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Interfacing Carbon Nanotubes with Living Mammalian Cells and Cytotoxicity Issues

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The unique structures and properties of carbon nanotubes (CNTs) have attracted extensive investigations for many applications, such as those in the field of biomedical materials and devices, biosensors, drug delivery, and tissue engineering. Anticipated large-scale productions for numerous diversified applications of CNTs might adversely affect the environment and human health. For successful applications in the biomedical field, the issue of interfacing between CNTs and mammalian cells *in vitro* needs to be addressed before *in vivo* studies can be carried out systematically. We review the important studies pertaining to the internalization of CNTs into the cells and the culturing of cells on the CNT-based scaffold or support materials. The review will focus on the description of a variety of factors affecting CNT cytotoxicity: type of CNTs, impurities, lengths of CNTs, aspect ratios, dispersion, chemical modification, and assaying methods of cytotoxicity.

Contents

1. Introduction

Exploitation of nanotechnology in biology has been an active research area in recent years. The discovery of carbon nanotubes (CNTs) has greatly advanced this area owing to their unique structures and properties. CNTs are hollow cylindrical carbon tubes made of a single, two, or several concentrically arranged cylindrical graphite layers capped by fullerenic hemispheres. Such materials are referred to as single-walled carbon nanotubes (SWCNTs), double walled carbon nantubes (DWCNTs), and multiwalled carbon nanotubes (MWCNTs), respectively (*1*). SWCNTs have diameters ranging from 0.4 to 3 nm (typically ∼1 nm) (*2*). The inner diameters of MWCNTs range from 1 nm to a few nanometers, and the outer diameters vary from 2 to 200 nm (*2, 3*), depending on the number of layers. The lengths of both SWCNTs and MWCNTs range from hundreds of nanometers to 20 cm (*4*), i.e., CNTs have extremely high aspect ratios (length/diameter). University of Cincinnati researchers also synthesized extremely long aligned carbon nanotube arrays, about 18 mm in length (http://www.uc.edu/News/NR.aspx-?ID=5700). CNTs, particularly SWCNTs, tend to form packed bundles, clumps and aggregates during the growth process (*5*).

Since the discovery of CNTs (*5*), considerable interest has been inspired by their unique structures, remarkable electrical (*1, 2, 6*) and mechanical properties (*7*), high chemical stability, and potential applications. They have been extensively employed in molecular electronics, sensing, gas storage, field-emission devices, catalytic supports, probes for scanning microscopy, components in high-performance composites, and in the biomedical field (*3, 8*−*13*). Remarkable advances have been made in the synthesis and functionalization of CNTs in the past decade. They arouse considerable interest in the applications of CNTs (*14*) in the fields of biomedical materials, biosensors, drug delivery, and tissue engineering. With their anticipated

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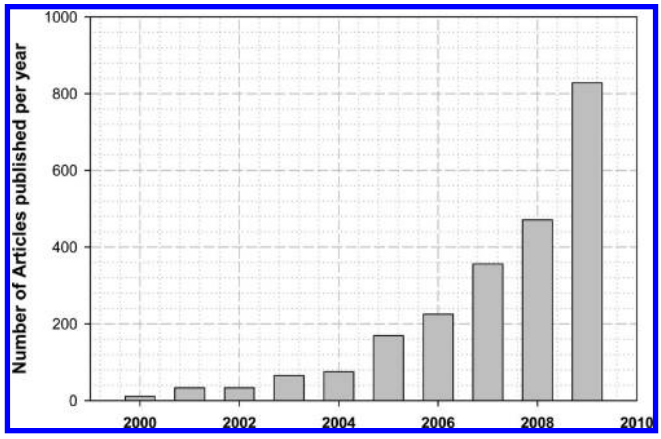

Figure 1. Number of articles published per year in the past decade pertaining to the use of CNTs for biomedical applications (on the basis of data taken from www.sciencedirect.com on December 30, 2009 using "carbon nanotubes" and "biomedical applications" in the advanced search option).

production and application at large scale, bioeffects and safety of CNTs to the environment and human health need to be addressed. The performance and properties of CNTs must be thoroughly investigated *in vitro* before they can be used *in vivo*. However, unlike chemical agents with well-defined structures and purity, CNTs are different in scale, structure, purity, and morphology, depending upon the preparation and purification methods. CNTs can be functionalized or oxidized to become more aqueous-soluble, and the cytotoxicity of such modified CNTs is different from pristine hydrophobic CNTs. Therefore, the interaction between CNTs and cells is very complicated, diverse, and somewhat unpredictable.

Cytotoxicity, the uptake mechanisms, and the subcellular localization of the CNTs have been investigated to assess the internalization of CNTs into the cells. The cellular uptake of CNTs has been studied in cell tracking and labeling, and intracellular transporting. The cell culture studies on CNT-based scaffolds or support materials have been focused on the biocompatibility of these materials and their potential applications in tissue engineering. The properties of CNTs such as solubility, size, length, dispersion, impurity, and functional groups available on the surface have great effect on their performance when they are interfaced with cells. Pristine CNTs are water insoluble and packed in bundles or aggregates in water. They usually have impurities, typically amorphous carbon, graphite nanoparticles, and transition-metal catalyst particles. The problem of impurity and insolubility has now been alleviated using various purification techniques and a wide variety of noncovalent and covalent methods for CNT functionalization (*15*−*21*). There have been continuously increasing research efforts pertaining to the use of CNTs for biomedical applications as reflected by the increasing number of articles published in the past decade (Figure 1).

This review will focus on the investigations of CNTs interfacing mammalian cells *in vitro*. The issues concerning the CNT interface with cells can be categorized into two major types: internalizing CNTs into cells and culturing cells on CNT-based scaffolds or support materials. Plausible cytotoxicity of pristine and modified CNTs is also discussed in detail.

2. Purification and Functionalization of Carbon Nanotubes (CNTs)

The purity of CNTs is an important issue to be addressed for interfacing CNTs with cells. As-synthesized CNTs prepared by arc-discharge, laser-ablation, and pyrolysis of hydrocarbon or organometallic precursors contain carbonaceous impurities and transition-metal catalysts (*22*), as the latter is necessary for the growth and formation of CNTs. Several purification strategies (*22*−*25*) have been developed after numerous research efforts that were pursued for more than a decade (Table 1).

In general, the chemical oxidation of CNTs by strong acids normally takes place at the end-cap of CNTs and the intrinsic defect sites at the walls of CNTs resulting in the introduction of functional oxygenated groups (*18*). This improves their water solubility and enables them to bind to many different types of chemical moieties by amide bonds (*18, 27, 28*) or ester bonds (*29*). The chemical modification at the nondefect sites of the sidewall of CNTs has also been realized. These occur by fluorination (*30, 31*): 1,3-dipolar cycloaddition of ozone (*32*) or azomethineylides (*33, 34*); the [2 + 1] cycloaddition of nitrenes (*35*); Diels−Alder cycloaddition of *o*-quinodimethane; fullerene cyclopropanation under the Bingel reaction condition; addition of nucleophilic carbenes (*35*); diazotization with aryl diazonium compounds (*36, 37*); and reduction reactions (*38, 39*).

CNTs can be modified by self-assembly of molecules or macromolecules to CNTs forming thermodynamically stable structures by noncovalent interactions such as hydrogen bond, $\pi-\pi$ stacking, electrostatic forces, hydrophobic interactions, and van der Waal forces. For example, SWCNTs can be solubilized in water by "wrapping" them with linear polymers such as poly(vinyl pyrrolidone) and poly(styrene sulfonate) (*40, 41*). These linear polymers bind rigidly and uniformly with the side walls of nanotubes by $\pi-\pi$ stacking, thereby disrupting the hydrophobic interface with water and the intertube interactions within the tubular aggregates. The wrapping of SWCNTs with single-stranded DNA (ssDNA) sequences has been employed to improve the separation of metallic CNTs from semiconducting nanotubes (*42, 43*). Molecular modeling suggests that ssDNA can bind to CNTs through $\pi-\pi$ stacking, thereby resulting in helical wrapping around the CNT surface (*42*). The covalent and noncovalent modification strategies thus play a vital role in enabling the dispersion, solubility, biocompatibility, tunable electronic property, and diameter- and chirality-based separation of the CNTs. These characteristics of CNTs are useful and attractive for their numerous applications including biomedical fields.

3. Internalization of CNTs into Living Mammalian Cells

Easily accessible organs such as skin, lungs, and blood borne cells are more vulnerable to CNTs; therefore, these organs and organ specific cell lines have been studied extensively (*44*−*58*). In addition, the nanotoxicity of CNTs has been investigated for other types of cell lines such as kidney cells (*59*), stem cells (*52*), and cancer cells (*44, 57*). CNTs have attracted considerable attention in biological sensing, cancer therapy, and drug/gene/vaccine delivery applications. CNTs can penetrate the mammalian cell membrane without any requirement of external transporter systems (*60*−*64*). The cellular uptake of CNTs depends on their length, size, surface chemistry, aggregation (*65*−*67*), and cell type (*67*). Dumortier et al. (*67*) exposed B and T lymphocytes and macrophages to two kinds of functionalized SWCNTs (f-CNT 2 and f-CNT 4), with the molecular structures shown in Figure 2. While f-CNT 2 is highly soluble in physiological conditions, f-CNT 4 has less solubility resulting in the formation of aggregates in the suspensions. The big bundles of f-CNT 4 are observed in the medium around B and T lymphocytes but not inside the macrophages. In contrast, well-dispersed f-CNT 2 is internalized by both lymphocytes and

Table 1. Methods for the Purification of CNTs and Their Effects on CNTs' Properties

methods for the purification of CNTs	effects on CNT's properties
high-temperature oxidation in air (*20*)	The high-temperature oxidation in air can convert amorphous carbon into CO_2.
heating in H_2 at elevated temperatures (*22, 23*)	Heating in H_2 at elevated temperatures can convert amorphous carbon into CH_4 (*22*).
acid refluxing (*7*)	The acid refluxing treatment can digest both the carbonaceous impurities and the transition-metal catalysts (*7*). However, the acid digestion of impurities also induces functional oxygenated groups such as carboxylic acid, ketone, and alcohol groups on the surface of CNTs (*18, 22*). For MWCNTs, heavy metal catalysts are present in the interior of the nanotubes and cannot be completely removed by concentrated acid or mixed acid. For example, the commercial MWCNTs purified by nitric and sulfuric acid treatment still contain 0.1% Ni and 0.2% Fe (*26*).

macrophages. Transmission electron microscopy (TEM) analysis reveals no evidence that SWCNTs dispersed in cell culture medium can be internalized by human A549 lung cells (*50*). There is no debundling or reduction in aggregation state upon the dispersion of SWCNTs in the medium. Typically, these aggregates can be of the order of micrometers, therefore reducing the likelihood of internalization. Similarly, there is no internalization of CNTs (wall number ranging from 1 to 6) inside human umbilical vein endothelial cells (*68*). The large length of the CNTs (likely ranging between 5 and 100 μm) might cause the rejection of the CNTs from crossing cell membranes. The effect of CNT length on their cellular uptake was investigated systematically by Becker et al. (*65*). The authors demonstrated a length-selective cellular uptake of deoxyribonucleic acid (DNA)-wrapped SWCNTs on several different cell populations including human alveolar basal epithelial cells, clonal murine calvarial cells, human fibroblast cells, and embryonic rat thoracic aorta medial layer myoblasts cells. The approximate uptake threshold is $\sim 189 \pm 17$ nm, i.e., shorter CNTs can be consumed and likely induce more toxicity. The *in vivo* implantation of MWCNT samples with an average length of 220 or 825 nm demonstrated that macrophages envelop the shorter CNTs more readily than the longer CNTs due to the greater coagulation tendency of the longer CNTs (*69*).

