ACCOUNTS
of chemical research

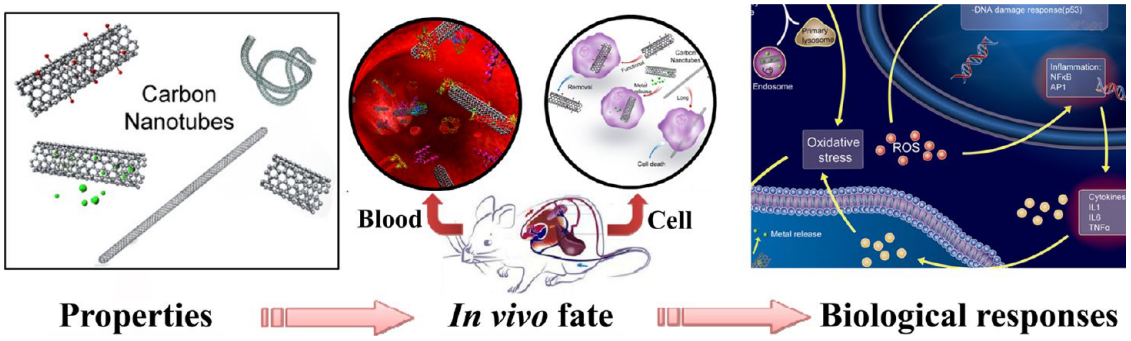
Understanding the Toxicity of Carbon Nanotubes

YING LIU,[†] YULIANG ZHAO,[†,‡] BAOYUN SUN,[*,‡] AND CHUNYING CHEN[*,†]

[†]*CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology of China, Beijing 100190, China, and* [‡]*CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, Institute of High Energy Physics, Chinese Academy of Sciences (CAS), Beijing 100049, China*

RECEIVED ON JANUARY 28, 2012

CONSPECTUS



Because of their unique physical, chemical, electrical, and mechanical properties, carbon nanotubes (CNTs) have attracted a great deal of research interest and have many potential applications. As large-scale production and application of CNTs increases, the general population is more likely to be exposed to CNTs either directly or indirectly, which has prompted considerable attention about human health and safety issues related to CNTs. Although considerable experimental data related to CNT toxicity at the molecular, cellular, and whole animal levels have been published, the results are often conflicting. Therefore, a systematic understanding of CNT toxicity is needed but has not yet been developed.

In this Account, we highlight recent investigations into the basis of CNT toxicity carried out by our team and by other laboratories. We focus on several important factors that explain the disparities in the experimental results of nanotoxicity, such as impurities, amorphous carbon, surface charge, shape, length, agglomeration, and layer numbers. The exposure routes, including inhalation, intravenous injection, or dermal or oral exposure, can also influence the *in vivo* behavior and fate of CNTs. The underlying mechanisms of CNT toxicity include oxidative stress, inflammatory responses, malignant transformation, DNA damage and mutation (errors in chromosome number as well as disruption of the mitotic spindle), the formation of granulomas, and interstitial fibrosis. These findings provide useful insights for de novo design and safe application of carbon nanotubes and their risk assessment to human health.

To obtain reproducible and accurate results, researchers must establish standards and reliable detection methods, use standard CNT samples as a reference control, and study the impact of various factors systematically. In addition, researchers need to examine multiple types of CNTs, different cell lines and animal species, multidimensional evaluation methods, and exposure conditions. To make results comparable among different institutions and countries, researchers need to standardize choices in toxicity testing such as that of cell line, animal species, and exposure conditions. The knowledge presented here should lead to a better understanding of the key factors that can influence CNT toxicity so that their unwanted toxicity might be avoided.

Introduction

Carbon is one of the elements that was discovered early and is readily available. Its allotropes include diamond, graphite, amorphous carbon, carbon nanotubes (CNTs),[1−6] graphene,[7,8] and fullerenes.[9,10] Since their accidental discovery and fabrication in 1991, CNTs have received considerable attention from scientific, industrial, and public communities because of their unique physical, chemical, electrical, and mechanical

Published on the Web 09/21/2012 www.pubs.acs.org/accounts
10.1021/ar300028m

TABLE 1. Basis of Carbon Nanotube Toxicity

	cell types/animals	type of CNTs	CNT toxicity	ref
metal impurities	H460	SWCNTs containing 19.4%Ni/5.49%Y; 14.3%Ni/2.09%Y; 3.15%Ni/9.21%Co; 22.8%Ni/4.79%Y; 24.1%Ni/4.17%Y ; 3.3%Co/1.27%Mo	nickel is bioavailable at toxicologically significant concentrations	16
	NR8383; A549	purified SWCNTs; SWCNTs containing 0.009%Fe/2.8%Co/4.2%Mo; purified MWCNTs; MWCNTs containing Ni	dose- and time-dependent increase of intracellular ROS; decrease of mitochondrial membrane potential	17
	RAW264.7	SWCNTs containing 26% Fe or 0.23% Fe	hydroxyl radical generation: loss of intracellular low molecular weight thiols; accumulation of lipid hydroperoxides	18
	HaCaT	SWCNTs containing 30% Fe	formation of free radicals; accumulation of peroxidative product; antioxidant depletion; loss of cell viability	44
surface charge and modification	HMMs	acid-treated, water-soluble SWCNTs	acid-treated SWCNTs are less aggregated within lysosomes and cytoplasm and cause no significant changes in cell viability or structure	25
	HUVEC	pristine SWCNTs; oxidized SWCNTs	functionalized and pristine SWCNTs have limited cytotoxicity	11
shape	normal mice	asbestos-like MWCNTs	length-dependent inflammation and formation of granulomas	27
	human primary macrophages	tangled CNTs; needle-like CNTs	tangled CNTs are swallowed into cells; long, needle-like CNTs activate secretion of IL-1α and IL-1β	28
length	normal mice	MWCNTs 15−20 μm or longer	long MWCNTs cause inflammation and granulomas	27
	human primary macrophages	short CNTs; long, tangled CNTs; long, needle-like CNTs	long, needle-like CNTs activate secretion of IL-1α and IL-1β	28
	THP-1; rat	MWCNTs 500 nm to 5 μm	MWCNT with an average length of 825 nm induce higher inflammation than those with an average length of 220 nm	29
	A549; THP-1; normal mice	MWCNTs: length 5−15 μm, diameter 20−60 nm; length 1−2 μm, diameter 60−100 nm; length 1−2 μm, diameter <10 nm SWCNTs: length 5−15 μm, diameter <2 nm	long and thick MWCNTs induce the strongest DNA damage and increase the total cell number in abdominal lavage fluid while similar SWCNTs caused little effect	30
	p53+/− mice	long MWCNTs 1−20 μm	Long MWNTs (short not included) can form fibrous or rod-shaped particles of length around 10-20 micrometer (μm) and induce mesothelioma	46
agglomeration	SPC; DRG	agglomerated SWCNTs; better dispersed SWCNT bundles	highly agglomerated SWCNTs significantly decrease the overall DNA content	31
	MSTO-211H	CNT agglomerates; CNT bundles	suspended CNT-bundles are less cytotoxic than asbestos, rope-like agglomerates	32
layer number	RAW 264.7	pristine graphene	depletion of the mitochondrial membrane potential and increase of intracellular ROS and apoptosis	7
	alveolar macrophage	SWCNTs; MWCNTs (diameters 10−20 nm)	SWCNTs > MWCNTs	33

