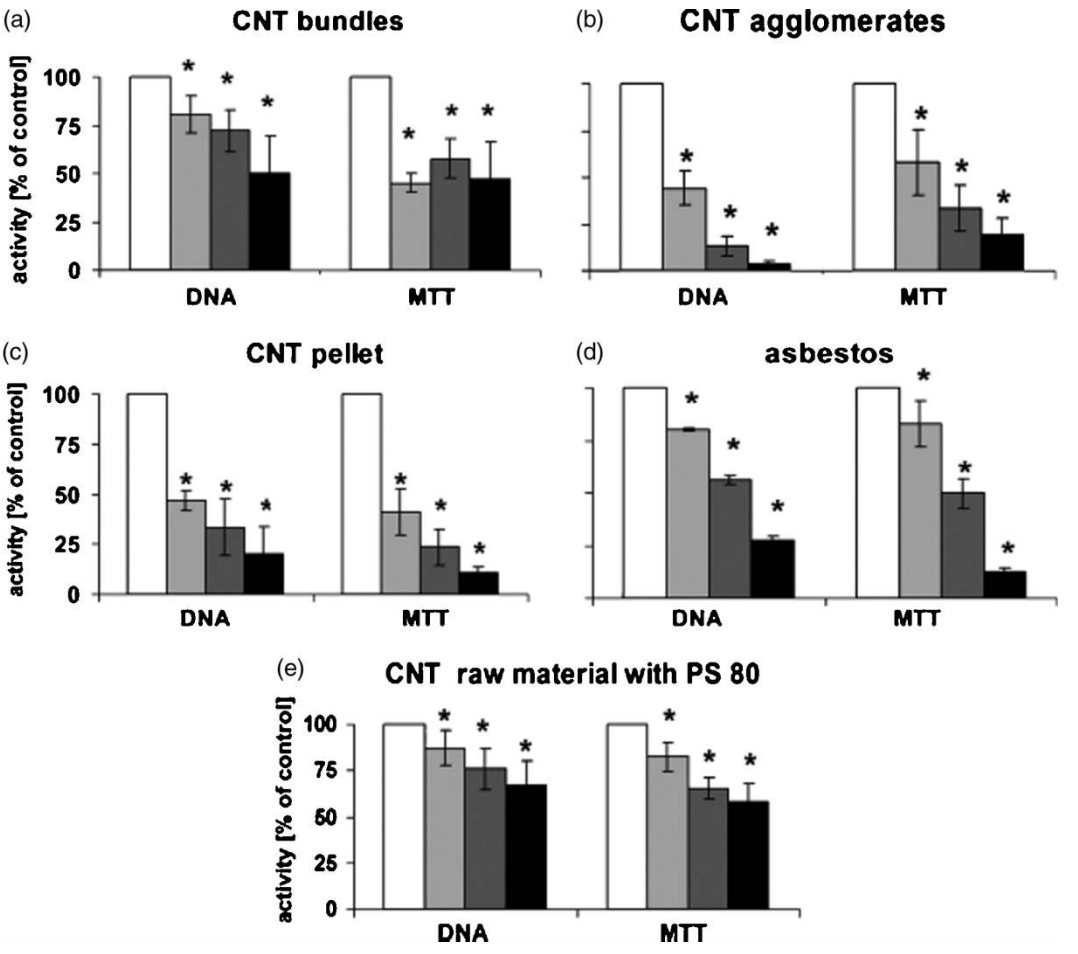


Figure 9. MSTO-211H cell proliferation (DNA) and cell activity (MTT) after 3 day of exposure to different fractions of CNTs. Cell culture treated with: (a) CNT-bundles, (b) CNT-agglomerates, (c) CNT-pellet fraction, (d) crocidolite asbestos, and (e) CNT raw material dispersed in 20 μg/ml PS80. White bar: control culture without particles; Grey bar: 7.5 μg/ml; Dark grey bar: 15 μg/ml; Black bar: 30 μg/ml of the corresponding CNT fraction. *denote significant different from control cell culture ($p<0.01$). Mean ± SEM over values of three independent experiments. Reprinted with permission from (Wick et al. 2007). Copyright (2007) Elsevier.

place in signaling molecules by asbestos. The absence of toxicity data for such an important commodity has concerned many (Gorman 2002). Concern about the potential for its workers to be exposed to materials of unknown toxicity prompted NASA to sponsor the pulmonary toxicity study.

Various works have been published regarding the *in vivo* toxicity of carbon nanotubes relating to its pulmonary toxicity, skin irritation and cytotoxicity of carbon nanotubes.

Lung toxicity

Various studies have been conducted into the pulmonary toxicity of carbon nanotubes (Huczko et al. 2001). All recent studies have found histological evidence of lung inflammation and granuloma formation (Lam et al. 2004; Warheit et al. 2004; Huczko et al. 2005; Muller et al. 2005). These studies have been compiled in Table II.

All studies that utilized intratracheal instillation reported major difficulties dealing with the agglomerative nature of CNT in aqueous solutions (Lam et al. 2004; Warheit et al. 2004; Huczko et al. 2005; Muller et al. 2005). Despite repeated use of surfactants (Lam et al. 2004; Warheit et al. 2004; Muller et al. 2005) and sonication (Muller et al. 2005; Huczko et al. 2005) histopathological examinations showed large CNT agglomerations in the lungs of treated animals. Hence, while initial evidence urges considerable caution in the handling of CNT, the actual toxicology of CNT cannot be verified until advances have been made in CNT delivery methods. Animal inhalation studies have been proposed to mimic respiration of airborne CNT particles, but only one investigation into this area has been performed (Maynard et al. 2004). Similar to intratracheal instillation techniques, pharyngeal aspiration showed evidence of large CNT agglomerations in the proximal alveolar regions of the lungs, as well as, fine fiber-type structures in the more distal

Table II. Summary of lung toxicity of CNTs.