The unique near-infrared (NIR) fluorescent and intense Raman scattering properties together with the cell penetration ability of CNTs have been utilized to explore their potential in cell tracking and labeling applications (*64, 70, 71*), and cancer therapy (*72*). The nanoscale dimensions of CNTs combined with their high aspect ratio make them promising candidates for the new class of transporting vehicles for high cargo loading (*73*). A variety of cargo molecules such as fluorescently labeled streptavidin (*73*), DNA (*73*), small interfering RNA (siRNA) (*73, 74*), an anticancer drug (*75*), and bioactive peptides (*76*) have been loaded onto CNTs by covalent or noncovalent bonds and delivered inside the mammalian cells. But the binding of different molecules onto the CNT surface, however, also modified the surface properties of CNTs, leading to the interaction of CNTs with cells.

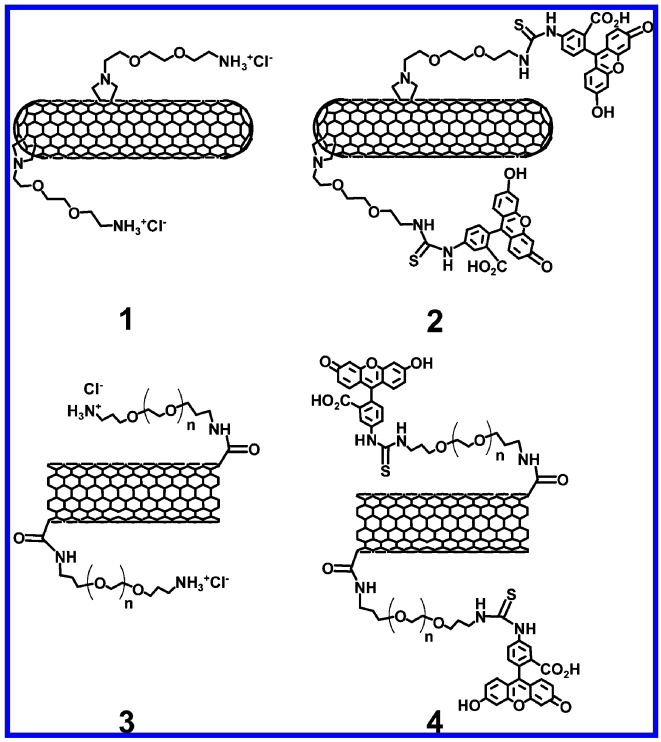

Figure 2. Molecular structures of functionalized SWCNTs (f-CNTs) used in the primary immune cell exposure study. Reprinted from Dumortier et al. (2006). Copyright 2006 American Chemical Society.

The cytotoxicity of CNTs and the effect of various factors such as hydrophilicity, impurity, and size of CNTs on their cytotoxicity have been extensively investigated (*59, 62, 67, 77*). With low density and nanosize, CNTs become airborne and are easily inhaled. Thus, the increasing industrial production of CNTs has aroused growing concern regarding their environmental and health effects, mainly on pulmonary and dermal toxicities. In addition to some pulmonary and dermal toxicity studies on animals (*45, 69, 78*), a number of *in vitro* cytotoxicity studies have also been performed on lung cells (*50*), alveolar macrophages (*47*), human epidermal keratinocytes (*44, 62*), and human fibroblasts (*56, 58*).

4. Cytotoxicity of CNTs

The cytotoxicity of CNTs with or without purification and functionalization has been studied in a variety of cell types *in vitro*. The reported cytotoxic effect of CNTs to mammalian cells has been controversial, with some reports demonstrating their cytotoxic effect (*44, 47, 59, 61, 62*), whereas the other reports demonstrating their biocompatibility (*18, 67, 68, 79*). Such contradictory reports could be due to different doses and properties of CNTs being employed, different cell populations, and different assessment methods. The incomplete characterization of the CNT materials following purification and functionalization could also be responsible for the contradictory results. A number of cell viability indicator dyes such as Commassie Blue, Alamar Blue, Neutral Red, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and WST-1 (a water-soluble tetrazolium salt), which interact with SWCNTs resulting in the reduction of the associated absorption/fluorescent emission, are used to evaluate the cytotoxicity of CNTs (*50, 80*). Briefly, the cytotoxicity can be related to several factors: metal impurities, length and size distribution, surface area, dispersion and aggregation status, coating or functionalization, immobilization, cellular uptake or internalization, and cell types.

4.1. Effect of Impurities in CNTs. *In vitro* studies have been performed to evaluate the cytotoxicity of both SWCNTs and MWCNTs by varying metal impurity contents. As shown in Table 2, the metal catalyst impurities may be mainly responsible for the cytotoxicity of the unpurified SWCNTs. Indeed, iron

Table 2. Effects of Impurities on Cell Behavior

CNTs used	effects on cell behavior
	CNTs with impurities
SWCNTs with 30% iron (*44*)	cause cell oxidative stress, loss of cell viability and ultra structural and morphological changes in human epidermal keratinocytes after 18 h of exposure
SWCNTs with 30% iron (*55*)	cause significant loss of intracellular low molecular weight thiols and the accumulation of lipid hydroperoxides in murine macrophages
SWCNTs, used as-purchased (no properties are given) (*59*)	inhibit the proliferation of human embryo kidney cells (HEK293) by inducing G1 arrest, cell apoptosis, and decreasing cellular adhesive ability
SWCNTs with 10 wt % iron (*50*)	*In vitro* cytotoxicity assessment of SWCNTs (10 wt % iron) on a human epithelial-like lung cell line, following 24 h exposure. A very low acute cytotoxicity, loss of cell membrane integrity, and inflammation response is observed. TEM studies further revealed that there is no intracellular localization of SWCNTs in the cells. The SWCNTs aggregate into large bundles and remain adhering to the cell surface even after several washes with PBS.
unpurified as-prepared MWCNTs (*58, 61*)	cause cytotoxic effects on human skin fibroblast and human epidermal keratinocytes
	CNTs with high purity
highly purified SWCNTs (*54*)	do not stimulate the release of the inflammatory marker nitric oxide in murine and human macrophages
purified SWCNTs (*79*)	Exhibit no evident short-term toxicity (within 3 days) and are biocompatible with cardiomyocytes in culture. In the long term (after 3 days) and after reseeding, the CNTs show negative effects, probably due to physical rather than chemical interactions.
MWCNTs (wall number ranging from 1 to 6), whose remaining oxide material and unprotected metal particles were removed (*68*)	exhibit no cytotoxicity on human umbilical vein endothelial cells, as tested by cell viability and cell metabolic activity.

Table 3. Plausible Cytotoxicity of Purified CNTs

properties of purified CNTs	some remarks
Refined SWCNTs (i.e., purified SWCNTs, in which the catalytic metals, i.e., iron, are removed by hot HCl treatment) are more toxic than their unrefined counterpart (*56*).	Surface area is suggested as the best determinant of the potential toxicity of the refined carbon nanomaterials where SWCNTs induce stronger cellular apoptosis/necrosis than MWCNTs.
	For comparable small surface areas, the dispersed carbon nanomaterials were observed to pose morphological changes and cell detachment upon apoptosis/necrosis, which could be attributed to the change in their surface chemistry.
Alveolar macrophages isolated from guinea pigs are exposed to SWCNTs (~90% purity with main impurity of amorphous carbon and trace amounts of metal catalysts) and MWCNTs (with a purity of >95% containing <3% amorphous carbon and ca. 0.6% Ni) for 6 h (*47*).	SWCNTs elicit a more toxic response than the MWCNTs. Although different impurities between the SWCNT and the MWCNT samples are the rationale behind the different cytotoxicity effects, it suggests that carbon nanomaterials with different geometric structures and sizes exhibit quite different cytotoxicities and bioactivities *in vitro*.
On the basis of the viability of rat aortic smooth muscle cells, the impact of cell culture media containing aggregations of purified SWCNTs is compared to that of the corresponding culture medium filtrations (*47*).	The culture medium filtrations have no visible micro- and macroscopic aggregates/particles but contain a large number of spherical graphitic (and amorphous carbon) particles (diameter ~20−60 nm) and some SWCNT bundles of diameter <5 nm. Large aggregates/particles and finely suspended nanoparticles of purified SWCNTs both result in a significant decline in cell viability.
	The adherence of SWCNT aggregates to cell surfaces most likely inhibits mass transport across the cell membrane (*82*). The nanomaterials in the filtration may have contributed partly to the inhibited cell growth either by inhibiting nutrient uptake by blocking the cell membrane or by random intracellular damage (*78*).

promotes free radical reactions in cells (*81*). The contribution of metal catalyst impurities to the cytotoxic effect of unpurified CNTs was demonstrated by Pulskamp et al. (*58*). They exposed rat macrophages (NR8383) and human A549 lung cells to SWCNTs and MWCNTs. The dose- and time-dependent increase of intracellular reactive oxygen species was detected along with the decrease in the mitochondrial membrane potential of both cell types. However, incubating the cells with purified CNTs had no cytotoxic effect. There are several conflicting reports on the cytotoxicity of purified CNTs, and indeed refined CNTs might be more toxic than their unrefined counterparts (Table 3).