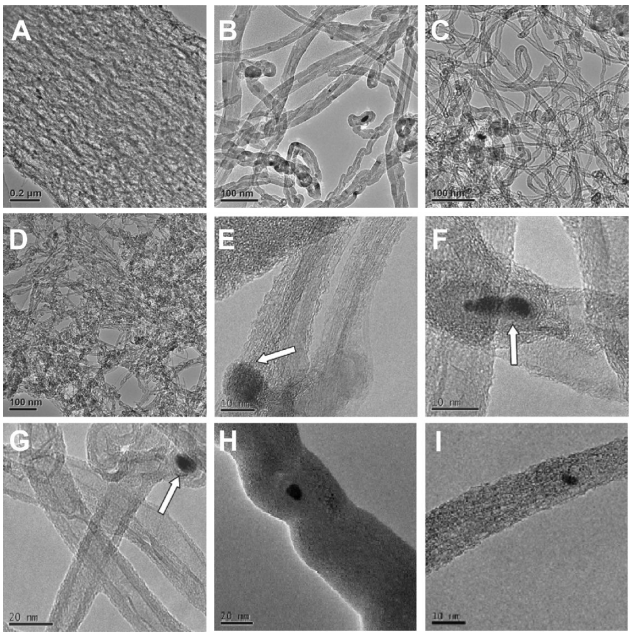


FIGURE 1. TEM images of (A) aligned MWCNTs,[5] (B) long carboxyl MWCNTs,[5] (C) short MWCNTs,[5] (D) short carboxyl MWCNTs,[5] (E) high Fe and Mo carboxyl MWCNTs,[6] (F) high Ni MWCNTs,[6] (G) high Fe and Mo SWCNTs,[6] (H) high Fe and Mo MWCNTs,[4] (I) high Fe, Yb, Ni, and Ce SWCNTs.[4] Reproduced from ref 4. Copyright 2008 American Chemical Society. Reproduced from refs 5 and 6. Copyright 2011 American Scientific Publishers.

properties. CNTs are classified in two main categories, single-wall carbon nanotubes (SWCNTs) and multiwall carbon nanotubes (MWCNTs), according to the number of layers. They all have an enormous potential for applications in nanotechnology, optics, electronics, materials science, biology, and medicine. CNTs have been widely used as biosensors, in antigen recognition, in enzyme reactions, and in DNA−DNA hybridization.[1] CNTs can induce proliferation and differentiation of neurons and osteoblasts and serve as drug and vaccine delivery vehicles for cancer treatment.[1]

With the development of potential applications and maturation of preparation technology, the production of CNTs has been increasing year after year. People have increasing opportunities to contact CNTs directly or indirectly. The extremely small size, fiber-like shape, large surface area, and unique surface modification of CNTs determine their distinctive chemical and physical characteristics and raise their potential hazards to humans. Therefore, the health and safety issues of CNTs have also attracted widespread attention.[11,12] However, there are a number of conflicting reports: some investigations have reported toxic effects following the exposure of several cell types to both SWCNTs and MWCNTs, while others demonstrate very low or insignificant cellular responses. This inconsistency could be explained by many external and intrinsic factors such as surface charge and modification, shape, length, agglomeration, or layer numbers, which can influence CNT toxicity.[13,14]

Among all the potentially influential factors, what is the crucial basis of CNT toxicity? At present there is no consensus. In order to better understand how to design safe CNTs for biomedical applications, this Account highlights the existing knowledge regarding the origin of CNT toxicity. More information and details are summarized in Table 1. We focus on a number of factors such as impurities, surface modification, structure, and exposure routes that may influence the toxicity of CNTs.

CNT Toxicity Derived from Impurities

More and more researchers have demonstrated that one of the most important factors leading to CNT toxicity is impurities, especially catalyst metal contaminants, such as the transitional metals Fe, Y, Ni, Mo, and Co, introduced during preparation and purification procedures. Other impurities, such as amorphous carbon and other carbon nanomaterials, might also contribute to the toxicity of CNTs. However, current results are inconsistent, and further research is needed for more accurate conclusions.

The presence of metal impurities could lead to conflicting data about the biocompatibility, toxicity, and risk assessment of CNTs and may limit their further industrial applications. Contamination of CNTs by catalyst residues is unavoidable during large-scale production by chemical vapor deposition techniques.[15] Moreover, it is impossible to entirely remove the metal impurities without destroying the structural integrity of CNTs because some metal particles are protected by graphitic shells.[4,15] However, in cell culture and human or animal bodies, the metal impurities may be released from the CNT structure to enhance their toxicity.[16]

Commercial SWCNTs and MWCNTs (with high contents of Fe, Co, Mo, and Ni), as well as acid-treated SWCNTs with reduced metal impurities, were able to cross the cell membrane.[17] Commercial CNTs had a dose- and time-dependent increase in intracellular reactive oxygen species (ROS) and a decrease in mitochondrial membrane potential, whereas purified CNTs had no effect. Nickel oxide in SWCNTs could affect the redox properties of the regulatory peptide L-glutathione, which is a powerful antioxidant protecting cells from oxidative stress.[11] Kagan et al. showed that neither purified nor nonpurified SWCNTs containing significant amounts of iron were able to generate intracellular production of superoxide radicals or nitric oxide in RAW 264.7 macrophages. Iron-rich SWCNTs were more effective than purified SWCNTs.[18] Metal in CNTs that acts as a catalyst

