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Review Article

Toxicity issues in the application of carbon nanotubes to biological systems

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Abstract

Carbon nanotubes (CNTs) have recently emerged as a new option for possible use in methodologies of cancer treatment, bioengineering, and gene therapy. This review analyzes the potential, through possible toxicologic implications, of CNTs in nanomedicine. Generally, proven success in other fields may not translate to the use of CNTs in medicine for reasons including inconsistent data on cytotoxicity and limited control over functionalized-CNT behavior, both of which restrict predictability. Additionally, the lack of a centralized toxicity database limits comparison between research results. To better understand these problems, we seek insight from currently published toxicity studies, with data suggesting postexposure regeneration, resistance, and mechanisms of injury in cells, due to CNTs.

From the Clinical Editor: Carbon nanotubes (CNTs) have recently emerged as a new option for cancer treatment, bioengineering, and gene therapy. Inconsistent data on cytotoxicity and limited control over functionalized-CNT behavior currently restrict predictability of such applications.

Key words: Biomedical applications; Carbon nanotubes; Functionalization; Toxicity

Carbon nanotubes (CNTs), in both the single-walled and multi-walled (SWCNT and MWCNT) forms, are widely considered to be the wonder materials of the 21st century and bring new paradigms to diverse fields including electronics,[1] structural integrity,[2] biomedical engineering,[3-5] tissue engineering,[5] drug delivery,[6] nanoinjectors,[7] neuroengineering,[8] gene therapy,[9] and biosensor technology.[10] Several reviews of CNTs regarding their methods of synthesis[11,12] and their superior mechanical, chemical, and electrical properties exist in the literature.[11,13-15] In this article, we focus on the attributes relevant for the practical application of CNTs in biomedicine.

An example of the utility of CNTs in biomedicine is their relatively large length-to-diameter aspect ratio (which can exceed 10^6, with an average length of 1 mm and diameter ~1 nm) with a very large surface area, which makes CNTs amenable for high-sensitivity molecular detection and recognition. Consequently, a large fraction of the CNT surface can be modified with functional groups of various complexities, which would modulate its in vivo and in vitro behavior.

In spite of such attractive features, the toxicity of CNTs is a prime concern, with several groups pointing to their similarity to asbestos fibers.[16] CNT toxicity in both in vivo and in vitro studies has been attributed to various factors, for instance, length, type of functionalization, concentration, duration of exposure, method of exposure, and even the dispersant used to solubilize the nanotubes. Yet many studies also seem to suggest that such attributes for CNT toxicity are unfounded. These inconsistencies seem to arise largely due to differences in experimental protocol, and whereas some points of view have been reconciled, most aspects of CNT toxicity remain uncertain. This review then aims to synthesize and further analyze representative data on toxicity by first considering how CNTs are designed for biomedical purposes, how this process itself can promote toxicity, and the behaviors of these CNTs when in clinical use, which all combine to explain the current toxicity profile of CNTs. A fresh evaluation of these studies yields new insight into CNT toxicity, with emphasis on issues such as cell-specific tolerance, rates of toxic events, mechanism of cell injury, and organ-specific biodistribution. Additionally, we hope that this review will stimulate further research into the

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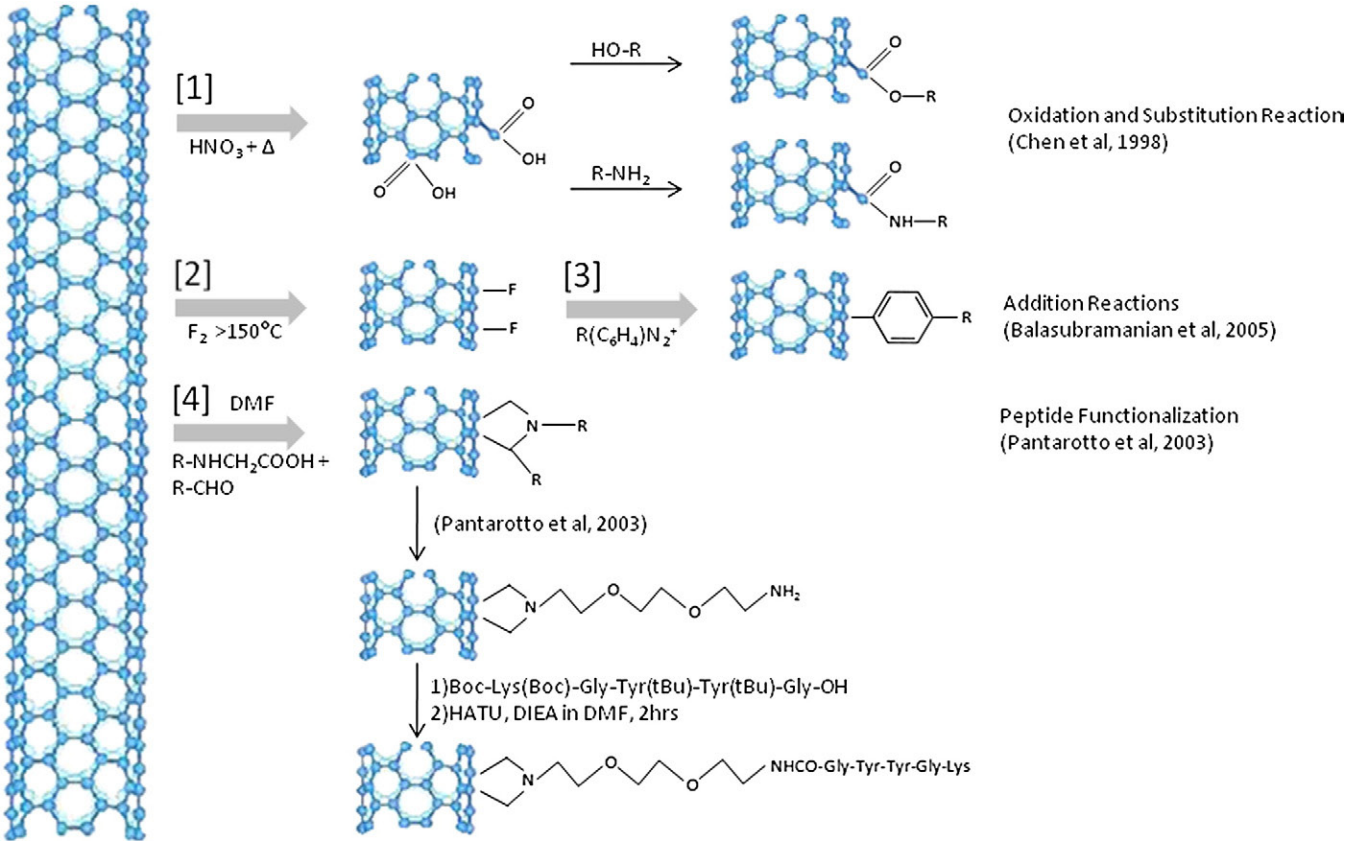

