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The biocompatibility of carbon nanotubes

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Abstract

Carbon nanotubes (CNT) are well-ordered, high aspect ratio allotropes of carbon. The two main variants, single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT) both possess a high tensile strength, are ultra-light weight, and have excellent chemical and thermal stability. They also possess semi- and metallic-conductive properties. This startling array of features has led to many proposed applications in the biomedical field, including biosensors, drug and vaccine delivery and the preparation of unique biomaterials such as reinforced and/or conductive polymer nanocomposites. Despite an explosion of research into potential devices and applications, it is only recently that information on toxicity and biocompatibility has become available. This review presents a summary of the performance of existing carbon biomaterials and gives an outline of the emerging field of nanotoxicology, before reviewing the available and often conflicting investigations into the cytotoxicity and biocompatibility of CNT. Finally, future areas of investigation and possible solutions to current problems are proposed.

Keywords: Carbon nanotube; Biocompatibility

1. Introduction

Carbon nanotubes (CNT) are unique, one-dimensional macromolecules, whose outstanding properties have sparked an abundance of research since their discovery in 1991 [1]. Single-walled carbon nanotubes (SWCNT) are constructed of a single sheet of graphite (diameter 0.4–2 nm), while multi-walled carbon nanotubes (MWCNT) consist of multiple concentric graphite cylinders of increasing diameter (2–100 nm) [2]. Both SWCNT and MWCNT possess high tensile strengths, are ultra-light weight and have excellent thermal and chemical stability. In combination with their metallic- and semi-conductive electronic properties, this remarkable array of features has seen a plethora of applications proposed.

One of the major areas of CNT research is the field of biomedical materials and devices. Many applications for CNT have been proposed including biosensors, drug and vaccine delivery vehicles and novel biomaterials [2]. CNT can be used as nano-fillers in existing polymeric materials to both dramatically improve mechanical properties and create highly anisotropic nanocomposites [3,4]. They can also be used to create electrically conductive polymers and tissue engineering constructs with the capacity to provide controlled electrical stimulation [5–7].

However, before such materials can be incorporated into new and existing biomedical devices, the toxicity and biocompatibility of CNT needs to be thoroughly investigated. For the purposes of this review, biocompatibility will be defined as "the ability of a material to perform with an appropriate host response in a specific application" [8]. Concurrent with the progress of research is the increase in exposure of the scientific community, and eventually the general public, to CNT [9]. Hence, it is also imperative that the occupational health and safety of CNT exposure be thoroughly investigated before the use of CNT-based materials becomes widespread. Despite the clear need for a full scientific assessment of CNT toxicology, early research into

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the safety of CNT was scarce. This oversight is perhaps founded on the excellent performance of existing carbon-based biomaterials and/or the general lack of the multidisciplinary expertise within CNT research groups required to perform such characterization. However, in the last 5 years, disturbing and often conflicting data have emerged concerning their safety. This review will present a summary of the excellent biocompatibility of existing carbon biomaterials, shed some light on some of the disturbing findings in the growing field of nanotoxicology, and critically review all reports concerning CNT toxicity and biocompatibility.

1.1. Existing carbon biomaterials

Carbon-based biomaterials are not new. Pyrolytic carbon has been used for several decades in biomedical implants and coatings, particularly in the manufacture of heart valve prostheses [10]. Pyrolytic carbons were initially developed for the aerospace and nuclear industries. Most are highly anisotropic, but isotropic coatings have also been developed using special chemical vapor deposition (CVD) processes, which were found to have excellent biocompatibility properties [11,12]. Initial studies demonstrated excellent blood compatibility of pyrolytic carbon heart valves, with good adherence of endothelial cells and minimal adherence and activation of platelets [13]. Subsequent decades of use have demonstrated the accuracy of these initial observations and pyrolytic carbon remains one of the most widely used biomaterials in mechanical heart valve prostheses. Conversely, a clinical study involving 420 patients, revealed pyrolytic carbon coated stents could not demonstrate a significant improvement in stent performance (restenosis rate, improvement in angiographic results, surgical re-intervention or death) over traditional high-grade stainless steel stents [14].

A more recent development in biomedical carbons is diamond-like carbon (DLC). This dense metastable form of amorphous carbon has several properties which make it desirable for biomedical applications. Most notable are its high hardness, low coefficient of friction, chemical inertness and good corrosion and wear resistance [15–17]. Many studies have investigated the biocompatibility of DLC coatings for orthopedic and cardiovascular applications. Initial biocompatibility studies reported no inflammatory response from macrophage cells in vitro [18–20] and no cytotoxic effects on fibroblasts or osteoblast cells have been observed [19,21]. Moreover, both osteoblast and fibroblast cells adhere well to DLC coatings, and osteoblast cells have been shown to proliferate well on DLC surfaces [15]. Subsequent tests investigating the activation of macrophages in a cardiovascular environment have shown DLC coatings did not affect the morphology or activation status of macrophages [20,22]. DLC coatings in cardiovascular stents have also been found to reduce platelet and macrophage activation in vitro when compared to uncoated stainless steel stents [22,23].

A small number of in vivo tests have been conducted on DLC coatings [21,24,25]. Implants of DLC-coated stainless steel rods had no adverse effects on bone or muscle tissue of sheep and were separated from surrounding tissue by a thin fibrous layer similar to non-DLC coated implants [24]. Similar studies involving DLC-coated CrCo rods in rats also showed no adverse effects under histological analysis [21].

Current carbon-based biomaterials clearly demonstrate excellent biocompatibility and it is hoped that the impressive properties of CNT can be exploited in biomedical devices safely and effectively.

1.2. Nanotoxicology

Particle toxicology is the study of the adverse effects of tissue exposure (typically through the lungs, digestive tract or skin) to particulate matter. The emergence of nanotechnology and nanoparticulate pollution has raised questions as to the effect of these nanomaterials on human health. Currently, the main route of exposure of the general public to nanoparticles comes in the form of air pollution [26]; however, this is not the only route. Nanoparticles are being introduced into epidermal creams such as sunscreens, and they are also being designed for use in a range of therapeutic and diagnostic applications (e.g. drug delivery) that will result in exposure to the digestive tract and possibly the blood stream [9]. With an obvious need for a toxicological evaluation of nano-sized particles and fibers, the field of nanotoxicology, currently defined as "science of engineered nanodevices and nanostructures that deals with their effects in living organisms" [27], was born [9].