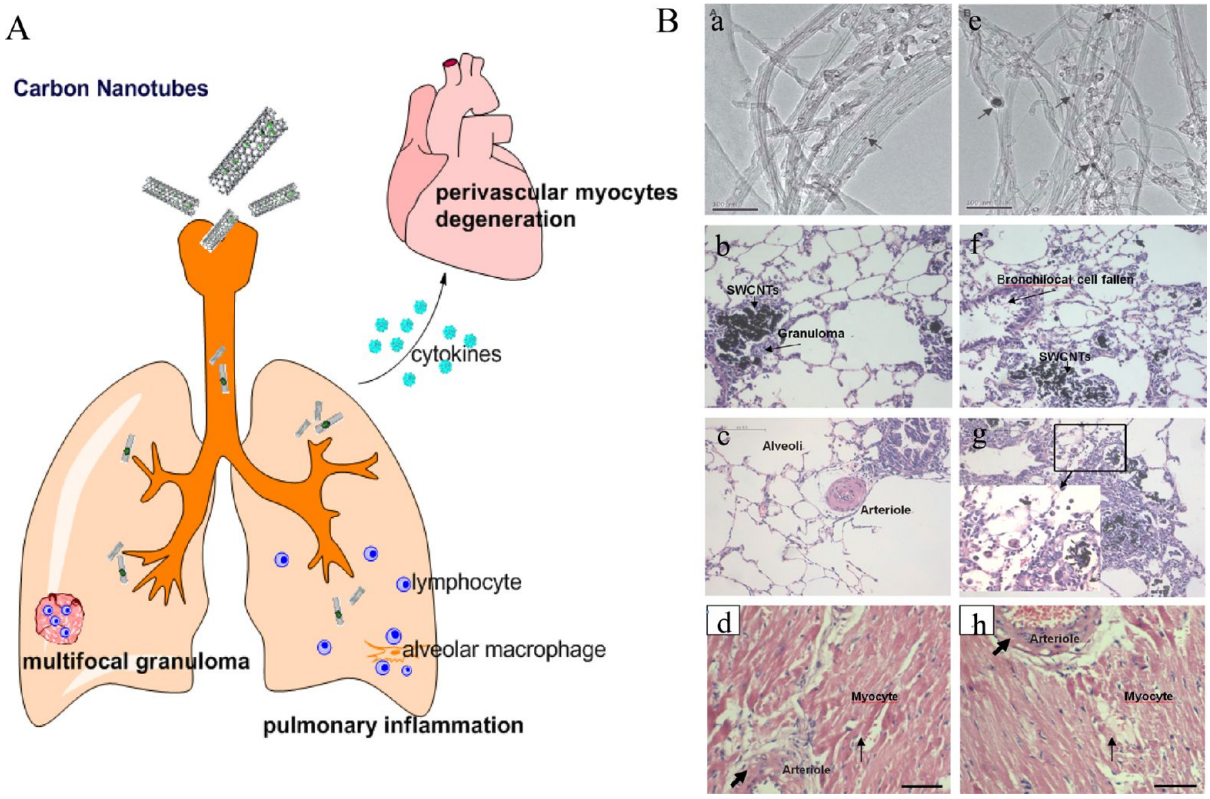


FIGURE 2. Respiratory exposure to SWCNTs could induce acute pulmonary and cardiovascular responses. (A) The schematic illustration of SWCNT exposure; (B) light micrographs of lung (b, c, f, g) and heart (d, h) tissue from male spontaneously hypertensive (SH) rats exposed to SWCNTs. TEM images of Fe-poor SWCNTs (a) and Fe-rich SWCNTs (e); (b, c, d) Fe-poor SWCNT groups after 24 and 72 h; (f, g, h) Fe-rich SWCNT groups after 24 and 72 h. Reproduced with permission from ref 3. Copyright 2012 Informa Plc.

of oxidative stress may be important in determining redox-dependent responses.

Our previous study indicated that respiratory exposure to SWCNTs containing different metal contents (Figure 1) could induce acute pulmonary and cardiovascular responses. The coexistence of metal residues in SWCNTs aggravated the adverse effects (Figure 2). Biomarkers of inflammation, oxidative stress, and cell damage in the bronchoalveolar lavage fluid (BALF) were increased significantly 24 h post-exposure to SWCNTs. The increased endothelin-1 levels in the BALF and plasma and angiotensin I-converting enzyme in plasma suggested endothelial dysfunction in the pulmonary circulation and peripheral vascular thrombosis.[3,6] Lam et al. also demonstrated that CNTs containing different types and amounts of metals induced dose-dependent lesions (such as epithelioid granulomas, interstitial inflammation, peribronchial inflammation, and necrosis) and a higher mortality rate.[19]

At present, metallic impurities in CNTs can be detected quantitatively using several analytical methods, such as neutron activation analysis and inductively coupled plasma mass spectrometry (Figure 1).[4,20] The constituent species and amounts of metal impurities in CNTs are quite different in materials obtained from different vendors, which makes it more complex to evaluate the toxicity of CNTs as influenced by impurities.

CNT Toxicity Derived from Their Chemical and Structural Characteristics

Surface Charge and Modification. For medical applications, it is a primary concern to remove impurities as cleanly as possible and disperse the CNTs well in water. In order to improve hydrophilicity, functional sidewall groups (such as hydroxyl and carboxyl) have inevitably been introduced onto the surface of the CNTs (Figure 1).[4−6] These groups can be also efficiently used to attach specific biomolecules to CNTs for delivery or therapeutic purposes via covalent binding.

Specific surface area and functional groups are highly related to pharmacokinetics, fate, and toxicity of CNTs. Hydroxylated SWCNTs (^{125}I-SWCNTols) are quickly distributed throughout the entire body and accumulate in the bone for a long time. Taurine covalently functionalized MWCNTs (^{14}C-tau-MWCNTs) predominantly accumulated in the liver

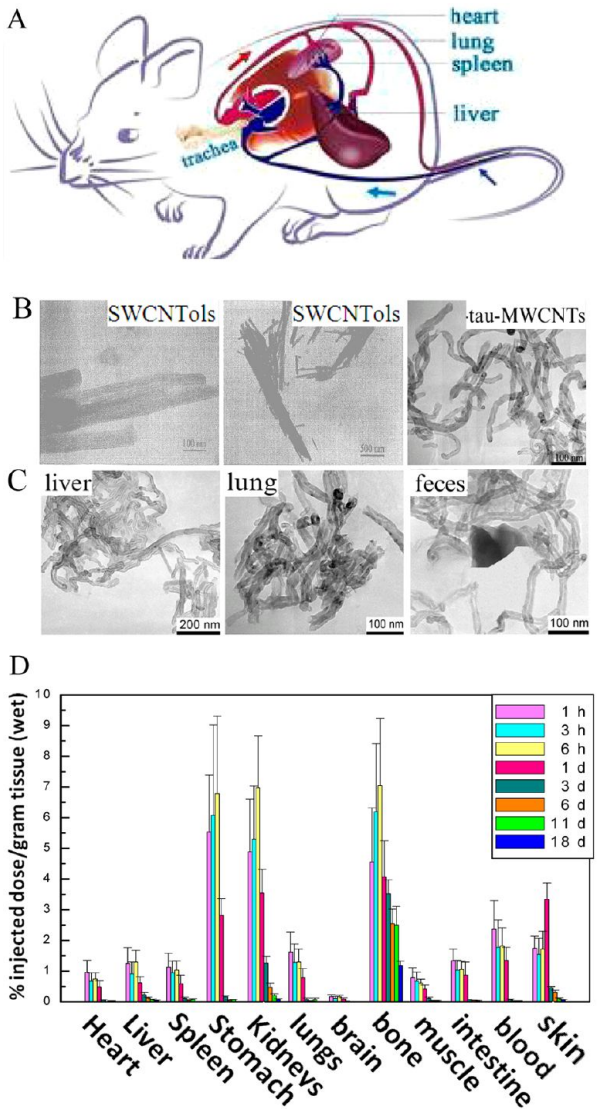


FIGURE 3. Distinct biodistribution of CNTs *in vivo* resulting from different surface modifications. (A) CNTs could arrive at almost all organs, including heart, lungs, liver, kidneys, spleen, stomach, and intestine; (B) TEM images of SWCNTols[21] and tau-MWCNTs[22]; (C) TEM images of MWCNTs in digested solutions of liver, lung, and feces after tail intravenous injection, intratracheal injection and gavage, respectively;[22] (D) ^{125}I-SWCNTols were distributed throughout the entire body quickly after intraperitoneal injection (ip), except brain, accumulated in the bone for a long time.[21] Reproduced from refs 21 and 22. Copyright 2004 American Scientific Publishers and Copyright 2007 by Elsevier B.V., respectively.