Figure 1. A diversity of functionalization schemes, either at the ends or sidewalls, can be used for engineering the solubility and dispersion of carbon nanotubes (CNTs). This includes (*1*) acid refluxing to open CNT ends, (*2*) addition of reactive species, such as fluorination, which could be further followed by (*3*) amidization and (*4*) attachment of biologically relevant groups such as amino acids and proteins.

fundamental aspects relevant for nanotube application, such as intrinsic defects, methods of synthesis, nature of the functional group, and so forth, all of which underlie biocompatibility issues and eventual widespread application.

Modifying CNTs for use in medicine

As CNTs are intrinsically not water soluble, modification through chemical functionalization using suitable dispersants and surfactants can enhance solubility to the range of g/mL[4] and is essential for their controlled dispersion. For example, constituent polar molecules can render CNTs soluble, whereas nonpolar moieties make CNTs immiscible. Such processes have proved especially important in that nonsolubilized CNTs have been found to cause cell death in culture.[17-19]

Additionally, the tubular, vesicle-like character of CNTs has been used for drug containment[20,21] and focused drug delivery in clinical trials (eg, for the dispersal of cancer drugs for localized tumor treatment[22]). Consequently, CNTs are also amenable for nano-sized platforms, whereby functional groups that would normally not coincide (eg, like antibodies,[23] polyethylene glycol,[24] and cancer medication[25]) can be brought together. Functionalization, through the attachment of different functional groups, has also made it possible to create nanotube-based moieties with complex behavior (eg, a drug-delivery vehicle that can traverse the plasma membrane, and release the drug in a target organelle[26]).

The underlying process of functionalization involves the selective breaking of C = C bonds in the CNT and is often done through oxidation (eg, refluxing in nitric acid or through electrochemical modification) resulting in carboxyl groups that could then be used as subsidiary sites for addition reactions. Whereas CNTs synthesized through arc discharge and laser ablation have *closed* ends, chemical vapor deposition (CVD) synthesized nanotubes have *open* ends with dangling bonds that are highly susceptible[13] to chemical reaction. These defect sites are often constituted by carboxyl and hydroxyl groups in aqueous solution.[4] A few examples of such functionalization schemes are illustrated in Figure 1 and representative toxicology studies depicted in Table 1. In addition to oxidative mechanisms, other means of modifying CNTs include substitution reactions with reactive species (eg, yielding fluorinated nanotubes[27]).

Most functionalization schemes depend on the presence of defects (both neutral and charged) along the CNT morphology, which determines the supporting reactions.[28-30] Figure 2 provides a schematic overview of the role of defects vis-à-vis their understood toxicity influence. Vacancies, nonhexagonal ring members (eg, pentagon-heptagon pairs constituting Stone-Wales type defects), interstitials, and rehybridization defects

Table 1
Toxicity study of various carbon nanotube solubilization/functionalization schemes of biologic interest

Solubilizing agent	Toxicity	Reference
Tetrahydrofuran	Tumorigen, mutagen	Hu et al [94]
Dichlorobenzene	Very harmful to aquatic organisms	Hu et al [94]
Dichlorocarbene	Harmful	Hu et al [94]
Anthracene	Possible tumor promoter	Hedderman et al [95]
Chitosan	Mostly safe	Tkac et al [96]
Pyrene	Carcinogenic, mutagenic	Guldi et al [97]
Polyethylene glycol	Acute oral and dermal toxicity, mutagenic	Zhao et al [98]
Lysozyme (ie, chicken egg white)	Unknown	Asuri et al [99]
Peroxidase (horseradish)	Unknown	Asuri et al [99]
Taurine	Safe up to ~28.57 mg/mL	Wang et al [100]
Helical amylase	Unknown	Kim et al [101]
Barbituric acid	Not pharmacologically active	Ikeda et al [102]
Sodium cholate	Unknown	Ishibashi et al [103]
Zn-porphyrin	Unknown but more toward the safe side	Cheng et al [104]
Poly (phenyleneethynylene)	Possible antimicrobial properties	Mao et al [105]
Poly(aminobenzene sulfonic acid)	Hazardous to blood, nervous system, liver	Zhao et al [98]
Poly(acrylic acid)	Severely irritating and corrosive	Liu et al [106]
Thiolated organosilane	Unknown	Bottini et al [107]
Phenyl ethyl alcohol	Topical irritant	Dumonteil et al [108]
n-octyl-beta-d-glucoside	Unknown	Ishibashi et al [103]
n-decanoyl-*N*-methylglucamide	Unknown	Ishibashi et al [103]
Triaminopyrimidine	Unknown	Roberts et al [109]
Lysophophatidylcholine	Unknown	Roberts et al [109]
Sulfonated polyaniline	Unknown	Zhang et al [110]

have been considered in terms of CNT electrochemistry,[31] as well as potential sites for bonding with biologics such as peptides and enzymes.[4] Electron transfer between the CNTs and the solution can also be used to generate highly reactive species in situ and for tailoring local polarity and/or hydrophobic/hydrophilic character. This can be done simply through the application of a constant current to a CNT-based electrode. As an example, polyurethanes can be coated onto amino-functionalized CNTs by in situ polycondensation of diisocyanate [4,4′-methylenebis(phenylisocyanate)] and 1,6-diaminohexane, followed by the removal of free polymer via repeated filtering and solvent washing.[32] In this case, the covalent attachment of molecules onto the CNT surface only relies on the exchange of electrons between the molecule and the CNT carbon bonds and less on the presence of defects.