The high aspect ratio of CNT makes them unusual for toxicological investigation in that they share shape characteristics with both nanoparticles and fibers [9]. This section introduces both particle and fiber toxicology and reviews current data concerning nanoparticulate carbon black and fullerenes.

There are three generally accepted factors which determine the potential of a particle to cause harm:

- The surface area/mass ratio of the particle—a large surface area gives the particle(s) a greater area of contact with the cellular membrane, as well as a greater capacity for absorption and transport of toxic substances.
- The particle retention time—the longer the particle stays in contact with the cellular membrane the greater the chance for damage. This factor also incorporates the concept of particle mobility, either through clearance or migration to surrounding tissue.
- The reactivity or inherent toxicity of the chemical(s) contained within the particle.

This list of factors was collated from studies involving lung inhalation [28–30]; however, the general concepts apply to all methods of exposure. Particles in the nano-sized range

will have their inherent toxic effects enhanced by virtue of the higher surface area/mass ratio [31]. Hence, as particles become smaller, the potential to cause harm is expected to become greater [9]. Limited data are currently available; however, concerns are currently being voiced about the impact of nano-sized particles on human health [32,33]. The following findings are of particular relevance to this issue:

- High purity carbon black with an average primary particle diameter of 14 nm increased oxidative stress in human type II alveolar epithelial cells in vitro and increased murine alveolar macrophage migration in fetal calf serum nearly two fold compared to high purity carbon black with an average primary particle diameter of 260 nm [34,35].
- Long-term, sub-chronic inhalation of carbon black with an average diameter of approximately 16 nm can cause the development of pulmonary tumors in rats [36,37].
- C_{60} fullerenes have not demonstrated significant toxicity [38], despite evidence that intravenous injection in rats leads to rapid distribution amongst many tissues (including the brain) and accumulation in the liver and spleen [39].

Fibrous materials present a different pathology to particulates. In particular, exposure via respiration is far more pathogenic than other methods of entry. Asbestos, perhaps the most infamous fibrous material, exemplifies the pathogenic characteristics of fibers and has been the main topic of research in fiber toxicology [40]. However, there is much heterogeneity in the pathological potential of different (conventional-sized) respirable fibers, and it has been research into synthetic vitreous fibers that has held the key to determining both the pathogenic characteristics of fibers and the mechanisms of pathogenicity [40].

There are three main characteristics of respirable fibers that determine their pathogenicity [40].

- Fiber dimensions
  - The dimensions of the fiber determine its respirability (the ability to penetrate into the centri-acinar region of the lungs). Essentially, the one-dimensional nature of fibers means that even long fibers (>15 μm) can be respired provided they are thin enough (<5 μm) [31].
  - Long fibers (>17 μm) become difficult for macrophages to phagocytose, which promotes chronic release of inflammatory mediators and contributes to fibrosis [41]. Short fibers (<7 μm), however, are usually easily phagocytosed and hence, cleared by the macrophages [42].
- Biopersistence
  - This is a key modifying factor in the toxicology of very long fibers, which are often difficult for macrophages to phagocytose. A high biopersistence increases the toxicity of all fibers. Fibers that are biosoluble are capable of having their structural components leached out. This weakens them over time until they eventually break into smaller fragments which are then phagocytosed and cleared [40]. A low biopersistance decreases the toxicity of even very long fibers (>20 μm).
- Reactivity or inherent toxicity
  - As with particulates, the toxicity of a fibrous material will also depend largely on the toxicity of its chemical components.

These characteristics apply to conventional diameter fibers and it is not yet known whether they will also be applicable to nano-fibers. Similarly, it is not clear how the inherent chemical stability of CNT [74] will impact on their biopersistence.

2. Toxicity of carbon nanotubes

One of the major concerns surrounding the use of CNT-based materials is the unknown impact on workers involved in their manufacture and handling. The majority of the work published, since the first study in 2001, has demonstrated that CNT could pose potential health problems. This section gives a detailed account of studies into lung toxicity, skin irritation and cytotoxicity.

2.1. Lung toxicity

Currently, there are five published in vivo studies assessing the potential impact of both unrefined and pristine CNT on the lungs [43–47]. Despite no initial indication of lung toxicity [43], all recent studies have found histological evidence of lung inflammation and granuloma formation [44–47]. The initial study in 2001 of Huczko et al. [43] investigated the effects of unrefined CNT on the pulmonary function of guinea pigs. The unrefined CNT were synthesized via the arc-discharge sublimation of a graphitic anode doped with Co/Ni catalyst. The authors did not specify whether the CNT produced were single- or multi-walled. The guinea pigs (Dunkin Hartley, male) were intratracheally instilled with 25 mg of unrefined CNT in 0.5 mL of saline solution, while control animals received 25 mg of CNT-free soot. Small amounts of surfactant were used to help disperse the nanotubes. Pulmonary function was investigated via non-invasive procedures 4 weeks after instillation. The animals were sacrificed at this time for further study. Neither the non-invasive procedures, nor the final bronchoalveolar lavage examination (BAL) showed any difference between the exposed or control groups, and it was concluded that "working with soot containing carbon nanotubes is unlikely to be associated with any health risk" [43]. Little was made of these preliminary findings, and it was not until the publication of two detailed studies into CNT lung toxicity [44,45] in 2004, that scientific focus shifted towards CNT toxicity.

Lam et al. [44] investigated the pulmonary toxicity of three batches of SWCNT (unrefined and purified samples

from iron catalyzed HiPco synthesis and unrefined SWCNT, containing nickel, synthesized via electrical arc-discharge) and compared them to carbon black and quartz particles. Mice were intratracheally instilled with 0, 0.1 or 0.5 mg of sample in 50 μL of mouse serum and then observed for either 7 or 90 days at which point they were sacrificed for histological examination. Investigators found that all three SWCNT products induced dose-dependent lung lesions, characterized by interstitial granulomas, regardless of the levels of metal impurities. They strongly believed that the presence of CNT resulted in granuloma formation, although they noted that the SWCNT containing Ni produced a higher mortality rate. The study also concluded that SWCNT were more toxic than carbon black, and CNT containing Ni was more toxic than quartz, the recognized positive inducer of lung toxicity.