over a period of 3 months with low liver toxicity (Figure 3).[21,22] ^{111}In-labeled diethylentriaminepentaacetic (DTPA) functionalized SWCNTs were not retained in any of the reticuloendothelial system organs (liver or spleen) and rapidly cleared from systemic blood circulation through the renal excretion route within 3 h.[23] Surface modification was able to change the interaction process of CNTs with the cellular lipid bilayer, which further changed the cell uptake and viability.[24] However, contrasting results were obtained that unpurified and acid-treated SWCNTs were frequently found inside lysosomes and cytoplasm of human monocyte-derived macrophages (HMMs) with no significant changes in cell viability or structure.[25] All pristine and functionalized SWCNTs had limited toxicity to endothelial cells *in vitro* as measured by growth, migration morphogenesis, and survival assays.[11]

Shape. CNTs are single-cylinder or multicylindrical structures that have a thin and elongated shape analogous to a fiber, according to the WHO fiber definition of a particle with an aspect ratio (length/diameter) greater than three.[26] So, CNTs might fit the "fiber toxicological paradigm"; namely, their toxic properties may be analogous to those observed with other fibrous particles such as asbestos. Poland et al. warned that CNTs introduced into the abdominal cavity had asbestos-like pathogenicity because of their needle-like fiber shape, including inflammation and the formation of granulomas.[27] Later, long and needle-like CNTs were reported to activate secretion of IL-1α and IL-1β from LPS-primed macrophages through activation of NLRP3, which depended on ROS production, cathepsin B activity, P2X(7) receptor, and Src and Syk tyrosine kinases.[28] It is of considerable importance to investigate the influence of the shape of CNTs further and to use great caution before introducing needle-like fiber-shaped products into the market if long-term harm is to be avoided.

Length. CNTs of different lengths caused various degrees of toxicity.[29,30] The degree of inflammation induced by 825 nm-long CNTs was stronger than that induced by 220 nm-long CNTs *in vivo* because macrophages could envelop 220 nm-long CNTs more readily.[29] Yamashita et al. concluded that long MWCNTs induced the strongest DNA damage and increased the total cell number in abdominal lavage fluid, while similar SWCNTs or short MWCNTs caused less effect.[30] MWCNTs of different lengths caused different degrees of granuloma formation, and injection of MWCNTs longer than 20 μm was significantly more serious than low-aspect-ratio, tangled nanotube aggregates because macrophages cannot completely engulf longer fibers (Figure 4).[27,29]

Agglomeration. The aggregation state can affect the shape and surface area of CNTs and is one of the essential factors in determining the toxic potential of CNTs. The effect of highly agglomerated SWCNTs was more pronounced in decreasing overall DNA content than better-dispersed SWCNT bundles.[31] Wick et al. also reported that suspended CNT bundles were less toxic than asbestos, while rope-like agglomerates of CNTs induced more pronounced