Toxicity at the level of fabrication and functionalization

Each CNT could be intrinsically different due to limitations on the fabrication of structurally identical CNTs with minimal impurities.[33] Subtle variations in local and overall charge, catalyst residue (typically Fe, Co, and Ni), and length of individual nanotubes are three representative issues that preclude precise use of CNTs in the biomedical sciences.

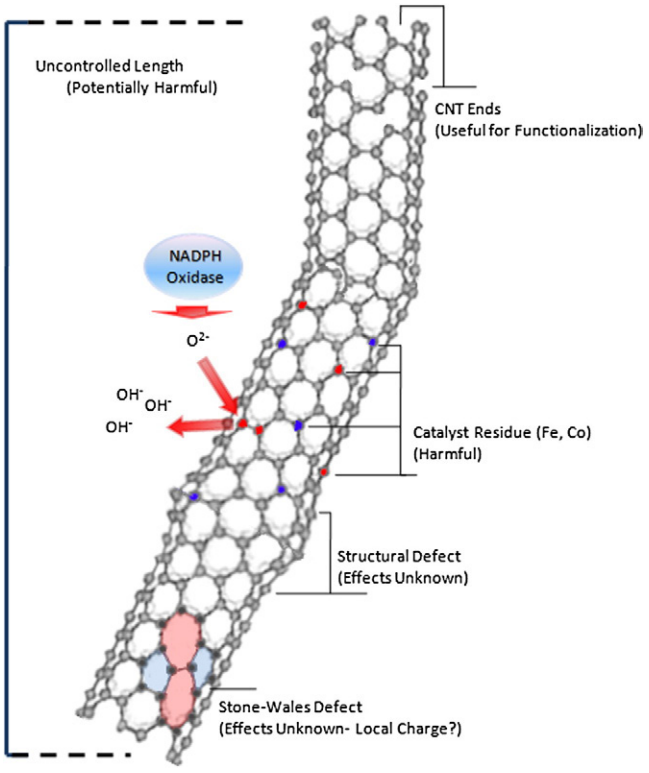

Figure 2. Defects and associated intrinsic/extrinsic charge on CNTs play a major role in their interaction with the environment and can be implicated in toxicity. As an example, Fe catalyst residue from CNT growth can catalyze generation of superoxide species that can damage cell components.

Uncontrolled binding of CNTs to charged biologics

It has been estimated that there is a 1% to 3% chance of finding nonhexagonal (seven- or five-membered) rings randomly distributed along a CNT surface over a length of 4 μm.[34] It has then been postulated that either deficit or excess charge may be present around the odd-membered rings, which causes deviations from neutrality. Although such charge modulation has been exploited for interesting applications,[35,36] studies have shown that the type of charge and charge density on a functionalized nanotube can affect cellular interaction.[37,38] For example, the amount of DNA, and the strength, with which DNA strands bind onto a CNT depends on nanotube charge density, which varies with fabrication.[39] Such effects can limit predictability (eg, if CNTs are used for gene therapy).

Inorganic residues act as catalysts in vivo

Extrinsic defects, such as catalyst residue, could also be harmful to biomedical application. As Fe and Ni catalysts used for CNT production can constitute 25% to 40% of the CNT by weight,[40] these embedded metals can catalyze oxidative species in cells and tissue through free radical generation.[17,33,41] The

oxidative species generated during a natural inflammatory response can interact with these transition metals to trigger redox-cycling cascades that deplete endogenous antioxidants and cause oxidative damage to tissue.[41,42] Such processes can occur after the CNTs are engulfed by macrophages; for example, when the enzyme nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) is activated inside the cell to produce superoxide O_2^- species—a highly reactive radical capable of killing bacteria and fungi. The Fe-based catalyst residue can then react with the superoxide to form highly reactive hydroxyl (OH^-) radicals (Figure 2), with the resulting oxidative stress damaging cell proteins, lipids, and DNA. CNTs with residual Co have noticeably caused chromosome damage at 4 μg/mL.[43] However, it was interesting to note that negligible cellular damage was found to be caused by Ni,[44,45] the reasons for which need further study.

Toxicity of chemicals used to increase solubility

It is generally agreed that functionalized CNTs (f-CNTs) constitute a major improvement over unmodified, nonfunctionalized (pristine) CNTs, as the latter are often reported to cause adverse reactions from living tissue, whereas the former could be much less toxic due to more biocompatible functional groups.[3,24,46] Consequently, using f-CNTs allows for experimentation with living cells through miscibility in cell culture, adequate distribution in solution, along with the prevention of aggregates. Whereas the lower toxicity of f-CNTs could make them more practical, some studies have shown that the chemical properties of some solubilizers and certain functional groups on a CNT can also cause toxicity, with the possibility of altered functionality. A representative compilation of the toxicity of various CNT solubilization/functionalization schemes of biologic interest is shown in Table 1.

In this regard, it was observed[19] that cytotoxicity was diminished as SWCNT sidewall functionalization increased, for example, using phenyl-SO_3H and phenyl-SO_3Na additives, and that even at high concentrations (>2 mg/mL) there was insignificant damage to cells. Further analysis indicates a greater decrease of toxicity for functionalization with phenyl-SO_3Na compared with functionalization with phenyl-SO_3H. However, SWCNTs with attached phenyl-$(COOH)_2$ groups manifested toxicity even at the 80 μg/mL level. The relative rate of increase/decrease in toxicity is important in indicating the extent to which the functionalization and safe dosage can be regulated.