Warheit et al. [45] investigated the lung toxicity of SWCNT in rats via intratracheal instillation. The study exposed 8-week old male rats to 1 or 5 mg/kg of unrefined SWCNT (synthesized via laser ablation with a composition of approximately 55–65% SWCNT, 30–40% amorphous carbon and 5% Ni and Co), quartz (as a positive control), carbonyl iron particles (as a negative control), graphite particles (with the same percentage Ni/Co as SWCNT) and phosphate buffered saline solution. The rats were studied using BAL fluid markers, cell proliferation assays and histopathological examination at 24 h, 1 week, 1 month and 3 months after instillation. Initially, a mortality rate of approximately 15% was observed in rats exposed to 5 mg/kg SWCNT. However, this was later attributed to mechanical blockage of the upper airways causing asphyxiation, rather than any inherent toxicity of the SWCNT soot. Researchers found that exposure to SWCNT produced only transient inflammation, as assessed by cell proliferation and cytotoxicity indices. Histological examination of exposed animals identified a series of non-dose-dependent multifocal granulomas. The granulomas were non-uniform in distribution and not progressive after 1 month. The presence of the granulomas was considered inconsistent with the lack of lung toxicity as determined by other parameters and the authors concluded that more research was needed.

Following their preliminary study in 2001 [43], Huczko et al. [46] published a follow-up study in 2005. In this investigation, five different samples of MWCNT (unrefined MWCNT produced by both CVD and arc-discharge, and commercially available CNT from Nanolab, Pyrograf and Showa Denko) were intratracheally instilled into guinea pigs. The animals were studied using BAL markers, lung resistance tests and histopathological examination at 90 days. Unlike the preliminary study, significant evidence of pulmonary toxicity was observed. These researchers found alveolar macrophage infiltration in the BAL of all non-control animals, except for those instilled with Pyrograf CNT. Abnormal lung resistance was observed in all animals exposed to CNT samples, while lung histology reported multiple lesions in all CNT-exposed animals. The authors concluded that, in conjunction with their previous report, exposure time was critical for induction of lung pathology.

Muller et al. [47] investigated the effect of intratracheally instilled MWCNT on the pulmonary function of rats. Animals were exposed to 0.5, 2 and 5 mg of purified and 'ground' MWCNT (purified MWCNT ground in an oscillatory ball mill to reduce CNT aggregation) and monitored for up to 60 days. Asbestos and carbon black were used for comparison. Rats (Sprague–Dawley, female) were studied using BAL, inflammatory and fibrotic markers, biopersistance tests, and histopathological examination. These researchers observed dose-dependent inflammation and granuloma formation, however, unlike previous studies [45] the inflammation was not transient, persisting for the full 60 days. In all cases, both MWCNT and 'ground' MWCNT were considered more inflammatory than carbon black, but less inflammatory than the asbestos fibers. Biopersistence tests revealed that the shorter 'ground' MWCNT were cleared faster than normal MWCNT. Although in both cases the clearance rates were slow, with material remaining in the lungs after 60 days. The authors urged the introduction of appropriate safety measures for handling CNT while calling for more studies to accurately establish the toxicology of CNT.

In the only study of its kind to date, Shvedova et al. [48] investigated the pharyngeal aspiration of SWCNT (synthesized via the HiPco process and purified to >99%) in mice (C57BL/6, female). The study found that SWCNT exposure (10, 20, 40 μg/animal) lead to a dose-dependent increase in inflammatory markers at 1–3 days post exposure, granuloma formation and progressive interstitial fibrosis and alveolar wall thickening up to 60 days post exposure. Contrary to intratracheal instillation studies [43–47], these authors believed that pharyngeal aspiration (a technique in which a 50 μL drop of SWCNT suspended in phosphate buffered saline solution is placed on the back of the mouse tongue, which is then held between forceps until the drop has been aerosolized) resulted in two distinct particle morphologies. The first particle morphology observed, compact SWCNT aggregates (>500 nm in diameter), was associated with acute inflammation and granuloma formation at the particle deposition sites. However, the second particle morphology, dispersed SWCNT structures (delicate fiber-like structures with average fiber diameter <50 nm in diameter), was associated with diffuse interstitial fibrosis and alveolar wall thickening in areas distant to SWCNT aggregate deposition (the proximal alveolar region) and in the absence of persistent local inflammation. These observations of a fibrogenic response to disperse aerosol SWCNT particles are unique and disturbing, suggesting that SWCNT exposure poses significant health risks for workers. The researchers concluded that more extensive inhalation studies were required to confirm these initial observations.

All studies that utilized intratracheal instillation reported major difficulties dealing with the agglomerative

nature of CNT in aqueous solutions [43–47]. Despite repeated use of surfactants [43,45–47] and sonication [44,47]; histopathological examinations show 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 [48]. 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 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 [49]. Current handling procedures employed by nanotube manufacturers do not produce significant quantities of airborne CNT [49]. However, the possibility of cumulative effects, especially if increased quantities are handled, justifies the introduction of safety measures.

2.2. Skin irritation

Studies into skin irritation by CNT are extremely limited at this time, with only one preliminary study published by Huczko and Lange [50]. They evaluated the potential of CNT to induce skin irritation 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" [50]. However, more recent in vitro studies involving human epidermal keratinocytes have raised concerns over this assessment [51,52]. Clearly this is an area requiring further scientific evaluation despite evidence of low skin exposure rates (estimated at 0.2–6 mg of CNT material per glove) during the manufacture and handling of CNT [49].

2.3. Cytotoxicity of carbon nanotubes

To date, there are six published studies focusing on CNT cytotoxicity [47,51–55]. Shvedova et al. [51] reported the first cytotoxicity study on CNT in 2003. These researchers investigated the effects of unrefined SWCNT on immortalized human epidermal keratinocytes (HaCaT). HaCaT cells were incubated for up to 18 h in media containing unrefined SWCNT (0.06–0.24 mg/mL). Exposure to SWCNT resulted in accelerated oxidative stress (increased free radical and peroxide generation and depletion of total antioxidant reserves), loss in cell viability and morphological alterations to cellular structure. It was concluded that these effects were a result of high levels (approximately 30%) of iron catalyst present in the unrefined SWCNT. They also warned of possible dermal toxicity in handling unrefined CNT, but stressed the role of SWCNT particle size and structure in these findings.