S No.	CNTs description	Amount of CNTs administered	Animal model	Mode of administration/ exposure condition	Duration of exposure	Toxicity	Specific comments	Reference
1	Unrefined CNTs(Synthesized via Arc- Discharge Sublimation Method)	25 mg in 0.5 ml of saline solution	Guinea Pig (Dunkin, Hartley male)	Intrathecal installation (dispersion in small amount of surfactants)	4 weeks (single dose 0.5 ml)	No measurable pulmonary dysfunction (as seen by either non-invasive procedure or BAL examination)	Working with soot containing carbon nanotubes is unlikely to be associated with any health risk.	(Huczko et al. 2001)
2	SWCNTs (three batches, i.e., raw and purified HiPco SWCNTs, Arc- SWCNTs)	0.1 & 0.5 mg/ mouse	Male miceB6 C3F1	Intrathecal installation (dispersion in mouse serum-heat inactivated)	7 & 90 days (single bolus dose-50 μl)	Dose-dependent lung lesion (interstitial granulomas)	A metal impurity does not affect the interstitial granulomas. Interstitial granulomas were ascribed to CNTs only. SWCNTs were more toxic than carbon black and CNTs containing Ni was more toxic than quartz.	(Lam et al. 2004)
3	Pristine laser SWCNTs	1 and 5 mg/kg (8 week old male rats)	Male Crl:CD® (SD)IGS BR Rats	Intracheal installation (in phosphate buffer saline solution with 1% Tween 80).	24 h, 1 week, 1 month and 3 months	15% initial mortality was seen, occurrence of only transient inflammation by SWCNTs and non-dose dependent tissue multimodal granulomas	Lung asphyxiation was attributed to the initial mortality. Multimodal granuloma was considered inconsistent with lack of lung toxicity.	(Warheit et al. 2004)
4	MWCNTs (produced by arc-discharge and CVD methods)	15 mg	Guinea Pig	Intratracheal instillation (suspension in sterile saline with SDS)	90 days (single bolus dose of 0.5 ml)	A significant evidence of pulmonary toxicity. Alveolar macrophage infiltration in BAL of all non-control animals. Multiple lesion were observed in CNTs exposed animals.	Alveolar macrophage infiltration was absent in pyrograph MWCNTs. Exposure time was critical for induction of pathology.	(Huczko et al. 2005)
5	MWCNTs (Purified and ground)	0.5, 2 and 5 mg/ rat	Females Sprauge-Dawley rats	Intratracheal instillation (suspension in sterile 0.9% NaCl with 0.1% Tween 80)	1 and 2 months (single bolus dose of 500 μl/rat)	Dose dependent granuloma formation persisted for whole 60 days. Both ground & purified MWCNTs were more inflammatory than carbon black and less inflammatory than asbestos fibres. Both types of nanotubes have cause pulmonary lesions at 2 months.	Clearance & biopersistance of carbon nanotubes appeared to modulated by its length. Appropriate safety measure should be taken while handling the CNTs.	(Muller et al. 2005)

Table II (*Continued*)

S No.	CNTs description	Amount of CNTs administered	Animal model	Mode of administration/ exposure condition	Duration of exposure	Toxicity	Specific comments	Reference
6	Metal-free SWCNTs	0–40 μg/mouse	Female C57BL/6 mice	Pharyngeal aspiration (suspension in PBS)	1, 3, 7, 28 and 60 days (single bolus of 50 μl)	Rapid progressive fibrosis and granulomas. Dose-dependent increase in expiratory time. Increased pulmonary resistance.	Exposure to respirable SWNT particles can be a risk to developing some lung lesions.	(Shvedova et al. 2005)

alveolar regions. However, this inhalation technique still does not mimic physiological respiration, bypassing the nose and delivering the CNT as a bolus dose. There is also evidence that it takes significant energy and agitation to release fine CNT particles into the air. Current handling procedures employed by nanotube manufacturers do not produce significant quantities of airborne CNT (Maynard et al. 2004). However, the possibility of cumulative effects, especially if increased quantities are handled, justifies the introduction of safety measures.

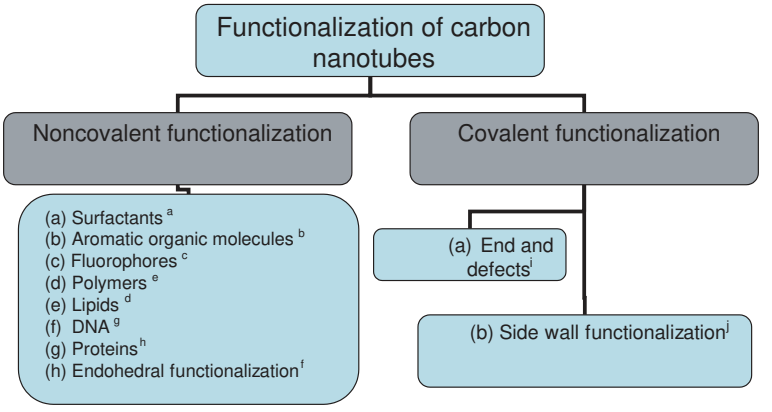
Skin toxicity

Very few skin irritation studies of CNTs have been reported with only one preliminary study published by Huczko and Lange (2001). CNT-induced skin irritation was evaluated by conducting two routine dermatological tests. Initially 40 volunteers with allergy susceptibilities were exposed to a patch test (filter paper saturated with water suspension of unrefined CNT (synthesized via the arc discharge process) for 96 h. Secondly a modified Draize rabbit eye test (using a water suspension of unrefined CNT) was conducted with four albino rabbits monitored for 72 h after exposure. Both tests showed no irritation in comparison to a CNT-free soot control and it was concluded that 'no special precautions have to be taken while handling these carbon nanostructures'.

Surface modification of carbon nanotubes

Functionalization of carbon nanotube. Many CNT applications require handling in solution-phase; however, CNTs have proven difficult to disperse in solvents (Ausman et al. 2000). Chemical modification of SWCNTs is often required for more versatile suspension capabilities and realization of certain applications. This has encouraged greater exploitation of their intrinsic properties, as well as the capability to modify these properties. The functionalization of CNTs is required for their aqueous suspension and to allow for molecular interactions with biological systems. In general, different *non-covalent* and *covalent* modification strategies can be used. Non-covalent methods *preserve the pristine CNT structure* while covalent modification *introduces structural perturbations*. Various methods for functionalization of carbon nanotubes (CNTs) are given in Figure 10.