One also needs to understand whether the underlying cause of toxicity in a certain f-CNT is due to the functional group, the CNT, or due to the combination. As an example, poly-ethylene glycol (PEG)—which by itself has been recognized to promote spinal cord recovery in guinea pigs[47]—when coupled to a CNT (ie, PEG-functionalized CNTs) activates[48] primary immune cells (macrophages) and increases proinflammatory cytokines in culture.[24] In an associated study,[49] 100% mortality was observed at ~12 hours in fat-head minnows after dosing of fullerenes solubilized in tetrahydrofuran (THF) where the primary cause of death was ascribed to the THF. Conversely, polyoxylethylene sorbitan monooleate (PS80) dispersed CNTs were used with human lung mesothelium (MSTO-211-H) cells with no toxicity due to the surfactant.[45] It is imperative to understand and catalog such instances for the insight they may provide into nanotube/tissue interactions so as to prevent accidental confounding of data due to dispersant toxicity.[50]

The size of the functional group also seems to matter. For example, it was indicated that SWCNTs functionalized with relatively large molecules (molecular weight >60 kDa) can increase toxicity.[51] Additionally, the uptake of large protein-SWCNT conjugates was found to be extremely poor, whereas the binding and intracellular transport of small to medium-sized conjugates (mostly <80 kDa) yields higher levels of uptake, as verified through cell cytometry.

Physical properties of the nanotube affect toxicity

The length and shape of the CNTs influence how well they traverse the membrane of macrophages and determine the resulting immunologic response.[52,53] For example, shorter CNTs (~0.22 μm in length) were found to be better integrated into macrophages and phagocytes than were the longer (>0.8 μm) CNTs.[54] It was found that most of the short CNTs injected into subcutaneous tissue in rats were in the cytosol of macrophages after 4 weeks, whereas the longer CNTs were still free floating and causing inflammation.[54] A complementary study arrived at similar conclusions after intraperitoneal injection of long- and short-length CNTs in mice.[16] However, through comparison, we see that the nontoxic length asserted in the later study[16] was ~10 μm, and the possible influence of defects along the CNT length and its influence on toxicity would be pertinent. In a related context, a lower concentration of larger-diameter amphotericin B (AmB) conjugated MWCNTs were found to be necessary to eliminate *Candida albicans* in a fungal infection test compared with that of smaller-diameter AmB conjugated SWCNTs.[55]

A major source of discrepancy could again arise from the differences from the defect and charge densities of the various CNTs used in the experiments. It would then be prudent to isolate CNTs with similar charge densities and lengths (eg, through using an electrophoresis-based process prior to testing).

Mechanisms of interaction

Although much progress has been made in understanding how CNTs traverse the lipid membrane of a given cell type, the details of the proposed mechanisms are still debated. Such considerations are important in that the failure to understand the uptake mechanisms of nanoscale materials and their influence on toxicity could create another level of unpredictability.[45] To date, two major mechanisms have been widely considered: (1) endocytosis/phagocytosis[56] and (2) nanopenetration (Figure 3).

Endocytosis[57,58] represents the engulfing of an extracellular particle by the cell, for example, viruses (~100 nm in size), through the creation of a vesicle that is then integrated into the cell. Phagocytosis is similar to endocytosis but usually involves uptake of larger particles, such as bacteria (~1 μm), and is characteristic to a subset of immune cells/phagocytes (eg, neutrophils, macrophages, dendritic cells). These processes are energy dependent and are hindered at low temperatures

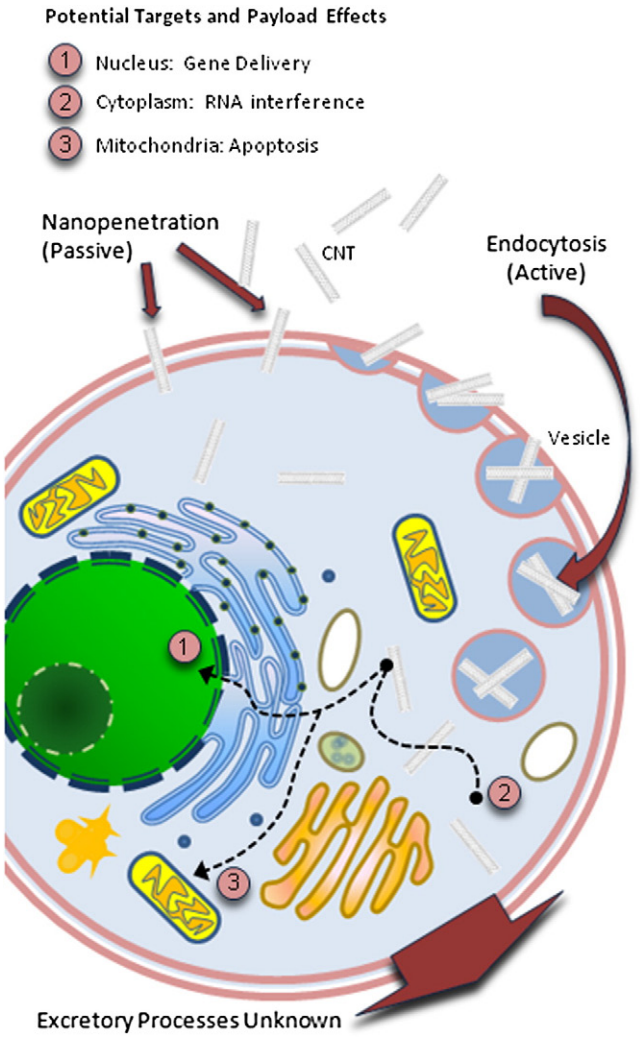

Figure 3. Either receptor-mediated endocytosis or nanopenetration, which is functionalization dependent, are suggested as possible mechanisms for CNT interactions with cells. Although preliminary studies have probed targeting capability of CNTs vis-à-vis distinct organelles, the mechanism of excretion is yet unknown.

and in low ATP environments.[59] Several studies implicate endocytosis/phagocytosis as the cellular uptake mechanism of CNTs.[51,60-62]

Nanopenetration is an energy-independent passive process, where the nanotubes diffuse across the cellular membrane.[37] In this respect, it is similar to passive diffusion of nano-needles from extracellular to intracellular space.[22,63,64] It was also posited that CNTs could behave similarly to cell penetrating peptides (CPPs), which represent poly-cationic sequences that enhance uptake of proteins into mammalian cells. An interesting study[65] in this context was the passive penetration of water-solubilized fluorescein isothiocyanate–attached CNTs and G-protein–functionalized CNTs into fibroblasts and keratinocytes at 37°C. Such investigations hint that f-CNTs that resemble CPPs in morphology and possess an overall charge may more likely penetrate the plasma membrane rather than undergo endocytosis. Further experimental testing is crucial in that the type of functionalization could ultimately determine the precise mechanism.