Similar dermal toxicity warnings were echoed in 2005, in a study which found that MWCNT initiated an irritation response in human epidermal keratinocyte (HEK) cells [52]. Purified MWCNT (synthesized via CVD) incubated (at doses of 0.1–0.4 mg/mL) with HEK cells for up to 48 h were observed to localize within cells (Figs. 1 and 2), elicit the production of the pro-inflammatory cytokine (IL-8) release and decrease cell viability in a time- and dose-dependent manner. The lack of catalyst particles led these authors to conclude that CNT themselves were a potential dermatological hazard, urging a full toxicological assessment before widespread public exposure.

In vitro evaluation of the inflammatory potential of CNT on peritoneal and alveolar macrophages was reported in 2005 [47,53]. Muller et al. [47] incubated peritoneal macrophages (taken from Sprague–Dawley rats) for up to 24 h in media containing purified MWCNT and purified

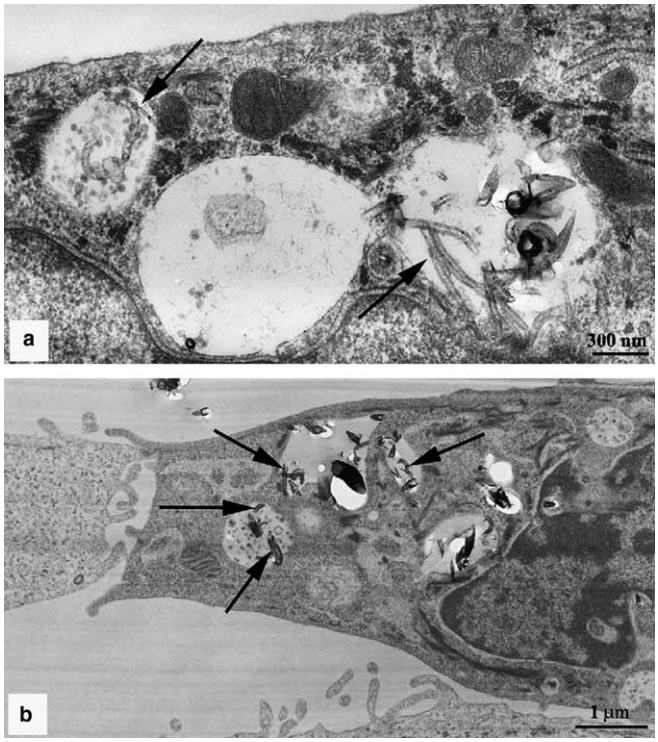

Fig. 1. Transmission electron micrograph of human epidermal keratinocytes: (a) intracellular localization of the MWCNT—arrows depict the MWCNT present within the cytoplasmic vacuoles of a HEK; (b) keratinocyte monolayer grown on a Permanox surface—arrow depicts the intracytoplasmic localization of the MWCNT. Reprinted with permission from [52]. Copyright (2005) Elsevier.

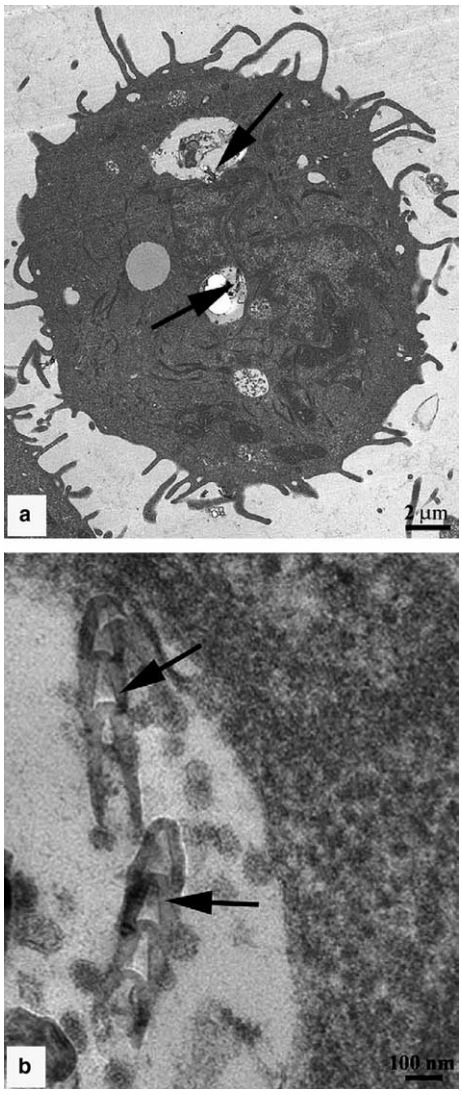

Fig. 2. Transmission electron micrograph of human epidermal keratinocytes: (a) low magnification of a keratinocyte for precise location of the vacuoles containing MWCNT (arrows); (b) high magnification of vacuole to demonstrate that multi-walled CNT structures resembling bamboo shoots are present inside keratinocytes. Reprinted with permission from [52]. Copyright (2005) Elsevier.

'ground' MWCNT (sample was ground in an oscillatory ball mill) at concentrations of 20, 50 and 100 μg/mL. Cytotoxicity was assessed by measuring lactate dehydrogenase (LDH) release 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. These researchers determined that 'ground' MWCNT had a similar capacity for inducing dose-dependent cytotoxicity and up-regulating TNF-α expression as asbestos and carbon black. In contrast, cytotoxicity and TNF-α expression in the 'un-ground' MWCNT sample were significantly lower than the 'ground' sample. It was postulated that the increased agglomeration observed in the 'un-ground' sample lead to a decrease in MWCNT availability to the cells, accounting for the lowered cytotoxic and pro-inflammatory response.

Jia et al. [53] investigated the effect of different carbonaceous nano-materials on the cytotoxicity of alveolar macrophages. These researchers exposed alveolar macrophages to SWCNT (1.4 nm in diameter, synthesized by electric arc-discharge and purified to 90%), MWCNT (10–20 nm in diameter, synthesized via CVD and purified to >95%) and C_{60} fullerenes (synthesized via electric arc-discharge and purified to 99.9%) for 6 h. Recognizing the tendency of CNT materials to aggregate, these researchers attempted to remove the effect of particle size by utilizing a modified dosing regime (1.41–226 μg/cm^2 for SWCNT and C_{60}, and 1.41–22.60 μg/cm^2 for MWCNT). 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 failings the study did show that SWCNT exhibited the most cytotoxic response, although both SWCNT and MWCNT 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. Disturbingly, SWCNT and MWCNT variants were found to have a greater negative impact on cell viability than the positive control, quartz.