Functionalization and toxicity of carbon nanotubes. Degree and kind of sidewall functionalization can affect toxicity of carbon nanotubes. The cytotoxic response of cells in culture is dependent on the degree of functionalization on the SWCNTs. Christie et al.



[a]Chen et al. 2003, Moore et al. 2003, Strano et al. 2003, Shim et al. 2002; [b]Zhao et al. 2003, Nakashima et al. 2002, Chen et al. 2001, Guldi et al. 2005; [c]Bottini et al. 2006b; [d]Richard et al. 2003; [e]Star et al. 2001; [f]Smith et al. 1991; [g]Zheng et al. 2003; [h]Balavoine et al. 1999; [i]Rey et al. 2006; [j]Bahr & Tour 2002.

Figure 10. Various methods of functionalization of carbon nanotubes.

(2006) examined the cytotoxicity of three different water dispersible SWCNTs samples in human dermal fibroblast cell cultures (Christie et al. 2006). Four water dispersible SWCNTs were used: SWCNTs-phenyl-SO_3H, SWCNTs-phenyl-$(COOH)_2$, SWCNTs-phenyl-SO_3Na and SWCNTs in 1% pluronic F108. Cells were exposed to 1% pluronic F108 as control. The direct evidence of covalent side wall functionalization was provided by Raman spectroscopy. The degree of side functionalization was determined by thermo gravimetric analysis from 80–800°C under the atmosphere of argon. The density of functionalization on the side wall of SWCNTs was reported as the ratio of SWCNTs carbon atoms to the addends (carbon/phenyl-SO_3X). The researchers found that with increase in the degree of sidewall functionalization, the SWCNTs became less cytotoxic. Elemental content of samples SWCNTs-phenyl-SO_3H and SWCNTs-phenyl-SO_3Na were determined by X-ray photon electron spectroscopy. Finally fluid atomic microscopy (AFM) provided a tool to image the interaction of water dispersible SWCNTs to the artificial phosphocholine membrane at nanometer level. It predicts the interaction of the tubes with the cell membranes. The differential cytotoxicity of cells was measured by MTT assay. The authors concluded that as the degree of sidewall functionalization of SWCNTs is increased the samples were substantially less cytotoxic than the surfactant stabilized SWCNTs. Even though cell death did not exceed 50% of cells dosed with side wall functionalized SWCNTs. Finally the optical and atomic force microscopy showed direct contact between cellular membrane and water dispersible SWCNTs, i.e., the SWCNTs in the aqueous suspension precipitated out and selectively deposited on the membrane.

Carbon nanotubes: A biocompatible module

All the methods used for the production of carbon nanotubes generate some impurities, which usually consist of carbon coated metal catalyst (contaminate HiPco produced nanotubes), carbon coated metal and carbon nanoparticles/amorphous carbon (in arc discharge-produced nanotubes) (Tasis et al. 2003). These metal impurities (e.g., iron from the nanotubes generation process contribute the observed effects) (Oberdörster et al. 2005a) when tested *in vivo*, revealed high acute effects, including mortality that was explained by large dose of instilled highly aggregated nanotubes, which caused death by obstructing the airways. This should not be considered as an effect of nanotubes (Oberdörster et al. 2005a) although toxic effects are due to the metal impurities that can be minimized by the purification of carbon nanotubes. All these treatments make the CNTs biocompatible. The main techniques for purification of nanotubes are oxidation of contaminate (Liu et al. 1998), flocculation and selective sedimentation (Bonard et al. 1997), filtration (Bandow et al. 1997), size exclusion chromatography (Duesberg et al. 1998) or microwave irradiation (Curran et al. 1998; Martinez et al. 2002).

In order to make CNTs safe for administration *in vivo*, i.e., to make them biocompatible, they should be completely dispersible. Nanotubes are completely insoluble in water and in any type of solvents and they are biologically non-degradable (Sen et al. 2004). Due to highly hydrophobic surface of carbon nanotubes, they have a tendency to aggregate in large bundles and ropes. Manipulation and characterization of indivisual CNTs is rendered difficult by their high molecular weight and strong intertubular forces (Vander walls forces and electrostatic forces).

This is the major concern in relation to the toxicity of CNTs, because dispersibility of CNTs directly affects its biocompatibility, and this is the only factor that ultimately dictates the presentation of carbon nanotubes into the cells.

Dispersion of carbon nanotubes in variety of solvents (organic solvents for polymer interaction and aqueous solvents for drug delivery) is critical for its efficient administration. Various attempts or modification have been done to enhance the dispersibility of CNTs in a system to increase their biocompatibility ultimately minimimising their toxicity. These are sonication, stabilization with surfactants, polymers and covalent functionalization as explained below.

Methods of dispersion

Sonication. Sonication is a commonly used method of dispersion of CNTs without the need of chemical modification. Ultrasonic bath and ultrasonic probe are the main methods of sonication. A stable dispersion of pristine has been produced when a highly polar solvent such as N, N-dimethylformamide is in conjugation with ultrasonic bath or probe. Although it is the simplest, it is not the most successful method for the dispersion of CNTs (Smart et al. 2006).

Surfactants. A large number of surfactants which are commonly used like anionic, cationic, nonionic are employed to disperse the CNTs. Sodium dodecyl sulfate (SDS) and Triton-X 100 are used to obtain stable suspension usually for one week in the concentration range 0.1 and 0.5 mg/ml, respectively (Islam et al. 2003). However a better stability has been achieved by employing sodium dodecyl benzene sulfonate (10 mg/ml) (Strano et al. 2003) and 1-pyrene butanoic acid activated as succnimidyl ester (Guldi et al. 2005). Although surfactants have been used to stabilize the dispersion of the CNTs and render them biocompatible *in vivo* yet surfactants may have their on toxicity profile on the cellular milieu, which limits their application.