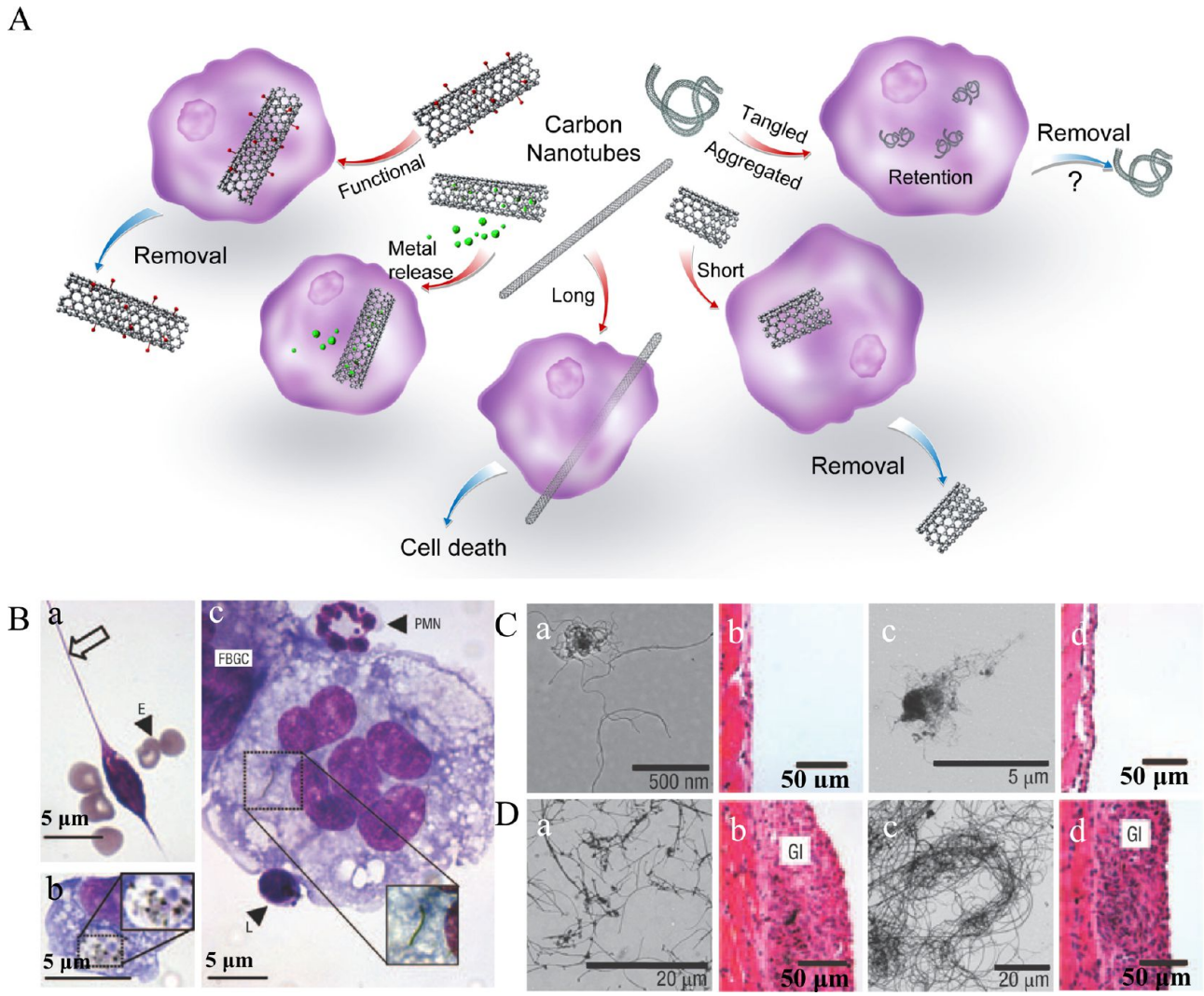


FIGURE 4. Physiochemical properties of CNTs can influence cell uptake and responses. (A) The varying types of CNTs can affect phagocytosis and cytotoxicity; (B) (a,b) long MWCNTs can lead to frustrated phagocytosis, while short MWCNTs are readily phagocytosed. (c) foreign body giant cells (FBGCs) are present after injection of long MWCNTs (PMN, polymorphonuclear leukocyte; L, lymphocyte); (D) diaphragms show the presence of granulomatous inflammation (GI) in mice exposed to long MWCNTs, while (C) a small granuloma response is seen in mice treated with tangled MWCNTs. Reproduced from ref 27. Copyright 2008 Nature Publishing Group.

cytotoxicity than asbestos fiber at the same concentrations. The level of toxicity was at least partially dependent on the agglomeration state of the tubes.[32] Agglomerated CNTs showed higher stiffness and rigidity, while the well-dispersed SWCNT bundles were softer and more flexible.

Layer Number. CNTs are manufactured to be either single- or multiwalled. Some reports show that more profound toxicity stems from SWCNTs than MWCNTs. SWCNTs significantly impaired phagocytosis of alveolar macrophages at a low dose of 0.38 mg/cm^2, whereas MWCNTs did so only at the higher dose of 3.06 mg/cm^2. The macrophages exposed to SWCNTs or MWCNTs showed characteristic features of necrosis and degeneration.[33] Our group found that pristine graphene sheets induced cytotoxicity through the depletion of the mitochondrial membrane potential and increase of intracellular ROS and then triggered apoptosis by activation of the mitogen activated protein kinase (MAPK) and TGF-β pathways (Figure 5).[7] Pristine graphene can also stimulate naïve macrophages to produce cytokines/chemokines via TLR- and NF-κB-related signaling pathways and further alter their adhesion and phagocytosis.[8]

Other factors, such as diameter and rigidity, could influence the potential toxicity.[34] Thin MWCNTs (diameter 50 nm) with high crystallinity showed cytotoxicity *in vitro* and subsequent inflammogenicity and mesotheliomagenicity *in vivo*, while thick (diameter 150 nm) or tangled (diameter 2−20 nm) MWCNTs were less toxic, inflammogenic, and carcinogenic.

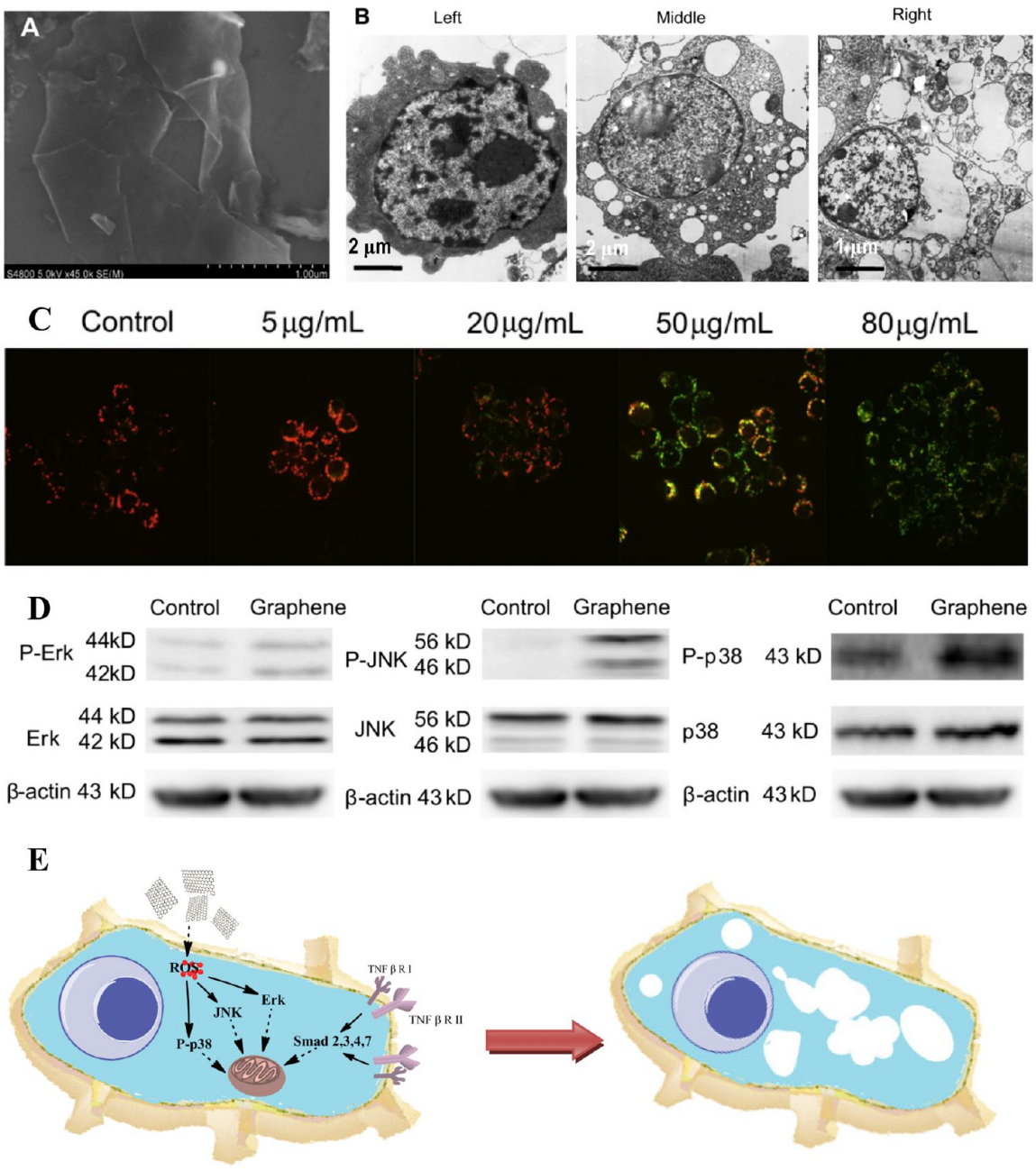


FIGURE 5. Pristine graphene caused cell apoptosis through ROS-activated MAPKs and TGF-β pathways. (A) The SEM image of pristine graphene. (B) Typical apoptotic cells with large phagocytic vesicles could be observed in pristine graphene-treated cells at higher concentrations: (left) control; (middle and right) graphene exposure. (C) Pristine graphene exhibited dose-dependent mitochondrial membrane depolarization. The mitochondrion-specific dye JC-1 was used to detect changes in mitochondrial membrane potential. Apoptotic cells mainly show green fluorescence, while healthy cells show red (yellow when merged). (D) Pristine graphene significantly increased phosphor-JNK (P-JNK), phosphor-Erk (P-Erk), and phosphor-p38 (P-p38). (E) The mechanisms of pristine graphene to induce cell damage. TNFβ R represents TNF-β receptor. Reproduced from ref 7. Copyright 2012 Elsevier B.V.