Cui et al. [54] investigated SWCNT cytotoxicity showing that SWCNT inhibited human embryonic kidney (HEK293) cells by inducing apoptosis and decreasing cellular adhesion ability. These researchers cultured HEK293 cells in media containing concentrations of SWCNT ranging from 0.78 μg/mL to 200 μg/mL in media. 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 G_1 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 SWCNT handling methods were given in this publication.

Tamura et al. [55] conducted a brief investigation into 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 compared to controls, while cell viability was observably decreased. Unfortunately, no details of CNT structure, synthesis or handling methods were provided, reducing the significance of this publication.

3. Biocompatibility of carbon nanotubes

Despite the evidence of CNT cytotoxicity, there have also been a number of published studies into CNT-based biomaterials, which support the biocompatibility of CNT and CNT-based materials. This section gives a detailed

discussion of investigations into interactions between CNT-based materials, neural cells, osteoblasts, fibroblasts, antibodies and the immune system, ion channels and cellular membranes.

3.1. Studies employing neuronal cells

The unique electronic properties of CNT have sparked several studies investigating the biocompatibility of CNT for neural applications [56–59] focusing particularly on neurite extension [56,58,59]. McKenzie et al. [57] investigated the effects of varying carbon nano-fiber diameter and surface energy on astrocyte proliferation and function. The study compared surfaces created from four different samples of carbon nano-fibers (CNF) (large MWCNT 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. Astrocytes, the cells largely responsible for the scar tissue formation seen with current implantable neural devices, were then seeded onto the surfaces for adhesion, proliferation and function studies. It was found that astrocytes preferentially adhered to and proliferated on larger diameter and higher surface energy CNF. The authors concluded that carbon nano-fibers with diameters <100 nm showed potential for neural applications due to a speculated reduction in neural scar tissue formation.

A follow-up study by Webster et al. [58] investigated the interactions between a series of polyurethane (PU)/CNF nanocomposites and astrocytes. The PU/CNF composites were synthesized by solvent-casting techniques, after PU/CNF sonication in chloroform. The CNF used in the study had an average diameter of 60 nm and were synthesized via CVD and purified by pyrolytic stripping. The mechanical properties of the nanocomposites were significantly poorer than those reported by other studies involving PU materials [4] suggesting that it was unlikely that the level of CNF dispersion was ideal. Neural cell seeding studies demonstrated that increased CNF loading in the nanocomposite resulted in both, decreased astrocyte adhesion and retarded neurite growth in rat pheochromocytoma cells by a small, but statistically significant amount. 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.

In contrast to the previous work, subsequent studies found that both pristine and chemically functionalized CNT have a positive impact on neuronal growth [56,59]. Confluent layers of neurons were seeded onto lithographically patterned CNT surfaces (islands of CNT grown via CVD on a quartz substrate). Over a time period of four days, neurons were observed to localize in CNT-rich regions, while their associated neurites and axons formed an interconnected network that replicated the pattern of the CNT template [59]. Similarly purified MWCNT that were chemically functionalized with carboxylic acid, ethylenediamine or poly-*m*-aminobenzene sulfonic acid (each holding a different ionic charge at physiological pH) were each observed to provide a substrate for neurite extension [56]. It was concluded that neurite extension was loosely based on the ionic charge, with positively charged ethylenediamine-MWCNT producing the most neurite extension. Neither the CNT-patterned surfaces, nor the functionalized MWCNT demonstrated cytotoxicity towards neuronal cells.

3.2. Studies employing osteoblast cells

A number of studies, similar to some of the neurological cell investigations, [57,58] have been conducted into the utility of CNT-containing materials as bone biomaterials, by examining the adhesion and function of bone-forming osteoblast cells [58,60,61]. The initial study in 2002 by Elias et al. [61] investigated the proliferation and function of osteoblast cells seeded onto four variants of compacted CNF (of diameters >100 nm or <100 nm, with each category further split into unrefined and pyrolytically stripped CNF). This study showed increased osteoblast proliferation on the nanophase (<100 nm) CNF. Alkaline phosphatase activity, intracellular protein synthesis and deposition of extra-cellular calcium, all increased in the smaller diameter CNF when compared with CNF whose diameter exceeded 100 nm. The authors concluded that CNF did not induce a cytotoxic response and that the nano-phase CNF demonstrated potential as orthopedic materials.

Two follow-up studies investigated the adhesion properties of osteoblasts, chondrocytes, fibroblasts and smooth muscle cells on PU/CNT nanocomposites [58,60]. As in previous studies [57,61], four variants of CNF were used to investigate the effect of surface energy and diameter on the adhesion properties of the cells. In addition, nano-sized CNF were incorporated at increasing concentrations into a PU nanocomposite. Price et al. [60] found that, in contrast to the larger diameter CNF, nano-sized CNF promoted osteoblast adhesion, while fibroblasts, chondrocytes and smooth muscle cells showed decreased adhesion on high energy CNF but were unaffected by diameter. These researchers also demonstrated increased osteoblast adhesion on CNF compared to the control materials (2 metal alloys currently used in orthopedic implants—Ti_6Al_4V and CoCrMo). In the nanocomposite, increasing concentrations of CNF led to increased osteoblast and decreased fibroblast adhesion. None of the nanocomposite formulations differed significantly from control materials. It was concluded that CNF did not show any cytotoxic effects and showed promise for use in orthopedic materials [58,60].

Osteoblast proliferation on nanocomposites of CNT under alternating current stimulation was also investigated [7]. Supronowicz et al. [7] fabricated conductive polylactic acid (PLA)/MWCNT nanocomposites at 10, 15 and 20%

w/w MWCNT (synthesized via electric arc-discharge). Osteoblast cells were seeded onto the surface and then exposed to alternating current stimulation. Control samples (PLA/MWCNT nanocomposite films) were run without electrical stimulation. Their results showed 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.