Polymers. Polymers are good alternatives to the surfactants for the dispersion of CNTs but have lower dispersion efficiency. They have the tendency to wrap around the CNTs. Suspensions of SWCNTs have been produced by substituted (metaphenylene vinylene) in the organic solvents. It wraps around the CNTs ropes. The steric repulsion plays a common role for the stabilization of CNTs suspension (Shvartzman-Cohen et al. 2004) where as in the nonionic surfactants the hydrophilic counter part plays an important role in the dispersion of CNTs. Steric stabilization also increase the suspendability. Various cationic copolymers (Didenko et al. 2005; Sinani et al. 2005) and fluorescent polymers have been conjugated to the CNTs surface e.g. poly vinylpyrrolidone (Dieckmann et al. 2003). It has been used to suspend the CNTs in 1% SDS to form the supramolecular complexes, which have the potential application as new molecular probes.

Biopolymers. In order to make CNTs more compatible with in the biological system it is advantageous that these CNTs should be dispersed or solubilized with the aid of biological components. For that a complex with DNA can be exploited to disperse the CNTs. DNA form the supramolecular complexes, which is based upon the π-stacking between the aromatic bases and CNTs surfaces (Balavoine et al. 1999). Sonication with single stranded DNA has been employed to solubilize the CNTs. Various amphiphilic peptides also have been employed efficiently to disperse the CNTs (Zorbas et al. 2004; Dieckmann et al. 2003). The selection of peptides could be done by phage- display peptide library (Wang et al. 2003).

Biocompatibility of carbon nanomaterials in vitro/in vivo

Although CNT have been proven cytotoxic, which limits their uses in the biological system, still number of studies have been performed which support the biocompatibility of CNT and CNT-based materials. Specific interaction between CNT-based materials and osteoblast, neural cells, fibroblasts, immune system, ion channels and cellular membranes has been studied.

Osteoblast cells interaction. Various neurological cell investigations support (McKenzie et al. 2004; Webster et al. 2004) the utility of CNT-containing materials as bone biomaterials, by examining the adhesion and function of bone-forming osteoblast cells (Price et al. 2003; Webster et al. 2004). Webster and co-workers (2004) investigated the proliferation and function of osteoblast cells (Elias et al. 2002). They selected the four variants of compacted carbon nanofibers CNF (of diameters >100 nm or <100 nm, with each category further split into unrefined and pyrolytically stripped CNF) as seeding material. This study revealed the increased osteoblast proliferation on the nanophase (<100 nm) CNF. Smaller diameter CNF was shown to have increased alkaline phosphatase activity, intracellular protein synthesis and deposition of extra-cellular calcium, as compared to the CNF (diameter >100 nm). Authors concluded that CNF did not induce a cytotoxic response and that the nano-phase CNF demonstrated potential as orthopedic materials. Same

group further investigated the adhesion properties of osteoblast, chondrocytes, fibroblasts and smooth muscle cells on PU/CNT nanocomposites (Price et al. 2003; Webster et al. 2004). Nano-sized CNF have been employed as an additional variant as in previous studies (Elias et al. 2002; McKenzie et al. 2004) to investigate the effect of surface energy and diameter on the adhesion properties of the cells. Webster and co-workers (2004) found that nano-sized CNF promoted osteoblast adhesion in contrast to the larger diameter CNF (Price et al. 2003), whereas fibroblasts, chondrocytes and smooth muscle cells showed decreased adhesion on high energy CNF but were unaffected by diameter. They also demonstrated increased osteoblast adhesion on CNF compared to the control materials, which was two metal alloys currently used in orthopedic implants – Ti_6Al_4V and Co Cr Mo. In the nanocomposite increased concentration of CNF led to increased osteoblast and decreased fibroblast adhesion with increasing concentrations of CNF. No significant difference was found between nanocomposites material and control material. They concluded that CNF did not show any cytotoxic effects and, on the contrary, showed promise for use in orthopedic materials (Price et al. 2003; Webster et al. 2004).

Another study indicated osteoblast proliferation on nanocomposites of CNT under alternating current stimulation (Supronowicz et al. 2002). The authors fabricated conductive poly lactic acid (PLA)/MWCNTs nanocomposites at 10, 15 and 20% w/w MWCNTs (synthesized via electric arc-discharge). Osteoblast cells were used as seeding material on the surface and then exposed to alternating current stimulation. Control samples were chosen as PLA/MWCNTs nanocomposite films. It was run without electrical stimulation. The results revealed an increase in osteoblast proliferation and extra-cellular calcium deposition on the nanocomposite compared with the control samples. Unfortunately, no comparison was made with a non-conductive or currently used orthopedic reference material under electrical stimulation.

Due to the excellent mechanical property of carbon nanotubes, it is expected to play the role as reinforcement for imparting strength and toughness to brittle hydroxyapatite (HA) bioceremic coating. It has been employed as enhancer of mechanical performance of HA coating. CNTs imparts the biocompatibility to the HA coating. Balania et al. (2007) distributed multiwalled CNTs reinforcement in HA coating using plasma spraying. It improves the fracture toughness (by 56%) and also enhances the crystallnity (by 27%). They also cultured osteoblast LFoB 1.19 cells onto the CNTs reinforced HA coating. This *in-vitro* experiment provided an insight to the biocompatibility of CNTs with the living cells. Cell culture study was performed with human osteoblast LFoB1.19, onto HA- 4 wt% CNTs plasma spraying coating. Differential cell growth was unrestricted and seen embedded in the HA matrix in the presence of CNTs. The biocompatibility of CNTs was revealed by good spreading of the LFoB1.19 osteoblast cells and unrestricted growth along the CNTs surface. Enhanced mineralization of the apatite was found over the CNTs surface, which has been proven to achieve stable scaffolds structure for the successful body implant performance. Finally, one important novelty was found in adding CNTs via the plasma spraying as revealed by three folds improvements in enhancing the fracture toughness of nanocomposites by 56%, aiding human osteoblast cell growth by its uniform dispersion and allowing precipitation and mineralization of apatite onto CNTs surfaces.