CNT Toxicity Derived from Surrounding Microenvironment and Analytical Methods

In addition to intrinsic physiochemical properties of CNTs, many external factors like cell types, experimental conditions, and assays can influence their apparent toxicity. First, it is known that CNT toxicity varies among different cell lines, which show cell-specific responses to the same CNTs. Among A549, the human bronchial epithelial cell line BEAS-2B, and HaCaT, SWCNTs inhibited cell proliferation effectively in all three cell lines, whereas only HaCaT and BEAS-2B cells showed decreased cell viability.[35] Generally, CNTs affect cell proliferation and adhesive ability in a

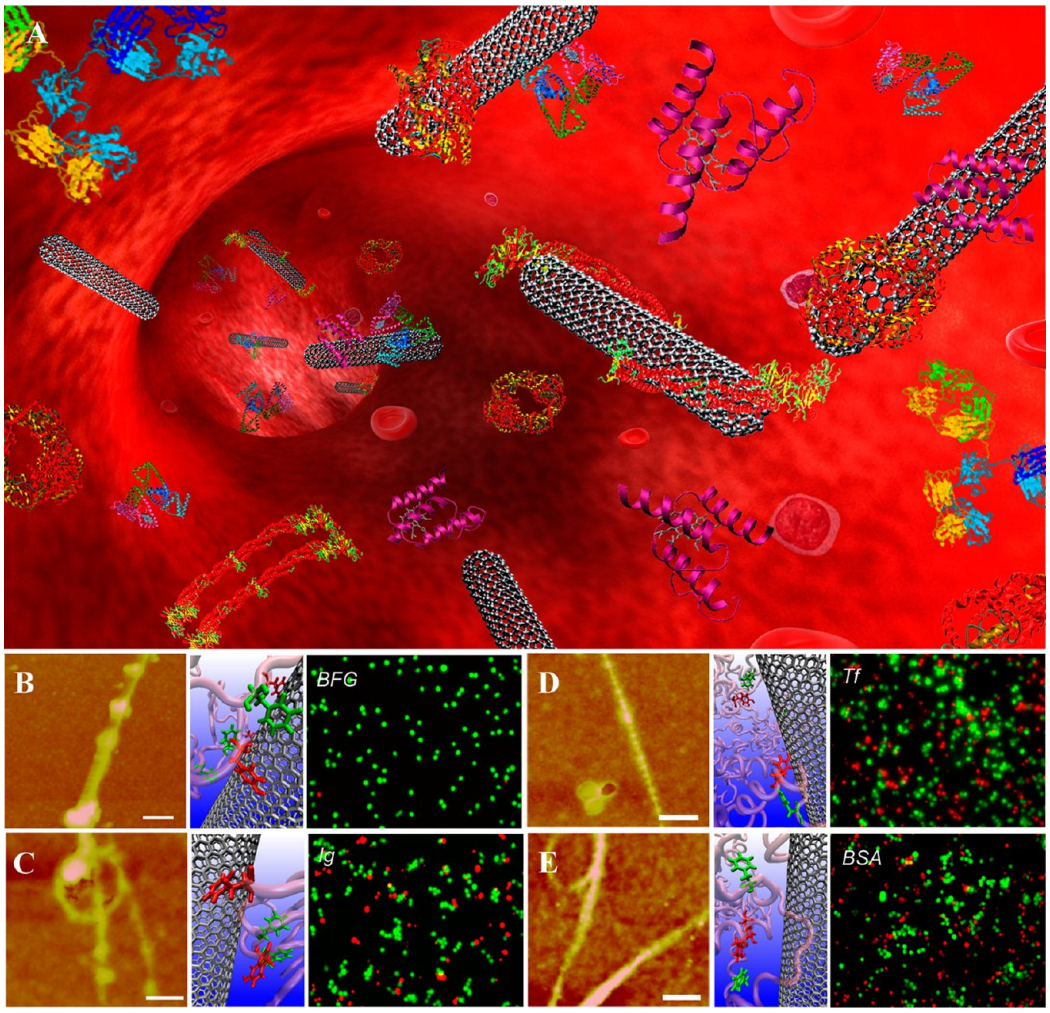


FIGURE 6. The competitive binding of blood proteins on the SWCNT surface could greatly alter their cellular interaction pathways and reduce the cytotoxicity of SWCNTs. (A) The schematic diagram of interactions between SWCNTs and human blood cells and proteins. (B–E, left) AFM images of proteins after incubation with SWCNTs for 5 h, including BFG (B), Ig (C), Tf (D), BSA (E). The interactions were estimated using both experimental (B–E, right) and theoretical (B–E middle) approaches. Abbreviations: BFG, bovine fibrinogen; Ig, gamma globulin; Tf, transferrin; BSA, bovine serum albumin. Reproduced from ref 2. Copyright 2011 National Academy of Sciences.

dose- and time-dependent manner. However, Tutak et al. observed that most primary calvariae osteoblastic cells died even when incubated with SWCNTs for a short time (at 24 h). But the proliferation ability of cells showed gradual recovery after 3 weeks.[36]

The surrounding environment is another important factor for evaluating CNT toxicity, for example, culture media and body fluids. Because of their huge specific surface area, CNTs easily adsorb proteins and small molecules onto their surface, which not only changes the surface features but also affects their toxicity. Meanwhile, cells lose some activity when the medium's nutritive value has been damaged. Casey et al. dispersed SWCNTs in commercial medium and subsequently removed them by centrifugation and filtration to obtain depleted medium, which was toxic to A549 cells. The removal of the nanotubes altered the composition of the medium to different degrees.[37] Our recent results demonstrated the interaction processes between SWCNTs and human blood proteins, fibrinogen, immunoglobulin, albumin, transferrin, and ferritin, using both experimental and theoretical approaches.[2,38] We found that proteins bound to the SWCNT surface with different adsorption capacities and packing modes. Additional cellular toxicity assays with a human acute monocytic leukemia cell line and human umbilical vein endothelial cells revealed that the competitive binding of blood proteins on the SWCNT surface could greatly alter their cellular interaction pathways and result in much reduced cytotoxicity (Figure 6). These findings are crucial to the design of safe CNTs, allowing comprehensive preconsideration of their interactions with human serum proteins.[2]

Another important factor is the accuracy and reliability of analytical methods. Cell viability is commonly assessed by (1) trypan blue staining, (2) MTT, WST, and CCK-8, (3) lactate dehydrogenase, (4) cell counting as detected by flow cytometry, or (5) Bradford measurements of protein concentrations. However, CNTs were found to interfere with the dyes causing variable results.[39] Thus, these methods might be not suitable for assessing CNT toxicity. Other evaluation methods without dyes have been used, such as a clone-forming method.[35,37] Therefore, more than one assay should be required to analyze CNT toxicity for hazard identification. It is important to establish a more standard and effective system for the accurate evaluation of CNT toxicity.