3.3. Nano-structured surfaces employing carbon nanotubes

Correa-Duarte et al. [62] investigated the effect of controlled MWCNT surface structures on the adhesion and proliferation of L929 mouse fibroblast cells. Unique 3D

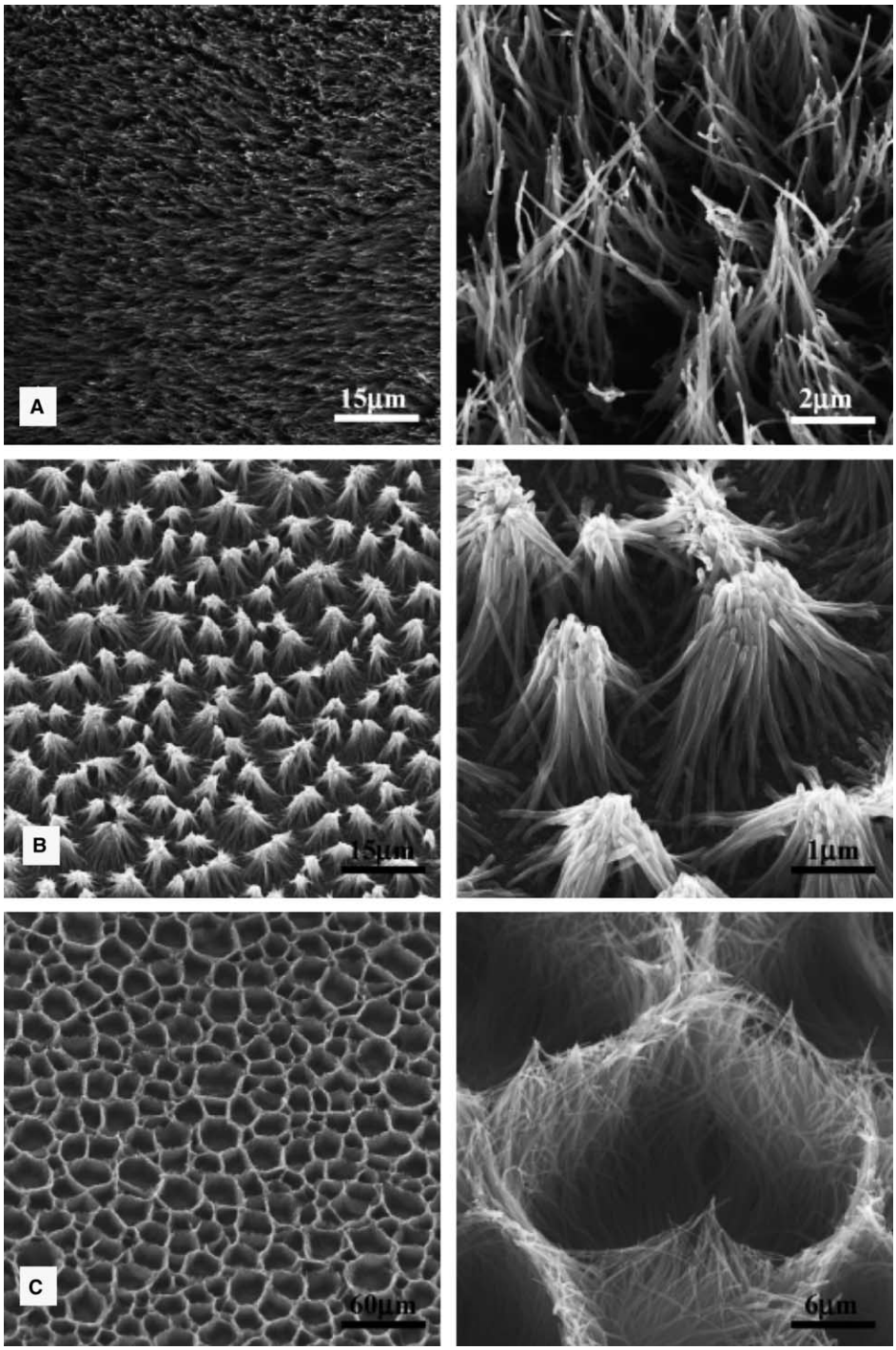

Fig. 3. Examination by SEM of the MWCNT-based structures: (A) perpendicular aligned carbon nanotubes; (B) the latter after a physicochemical treatment forming pyramid-like structures; (C) network of cross-linked carbon nanotube walls forming cavities. Reprinted with permission from [62]. Copyright (2004) American Chemical Society.

surface morphologies were created from aligned MWCNT grown on silicon substrates (via CVD using a Ni catalyst), by means of an oxidation process [63], which functionalizes the MWCNT 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 (as seen in Fig. 3), that are dependent on the length of the aligned MWCNT.

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 shown in Fig. 4). 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 [62].

Controlled CNT surface structures have also been used for non-inheritable genetic modification of Chinese hamster ovary (CHO) cells [64]. 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.

3.4. Antibody interactions

The generation of fullerene-specific anti-bodies was first determined in 1998 by Chen et al. [65]. This was achieved by the immunization of BALB/c mice with a C_{60} fullerene–thyroglobulin conjugate, which yielded a polyclonal IgG antibody that could bind to both C_{60} and C_{70} fullerene derivatives.

A follow-up study in 2001, investigated the binding of C_{60}-specific monoclonal antibodies to SWCNT [66]. This study showed that the antibodies bound to aqueous SWCNT ropes. Similarly, Naguib et al. [67] demonstrated that the binding of monoclonal antibodies to CNT is influenced by the hydrophilicity and surface disorder of the fibers. Poly-L-lysine adsorbed onto CNT was also shown to enhance antibody and protein binding. These studies demonstrate the possibility of generating antibody-coated SWCNT 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.