Neuronal cells interaction. The biocompatibility of CNT was demonstrated by its use for neural applications which particularly focuses on Neurite extension (Hu et al. 2004; Webster et al. 2004), astrocytes proliferation and function was studied with varying carbon nano-fiber diameter and surface energy by McKenzie et al. (2004). Four different surfaces were created from samples of carbon nanofibers (CNF) (large MWCNTs with diameters 60–200 nm synthesized via CVD). Two CNF samples (with diameters 100 and 200 nm) were used unrefined (classed as low surface energy CNF), whilst the other two CNF (with diameters 60 and 125 nm) were subjected to pyrolytic stripping to remove the outer hydrocarbon layer. Commonly astrocytes cells are largely responsible for the scar tissue formation seen with current implantable neural devices. They were than seeded onto the surfaces for adhesion, proliferation and function studies. The result showed that astrocytes preferentially adhered to and proliferated on larger diameter and higher surface energy CNF. They concluded that carbon nano-fibers with diameters <100 nm showed potential for neural applications due to a speculated reduction in neural scar tissue formation.

Another study was performed by Webster et al. (2004) which investigated the interactions between a series of polyurethane (PU)/CNF nanocomposites and astrocytes. The nanocomposites were synthesized by solvent-casting techniques, after PU/ CNF sonication in chloroform. The average diameter of CNF used in the study was 60 nm and were synthesized via CVD and purified by pyrolytic stripping. A small decrease (statistically significant amount) in astrocytes adhesion and retarded neurite

growth was observed in rat pheochromocytoma cells with increase in CNF loading in the nanocomposite in neural cell seeding studies. The authors concluded that these findings, coupled with ability to tailor the electrical resistance of CNF/PU nanocomposites, warranted further investigation into their use in neural probe applications.

Contrary to the previous studies subsequent studies found that both pristine and chemically functionalized CNT have a positive impact on neuronal growth (Erlanger et al. 2001; McKnight et al. 2003). Lithographically patterned CNT surfaces (islands of CNT grown via CVD on a quartz substrate) were seeded by confluent layers of neurons (Gabay et al. 2005). Neurons were observed to localize in CNT-rich regions after four days, while their associated neurite and axons formed an interconnected network that replicated the pattern of the CNT template (Erlanger et al. 2001). Neurite extension depends upon surface charge of carbon nanotubes as revealed by various functionalized CNTs (chemically functionalized with carboxylic acid, ethylenediamine or poly-m-aminobenzene sulfonic acid). Each holding a different ionic charge (at physiological pH) was observed to provide a substrate for neurite extension (McKnight et al. 2003). It was also concluded that positively charged ethylenediamine-MWCNTs produces the most neurite extension. Both the CNT-patterned surfaces and the functionalized MWCNTs showed no sign of cytotoxicity towards neuronal cells, suggesting the biocompatibility of carbon nanotubes.

Nano-structured surfaces employing carbon nanotubes. Correa-Duarte et al. (2004) investigated the effect of controlled MWCNTs surface structures on the adhesion and proliferation of L929 mouse fibroblast cells. Examination by SEM of the MWCNTs-based structures revealed: (a) Perpendicular aligned carbon nanotubes; (b) the latter after a physicochemical treatment forming pyramid-like structures; (c) network of cross-linked carbon nanotube walls forming cavities (Correa-Duarte et al. 2004) (Figure 11).

Surface morphologies were created from aligned MWCNTs grown on silicon substrates (via CVD using a Ni catalyst), by means of an oxidation process (Lui et al. 2004), which functionalizes the MWCNTs with –COOH groups, as well as creating capillary and tensile forces. The end result is a surface morphology composed of honeycomb-like polygons or pyramid-like structures that are dependent on the length of the aligned MWCNTs. Mouse fibroblast cells were seeded onto the various surfaces and incubated for up to 7 days. SEM analysis of cellular morphology showed isolated cells (one cell per polygon) after 1 day and a confluent layer of cells after 7 days. SEM images are shown in Figure 12.

Initial attachment was observed to be via elongated cytoplasmic projections to the walls and floor of the polygonal cavity. The authors reported no cytotoxicity at either the 1 or 7 day time points (Correa-Duarte et al. 2004). Controlled CNT surface structures have also been used for non-inheritable genetic modification of Chinese hamster ovary (CHO) cells (McKnight et al. 2003). Vertically aligned carbon nano-fiber (VACNF) arrays were constructed from 500 nm nickel catalyst dots imprinted at 5 μm intervals on silicon wafers. Plasma enhanced CVD was used to grow conical bundles (tip approximately 20–50 nm in diameter) of nitrogen-doped CNT. Plasmid DNA encoding the green fluorescent protein (GFP) was then attached (either covalently or by simple drying) to the VACNF arrays. CHO cells were impaled onto the VACNF arrays via centrifugation and pressing. Cytotoxic responses were not reported for the seeded cells, although plasmid retention was poor and only minimal GFP production was reported. Plasmid expression ceased when the cells were no longer in contact with the nano-fibers and cell progeny were found not to contain the plasmid. This suggests that the cells, which received plasmid DNA through contact with the CNT, may have been sufficiently damaged in the process to result in cell death.

Antibody interactions. Chen et al. (1998) determined the generation of fullerene-specific anti-bodies. This was the first work regarding the interaction of fullerene with antibodies. Immunization was carried out in BALB/c mice with a C60 fullerene-thymoglobulin conjugate; it resulted into generation of the polyclonal IgG antibody that could bind to both C60 and C70 fullerene derivatives. A similar study investigated the binding of C60-specific monoclonal antibodies to SWCNTs (Erlanger et al. 2001). The study revealed the binding of antibodies to aqueous SWCNTs ropes. Similarly, hydrophilicity and surface disorder of the fibers of CNTs affects it's binding to the monoclonal antibodies as demonstrated by Naguib et al. (2005). Poly-L-lysine adsorbed onto CNT was also shown to enhance antibody and protein binding. These studies demonstrate the possibility of generating antibody-coated SWCNTs cellular probes and drug delivery vehicles. It also demonstrates the versatility of the immune system and raises questions about potential health effects of long-term exposure in the form of allergies and hypersensitivity (Naguib et al. 2005).