How do CNTs cause toxicity?

In cases where CNTs have a toxic interaction with cells, the mechanisms of toxicity are coming into focus. Results suggest CNTs may cause harm to cells by activating many pathways at once, mostly involving DNA damage.[66] In one study, mesothelial cells exposed to SWCNTs at concentrations ~25 μg/cm^2 activated DNA recovery along with changes in the cell cycle and generation of apoptotic signals. Another approach[67] showed that most cells incubated with CNTs halt at the G1 phase of the cell cycle. It was also observed that CNT/DNA interaction was the preferred route of toxicity in a 3-hour incubation study with 96 μg SWCNT/cm^2, which induced DNA damage (through micronucleus generation) in lung fibroblasts.[68] It should be possible, through the observation of specific toxic events that result from incubations with different types of f-CNTs, to test for functional groups that reduce the severity of such events.

Differential sensitivity of tissue to CNTs

In another study[69] aimed at understanding cytotoxicity of pristine SWCNTs in the liver, spleen, and lungs, it was observed that indicators for oxidative stress due to SWCNTs [eg, malondialdehyde (MDA) and glutathione (GSH) levels] increased in a dose-dependent manner in the liver and lung, whereas the stress remained relatively constant in the spleen as nanotube dosage increased. If certain organs are sensitive to CNTs in different ways, this creates another facet to consider during the search for safe in vivo dosage.

Another area of research indicates that tumor cells interact differently with CNTs than do wild-type cells.[66] It was seen that malignant mesothelial cells were able to maintain a dose-dependent increase of stress-response proteins [activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)] as a reaction to increasing toxicity due to longer incubation with CNTs in culture, whereas normal mesothelial cells had higher sensitivity (ie, produced a higher level of similar proteins initially) but were unable to maintain production under longer incubation. The latter are also more sensitive to DNA damage by CNTs as confirmed by significantly higher levels of the DNA repair protein poly(ADP-ribose) polymerase (PARP). These studies suggest that cancer cells could be bioactively prepared for an assault by CNTs and caution our understanding of correct dosage.

In vitro toxicity: Dosage, tolerance, and regeneration

As with most biomedical research, in vitro tests for toxicity pave the way for future applications in vivo. However, for CNT application, it seems that in vivo studies have been attempted while many questions from in vitro studies have been left unanswered (eg, issues on overall safe CNT dosage for a particular task and the sensitivity of particular cell lines). The probing of such issues is relevant as there may not be a linear relationship between mass and toxicity tolerance.

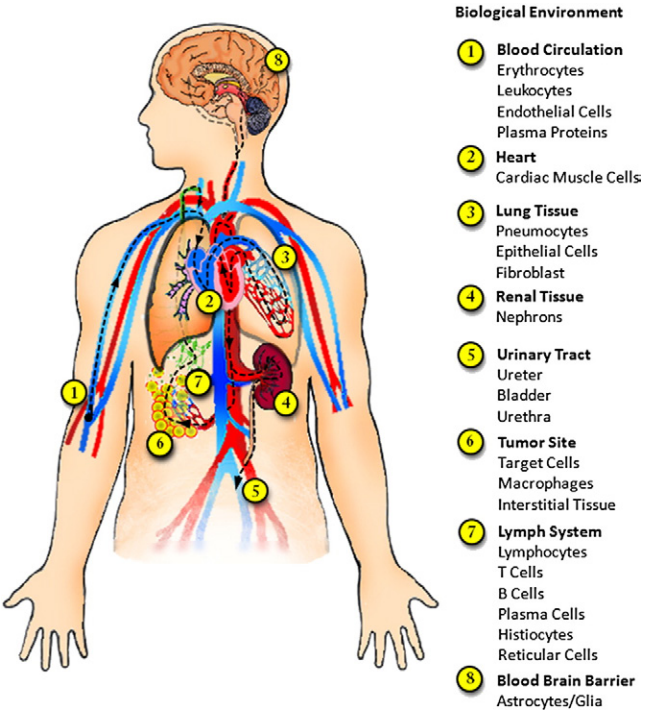

Figure 4. Toxicity studies using different cell types remain inconsistent because all studies used different nanotubes and did not compare CNT toxicity between cell types. The illustration presents a possible intravenous route of CNT circulation after injection: (*1*) Contact with flowing red blood cells and plasma, and with vascular endothelium; (*2*) interaction with contractile fibers of the heart wall; (*3*) ingestion into alveolar capillaries (lung) and travel through the arteries via the heart; (*4*) localization to the kidneys for excretion in urine; (*5*) interaction with cells of the ureter and urethra; and (*6*) delivery close to tumor sites for CNT action (ie, drug and gene delivery). As CNTs move into the interstitial space of tissue, excess fluids, along with CNTs, are collected into the (*7*) lymphatic system and recycled back into the blood to complete the circuit. The effect of CNT interactions at the (*8*) blood-brain arrier is still unknown.

Tests for a safe dosage

Table 2 presents a representative compilation from the literature of cell viability subsequent to interaction with various types and concentrations of f-CNTs. A few representative results will now be discussed. Bianco et al[3] observed death in 50% of HeLa (Henrietta Lacks) cells in culture, after a 6-hour incubation with increasing doses of f-SWNTs and f-MWNTs at a concentration of 5 to 10 mg/mL. This dosage was shown to be excessively high by Pantarotto et al.[70] On the other hand, a safe concentration[71] of CNTs seems to be around 40 μg/mL as was suggested through tests on T lymphocytes. Such a concentration translates to approximately 10 individual CNTs per cell, based on a mean length of 1 μm and diameter of 40 nm.