4.2. Effect of Length of CNTs. Sato et al. (*66*) investigated the length effect of MWCNTs (with diameter of 20−40 nm) on the human acute monocytic leukemia cell line *in vitro* and subcutaneous tissue of rats *in vivo*. MWCNTs with an average length of 220 and 825 nm, referred to as 220-CNTs and 825-CNTs, respectively, were taken. They possess similar surface areas and are in dilute solutions without any noticeable coagulation. Both MWCNTs induce very slight and similar activities *in vitro*. Becker et al. (*65*) demonstrated a length-selective cellular uptake of DNA-wrapped SWCNTs on several different mammalian cell populations. Nanotubes shorter than (189 ± 17) nm are consumed and likely induce more cytotoxicity. This discrepancy about the effects of length of SWCNTs may be due to the inconsistencies in other factors such as coagulation and cell population.

4.3. Effect of Surface Chemistry. The surface chemistry of CNTs also plays a pivotal role in biocompatibility for cellular uptake. Pristine CNTs cannot readily disperse or dissolve in water due to their hydrophobic nature and $\pi-\pi$ interactions between them. Insolubility of CNTs is responsible for their limited applications in biological systems as it causes the accumulation of CNTs into cells, organs, and tissues with dangerous effects (*83*). In addition, as vectors for drug delivery, CNTs must be soluble so that they are capable of penetrating the cell membranes and thus distributing the drug to specific target cells (*62*). The problem of insolubility of CNTs has now

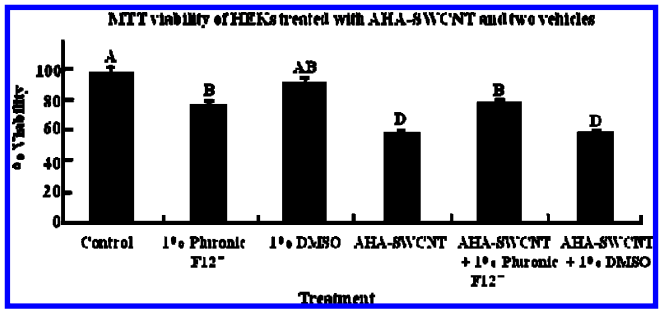

Figure 3. MTT viability of human epidermal keratinocytes treated with AHA-SWCNTs alone, 1% Pluronic F127, 1% Pluronic F127 + AHA-SWCNTs, 1% DMSO, and 1% DMSO + AHA-SWCNTs at 24 h. Histograms with different letters (A, B, C, and D) denote the mean values that are statistically different at $p < 0.05$. Data represent the means ± SEM. Reprinted with permission from Zhang et al. (2007). Copyright 2007 American College of Toxicology.

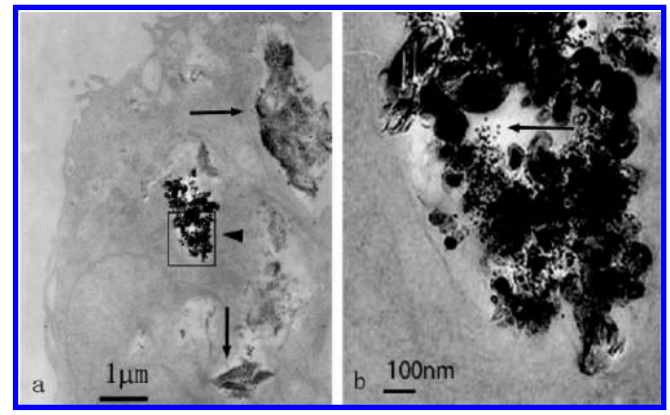

Figure 4. TEM images of human epidermal keratinocytes dosed with 0.05 mg/mL of AHA-SWCNTs at 24 h. (a) Note several vacuoles containing 0.05 mg/mL AHA-SWCNTs (arrow and arrowhead). (b) Higher magnification of the arrowhead region showing large aggregations and small fine aggregates (arrow). Reprinted with permission from Zhang et al. (2007). Copyright (2007) American College of Toxicology.

been solved using noncovalent and covalent methods for CNT functionalization (*16, 17, 41, 84*) to improve their aqueous solubility (*67*). The functionalization of CNTs can also lead to a dramatic reduction in toxic effects (*18, 67, 85*). Indeed, the cytotoxic response of human dermal fibroblast cells is dependent on the degree of functionalization of SWCNTs (*85*). The cells were exposed to a set of water-dispersible SWCNTs including SWCNT-phenyl-SO_3H, SWCNT-phenyl-$(COOH)_2$, and underivatized SWCNT stabilized in 1% anionic surfactant Pluronic F108. In general, the higher the degree of sidewall functionalization, the lesser is their cytotoxic effect. The sidewall functionalized SWCNT samples are substantially less cytotoxic than the surfactant stabilized SWCNTs. Cells of the immune system (including B and T lymphocytes, and macrophages) are exposed to two kinds of amino group-functionalized SWCNTs, one being highly water-soluble and the other forming mainly stable suspensions in water. The highly soluble CNTs do not influence the functional activity of immunoregulatory cells, whereas the CNT suspension preserves the lymphocytes' functionality but provokes secretion of proinflammatory cytokines by macrophages (*67*). CNTs can be coated with a biomimetic glycopolymer designed to mimic cell surface mucin glycoproteins (*18*). The functionalized CNTs are nontoxic to Chinese hamster ovary (CHO) cells and Jurkat human T-lymphoma cells, whereas the unmodified CNTs induced cell death.

4.4. Effect of Dispersion. The methods used to disperse CNTs could also affect their cytotoxicity. Zhang et al. (*62*) measured the effects of various dispersion methods employing dimethylsulfoxide (DMSO) and a 1% anionic surfactant Pluronic F127 on the cytotoxicity of 6-aminohexanoic acid derivatized SWCNTs (AHA-SWCNTs). The surfactant treatment causes the dispersion of the AHA-SWCNT aggregates in the culture medium, thus contributing to less cytotoxicity. 1% Pluronic F127 is a nonionic surfactant which is capable of shielding AHA-SWCNT cytotoxicity by coating its surface and by forming micelles in the medium that altered the AHA-SWCNTs surface properties. It may be capable of decreasing the AHA-SWCNT adsorption to the cell membrane by shielding some of the cell membrane receptors. DMSO does not effectively disperse the aggregates, and therefore, it does not affect the biocompatibility of AHA-SWCNTs. Figure 3 shows the effects of different dispersion methods on the viability of human epidermal keratinocytes as analyzed by the MTT viability assay. The AHA-SWCNTs can penetrate the cell membrane of human epidermal keratinocytes without any need of an external transporter system. Figure 4 shows the TEM images of human epidermal keratinocytes incubated in 0.05 mg/mL of AHA-SWCNTs alone for 24 h. Large as well as small fine aggregates of AHA-SWCNTs localized within the cytoplasmic vacuoles can be observed.

4.5. Effect of Interaction between Various Factors. Several factors also affect the cytotoxicity of CNTs including the amount of CNTs used, their properties, types of cells used, and the methods used for cytotoxicity assessment. All of these factors could interact with each other. Therefore, some of the published data citing the *in vitro* toxicity of CNTs is inconsistent and disputable. However, the noncytotoxic reports of highly purified CNTs with or without functionalization have made this unique nanomaterial very promising for biological applications. Particular attention should be paid to the interactions of these factors when cytotoxic studies *in vitro* and *in vivo* are designed, and when the experimental results are interpreted. Apparently, nonpurified pristine CNTs with catalyst metal particles could cause severe cytotoxic effects to the exposed skin and lung cells. *In vivo* toxicity of CNTs has been reviewed by Lacerda et al. (*86*). As discussed later, pristine CNTs with or without purification cause pulmonary toxicity and respiratory distress. Therefore, persons involved in CNT preparation need to be very well protected to reduce the possibility of skin contact and inhalation of CNTs. The quality control and manufacturing guidelines should ensure biosafety and environmental protection.

4.6. Type of CNTs. CNTs consist of mainly single-walled carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs), albeit double-walled CNTs have also been commercially available recently. In general, the nanotoxicological effect is related to the specific surface area (*87*), and SWCNTs have greater specific surface areas compared to those of MWCNTs. However, SWCNTs usually form bundles due to relatively stronger van der Waals forces between their side walls which in turn reduce their effective specific surface areas. MWCNTs have slightly lower specific surface areas. There are also many defects with high chemical activity (usually carboxylate group) along their sidewalls. Therefore, the aggregation ability of MWCNTs is lower than that of SWCNTs. It is somewhat difficult to compare the cytotoxicity of MWCNTs and SWCNTs since it is not clear whether nanotoxicity should be related to the same mass concentration of CNTs or the same total surface area. SWCNTs (1.4 nm in diameter) can easily induce serious damage of alveolar macrophages even at 0.38 $\mu g/cm^2$ compared to 3.06 $\mu g/cm^2$ for MWCNTs (10−20 nm in diameter) (*47*). However, Fiorito et al. (*54*) reported that fewer

Table 4. Some Key Advantages of the Optical Property of CNTs over Fluorophores and Quantum Dots

1. Organic fluorophores easily undergo photobleaching (*89*).
2. Quantum dots are highly luminescent semiconducting nanoparticles and more resistant to photobleaching than organic fluorophores (*90*). However, their signal attenuates significantly under prolonged excitation (*71*). They have a limited lifetime in aqueous solution (~7−12 days), and their cores, often composed of cytotoxic cadmium selenide, can be difficult to shield from the cellular medium (*71, 91, 92*).
3. The 1-D electronic structure of nanotubes results in sharp interband transitions of SWCNT absorption spectra as well as photoluminescence of semiconducting nanotubes in the NIR region (800−1600 nm) (*70*). This unique NIR intrinsic fluorescence of pristine CNTs is important in optical cell labeling and optical biosensors.
4. The NIR region includes the tissue-transparent region of the electromagnetic spectrum (800−1400 nm) in which radiation passes through live cells without significant scattering, absorption, heating, or damage to tissues (*70*−*72*).
5. The fluorescence of CNTs is extremely photostable without blinking or photobleaching even after prolonged exposure to excitation at high fluorescence (*64, 71*).
6. Semiconducting SWCNTs show photoluminescence in the NIR region (800−1600 nm) (*70*). In addition, both metallic and semiconducting SWCNTs exhibit intense Raman scattering with resonance enhancement at NIR absorption transitions (*71*).
7. Strong Raman scattering of nanotubes is easily detectable, unmistakable, and often of similar intensity as that of fluorescence events. There is no blinking, quenching, or diminishing after prolonged excitation (*15, 71, 93*).