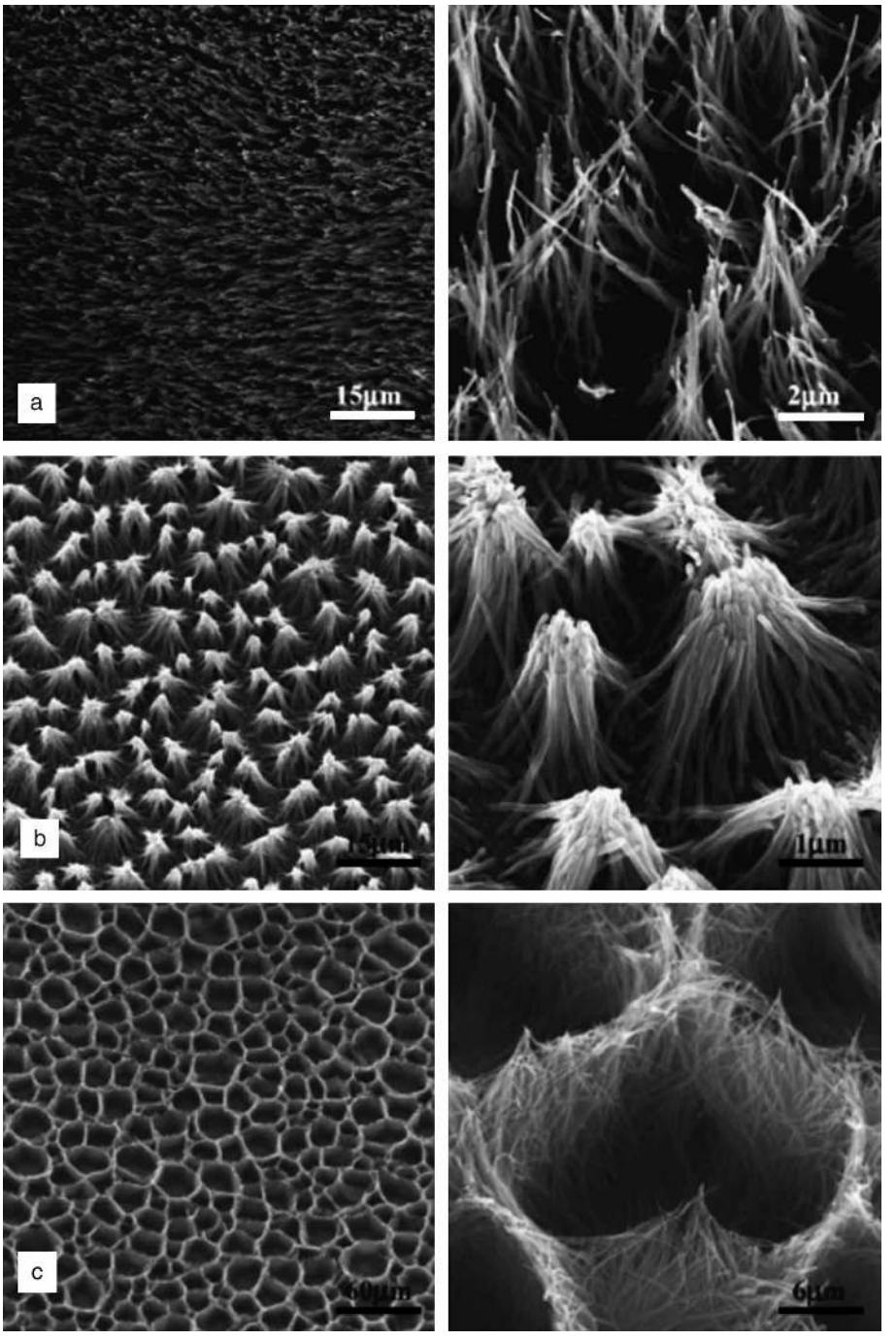


Figure 11. Examination by SEM of the MWCNTs-based structures. (a) Perpendicular aligned carbon nanotubes; (b) Pyramid-like structures which are formed after a physicochemical treatment; (c) Network of cross-linked carbon nanotube walls forming cavities. Reprinted with permission from (Correa-Duarte et al. 2004). Copyright (2007) American Chemical Society.

Ion channel interactions. A specific interaction was investigated by Park et al. (2003) between SWCNTs (synthesized via CVD using a Co/Mo catalyst and later purified), MWCNTs (synthesized via a catalyst free arc-discharge technique), fullerenes (C60 and hyper fullerenes) and ion channels. They carried out the study (Park et al. 2003) using several 'different pore-forming ion channel subunits, heterogeneously expressed in mammalian (CHO) cells'. SWCNTs were found to block channels, formed from EXP-2, KVS-1, human KCNQ1, Kv4.2 and HERG potassium channels, but in a dose-dependent manner. Fullerenes were shown to be less effective channel blockers than CNT. Authors concluded that the