Intrinsic cellular tolerance to toxicity

According to the published literature, toxicity and resistance can occur within a CNT concentration range spanning six orders of magnitude (ie, from 5 ng CNT/mL[22] to 10 mg CNT/mL[18])! While differences in experimental methods are commonly blamed for these inconsistencies, the reasons could be simpler: CNTs used for experimentation span a large range of lengths from nanometers to micrometers, with a corresponding range of toxicity.[54] An interesting issue in such studies is whether all the CNTs were indeed in contact with the cell types.

Whereas a few in vitro studies assert that oxidative stress from CNTs is a major result of toxicity, others suggest that certain cells may have a tolerance level that can be directly measured. For example,[72] fibroblast cell cultures had an exponential increase in reactive oxidative particles and DNA damage after incubation in concentrations above 5 μg CNT/mL culture, but any uptrend in toxicity before and up to the 5 μg CNT/mL point was low. Other data[73] shows a trend of lactate dehydrogenase (LDH) release with low variability as CNT incubation concentrations increased up to a concentration of 50 μg CNT/mL at which point a strong uptrend in LDH release was observed. Similar trends of negligible toxicity in the liver and lung after intravenous exposure to CNTs of increasing concentration were indicated[74] through constant MDA levels except in one condition (between the 200 μg/mouse and 1 mg/mouse condition) when MDA significantly increased. The same sudden upward inflection of MDA levels in primary mouse embryonic fibroblast (PMEF) cells, after a steady level of low toxicity that did not change with increasing CNT dosage, were also observed in another study.[72] Such a drastic relationship between toxicity and CNT concentration could imply a distinct boundary between low toxicity and a major toxic event when a critical concentration specific to each cell type is reached, indicating an innate degree of CNT tolerance.

Regeneration of cells postexposure

More fascinating than CNT tolerance is data revealing what seems to be cellular regeneration postexposure. For example, in one study,[67] mutant lung epithelial cell line (FE1-MML type) cells were exposed to 100 μg CNT/mL concentrations in media incrementally, where the CNTs were refreshed every 3 days. Between 3 and 69 days, the number of viable cells were on average 48% less than control, but in the final 3 days of the study (days 69 to 72), cell count returned to the control level. It was not mentioned whether stabilization was ultimately achieved. Complementary data also show[75] increasing numbers of viable albino BALB/c mouse macrophage-like cells (J774.1) after an initial downtrend, but this was plausibly due to changes in concentration as the cell count was higher when incubated in a 1 mg CNT/mL culture condition compared with that in the 100 μg CNT/mL media condition. Other studies[76] reinforce the possibility that cells incubated with CNTs in culture can lead to cycles of initial senescence with subsequent proliferation after a certain critical concentration is reached. These findings give rise the possibility that cells have reaction cascades that can resist toxicity induced by CNTs, and are environmentally dependent. However, the detailed mechanisms still remain elusive. It was also recently observed that CNTs can be actively ingested and excreted from cells without any observable toxicity effects (eg, as in the *Tetrahymena thermophila* bacteria).[77]

Further insight into CNT-cell interactions may benefit through an analysis of the trends and rates of toxicity. The fourth column of Table 2 presents data on dosage concentrations

Table 2
A compilation of cellular and tissue toxicity studies of pristine or functionalized CNTs

Nanotube	Biological System	Dosage	Toxicity	Reference
Plasmid DNA-SWCNT and Plasmid DNA-MWCNT	f-CNTs: HeLa cell lines in vitro	10 mg/mL	50% survival of HeLa cells	Pantarotto et al [70]
Fluorescein isothiocyanate-SWCNT and fluorescein isothiocyanate-MWCNT	f-SWCNT and f-MWCNT: HeLa cell lines in vitro	5–10 mg/mL	50% survival of HeLa cells	Bianco et al [3]
Pristine SWCNT	SWCNT: Mesothelioma cell line MSTO-211H in vitro	7.5 μg/mL water	10% decrease in cell proliferation and activity	Wick et al [45]
Ammonium chloride-SWCNT, and poly(ethylene glycol)-SWCNT	Macrophages, B and T lymphocytes from BALB/c mice spleen and lymph nodes in vitro	10 μg/mL water	5% decrease in viability of B lymphocytes, but no adverse effects on T lymphocytes and macrophages	Dumortier et al [24]
RNA-polymer SWCNT conjugate	MCF-7 breast cancer cells in vitro	1 mg/mL	No significant cell damage	Lu et al [18]
[^{111}In] DTPA-SWCNT and [^{111}In] DTPA-MWCNT	Intravenous injection, systemic, female BALB/c mice in vivo	20 μg/μl PBS	No acute toxicity after single 200 μL dose	Singh et al [38]
Pristine MWCNT	Human T lymphocytes in vitro	40 μg/mL	Should have no toxicity on human T lymphocytes	Bottini et al [71]
Pristine SWCNT	Intravenous injection, systemic, rabbit in vivo	7.5 mL of 20 μg/kg body mass	No toxicity	Cherukuri et al [78]
^{125}I-SWCNT (OH)	Intraperitoneal, intravenous, subcutaneous, in male KM mice in vivo	1.5 μg/mouse	Accumulate in bone, but good biocompatibility	Wang et al [91]
Glucosamine-MWCNT	Intraperitoneally into female Kunming mice in vivo	300 μL single dose, suspension concentration unknown	Good biocompatibility	Guo et al [82]
pEGFP-c1 plasmid DNA-SWCNT	Mouse B-cells and cortical neurons in vitro	0.1 pM/10 mL serum-free medium	~10% of cells were no longer viable	Cai et al [63]
6-Aminohexanoic acid–derivatized SWCNT	Human epidermal keratinocytes (HEK) in vitro	Multiple tests from 0.00000005 to 0.05 mg/mL	Highest concentration that can interact with HEKs without toxicity, 0.000005 mg/mL for 24 hr	Zhang et al [17]
DNA-Cy3 (fluorescent label)-SWCNT	HeLa cell line in vitro	2.5–5 mg/L water	No toxic effects, after six pulses of 10-s, 808-nm laser radiations at 1.4 W cm^2	Kam et al [22]
Streptavidin-SWCNT	HL60 and Jurkat cells in vitro	0.025 mg/mL	No adverse effects	Kam et al [60]
SWCNTs dispersed in DMEM with 5% (vol/vol) fetal bovine serum	Human epithelial-like HeLa cells *in vitro*	100 μg/mL	No effect on growth rate	Yehia et al [92]
0.5 DMSO pristine SWCNT	Human embryo kidney (HEK 293) cells in vitro	25 μg/mL	G1 cell arrest and apoptosis	Cui et al [93]

that may be useful in further testing functionalized-CNT toxicity through examples of lethal and safe dosage.