SWCNTs, synthesized via chemical vapor deposition, can be internalized into murine macrophages (J774). The SWCNTs only show slight toxicity at high concentrations (30 and 60 μg/mL).

5. CNTs for Optical Cell Imaging

Optical cell imaging *in vivo* is attractive because optical detection is a simple and direct method for diagnostics and biomedical imaging (*88*). It can be used in tissue engineering to track the implanted cells; to monitor the progress of *in vivo* tissue formation; to understand the biodistribution and migration pathways of transplanted cells; and as optical sensors to understand and sense biological events in the cells. For *in vivo* cell imaging, a robust cellular marker should be biocompatible, have a narrow emission band, and no blinking or photobleaching under prolonged excitation. The tissues in the body should not absorb or scatter the excitation and the emission light (*88*).

CNTs are promising agents for cell-labeling compared to fluorophores and quantum dots. Some important optical properties of CNTs are summarized in Table 4. Cherukuri et al. (*64*) dispersed SWCNTs in a Pluronic surfactant and exposed the CNT suspension to mouse peritoneal macrophage-like cells. With NIR fluorescence excitation at 660 nm, macrophage cells can actively ingest significant quantities of SWCNTs without showing toxic effects, and the ingested nanotubes remain fluorescent and can be imaged through NIR fluorescence microscopy at wavelengths beyond 1100 nm. Figure 5 shows the SWCNT NIR specific fluorescence intensity as a function of incubation time with 7.3 μg/mL of SWCNTs (a) or as a function of SWCNT concentration after 24 h incubation (b). The smooth increase of fluorescence intensity versus time indicates a steady rate of CNT uptake by the cells.

DNA-encapsulated SWCNTs show persistent Raman scattering and changing fluorescence spectra within living 3T3 fibroblasts and murine myoblast stem cells, thereby functioning as cell markers for up to three months in culture (*71*). The CNT aggregates remain in the cells during repeated cell divisions as evident by long-term experiments. This leads to the possibility of new experiments concerning cell proliferation and stem cell differentiation, long-term labeling of cell populations, and continuous monitoring of the nanotube environment within vesicles. Spectral changes in nanotube fluorescence with the changes in the surrounding microenvironment (*41, 64, 71*) may be utilized to fabricate long-term optical sensors *in vitro* and *in vivo*.

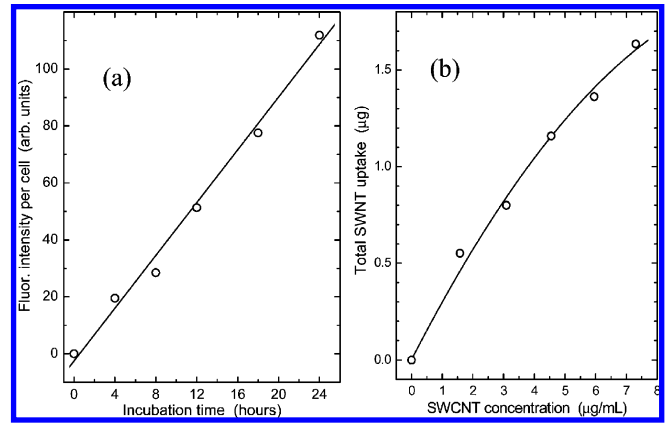

Figure 5. SWCNT fluorescence intensity (integrated from 950 to 1500 nm) from incubated, washed samples of 10^7 macrophage cells: (a) as a function of incubation time with 7.3 μg/mL of SWCNTs; (b) as a function of SWCNT concentration after 24 h incubation. The solid lines show linear (a) and quadratic (b) fits to the data. Reprinted from Cherukuri et al. (2004). Copyright 2004 American Chemical Society.

Besides the intrinsic NIR fluorescence and intense Raman scattering properties with pristine CNTs, some functionalized CNTs have been demonstrated to exhibit intrinsic fluorescence in the ultraviolet/visible region (*71, 86, 94*). For example, 1,3-dipolar cycloaddition of azomethineylides on the side walls and the tips of CNTs with the introduction of covalently linked aliphatic appendages terminated in some chemical moieties, such as an amino group (*63, 94, 95*) and an acetamido group (*63, 95*), not only allows the development of water-soluble CNTs but also leads to the emergence of a luminescence signal with characteristic broad 395/485 nm (excitation/emission) peaks detected by fluorescence spectrophotometry (*95*). The intrinsically luminescent functionalized-CNTs have been directly tracked and imaged in a wide variety of live cells, including adherent mammalian cell monolayers (*63, 94*), mammalian cell suspension, fungal cells, yeast, and bacteria (*63*) by confocal laser scanning microscopy. The tracking of intrinsically luminescent functionalized-CNTs in live cells is useful to elucidate the intracellular transporting properties of CNTs, avoiding any misinterpretation that may be introduced by coating or conjugating fluorescently labeled macromolecules onto the surfaces of CNTs.

6. CNTs as Transporting Vehicles for Intracellular Delivery of Bioactive Molecules

Therapeutic effects of proteins, drugs, genes, etc. depend greatly on the bioavailability of these molecules at the targeted tissues as well as the ability of these molecules to cross cellular barriers. To facilitate the delivery of these molecules, transporting vehicles that can carry the cargos through cell membranes without impairing their biological functionalities are being developed in novel biomedical therapies. The transporting vehicles that have been explored extensively include viral vectors, liposomes, cell-penetrating peptides, and other small molecules (*73*). With the progress in understanding and ap-

Table 5. CNT−Biomolecule Conjugates and Their Interaction with Cells

CNT−biomolecule conjugates	interaction with cells
Amino-group functionalized SWCNTs are able to associate with plasmid DNA through electrostatic interactions (*97*).	The CNT−DNA complexes are taken up into the mammalian HeLa cells after interaction, and the associated plasmid DNA is delivered inside the cells efficiently. Gene expression levels up to 10 times higher than those achieved with DNA alone can be observed.
The intracellular delivery of various proteins adsorbed on the sidewalls of acid-oxidized SWCNTs (*19*).	The proteins are readily transported inside various mammalian cells, and the bioactivity preservation of the transported proteins inside cells is evinced by apoptosis induction by transported cytochrome *C*.
Mediated *in vivo* and *in vitro* delivery of small interfering RNA (siRNA) by positively charged $SWCNTs^+$ (amino-group functionalized SWCNTs) into antigen-presenting cells (*74*). The positively charged $SWCNTs^+$ absorb siRNA designed against antigen CD80 to form complexes of siRNA with $SWCNTs^+$, which are taken up by dendritic cells *in vitro*.	These complexes specifically reduce surface CD80 expression and the level of mRNA. As a control, naked siRNA (or fluorescence labeled siRNA) alone could neither be taken up nor cause significant CD80 RNAi (RNA interference) in the limited time of this assay *in vitro*. Infusion of $SWCNTs^+$ carrying siRNA designed against SOCS1 (an intracellular signal regulator) reduces the SOCS1 expression and retards the growth of established B16 tumor in mice.
Amino-group functionalized SWCNTs could facilitate the coupling of siRNA that specifically target murine TERT expression to form the mTERT siRNA:$SWCNT^+$ complex (*98*).	This complex can rapidly enter various cultured tumor cell lines (human HeLa cells, mouse ovarian surface epithelial cell line 1H8, mouse cervical cancer cell line TC-1, and Lewis lung carcinoma tumor cells), suppress mTERT expression, and produce growth arrest. Injection of mTERT siRNA:$SWCNT^+$ complexes into s.c. Lewis lung tumors reduced tumor growth.
SWCNTs covalently bound to bioactive peptides (*76*)	SWCNT-peptides are interfaced to a variety of mammalian cell lines (human 3T6 and murine 3T3 fibroblasts, and human keratinocytes). The peptide-functionalized CNTs are able to cross the cell membrane and distribute inside the nucleus. But the mechanism for this nuclear localization is still unknown.

plications of nanotechnology, various nanomaterials have been interfaced and applied with biological systems. Some nanoparticles have been explored as transporting vehicles to deliver biomolecules/drugs into the living cells (*96*). CNTs, very unique and important nanomaterials, have been reported to be capable of penetrating mammalian cells (*60−64*). Highly purified CNTs with or without functionalization can be biocompatible *in vitro* and *in vivo* (*18, 66−68, 85*). Different groups can be introduced on the side walls and the tips of CNTs after functionalization, which allow covalent or noncovalent loading of drugs/biomolecules onto CNTs (*73−76, 97−99*). The nanoscale dimensions of CNTs combined with their high aspect ratio make them ideal for high cargo loading. Hence, CNTs have emerged as a new class of promising transporting vehicles (*73*) with all the desired characteristics for a plethora of applications (Table 5).