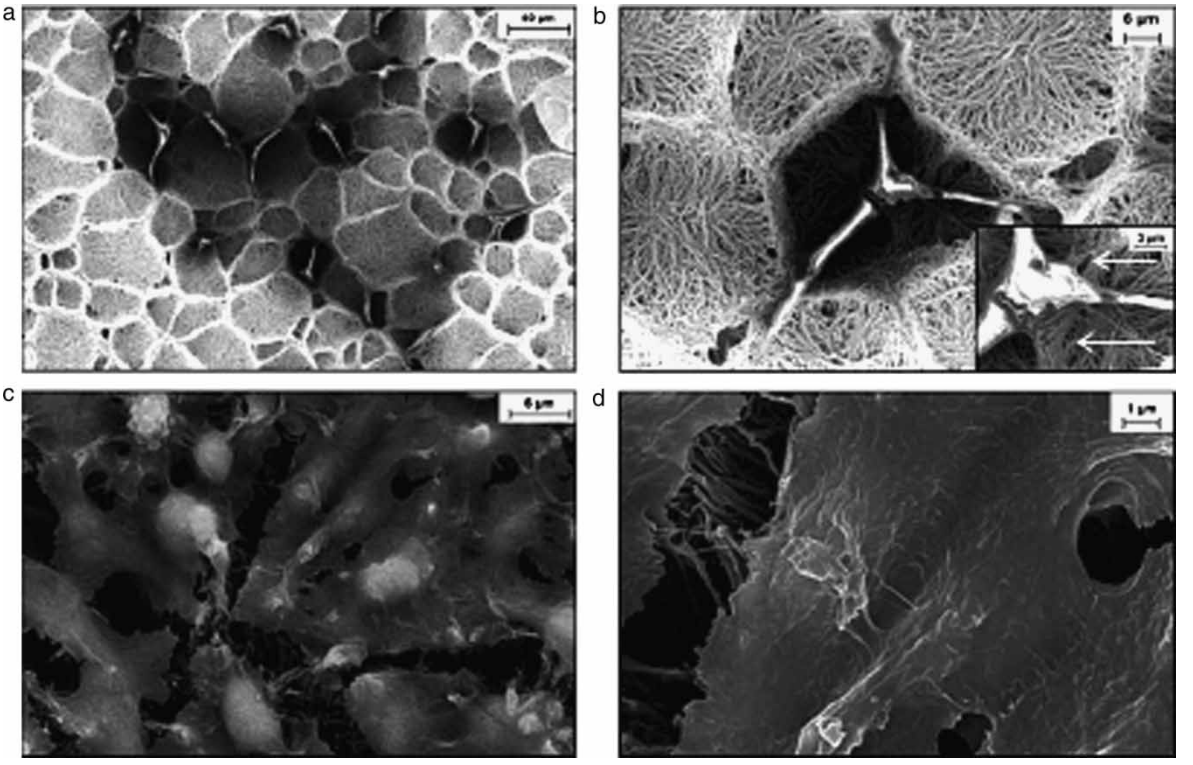


Figure 12. Scanning electron micrographs of L 929 mouse fibroblasts growing on MWCNTs-based network. (a) and (b): After 1 day; (c) and (d): After 7 days. Reprinted with permission from (Correa-Duarte et al. 2004). Copyright (2007) American Chemical Society.

interaction mechanism was solely dependent on the size and shape of the nanoparticles; and that the electrochemical interactions between CNT and the ion channels were absent, in contrast to conventional channel blockers.

Protein interaction. Since inhalation route is the main portal of entry of carbon nanotubes among the various exposure pathways, it constitutes the highest risk to the physiological system as it is notably difficult to deal reliably with suspension in air of extremely small particles. Carbon nanotubes tend to form aggregates, many of which will not penetrate to the lung alveoli when inhaled. However, non-aggregated carbon nanotubes may, because of their small size, penetrate to the alveoli of the lungs, where they may interact with surfactant proteins and lipids. Morales et al. (2007) described the interaction of carbon nanotubes with host pulmonary system at the molecular level. They (Morales et al. 2007) reported the interaction of two surfactant proteins SP-A and SP-D (collectively known as collectins that contain a collagenous region and a C-type lectin domain.). A major biological role of the collectins is to bind to targets (e.g., microorganisms and allergens) by recognizing patterns of carbohydrate distribution at their surface and to enhance phagocytosis/clearance of the targets. Thus, they play an important role in first-line immune defenses within the lung. Catalytic vapor deposition double walled carbon nanotubes were used as sample that was characterized by plasma emission spectroscopy, transmission electron microscopy (TEM), themoquest instrument and FTIR instrument. Bronchoalveolar Lavage Fluid (BALF), which was obtained from protienosis patients (have high concentration of lung surfactant proteins). It was characterized for protein content by Bradford method. They employed SDS-PAGE, mass spectrometry and N-terminal sequence analysis for the characterization of binding of human plasma proteins including C1q, fibrinogen and high-density lipoproteins to carbon nanotubes. The researchers found that these binding reactions did not require divalent metal ions, which were contrary to the selective binding of BALF supernatant SP-A and SP-D to carbon nanotubes that is Ca^{2+}-ion dependent. The binding of SP-A and SP-D to carbon nanotubes can be stopped by using Ca^{2+}-ion chelators such as EDTA. Through various studies the researchers tried to correlate the different inflammatory response like the formation of bronchiolar granulomas around focal aggregate of CNTs, production of TNF-α by carbon nanotubes, and the accumulation of collagen in the lungs of animals treated with carbon nanotubes and ground carbon nanotubes, to the SP-A and SP-D functions.