Mechanisms of CNT-Induced Toxicity

After CNTs enter the body via inhalation or dermal or oral routes, the underlying mechanisms of CNT toxicity are manifested as oxidative stress, inflammatory responses, malignant transformation, DNA damage and mutation, formation of granuloma, and interstitial fibrosis.

CNT-induced oxidative stress has been regarded as the most acceptable mechanism. Increased intracellular ROS can react with cellular macromolecules including DNA, proteins, and lipids and disturb the homeostasis of the intracellular milieu. Numerous studies have documented that transition metals released from CNTs have the potential to cause the conversion of cellular oxygen metabolic products such as H_2O_2 and superoxide anions to hydroxyl radicals. However, highly purified MWCNTs also caused cells to generate ROS, which was promoted by the high surface area.[40] CNTs could activate the specific molecular signaling associated with oxidative stress, including activator protein-1 (AP-1) and nuclear factor κB (NF-κB) and MAPK, which leads to the release of proinflammatory cytokines together with the depletion of antioxidant defenses, poly(ADP-ribose) polymerase 1 (PARP-1), protein p38, and protein serine-threonine kinase (Akt).[41] However, CNTs could be very efficient as free-radical scavengers. Watts et al.[42] first reported that MWCNTs and boron-doped CNTs can act as antioxidants, and the acceptor-like electronic properties of CNTs were mainly responsible for the radical termination. Incorporation of boron could further enhance the radical-accepting capacity of the CNTs. Fenoglio et al.[43] also demonstrated that MWCNTs exhibited a remarkable scavenging capacity against an external source of hydroxyl or superoxide radicals. However, this research is still in its initial stage and much more work should be done before practical applications.

When MWCNTs were injected intraperitoneally into mice, inflammation was observed.[27] *In vitro*, MWCNTs were also able to activate the NF-κB signaling pathway to increase the secretion of cytokines and chemokines (TNF-α, IL-1β, IL-6, IL-10, and MCP1) that promote inflammation.[41] *In vivo*, neutrophils accumulated and pro-inflammatory cytokines (TNF-α, IL-1β) were secreted, followed by the appearance of lymphocyte and macrophage and elevation of fibrogenic transforming growth factor TGF-β.[44] Moreover, CNTs developed a robust pulmonary inflammatory response culminating in the development of multifocal granulomatous pneumonia and interstitial fibrosis.[44] Exposure of lung fibroblasts to SWCNTs resulted in an increase in cell proliferation and collagen production without producing cell damage. One of the major matrix metalloproteinases, MMP-9, was involved in the fibrogenic process both *in vitro* and *in vivo*.[45] The mechanisms by which CNTs induce cell damage and inflammation are depicted as shown in Figure 7.

Carcinogenicity of CNTs is also a major concern but has not been well addressed. Chronic exposure to SWCNTs caused malignant transformation of human lung epithelial cells and induced tumorigenesis in mice.[45] Furthermore, recent studies supported the observation that mesothelial cells were sensitive to CNTs[41] and mesothelioma was induced 6 months after intraperitoneal injection in MWCNTs-exposed mice.[46] These findings are striking, and more research should focus on the issues of whether acute inflammatory responses would persist and progress to mesothelioma after human exposure to CNTs and whether inhaled CNTs would be able to migrate to other organs and affect the mesothelium.

Genotoxicity is the deadliest cause of cytotoxicity. CNTs not only entered the cytoplasm but also localized within the cell nucleus[25,42] and caused cell mortality by activating the tumor suppressor protein p53, enhancing the expression of 8-oxoguanine-DNA glycosylase 1 (OGG1) and double-strand break repair protein Rad 51, the phosphorylation of H2AX histone at serine 139, and the SUMO modification of XRCC4.[47] High-purity SWCNT induced DNA strand breakage and some but not significant micronucleus induction. However, the underlying mechanism has not been studied and remains unknown. If small enough, CNTs may pass through cellular membrane and nuclear membrane and interact directly with DNA. CNTs may still come into contact with DNA when nuclear membrane breaks down during mitosis if they accumulate in cells but do not necessarily gain access to the nucleus. On the other hand, they may cause DNA damage indirectly by promoting oxidative stress and inflammatory responses.

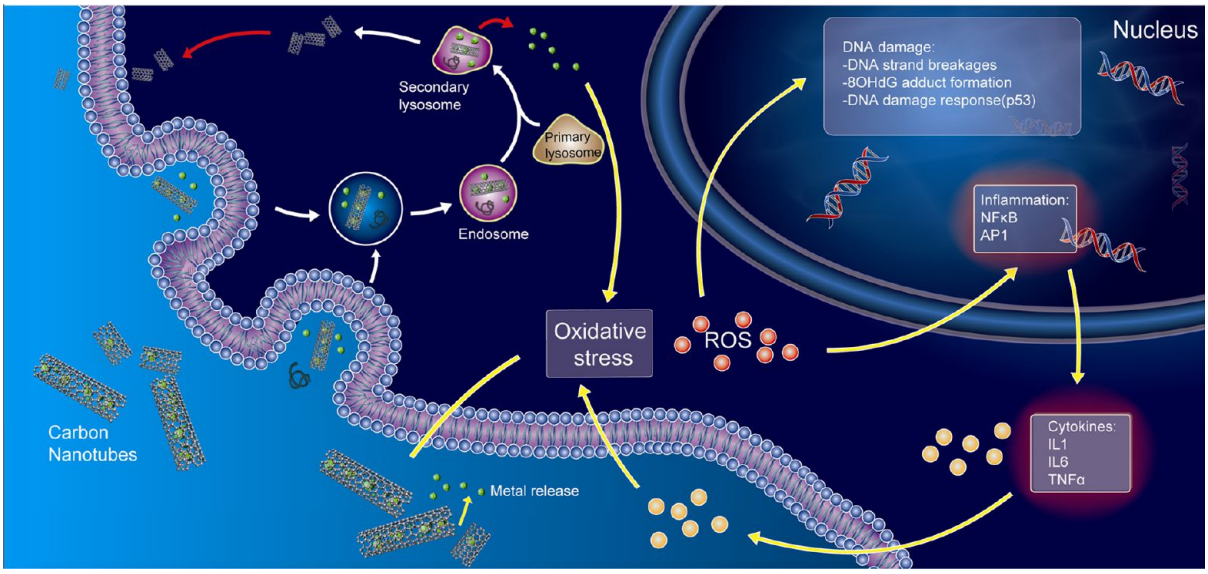


FIGURE 7. The mechanisms by which CNTs induce cell damage and inflammation.