Several researchers reported that polymer-coated or chemically functionalized CNTs are internalized inside cells by endocytosis (*19, 60, 72, 75*). For the cargos loaded on CNTs to be therapeutically effective, they must escape from the endosomes once they are internalized by endocytosis. Some schemes have been designed and investigated for this behavior. Feazell et al. (*75*) covalently attached a platinum(IV) compound, an anticancer prodrug which is nearly nontoxic to cancer cells, to the surface of amino-group functionalized soluble SWCNTs. The soluble nanotubes internalized the platinum(IV) compound through endocytosis into the testicular carcinoma cell line NTera-2, providing six times the concentration that the untethered complex was able to achieve on its own. The distribution of platinum throughout the cell interior and the trapped nature of the SWCNTs can be verified by fluorescence microscopy. Therefore, the platinum(IV) prodrug can be reductively released in the acidic and reducing environment of endosomes in the form of an active platinum(II) species by reduction and concomitant loss of the axial ligands by which it is tethered to the SWCNT surface. This SWCNT-Pt (IV) conjugate shows a substantial increase in toxicity compared to that of the free platinum(IV) compound, which even surpasses that of the active platinum(II) species when compared on a per platinum basis. In two other reports (*73, 100*), fluorescence-labeled DNA and siRNA are attached to SWCNTs through disulfide bonds. The nuclear translocation of the DNA molecule is observed in the presence of the disulfide bond, thereby indicating the detachment of the DNA from the nanotube (*100*). Disulfide bonds can be broken under an acidic pH environment, especially in the lysosomal compartment of cells (*101*). Thus, the breaking of the disulfide bonds leads to the detachment of the DNA cargo from the nanotube vehicles. For the siRNA (specifically against lamin A/C) attached to SWCNTs through a disulfide bond, a dramatic reduction in the level of lamin A/C can be detected in HeLa cells following treatment with the SWCNT-siRNA conjugate (*73*). Incubation of cells with SWCNT-siRNA conjugates that do not contain the disulfide bond shows an inferior level of silencing, indicating that the disulfide bond is necessary for cargo release. These reported successful and efficient intracellular deliveries of DNA, anticancer drugs, proteins, and siRNA with preserved biological activity indicate that CNTs may be developed as novel biomedical transporting vehicles. The applications of CNTs in drug delivery, gene therapy, and cancer therapies are continuously emerging and need to be fully explored in a variety of systems and eventually in *in vivo* settings to fully establish their potential in the biomedical realm (*73*).

7. Cellular Mechanisms for CNTs Uptake

The mechanism of CNT internalization inside cells needs to be carefully understood for the controlled uptake of CNTs and for devising new strategies that can allow them not only to carry cargos inside the cells but also to have the ability to release them. The reported mechanisms so far have discrepancies. Some researchers (*63, 76*) have demonstrated that functionalized CNTs are able to cross the cell membrane in a passive and endocytosis-independent way. In contrast, other researchers (*19, 60, 72, 75*) proposed an energy-dependent endocytosis mechanism. Endocytosis is known as a general entry mechanism for various extracellular materials. As an energy-dependent uptake, it is hindered at low temperature (4 °C instead of 37 °C) or in ATP (adenosine triphosphate) depleted environments (*102*). Other researchers observed considerable reduction in the cellular uptake of the fluorescently labeled macromolecules adsorbed on CNTs under endocytosis-inhibiting conditions (*19, 72, 103*). In another report, a smaller amount of fluorescently labeled amino-group functionalized MWCNTs can be detected in both the multidrug-resistant cells (K562A) and the parent cells

(K562S) after incubating at 4 °C rather than at 37 °C, suggesting that the fluorescently labeled functionalized CNTs may traverse cellular membranes by endocytosis (*60*). Observation of the intracellular confinement of CNTs in small vesicles is also confirmed by the endocytosis mechanism. Feazell et al. (*75*) incubated the testicular carcinoma cell line NTera-2 with SWCNT-Pt (IV) conjugates (tethered with a fluorophore). Fluorescent SWCNTs are readily located within small (diameter ~2 μm) vesicles in the cell after 1.5 h of incubation. Heller et al. (*71*) observed the perinuclear accumulation of DNA coated SWCNTs within membrane-enclosed vesicles. In contrast, some research reports suggest that endocytosis is not evoked. Pantarotto et al. (*76*) incubated several mammalian cell lines (including human 3T6 and murine 3T3 fibroblasts) and human keratinocytes with fluorescently labeled amino-group functionalized SWCNTs or SWCNTs covalently bound to a bioactive peptide. The internalization is not affected by temperature or the presence of endocytosis inhibitors. Similarly, various types of functionalized CNTs (including SWCNTs and MWCNTs) can be internalized by Jurkat human T-lymphoma cells (a mammalian cell suspension), and the internalization is not affected by temperature and the presence of endocytosis inhibitors (*63*). In addition to mammalian cells, the functionalized CNTs exhibit a capacity to be taken up by fungi and yeast cells, which contain a capsule, composed primarily of a high molecular weight polysaccharide lacking the capability for endocytosis. Such results imply that the observed cellular internalization of the functionalized CNTs is not solely dependent on endocytosis. Discrepancies in the reported internalization mechanisms can be attributed to sharp differences in the characteristics of CNT constructs (*63*). Indeed, macromolecules acting as solubilizing agents can play a critical role in determining the type of ensuing interactions with cells and the mechanisms of cell uptake. However, other factors such as the nanotube length and aggregation should also contribute to such discrepancies, considering the discrepancies for the same kind of functionalized CNTs (i.e., fluorescently labeled amino-group functionalized CNTs) between the reports by Kostarelos et al. (*63*) and Xiao et al. (*60*).

8. CNT-Based Cell Culture Scaffolds for Tissue Engineering

Tissue engineering is an interdisciplinary field that aims to develop scaffolds, which can direct or control cellular behavior to create biological constructs capable of replacing or regenerating damaged or diseased tissues (*104, 105*). This engineered scaffold provides an artificial extracellular matrix (ECM) to provide support and be the substrate for cell adhesion, proliferation, and migration. It also guides tissue regeneration by the host or other transplanted cells. The natural ECM is a 3D structure enriched with nanoscale features such as interconnecting pores, ridges, and fibers (*106*−*108*). Materials for artificial 3D scaffolds used in most of the previous works have been biodegradable synthetic polymers such as poly(L-lactic acid), poly(glycolic acid), or biopolymers such as collagen and fibroin (*109*). Recently, scaffolds based on CNTs have been explored in neural, bone, and dental tissue engineering due to their nanoscale, 1D structure, lightweight, high mechanical strength, electrical conductivity, thermal conductivity, and tunable surface functionalities. By adjusting the loading of CNTs inside the appropriate polymer, the mechanical, electrical, and thermal properties of the composite biomaterials can be fine-tuned to suit specific biological applications. When used as a scaffold material, CNT composites will come into contact with the surrounding tissues. The microstructures of the 3D constructs and the surface properties of CNTs are key factors to determine cell adhesion, proliferation, and migration for guided tissue regeneration. The cell growth behavior of different kinds of cells has been investigated on a variety of CNT 2D or 3D structures constructed from modified or unmodified CNTs (*109*−*114*).

8.1. Scaffolds for Neuron Cell Culture. In mammalian nervous systems, neurons communicate with each other through electrical signals, which propagate through neurites (axons and dendrites) in the neural network. The neural network is made of millions of interconnected neurons. Neurons are unable to divide or regenerate, leaving the injuries and neurodegenerative disorders of nervous systems incurable. Thus, the formidable challenge is to find means to cure the disabilities arising from the injuries and disorders of nervous systems by stimulating inactive neurons and regulating their growth in a proper way (*115*). The diameters and aspect ratios of CNTs are compatible to those of small nerve fibers (postganglionic fibers and sensory fibers type IV with diameters ranging from 200 nm to 1,500 nm), which make them a suitable scaffold material in neural tissue engineering for neuron growth. They ensure tight binding with neurons, controllable branching of neurites, long-term cell viability, and the capability of forming a highly directed neuron network. Hu et al. (*112*) demonstrated that both the as-prepared MWCNTs and chemically functionalized MWCNTs with different charges provide permissive substrates for neurons (hippocampal neurons from 0- to 2-days old Sprague−Dawley rats). More importantly, neurite outgrowth is greatly dependent on the surface charge of MWCNTs, as characterized by the presence of more numerous growth cones, longer average neurite length, and elaborate neurite branching for neurons grown on positively charged MWCNTs as opposed to neutral or negatively charged MWCNTs. Similarly, neurons grown on positively charged and branched polyethyleneimine (PEI) functionalized SWCNT graft copolymer exhibit more decorated (branched) neuritis, more numerous growth cones, and longer average neurite length than neurons grown on as-prepared MWCNTs (*113*). By culturing dissociated cortical cultures from 1-day old Charles River rats on quartz substrates patterned with CNT islands, electrically viable neuronal networks consisting of interconnected ganglion-like clusters of neurons are formed following the exact pattern of the CNT templates (*116*). In this case, neurons and glia cells are preferentially accumulated and adhered on the CNT-coated regions. Subsequently, the gangliated neurons send neurites to form interconnected networks with predesigned geometry and graph connectivity. MWCNT substrates can boost neuronal electrical signaling, reflected by a significant increase in the frequency of spontaneous postsynaptic currents for hippocampal cultures compared to that in the cultures on glass substrates (*117*). The increase in the efficacy of neural signal transmission may be related to the specific properties of CNT materials such as high electrical conductivity (*117*). A carboxyl group-functionalized MWCNT mat deposited on a track-etch membrane is a permissive substratum for somatosensory neurons, i.e., primary cultures of dorsal root ganglion neurons (*115*). SEM imaging reveals obvious neurite outgrowth from the cultured neurons plated on a functionalized CNT mat and an intertwinement between the neurites and underlying CNTs (Figure 6). Such functional groups may act as anchoring seeds that enhance the adhesion of neurons as well as neurites, thereby promoting neurite growth.

The studies so far indicate that CNT-based scaffolds could provide permissive substrates for neurons; serve to ensure tight binding with neurons; boost neuronal electrical signaling; and

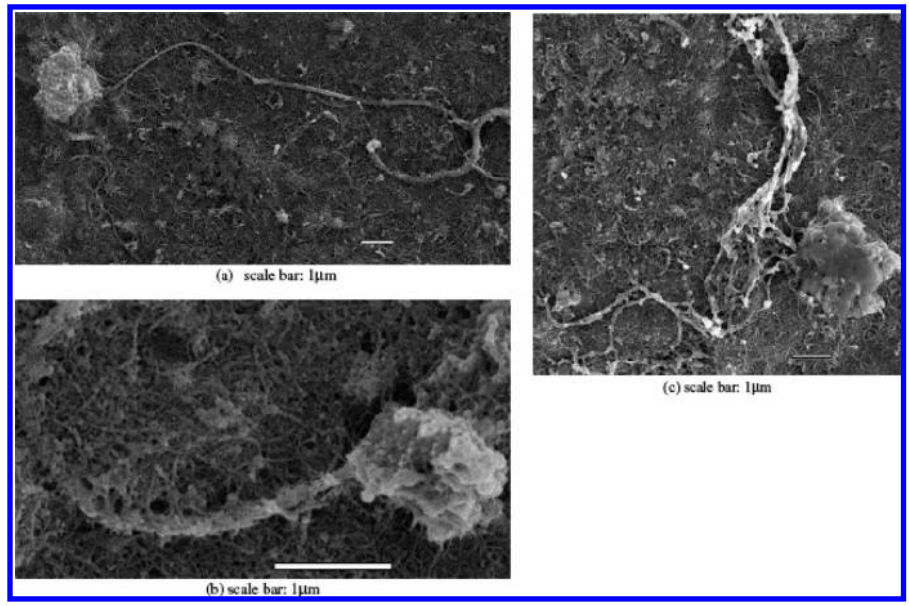

Figure 6. SEM micrographs of neurite growth from neurons on a functionalized carbon nanotube mat after two days in culture: (a) long neurite grown from a neuron, (b) higher magnification image of a neuron, and (c) multiple neurites growing out in a bundle from one neuron. Reprinted with permission from Xie et al. (2006). Copyright 2006 IOP Publishing Ltd.

form a highly directed neural network. The branching and length of neuritis can be controlled by adjusting the functionalities on the CNT surfaces. This biocompatibility for neuron culture together with the CNT properties such as lightweight and high mechanical strength should lead to the use of CNTs as a very promising scaffold material for neural tissue engineering.