In vivo toxicity: new insights

Currently, insights into how CNTs behave in the human body (Figure 4) are obtained through recent studies of systemic toxicity, as representatively illustrated in Table 2. To date, the number of studies suggesting CNTs to be nontoxic in vivo outnumbers those proposing otherwise. For example, doses of 20 μg diethylene triamine pentaacetic acid (DTPA)-MWCNT/μL phosphate buffer saline (PBS) and 20 μg DTPA-SWCNT/μL PBS were administered in different mice intravenously with no acute toxicity observed.[38] As yet another example, an intravenous injection of a ~20 μg SWCNT/kg body weight concentration into specimens confirmed safety of this dosage after a 24-hour period.[78]

Laboratories around the world are tackling salient issues involving CNTs such as chronic toxicity and organ localization. As new research continues to support a positive outlook, other promising routes may be found through a careful look at data. A recent update[79] expanded our understanding of chronic toxicity of CNTs by asserting negligible toxicity in a sample of mice after 4 months of treatment. New insight arises from the observation that the changes in neutrophil count for mice treated with PEGylated oxidized SWCNTs were larger than counts from those mice treated with PEGylated SWCNT, which suggests that varying functionalization can modify toxicity. A recent in vivo cancer therapy study using CNTs originally designed as drug-delivery enhancers was able to demonstrate[25] that tumor cells respond to toxicity differently than do wild-type cells. In this study, SWCNTs conjugated with paclitaxel (a common chemotherapy drug) markedly decreased breast cancer tumors in mice, far more than by using paclitaxel alone. However, the data also shows that under some conditions, a few tumor-bearing mice treated with nonfunctionalized SWCNTs had a similar rate of tumor growth as that of the untreated control. This study then suggests that cancer cells may have a resistance mechanism

against CNTs. If this was indeed correct, effective chemotherapy dosages, using CNTs, may have to be higher than what is currently known to be safe in order to be useful in drug delivery. This is of particular relevance as on average, SWCNT-paclitaxel causes more liver damage than does paclitaxel alone. Concomitantly, no psychological stress (ie, aggressive behavior, weight gain/loss) was observed in animals injected with SWCNTs, even during the process of liver damage.[25,74,79]

Prospects for CNT use in vivo have recently become concrete, but nanotubes are no exception to the pitfalls inherent in the field of nanomedicine. Although experiments carried out in small animals in vivo suggest the efficacy of CNTs for drug-delivery vehicles, it is not clear whether such processes can be scaled up to larger organisms. A case in point is a study[63] where drug-carrying CNTs injected into a tumor site are manipulated by a system of magnets to penetrate tumor cells in vivo. When similar procedures are adapted for larger systems, sites further from the external magnet are more difficult to target, as also observed in other nanoscale drug-delivery schemes.[80] Potentially, the simple increase in total body mass that is obvious between small and large animals may practically demand higher quantities of f-CNTs.

Pharmacokinetic profile

The map of CNT pharmacokinetics and biodistribution seems to be developing rapidly. The majority of intravenously injected CNTs in mice mainly seem to be emptied in the urine, with far less found in the liver, spleen, and lungs.[74] However, other studies indicate the liver and spleen to be the main sites of CNT accumulation.[79] Studies have also found CNT deposits mostly in the excretory systems like the bladder, kidneys, and intestines, in feces,[81] and again in the kidneys.[69] A contributing factor to this tendency is that SWCNTs can often be trapped in capillaries, a mechanical cause of toxicity that may explain distribution of residual CNTs.[38,78,79,81] For example, the liver might be a preferred site for CNT accumulation due to its greater vascularity.

Functional groups modify site of CNT deposits

Studies indicate that f-CNTs interact differently with cells depending on the conjugated moiety. In fact, the biodistribution of most functional compounds can be modified due to their attachment with a CNT, for example, paclitaxel conjugated with SWCNTs seems to localize more in intestines and liver, whereas when conjugated with PEG, localization occurs more frequently in the lung.[25] Similarly, higher concentrations of rituximab-conjugated CNTs were found in the liver compared with that when only rituximab was used.[23] It is to be noted that in all the biodistribution studies mentioned, the concentration of CNTs remaining in an injected host never reached 0%, even in chronic studies. The consequent implications on drug delivery where repeated exposure is necessary would then have to be thoroughly understood, especially due to the fast rate of f-CNT clearance from intravenous and intraperitoneal injections[38,78,82] along with the minimum dosage of drug-carrying f-CNTs that need to be circulated in order to yield a positive result.

Testing protocol: An unforeseen obstacle

The crux of the experimental method is the process of testing, but what implications will it have on our current understanding of CNT toxicity if some results could arise from internally invalid tests? The argument for increasing testing confidence stems from an in vitro study[83] where it was revealed that common amino acids and vitamins (eg, phenylalanine and folate) passively adsorb onto CNTs. Concentrations of SWCNTs as low as 0.01 to 0.1 mg/mL culture was able to deplete 2 nM of folic acid from solution. Future research should take caution from such findings.