3.5. Ion channel interactions

Park et al. [68] investigated the interactions between SWCNT (synthesized via CVD using a Co/Mo catalyst and later purified), MWCNT (synthesized via a catalyst free arc-discharge technique), fullerenes (C_{60} and hyperfullerenes) and ion channels. They carried out the study

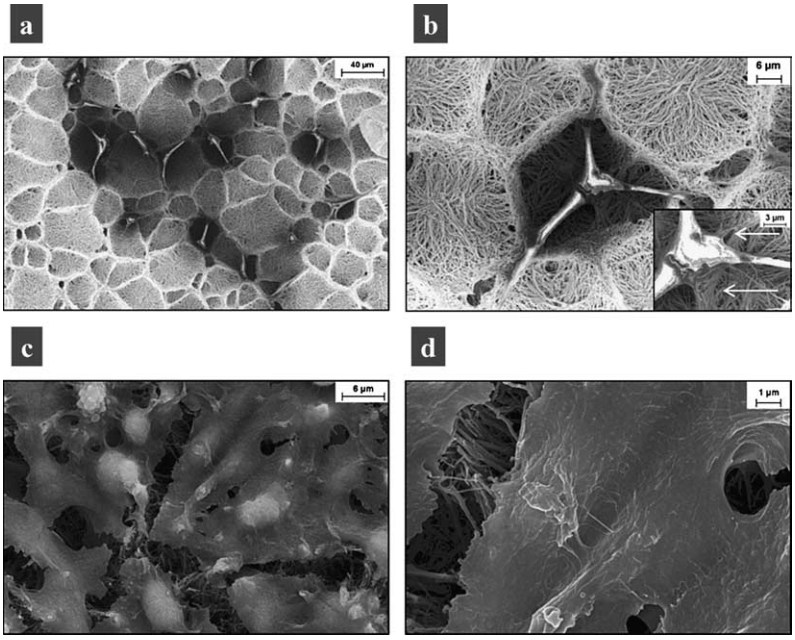

Fig. 4. Scanning electron micrographs of L929mouse fibroblasts growing on MWCNT-based network: (a,b) after 1 day; (c,d) after 7 days. Reprinted with permission from [62]. Copyright (2004) American Chemical Society.

using several "different pore-forming ion channel subunits, heterogeneously expressed in mammalian (CHO) cells" [68]. SWCNT were found to block channels, formed from EXP-2, KVS-1, human KCNQ1, Kv4.2 and HERG potassium channels. In fact, it was determined that SWCNT blocked potassium channels in a dose-dependent manner. Fullerenes were discovered to be less effective channel blockers than CNT. The authors postulated that the mechanism was solely dependent on the size and shape of the nanoparticles. They also concluded that electrochemical interactions between CNT and the ion channels were absent, in contrast to conventional channel blockers.

3.6. Carbon nanotubes as drug and vaccine delivery vehicles

Functionalization of CNT has been used to address the problem of CNT insolubility in aqueous media and, in many cases, has permitted linking of biologically active peptides and medicinal drugs to the CNT side-wall [69–72]. These properties have generated interest in using CNT as drug or vaccine delivery vehicles and to this end there have been several studies conducted on CNT functionalization with vaccine or drug molecules [70–76].

Two studies have used functionalized SWCNT 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 SWCNT via 1,3-dipolar cyclo-addition [70,71]. Both studies demonstrated that the conformation of the peptide sequence was maintained and recognized by mono- and poly-clonal antibodies, and that the SWCNT-FMDV peptide complex induced a specific anti-body response in vivo. The authors also concluded that there was no cross reactivity (immune response) to the SWCNT in vivo, suggesting that vaccine delivery is a viable application for CNT [71].

Pantarotto et al. [72] studied the translocation of functionalized SWCNT across human 3T6 and murine 3T3 cell membranes. Investigators attached the peptide fragment from the α subunit of the G_S protein to the purified SWCNT via 1,3-dipolar cyclo-addition (method outlined in [69]). The SWCNT-α_S complex was also fluorescently labeled with fluorescein isothiocyanate (FITC). Control samples consisted of SWCNT samples labeled with FITC (FITC-SWCNT), FITC-α_S and unbound FITC. The FITC-SWCNT-α_S complex was able to cross the cell and nucleic membranes, while FITC-SWCNT was only able to cross the cellular membrane and accumulated in the cytoplasm. In contrast, FITC-α_S and unbound FITC were unable to enter the cells. The authors were unable to determine the mechanism for cellular FITC-SWCNT-α_S uptake or localization within the nucleus. It was postulated that passive uptake mechanisms, such as endocytosis, were not responsible, rather the FITC-SWCNT-α_S 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-SWCNT-α_S 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-SWCNT-α_S complex induced cell death in 80% of cells. The translocation across the cellular membrane indicates that SWCNT may be a promising carrier for drug-delivery applications.

Kam et al. [73] studied the uptake of SWCNT (synthesized via laser ablation, purified and shortened by sonication) and SWCNT–streptavidin conjugates into human promyelocytic leukemia cells and human T cells via the endocytosis pathway. In contrast to Pantarotto et al. [72] this study demonstrated that SWCNT were taken up into cells via a mechanism consistent with endocytosis. No cytotoxicity was observed for the pristine SWCNT. SWCNT–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 [75,76]. CNT-specific cytotoxicity was not observed in either study.

Cherukuri et al. [74] investigated the uptake of pristine SWCNT into the mouse J774.1A macrophage-like cell line via near infrared fluorescence microscopy. The study reported that the macrophage-like cells appeared to phagocytose SWCNT at a rate of approximately one SWCNT per second, without any apparent cytotoxicity [74]. The SWCNT 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 [47,53]. It should be noted that prolonged or incomplete breakdown of foreign material often leads to chronic macrophage activation, and in turn to chronic inflammation [40].

4. Dispersion: the common problem

One of the major and recurring problems encountered by researchers in the investigation of CNT toxicity and biocompatibility is the tendency of CNT to aggregate in large bundles and ropes. The manipulation and characterization of large numbers of individual CNT is a difficult task because high molecular weights and strong intertubular forces (both van der Waals and electrostatic) promote the formation of such bundles and ropes [77]. This is especially true for the saline, media or serum solutions commonly used in toxicology testing. Indeed, it is very likely that a large portion of the discrepancies in toxicity and biocompatibility data are due to differences in CNT dispersion, the factor that ultimately dictates the presentation of CNT to the cells. Since many applications of CNT (biomedical or otherwise) require their dispersion in a variety of solvents (for example organic solvents for polymer interactions and aqueous solvents for drug delivery) there have been many investigations into improving CNT solubility. This section discusses some of the successful and popular methods, namely: sonication, stabilization with surfactant and covalent functionalization.