8.2. Scaffolds for Bone Cell Culture. Bone tissues include cell types such as osteoblasts, osteoclasts, and osteocytes, which are embedded in a mineralized extracellular matrix consisting of collagen and a number of noncollagenous proteins (*118*). Osteoblasts are the bone-forming cells that proliferate on the bone surface. They produce and secrete bone matrix proteins. As they progress through the stages of cell differentiation under hormonal control, they mineralize the matrix by means of production of hydroxyapatite (HA) crystals (*114*). The high electrical conductivity of CNTs has been demonstrated as a useful property for bone scaffold materials. When an alternating current is applied to the nanocomposites of polylactic acid and MWCNTs (*28*), osteoblasts cultured on nanocomposite surfaces exhibit a 46% increase in cell proliferation after 2 days, a 307% increase in the extracellular calcium concentration after 21 consecutive days, and an upregulation of mRNA expression for collagen type-I (a major component in organic bone formation). Zanello et al. (*114*) cultured osteosarcoma ROS 17/2.8 cells (fully mature osteoblasts) on as-prepared and chemically modified SWCNTs and MWCNTs. CNTs carrying neutral electric charge (as-prepared and polyethylene glycol-functionalized) sustain higher cell growth than those carrying net negative and zwitterionic electric charges. In addition, osteoblasts grown on as-prepared SWCNTs produce plate-shaped crystals, similar in shape to the HA crystals found in woven bone. Owing to its bioactivity and biocompatibility, HA coatings have long been applied to dental implants, bone repair scaffolds, skeletal implants, and body/bioinsert materials (*119*). The microstructure, crystallinity, and phase composition of HA coatings are critical in cell interaction and mechanical performance as high crystallinity contents could lead to increased implant life (*120*). MWCNTs can be distributed in HA coating using plasma spraying, to enhance fracture toughness by 56% and crystallinity by 27% (*120*). Unrestricted growths of human osteoblast hFOB 1.19 cells are observed near CNT regions, claiming assistance by CNT surfaces to promote cell growth and proliferation. MWCNTs grown on quartz substrates are indeed biocompatible to human osteoblastic cells (*105*). The cells attach and survive on the MWCNT constructs with higher metabolic activity on the MWCNT constructs compared to that of the control highly ordered pyrolytic graphite (HOPG) flat surfaces. The metabolic activity of the human osteoblastic cells is inversely proportional to the MWCNT diameter. CNTs have been proven as a biocompatible scaffold material for osteoblasts to promote cell growth and proliferation, thereby ensuring the production of HA crystals for matrix mineralization. CNTs with high electrical conductivity and mechanical strength could be utilized to promote cell growth and proliferation to enhance the production of bone forming materials as well as to increase the mechanical strength and implant life of bone tissue scaffolds.

8.3. Scaffolds for Other Cell Cultures. Besides neurons and bone cells, CNT-based 2D or 3D scaffolds have been investigated for other cell types. A novel type of CNT material known as nonwoven SWCNTs, which has nanotopographic structure and macroscopic volume (*109*), has been fabricated. Impurities in the as-synthesized CNTs are removed by high temperature oxidation in air followed by hot-acid treatment. The use of such nonwoven SWCNTs as a cell growth scaffold is investigated for 3T3-L1 mouse fibroblasts. The nonwoven SWCNTs exhibit significant enhancement of cell adhesion and proliferation in at least 3 weeks. Of interest is the creation of a honeycomb-like 3D structure from vertically aligned MWCNTs growing on a silicon substrate using a CNT functionalization process in (1:3 v/v) nitric/sulfuric acid solution (*110*). SEM micrographs of the honeycomb-like 3D structure are shown in Figure 7. Carboxylic groups are formed at the ends and in the defects of the side walls of the MWCNTs, resulting in a 3D assembly by the spontaneously generated capillary and tensile forces between the aligned tubes in the acid solution. Extensive growth, spreading, and adhesion of common mouse fibroblast cell line L929 are demonstrated in the 3D CNTs. Figure 8 shows the SEM images of L929 mouse fibroblasts growing on the MWCNT-based 3D network. Rat aortic smooth muscle cells can be seeded inside collagen-SWCNT composite scaffolds with different amounts of CNTs (*111*). The SWCNTs used for these constructs are functionalized with carboxyl groups and exhibit

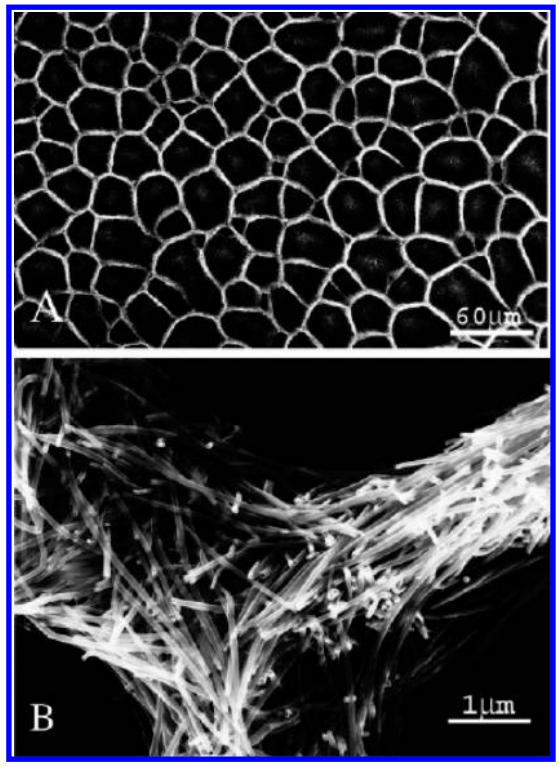

Figure 7. Examination by SEM of (A) the 3D networks of 50 μm length carbon nanotubes and (B) high magnification image of intercrossed carbon nanotubes in the walls of the cavities. Reprinted from Correa-Duarte et al. (2004). Copyright 2004 American Chemical Society.

no effect on cell viability and morphology. The cell numbers at day 7 are also not statistically affected by the presence of CNTs, though those at day 3 in CNT-containing constructs are lower than the control constructs. Thus, CNT-based scaffold materials have been reported as biocompatible to a variety of cell populations. They could accelerate and direct cell growth, and conduct electricity to enhance cell growth. Therefore, CNTs may be able to provide the initial structural reinforcement needed for a variety of tissue scaffolds. Table 6 summarizes the latest advances pertaining to the cellular toxicity of CNTs in different biological systems.

9. Some Remarks about Cytotoxicity of CNTs

Of importance is also the method used for studying cytotoxicity. To date, the MTT assay is the most commonly used method in cytotoxicity assessment. In this procedure, the yellow tetrazolium salt (MTT) is reduced by mitochondrial dehydrogenase from viable cells into blue formazan crystals, which are soluble in organic solvents. Hydrophobic CNTs with a very high specific surface area and high chemical activity might interact with the dye to affect the experimental data. As an example, the cytotoxicity measured by the MTT assay for human epithelial A549 cells exposed to SWCNTs for 24 h reaches up to 50%, whereas the lactate dehydrogenase (LDH) assay reveals no cytotoxicity (*149*). Indeed, SWCNTs are known to bind various organic molecules such as sugars, proteins, culture medium components (*150*−*152*), and lung surfactant proteins (*153*). Thus, a reliable protocol for cytotoxicity assessment of CNTs remains to be established and requires further intensive validation. Of course, the most important issue is the long-term toxicity and *in vivo* testing using different animal models and doses on large scales.

Several factors relevant to the cytotoxicity of CNTs are addressed in this review, and the topic of CNT cytoxocity remains controversial and confusing. However, a close look at such reports reveals that when cells are used as a suspension in cell culture, CNTs appear toxic, whereas they are not toxic if immobilized to a matrix or to a culture disk for cytotoxicity testing. Biomaterials incorporated with CNTs are noncytotoxic (*154*−*157*), and the use of embedded CNTs in nondegradable nanocomposite scaffolds is advantageous over unattached CNTs from a toxicological point of view (*154*).

Another technical issue is the hydrophobicity of CNTs. Well-dispersed CNTs in a cell culture medium will absorb the nutrient biomolecules, leading to the depletion of such components and affecting the viability test. Spotting the CNTs entering the cells and differentiating them from cell structures such as membranes are time-consuming and tedious. Thus, there is a critical need for the development of more effective ways to study the uptake and cytotoxic effects of CNTs in cells. In this context, NIR and fluorescence properties of CNTs should be exploited in cell imaging as discussed earlier. In particular, SWCNTs have potential uses as photoluminescence, Raman, and photoacoustic contrast agents for cell imaging.

The effect of catalyst metal impurities in CNTs is always problematic since their complete removal always affects the structure entity of CNTs, particularly with MWCNTs. Both positive and negative toxicities are found with highly refined CNTs, which remains a dilemma, and therefore, further studies are needed to characterize the structural integrity and physiochemical properties of such refined CNTs compared to those of their parental CNTs.