Applicability of biocompatibility of carbon nanotubes

Functionalization of CNTs has been used to address the problem of CNTs insolubility in aqueous media and, in many cases, has permitted linking of biologically active peptides and medicinal drugs to the CNTs side-wall (Georgakilas et al. 2002; Pantarotto et al. 2003a,b; 2004; Cherukuri et al. 2004). These properties have generated interest in using CNTs as drug or vaccine delivery vehicles. Several studies had been conducted on CNTs functionalization with vaccine or drug molecules (Georgakilas et al. 2002; Pantarotto et al. 2003a; Pantarotto et al. 2003b, 2004; Cherukuri et al. 2004; Kam et al. 2004, 2005). Two studies have used functionalized SWCNTs to create a vaccine delivery device by attaching a small peptide sequence from the foot and mouth disease virus (FMDV) to the side-wall of purified SWCNTs via 1,3-dipolar cycloaddition (Georgakilas et al. 2002; Pantarotto et al. 2003a). Both studies demonstrated that the conformation of the peptide sequence was maintained and recognized by mono- and poly-clonal antibodies, and that the SWCNTs-FMDV peptide complex induced a specific anti-body response *in vivo*. No cross reactivity (immune response) observed was to the SWCNTs *in vivo*, suggesting that vaccine delivery is a viable application for CNT (Pantarotto et al. 2003b). Pantarotto et al. (2004a) studied the translocation of functionalized SWCNTs across human 3T6 and murine 3T3 cell membranes. Investigators attached the peptide fragment from the subunit of the GS protein to the purified SWCNTs via 1, 3-dipolar cyclo-addition. The SWCNTs-αS complex was also fluorescently labeled with fluorescein isothiocyanate (FITC). Control samples consisted of SWCNTs labeled with FITC (FITC-SWCNTs), FITC-αS and unbound FITC. The FITC-SWCNTs-αS complex was able to cross the cell and nucleic membranes while FITC-SWCNTs were only able to cross the cellular membrane and accumulated in the cytoplasm. In contrast, FITC-αS and unbound FITC were unable to enter the cells. The authors were unable to determine the mechanism for cellular FITC-SWCNTs-αS uptake or localization within the nucleus. It was postulated that passive uptake mechanisms, such as endocytosis, were not responsible, rather the FITC-SWCNTs-αS complex behaved like a cell-penetrating peptide, despite lacking the cationic character or amino acid sequences usually associated with translocation to the nucleus. It was also discovered that the FITC-SWCNTs-αS complex could accumulate in the cytoplasm, or cross the nucleic membrane to a concentration of 10 μM before being toxic to the cells. At 10 μM, the FITC-SWCNTs-αS complex induced cell death in 80% of cells. The translocation across the cellular membrane indicates that SWCNTs may be a promising carrier for drug-delivery applications. Kam et al. (2004) studied the uptake of SWCNTs (synthesized via laser ablation, purified and shortened by sonication) and SWCNTs–streptavidin conjugates into human promyelocytic leukemia cells and human T cells via the endocytosis pathway. In contrast to Pantarotto et al. (2004) this study demonstrated that SWCNTs were taken up into cells via a mechanism consistent with endocytosis. No cytotoxicity was observed for the pristine SWCNTs. SWCNTs–streptavidin conjugates caused extensive cell death, which was attributed to the delivery of streptavidin to the cells. In 2004 and 2005, similar trials also demonstrated the successful transportation (via endocytosis after attachment to SWCNT) of RNA polymer and cytochrome-c into the cytoplasm and nucleus of cells (Lu et al. 2004; Kam et al. 2005). CNT-specific cytotoxicity was not observed in either study. Cherukuri et al. (2004) investigated the uptake of pristine SWCNTs into the mouse J774.1A macrophage-like cell line via near infrared fluorescence microscopy (Cherukuri et al. 2004). The study reported that the macrophage-like cells appeared to phagocytose SWCNTs at a rate of approximately one SWCNT per second, without any apparent cytotoxicity. The SWCNTs remained fluorescent, suggesting that the macrophage-like cells were not capable of breaking them down within the time period of study. This result is inconsistent with previous macrophage investigations (Jia et al. 2005; Muller et al. 2005). It should be noted that prolonged or incomplete breakdown of foreign material often leads to chronic macrophage activation, and, in turn, to chronic inflammation (Donaldson & Tran 2004). Another group of scientists (Wu et al. 2005) reported the efficient utilization of functionalized carbon nanotubes for the targeted delivery of Amphotericin B. They functionalized the MWCNTs by orthogonal double functionalization method employing the conjugation of Amphotericin B and FITC to the nanotubes. FITC was employed for efficient cellular localization. Biological activity of doubly functionalized MWCNTs was determined by incubating the Human Jurkat Lymphoma cells with functionalized MWCNTs in RPMI medium at 37°C. AmB was used as a control. They observed that the conjugation of Amphotericin B to the MWCNTs not only reduced the toxic effects of the antibiotic on the mammalian cells but also increased the internalization of the antibiotic into the cells. The scientist also found that internalization of antibiotic into the cells was dose-dependent. They also reported the antifungal activity of CNTs functionalized with AmB,

against three species of fungi, i.e., *Candida parapsilosis* ATCC 90118, *Cryptococcus neoformans* ATCC 90112 and *Candida albicans*. AmB was covalently linked to the ammonium functionalized single and multiwalled nanotubes. The minimum inhibitory concentration (MIC) was calculated after 48 h of incubation with different doses of AmB, unconjugated CNTs and CNTs-AmB conjugates. They observed that the ammonium functionalised CNTs (free of AmB) were inactive up to a maximal concentration of 80 μg/ml, against all microorganisms. They also revealed that the activity of the drug was not marred by the covalent binding to the SWCNTs and MWCNTs as indicated by the high activity of SWCNTs-AmB and MWCNTs-AmB conjugates. The increased activity was attributed to the increased solubilization of AmB by conjugation to the CNTs, presence of multiple copies of AmB on CNTs which provide the efficient interaction to fungal membrane. Finally the authors concluded that multifunctional and conjugated CNTs could be utilized for the delivery of bioactive antibiotics to the cellular milieu by a specific transport mechanism and for modulating the therapeutic action of the drug, respectively.

Conclusion

Research on a unique nanomaterial such as carbon nanotubes has provided the new opportunities to the drug delivery scientist to utilize them as an efficient targeting module both at the cellular and the intracellular level. It has unique chemical and electronic properties. Certain new classes of carbon nanotubes have been developed for biomedical applications, while cytotoxicity may be mitigated by chemical functionalization. However certain drawbacks of carbon nanotubes such as nonbiodegrability, inherent toxicities on lungs, skin, and eye irritation might limit their usefulness. Although a number of *in-vitro* and *in-vivo* toxicological studies have been performed, the results of these studies are not unequivocal to the extent of being controversial. Some area of investigation regarding the size distribution of CNTs, respirability of aerosolized CNT particles, accurate information on the dermal cytotoxicity, macrophage response of CNTs, toxicities in relation to the properties of carbon nanotubes and possible *in-vivo* biokinetics of carbon nanotubes should be evaluated with objective experimental design. Safety of a drug/vaccine delivery system being a paramount consideration, it requires more intense, specific and conclusive research efforts. In view of the extensive application potential of carbon nanotubes, it is highly desirable that this material is adjudged as GRAS or otherwise. Proper handling procedures and the introduction of safety measures in the manufacturing premises and research laboratories would pave the way for the possible exploitation of CNTs in a wide spectrum of applications including pharmaceuticals.

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