Sonication is a commonly used method for separating CNT aggregates in solution, because it quickly disperses CNT without the need for any chemical modification [78]. There are two main methods of sonication, the ultrasonic bath and the ultrasonic probe (also known as a horn or wand), both of which utilize a bubble nucleation and collapse mechanism [78]. The frequency of ultrasound determines the maximum bubble size, with increasing frequencies reducing bubble size [78]. Ultrasonic baths typically have higher operating frequencies (40–50 kHz) and no defined cavitation zone, while ultrasonic probes operate at frequencies around 25 kHz with a conical bubble nucleation zone extending from the probe tip. Only highly polar organic solvents such as *N*,*N*-dimethylformamide, when used in conjunction with ultrasonic baths or probes, have been shown to produce stable dispersions (for time periods greater than 1 h) of individual pristine CNT [79–81]. Despite being the most commonly used method of dispersion in toxicological testing to date, many researchers still observed CNT aggregates after sonication in aqueous solution [44,47,53,70–76].

Organic synthetic surfactants have been commonly used to improve CNT solubility in aqueous solutions, because of their ready commercial availability, low cost and the relative simplicity of experimental procedures that utilize them [2]. Sodium dodecyl sulfate (SDS) and Triton^{TM} X-100 are two of most popular surfactants [2], although many studies have used the commercially available Pluronic^{TM} surfactant varieties [82]. Biomolecules such as DNA [83,84], carbohydrates [85–88] and peptides [89] are also commonly studied as surfactants to solubilize CNT in aqueous solution. The wide variety of synthetic and biological surfactants makes them an attractive proposition for tailoring solubility towards specific applications. Unfortunately, some processing conditions have been found to cause surfactant dissociation from CNT [2].

Chemical functionalization of CNT is another commonly employed technique for improving solubility [77]. Typically, this involves covalently attaching appropriate molecules such as peptides [69–72,77,90], acids, amines, polymers [91,92] and poly-L-lysine [93] to the side-walls of CNT. This is most commonly achieved either by amidation or esterification of the –COOH groups present after CNT purification [90–93] or by 1,3-dipolar cyclo-addition [69–72,77]. By their reactive nature, many of these methods are associated with uneven surface coverage, or inter-CNT coupling.

In the context of biomedical applications, the chemical functionalization of CNT or adsorption of biomolecules shows the most promise as a dispersion technique, since no CNT-specific cytotoxicity was observed in studies into drug or vaccine delivery [70–76]. Unfortunately, there are no reports on the long-term stability of chemically functionalized CNT at this time.

Problems with dispersion arise not only in suspensions of CNT in solution, but also in inhalation studies. Evaluation of lung toxicity is best achieved by using the particle size distributions seen when CNT materials are aerosolized. However, the significant energy and agitation required to release fine CNT particles into the air [49], has hampered investigations. Instead, in vivo studies into lung toxicity have used intratracheal instillation of CNT in an attempt to mimic CNT particle distribution, but have only met with limited success.

5. Future areas of investigation

This review illustrates that there is still much work to be done in establishing the toxicity and biocompatibility of CNT. We feel the following issues are important areas requiring major research efforts and that, once thoroughly understood, they should provide a more complete picture of CNT toxicity. Perhaps most importantly, it is imperative that a more coordinated global approach to the following aspects is undertaken, where CNT ''standards'' are utilized in order to more easily correlate data generated from future studies.

- Lung toxicity studies
  - Current studies into lung toxicity need to be augmented with investigations into aerosolized CNT size distributions and the respirability of CNT particles. Given the apparent toxicity of other carbon nanoparticles [34–37], it is imperative that an adequate image of CNT lung toxicity is established quickly.
- Skin irritation
  - At present, very little is known about dermal exposure to CNT material. Accurate information on the cytotoxicity of CNT to dermal cells and the ease with which CNT material can be absorbed through the skin is vital for addressing issues of CNT handling.
- Macrophage response
  - Studies have shown that macrophages react to CNT in an inflammatory manner [47,53]. This reaction and the ability of macrophages to phagocytose and digest CNT over extended time periods will provide clues about the inflammatory nature of CNT materials.
- Effect of CNT properties on cytotoxicity
  - CNT materials vary greatly in composition depending on production and purification methods. Apart from the obvious SWCNT/MWCNT distinction, they also contain varying percentages of catalyst and other impurities. The impact of these factors on CNT toxicity has not yet been determined and it is important that this be addressed in a controlled and scientific manner.
- In vivo absorption, distribution and excretion
  - There have been no investigations into the ability of CNT to migrate or accumulate in vivo, despite their apparent potential as drug delivery vehicles. Previous studies have demonstrated the efficacy and speed with which fullerenes can be distributed and accumulated in vivo [34]. Assessing whether this is also true for

CNT is important, not only for determining the potential of CNT-based drug delivery, but also for calculating the impact of leaching and wear on CNT-reinforced nanocomposite biomaterials.

- Efficacy of chemical functionalization at biocompatibilizing CNT materials
  - Current data indicate that CNT functionalized for drug or vaccine delivery exhibit lower toxicity than unmodified or unrefined nanotubes. Further research needs to be conducted to confirm that this is the case, and to establish the long-term stability of these conjugates in vivo. If such investigations yield positive results, it is also of interest to determine which functionalizations are most effective at enhancing CNT biocompatibility for specific applications.

6. Conclusion—are carbon nanotubes safe?

CNT are unique materials with exceptional chemical and electronic properties. There have been a plethora of applications proposed in the biomedical field alone. However, before such materials can be successfully incorporated into biomedical implants, drug/vaccine delivery vehicles or biosensors, there is a need to establish their biocompatibility. Other carbon-based biomaterials have demonstrated excellent long-term biocompatibility and biological performance in medical device applications. Early biocompatibility data for CNT and novel nano-structured biomaterials suggest that the scientific community could remain cautiously enthused by potential biomedical applications of CNT-based materials.

Despite the importance of determining if CNT have associated toxicity in vivo, relatively few studies have been devoted to this topic. Furthermore, the results of these studies are often inconclusive or contradictory. The data presented in this review indicate that unrefined CNT possess some degree of toxicity (in vivo and in vitro), predominately due to the presence of transition metal catalysts. Exposure to pristine CNT has been shown to cause minimal cytotoxicity at higher concentrations (both in vivo and in vitro), while chemically functionalized CNT enhanced for drug delivery have not demonstrated any toxicity thus far. However, CNT aggregation has plagued research in this area and the impact of this key variable is unclear at this stage.

Clearly, these initial results urge caution when handling CNT, and the introduction of safety measures in manufacturing facilities and laboratories should be seriously considered. Most importantly, the success of CNT technology is dependent upon the continuation of research into the toxicology of CNT and CNT-related materials.

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