Highly water-soluble and serum stable nanotubes are biocompatible and nontoxic or very low toxic as confirmed by several *in vitro* and *in vivo* toxicity studies. The preparation of a stable suspension of CNTs in water is also challenging due to the uncontrollable aggregation behavior of CNTs. This is critical for the investigation of the alleged nanosize effect of CNTs. Although several surfactants can be used to suspend CNTs in the solution, they are also toxic per se and must not be used in toxicity assays. For example, polyoxyethylene sorbitan monooleate can well disperse CNTs, and this surfactant is considered to be nontoxic (*158*). CNT solubilization through functionalization avoids the use of surfactants, thereby eliminating the effect of such surfactants in cytotoxicity studies. Functionalized CNTs are more aqueous-soluble and suitable for a given application; however, they might be taken up more rapidly than the pristine counterpart and this point should be taken into consideration from a toxicity viewpoint. In general, nonfunctionalized, hydrophobic CNTs exhibit toxicity (*45, 46, 58, 59, 159, 160*), whereas CNTs with biocompatible coatings are virtually nontoxic to cells *in vitro* (*18, 57, 64, 67, 72, 161*−*164*) and mice *in vivo* within the tested dose ranges (*165, 166*). Surface chemistry and surface reactivity at the CNT interface play an important role in the cytotoxic effect of CNTs. Nanoscale materials are expected to possess more reactive surfaces since with a reduction in particle size, surface atoms are proportionately enhanced compared to the proportion inside its volume. Reactive groups present on surfaces may have some plausible biological effects, another important parameter in toxicity assays.

Several cytotoxicity mechanisms for CNTs have been proposed: (i) disruption of intracellular metabolic pathways, (ii) oxidative stress and physical membrane damage causing ruptures, and (iii) the generation of reactive oxygen species (ROS) with oxidative stress as the most developed paradigm for probing the CNT effect on mammalian cells. MWCNTs are reported to induce cell-cycle arrest, increase apoptosis/necrosis, perturb

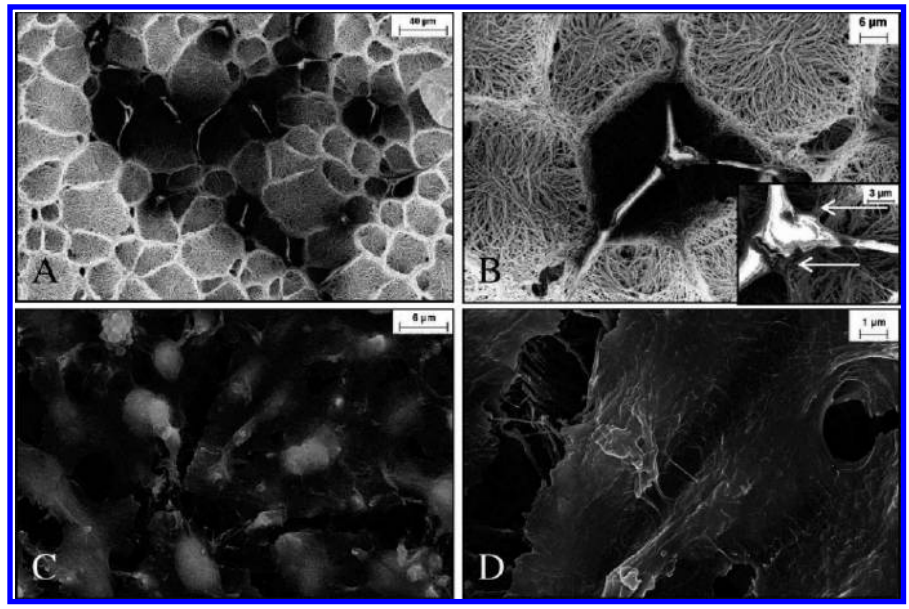

Figure 8. SEM images of L929 mouse fibroblasts growing on MWCNT-based 3D network after 1 day (A and B) and after 7 days (C and D). Reprinted from Correa-Duarte et al. (2004). Copyright 2004 American Chemical Society.

multiple cellular pathways, activate genes involved in cellular transport, metabolism, cell-cycle regulation, and stress response (*58*). Cell behavior is observed when HEK cells are exposed to SWCNTs (*59*): (i) secretion of some 20−30 kDa proteins, (ii) aggregation of cells attached by SWCNTs, (iii) G1 arrest and cell apoptosis, (iv) up-regulation expression, and (v) down-regulation expression. Cytotoxicity data obtained for MWCNTs reveal that the mechanism might be linked to ROS generation (*167*). The DNA damage response to MWCNTs in mouse embryonic stem (ES) cells has been reported (*168*). MWCNTs can accumulate and induce apoptosis in mouse ES cells and activate the tumor suppressor protein p53 within 2 h of exposure (*169*). Therefore, the genotoxicity of MWCNTs must be carefully re-evaluated, although some previous reports did confirm their limited or nontoxicity.

10. *In Vivo* versus *in Vitro* Cytotoxicity

In vivo versus *in vitro* cytotoxicity is another important issue and deserves some attention since cell culture studies measure cytotoxicity end points with high doses, and such information might have little relevance for human health effects. Thus, it is of importance to highlight some important findings with *in vivo* cytotoxicity in this review. With rat models, the pulmonary toxicity is due to mechanical blockage of the large airways (*45, 48*). The physiological relevance of these findings remains to be elucidated since the SWCNTs have a strong tendency to agglomerate following intratracheal exposures (*45*). A similar finding in mice confirms that SWCNTs are found to induce dose-dependent interstitial granulomas and pulmonary injuries (*46*). Further study reveals that besides mechanical blockage and distinct granulomas, the pharyngeal aspiration of CNTs in animal models results in a pronounced cellular response with increases in various cytotoxicity and inflammatory markers in the lungs (*45*). There is a significant increase in total broncho-alveolar lavage (BAL) cells and polymorphonuclear leukocytes, and also protein, lactate dehydrogenase (LDH), tumor necrosis factor (TNF)-α, interleukin (IL) 1β, and mucin levels (*45*). The long exposure of MWCNTs (length 10−50 μm, diameter 80−160 nm) to the mesothelial lining of the body cavity of mice results in asbestos-like, length-dependent, pathogenic behavior including inflammation and granuloma formation (*160*). Length-dependent pathogenicity is observed, but no obvious toxic effect is observed for shorter and smaller MWCNTs (length 1−20 μm, diameter 10−14 nm). Such results imply that the toxicology profiles of CNTs may significantly differ between CNTs of various sizes (diameter and length) as discussed earlier. Thus, functionalized SWCNTs might be more suitable with respect to their length (50−300 nm) and diameter (1−2 nm). Recently, the effects of SWCNTs as a function of dose, length, and surface chemistry in Swiss mice was reported (*170*). Neither death nor growth nor behavioral trouble is observed at a dose level of 1000 mg/kg body weight (b.w.). After intraperitoneal administration, SWCNTs, irrespective of their length or dose (50−1000 mg/kg b.w.), can coalesce inside the body to form fiber-like structures. When structure lengths are above 10 μm, they irremediably induce granuloma formation. Smaller aggregates do not induce granuloma formation, but persist inside cells for up to 5 months after administration. Short (<300 nm) well-individualized SWCNTs can escape the reticuloendothelial system to be excreted through the kidneys and bile ducts. Thus, for medical applications, CNTs must be engineered into discrete, individual "molecule-like" species. Nevertheless, the long-term toxicity of CNTs needs to be fully addressed and requires systematic investigations using different animal models on larger scales with various doses. Although SWCNTs have been widely used in biomedical applications, MWCNTs with bigger sizes could offer different platforms for different intended applications, e.g., delivery of large biomolecules including DNA plasmids into cells (*97, 171−173*).

11. Conclusions and Future Perspectives

CNTs have potential biomedical applications based on their unique properties. These applications include optical biosensing; cell tracking and labeling; and serving as scaffolds for tissue engineering as well as vehicles for intracellular transporting drugs, gene,s and proteins. The unique NIR intrinsic fluorescence of pristine CNTs and the changing of the fluorescence with the microenvironment may create significant advances in biosensing and noninvasive *in vivo* monitoring of engineered tissues. The efficient intracellular delivery of anticancer drugs, siRNA, proteins, and DNA combined with their corresponding effective therapeutic effects suggest that CNTs may provide a promising