Additionally, results from imaging assays and biochemical tests also require skepticism. A chronic toxicity study claimed that image interpretations of SWCNTs in the spleen can be obfuscated by other compounds endogenous to cells like the hemosiderin in splenic macrophages. Raman spectroscopy mapping can also be considered inadequate because signals below background do not necessarily imply an absence of SWCNT.[79] The accuracy of the LDH assay often used in CNT toxicity studies is also debatable. The amount of LDH release in culture is used to measure cell death[43,72,74] after exposure to CNTs, but such a test does not discriminate necrosis from apoptosis, leaving the specific mechanism and any trends in toxicity difficult to establish. A number of biochemical markers for toxicity after long-term intravenous exposure to CNTs in mice have been measured, and while tests for liver damage [alanine transaminase (ALT) and aspartate transaminase (AST) indices] showed dose-dependent toxicity, the LDH-based test had no such dependency.[74]

Future testing would then benefit by using mutually exclusive assays in order to reconcile variations in toxicity. A major complication is the chance that in vivo toxicity may not always be realized as just a pronounced inflammatory response.[41] Other reactions like white blood cell buildup and fibrinogenesis may also occur, and tests must be configured to consider these changes.

Conclusions

CNTs have been proposed for use in medicine chiefly for (1) their ability to be functionalized, both covalently and noncovalently, with various moieties, and (2) their aspect ratio and geometry, which enables penetration into the cell. In our survey of existing work and methods, we have found that considerably more fundamental and applied research must be carried out before the viability of CNTs can be realized. One of the major issues affecting nanotube biomedical application is that of charge control for enabling predictable interactions with the environment. In addition to variable geometries,[54] the inevitable presence of impurities [both intrinsic (vacancies, charged defects, etc.) and extrinsic (catalyst residue, etc.)] has to be controlled and understood—only then can one artificially introduce defects into the nanotubes so as to achieve predictability.[28] CNT length has also been implicated in toxicity studies many times over, implying a need for reliable techniques to produce CNTs with consistent properties. Additionally, the

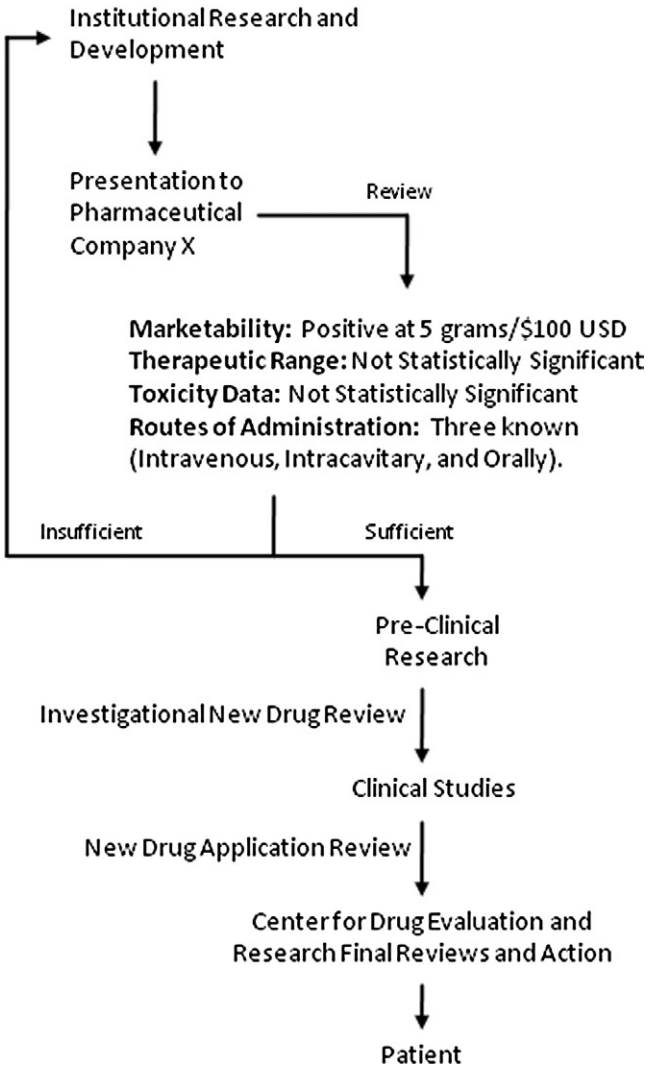

Figure 5. The long and winding path for CNT applications to drug delivery. The fundamental issues that must be resolved should include the unique aspects of nanotubes for drug delivery and a cataloging of their physiologic interactions (toxicity, administration, etc.) to enable pharmaceutical selection.

lack of a comprehensive catalog of each different f-CNT and its toxicity profile (ie, cellular interaction, pharmacokinetics, etc.) will translate to stalled deployment to the public and missed opportunities for patient care.

From an applied perspective, an important question that should be probed is why CNTs would be efficacious at all. For example, rituximab-antibody conjugates have been used in conjunction with other cancer drugs to target and effectively destroy non–Hodgkin's lymphoma with great success, and such a method may not need replacing. What then would be the niche areas for CNTs? What are the relative advantages of CNTs for drug delivery compared with, for example, those of nanospheres from biodegradable polymers[84,85] or even nanotubes made of other materials, like silica, peptides, or lipids?[86-89]

The primary purpose of CNT biomedical research should then be to first determine the tasks for which CNTs are particularly valuable. A strong case must be made for CNTs in medicine to enable pharmaceutical selection (Figure 5). To date, CNT drug-delivery research may fall short of the election criteria for funded research and development by pharmaceutical companies. Alternative paths toward development can bypass this issue but will nevertheless face scrutiny by not just the Center for Drug Evaluation and Research (CDER), but also tracking agencies such as the Environmental Protection Agency (EPA), International Center for Technology Assessment (ICTA), and the Project on Emerging Nanotechnologies (PEN).

However, we think the safety concerns regarding CNTs can be ameliorated. In this context, it is important to put the known hazards of CNTs into perspective. The average person consumes an estimated 5000 to 3,000,000 particles/cm^3 daily due to incidental nanoparticles from the ambient environment.[90] The safe systemic dose of CNTs, if it can be made to conform to such numbers, would then make current toxicity reports on biological risk seem overestimated. Only through a relative comparison can one understand the dangers of functionalized CNT administration against other treatment options. If the queries raised to date can be satisfactorily answered in its favor, then the use of CNTs in biomedicine may indeed be feasible.

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