Table 6. Toxicity of CNTs in Biological Systems

biological system employed	results of toxicity analysis	reference
human epidermal keratinocytes	Cell oxidative stress, loss of cell viability, and ultra structural and morphological changes in cells were observed following 18 h exposure to iron-rich (30 wt % iron) SWCNTs.	Shvedova et al. (*44*)
human embryo kidney cells (HEK293)	SWCNTs, used as purchased, inhibited cell proliferation by inducing G1 arrest, cell apoptosis, and decreasing cellular adhesive ability.	Cui et al. (*59*)
lungs of Sprague−Dawley rats *in vivo* and macrophages *in vitro*	Purified MWCNTs were toxic.	Muller et al. (*121*)
human skin fibroblast	Unpurified as-prepared MWCNTs had cytotoxicity.	Ding et al. (*58*)
human epidermal keratinocytes	Unpurified as-prepared MWCNTs had cytotoxicity.	Monteiro-Riviere et al. (*61*)
human acute monocytic leukemia cell line *in vitro* and subcutaneous tissue of rats *in vivo*	MWCNTs with an average length of 220 and 825 nm induced very slight and similar activities *in vitro.*	Sato et al. (*66*)
alveolar macrophages from guinea pigs	SWCNTs had more toxicity than MWCNTs.	Jia et al. (*47*)
cardiomyocytes	Purified SWCNTs had no cytotoxicity in both short-term (within 3 days) and long-term (after 3 days and after reseeding).	Garibaldi et al. (*79*)
human umbilical vein endothelial cells	Different CNT samples with the wall number ranging from 1 to 6 had no cytotoxicity.	Flahaut et al. (*68*)
murine RAW 264.7 macrophages	Iron-rich SWCNTs (26 wt % iron) resulted in a significant loss of intracellular low molecular weight thiols and an accumulation of lipid hydroperoxides in murine macrophages	Kagan et al. (*55*)
human fibroblast cells *in vitro*	All CNTs (refined SWCNTs and MWCNTs, and unrefined SWCNTs) were toxic. Refined SWCNTs were more toxic than unrefined SWCNTs.	Tian et al. (*56*)
mice	All CNTs employed [SWCNTs, MWCNT-I (dia 20−70 nm), MWCNT-II (dia50−150 nm) and Cup-stacked CNT] were not so toxic.	Koyama et al. (*122*)
murine and human macrophage cells *in vitro*	Highly purified SWCNTs and C-fullerenes had very low toxicity.	Fiorito et al. (*54*)
human dermal fibroblast cells	Sidewall functionalized SWCNTs were substantially less cytotoxic than surfactant stabilized SWCNTs.	Sayes et al. (*85*)
B and T lymphocytes, and macrophages	soluble amine-functionalized SWCNTs did not influence the functional activity of immunoregulatory cells, whereas amine-functionalized SWCNTs forming stable suspensions in water preserved the lymphocytes' functionality but provoked secretion of proinflammatory cytokines by macrophages.	Dumortier et al. (*67*)
Chinese hamster ovary (CHO) cells and Jurkat human T-lymphoma cells	CNTs coated with biomimetic glycopolymer were nontoxic, whereas unmodified CNTs induced cell death.	Chen et al. (*18*)
different mammalian cells	DNA-wrapped SWCNTs shorter than (189 ± 17) nm were consumed and likely induced more toxicity.	Becker et al. (*65*)
human alveolar carcinoma epithelial cell line (A549), human bronchial epithelial cell line (BEAS-2B), and human keratinocyte cell line (HaCaT)	All SWCNTs employed (HiPco SWCNT and arc discharge SWCNT) were toxic.	Herzog et al. (*123*)
human lung cell line (A549)	SWCNTs had very low acute toxicity.	Davoren et al. (*50*)
rat macrophages (NR8383) and human A549 lung cells	All CNTs employed (Commercial SWCNTs, MWCNTs, CNTs, and acid-treated SWCNT with reduced metal catalyst content) had no acute toxicity.	Pulskamp et al. (*58*)
A549 human alveolar carcinoma cell line	Flaws in cytotoxicity testing were identified, which led to inconclusive results with SWCNTs.	Casey et al. (*51*)
rat aortic smooth muscle cells (SMC)	SWCNTs were toxic.	Raja et al. (*78*)
human MSTO-211H cells	Dispersed SWCNTs were less cytotoxic, whereas agglomerated SWCNTs were more cytotoxic.	Wick et al. (*124*)
human epithelial lung cells	SWCNTs had no acute toxicity.	Pulskamp et al. (*77*)
BV2 microglia and GL261 glioma cells	MWCNTs had no acute toxicity.	Kateb et al. (*125*)
human epidermal keratinocytes	The surfactant treatment (1% anionic surfactant Pluronic F127) caused the dispersion of 6-aminohexanoic acid-derivatized SWCNTs (AHA-SWCNT) aggregates in the culture medium, thus contributing to less cytotoxicity.	Zhang et al. (*62*)
A549 human pneumocytes *in vitro*	MWCNTs had cytotoxic effects, which were unaffected by the length and presence of metal catalyst impurities.	Simon Deckers et al. (*126*)
Calu-3 human airway epithelial cells	Commercial MWCNTs affected cell functions, while commercial SWCNTs had no toxicity.	Rotoli et al. (*127*)
skeletal rat muscle *in vivo*	There were some undesirable effects such as the presence of abundant multinucleated cells attached to the MWCNT agglomerations and the transport of SWCNTs from the implant sites to the lymph nodes, which might be related to cytotoxicity.	Fraczek et al. (*128*)
subcutaneous tissue of rats *in vivo*	MWCNTs had no toxicity.	Sato et al. (*66*)
L929 mouse fibroblast cells	MWCNTs films grown on Si/SiO2/Ni substrate had no toxicity compared to those grown on Ti/TiN/Fe samples.	Lobo et al. (*129*)
mouse macrophages (J774.1)	MWCNTs triggered cytotoxic effects by associating with the plasma membrane of macrophages via macrophage receptor with collagenous structure (MARCO) and rupturing the plasma membrane.	Hirano et al. (*130*)

Table 6 Continued

biological system employed	results of toxicity analysis	reference
HT1080 cell line (human Fibrosarcoma) and red blood cells	MWCNTs-polyglycerol (PG) hybrid material had no toxicity.	Adeli et al. (*131*)
fibroblast cell line (L-929)	SWCNTs had no toxicity.	Thomson et al. (*132*)
lung epithelial cells (A549 cells) and macrophages (stimulated THP-1 cells)	MWCNTs were toxic, and it was observed that the culture conditions can significant affect the cytotoxicity determination.	Geys et al. (*133*)
primary cultures derived from chicken embryonic spinal cord (SPC) or dorsal root ganglia (DRG)	SWCNT suspensions induced acute toxic effects, which were partially dependent on the SWCNTs' agglomeration state with greater toxicity in the case of highly agglomerated SWCNTs as compared to well dispersed SWCNTs.	Belyanskaya et al. (*134*)
human aortic endothelial cells	SWCNTs and MWCNTs were cytotoxic at 24 h postexposure.	Walker et al. (*135*)
Caco-2 cells, derived from human intestinal adenocarcinoma	COOH-SWCNTs induced toxic effects in nondifferentiated and differentiated Caco-2 cells mainly at concentrations higher than 100 μg/mL. The cytotoxic effects were higher on the Caco-2 cells with differentiated cultures.	Jos et al. (*136*)
in vitro and *in vivo* skin of rabbits	MWCNTs had no cytotoxicity as shown by ocular and dermal irritation.	Kishore et al. (*137*)
human macrophages	Purified (by annealing at 2400 °C) and better electroconductive MWCNTs, i.e., α-MWCNTs were less cytotoxic than as-prepared MWCNTs.	Fiorito et al. (*138*)
mice *in vivo*	Purified carboxylated MWCNTs with higher agglomeration were retained in lungs and liver, and were not eliminated completely even after 28 days, which was responsible for temporary organ injury in the lungs and liver even though there was no acute general toxicity.	Qu et al. (*139*)
GL261 murine intracranial glioma model	MWCNTs had no significant cytotoxicity.	VanHandel et al. (*140*)
dendritic cells (DCs)	Carboxylic-MWCNTs, irrespective of their diameters, were generously phagocytosed by DCs without any cytotoxicity.	Wang et al. (*141*)
Arabidopsis T87 suspension cells (plant cells)	MWCNT agglomerates of smaller size induced stronger toxicity than MWCNT agglomerates of larger size.	Lin et al. (*142*)
mouse macrophages (RAW 264.7 cell line) and murine bone marrow-derived dendritic cells (bmDC)	MWCNTs and SWCNTs showed the least toxicity and activation ability of these antigen presenting cells (APCs) suggesting that APCs might not be the target cells for CNTs.	Palomaki et al. (*143*)
human monoblastic leukemia cells (U937)	As-grown MWCNTs and MWCNTs thermally treated at 1800 °C (HTT1800) were cytotoxic, whereas MWCNTs thermally treated at 2800 °C (HTT2800) were not cytotoxic.	Haniu et al. (*144*)
mice (*in vivo*)	MWCNT exposure in mice caused dose- and time-dependent pulmonary inflammation, and damage peaked at 7 days postexposure.	Porter et al. (*145*)
human neuroblastoma cells SH-SY5Y (*in vivo*)	MWCNTs [pure MWCNTs (99% purity), 97% purity MWCNTs, and acid treated MWCNTs (97% purity, surface oxidation 8%)] had no significant cytotoxicity.	Vittorio et al. (*146*)
immortalized and primary human lung epithelial cells (A549 and NHBE)	HiPco and arc discharge SWCNTs had no acute toxicity but resulted in moderate to low oxidative stress. SWCNT dispersion in A549 medium was improved by dipalmitoylphosphatidylcholine (DPPC), which increased the particle toxicity, but it had no effect on NHBE cell.	Herzog et al. (*147*)
E.coli and WB-F344 rat liver epithelia cells	SWCNTs solubilized by Triton X-100 are cytotoxic due to the toxicity of the solubilizer.	Aplatova et al. (*148*)

novel transporting vehicle for bioactive molecules in the new generation of biomedicine. CNTs may also serve as an important component in engineered tissues to impart important features such as lightweight, greater mechanical strength, and good electrical and thermal conductivity, and to guide cell growth and tissue regeneration. Cytotoxicity appears to depend on material preparation, impurities, geometry, and surface functionalization. Although cytotoxicity has been reported in some studies and remains contradictory in some future reports, purification and functionalization could greatly reduce the toxicities of CNTs, resulting in virtually biocompatible nanomaterials. There is a strong need to establish an international set of guidelines and protocols for the determination of the cytotoxicity of CNTs *in vitro* and *in vivo*, which need to be universally followed to gather highly valuable data that can elucidate the scientific facts and mechanisms of CNTs cytotoxicity. Future studies should focus on developing well-dispersed CNTs with minimized aggregations. Systematic studies are required to elucidate the effects of the different factors on cell interfacing without any inferences from other factors. The controlled release of bioactive molecules can be combined with targeted tissue delivery for potential biomedical applications. The use of an appropriate amount of properly functionalized CNTs in combination with other biomaterials may lead to the development of novel tissue scaffold materials for different applications. However, CNT samples used for testing are often heterogeneous mixtures of nanotubes with different lengths, diameters, and chiralities. The CNT length may affect *in vitro* cellular uptake and *in vivo* pharmacokinetics. CNTs with homogeneous length, diameter, and chirality distributions are ideal for studies in biomedicine, *in vivo* toxicity, drug delivery, and targeting.

In conclusion, the unique properties of CNTs together with the functionalization strategies may lead to potential applications of CNTs in biological sciences. To date, nanotoxicity studies often regard CNTs as nanoscale chemical substances using conventional toxicology methods. However, many experimental results obtained so far could not be sufficiently explained by this model. Thus, physical and chemical mechanisms of CNT cytotoxicity must be investigated toward the development of assaying methods and evaluating criteria for nanotoxicity. CNTs of varying lengths must be carefully examined to decipher size

and shape effects on nanomaterial for potential therapeutic approaches. The long-term effects of f-CNTs in animals and their swift clearance from such animals remain to be investigated. Intensive efforts are required to optimize the surface chemistry of CNTs, to further enhance biocompatibility. Surface coating and functionalization of CNTs are essential for biomedical applications. CNTs with a distinctive 1D structure and tunable length provide an ideal platform to investigate size and shape effects *in vivo*. The intrinsic physical properties of SWCNTs including resonance Raman scattering, photoluminescence, and strong NIR optical absorption can be used for tracking, detecting, and imaging.

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