Conclusions and Perspectives

With the rapid development of nanotechnology, humans will be exposed to nanoparticles through inhalation, ingestion, skin uptake, and vein injection. Although CNTs are highly promising nanomaterials, their health impact on humans is poorly understood. Moreover, conflicting results have been noticed because of the use of varying types of CNTs, different evaluation methods, and even different exposure conditions.

Though the diversity of CNT materials themselves makes it difficult to evaluate their toxicity, in this Account we preliminarily clarified the experimental contradictions of CNT toxicity coming from different factors. We showed how the surface charge, shape, length, diameter, agglomeration, and purity, which are hard to keep consistent due to different preparation and purification procedures of CNTs, influence their toxicity. To make experimental results comparable, we first need to establish a recognized standard CNT samples in toxicity testing, because it seems that impurities and not CNTs themselves are responsible for the toxicity. Second, the traditional methods of cell biology are not always suitable for detecting CNT toxicity. For example, CNTs can interfere with the dyes to cause many variables in results.[39] Thus, to establish standardized and reliable methods for evaluating CNT toxicity is the key foundation of accurate and reproducible measurements of CNT toxicity. Third, because of the low solubility, CNTs usually deposit on the cell surface without proper dispersion in the culture medium, which makes that the exact concentration of CNTs interacting with the biological system difficult to know. Therefore, it is of profound significance to establish appropriate dosimetry for CNTs in toxicity research. Fourth, a thorough understanding of the toxicology kinetics of CNTs is necessary for risk assessment and for minimizing any unwanted impact to human health and the environment.

We thank the Ministry of Science and Technology of China (Grants 2011CB933401, 2012CB93403, and 2012CB932601) and National Science Foundation of China (Grants 10975040 and 21001034) for their financial support. The authors are indebted to Dr. Ann Motten, NIEHS/NIH, for critical reading of the manuscript.

BIOGRAPHICAL INFORMATION

Ying Liu is currently a research fellow at CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology of China. She got her B.Sc. degree in Clinical Medicine from Tianjin Medical University and Ph.D. degree in biology from Academy of Military Medical Science. She is now working on the biological effects of nanoparticles.

Yuliang Zhao received his Ph.D. at Tokyo Metropolitan University and worked at RIKEN (Japan); he moved to Chinese Academy of Sciences and became the full professor as a Hundred Elite Professorship in 2001. His research interests include nanotoxicology, nanobioanalytical chemistry, nanotoxicological chemistry, cancer nanotechnology, and radiochemistry. He has published more than 200 papers and authored/edited 10 books. The "Nanotoxicology" published in USA (2007) is the first textbook worldwide in the field. He has delivered 125 invited lectures and is serving as Associated Editor or Editorial Broad Member for 8 international SCI journals in USA and Europe.

Baoyun Sun is a professor at the CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, Institute of High Energy Physics of CAS, and the National Center for Nanoscience and

Technology of China. She got her Ph.D. degree at Peking University and then worked at Department of Chemistry, Nagoya University, as a postdoctoral fellow (JSPS) and a researcher (JST). Then she moved to Leibniz Institute for Solid State and Materials Research, Germany, to continue her research. From 2006, she has worked at CAS as a Hundred Elite Professor. Prof. Sun's research interest is in chemistry and physics of fullerenes, nanotubes, and other carbon-based materials.

Chunying Chen is a principal investigator at CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology of China. Her research interests include the potential toxicity of nanoparticles, therapies for malignant tumors using theranostic nanomedicine systems that carry chemotherapeutics and imaging tags, and vaccine nanoadjuvants using nanomaterials as potential nonviral adjuvant. Her research is supported by the China MOST 973 Program, Natural Science Foundation of China, EU-FP6 and FP7, and IAEA. She has authored/coauthored over 90 peer-reviewed papers, 2 books, and 10 book chapters.

FOOTNOTES

*To whom correspondence should be addressed. E-mail: chenchy@nanoctr.cn; sunby@ihep.ac.cn. Mailing address: CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology of China, Beijing 100190, China. Telephone: +86-10-82545560. Fax: +86-10-62656765.
The authors declare no competing financial interest.

REFERENCES

1 Gorityala, B.; Ma, J.; Wang, X.; Chen, P.; Liu, X. Carbohydrate Functionalized Carbon Nanotubes and Their Applications. *Chem. Soc. Rev.* **2010**, *39*, 2925–2934.

2 Ge, C.; Du, J.; Zhao, L.; Wang, L.; Liu, Y.; Li, D.; Yang, Y.; Zhou, R.; Zhao, Y.; Chai, Z.; Chen, C. Binding of Blood Proteins to Carbon Nanotubes Reduces Cytotoxicity. *Proc. Natl. Acad. Sci. U S A* **2011**, *108*, 16968–16973.

3 Ge, C.; Meng, L.; Xu, L.; Bai, R.; Du, J.; Zhang, L.; Li, Y.; Chang, Y.; Zhao, Y.; Chen, C. Acute Pulmonary and Moderate Cardiovascular Responses of Spontaneously Hypertensive Rats After Exposure to Single-Wall Carbon Nanotubes. *Nanotoxicology* **2012**, *6*, 526–542.

4 Ge, C.; Lao, F.; Li, W.; Li, Y.; Chen, C.; Qiu, Y.; Mao, X.; Li, B.; Chai, Z.; Zhao, Y. Quantitative Analysis of Metal Impurities in Carbon Nanotubes: Efficacy of Different Pretreatment Protocols for ICPMS Spectroscopy. *Anal. Chem.* **2008**, *80*, 9426–9434.

5 Du, J.; Ge, C.; Liu, Y.; Bai, R.; Li, D.; Yang, Y.; Liao, L.; Chen, C. The Interaction of Serum Proteins with Carbon Nanotubes Depend on the Physicochemical Properties of Nanotubes. *J. Nanosci. Nanotechnol.* **2011**, *11*, 10102–10110.

6 Ge, C.; Li, W.; Li, Y.; Li, B.; Du, J.; Qiu, Y.; Liu, Y.; Gao, Y.; Chai, Z.; Chen, C. Significance and Systematic Analysis of Metallic Impurities of Carbon Nanotubes Produced by Different Manufacturers. *J. Nanosci. Nanotechnol.* **2011**, *11*, 2389–2397.

7 Li, Y.; Liu, Y.; Fu, Y.; Wei, T.; Le Guyader, L.; Gao, G.; Liu, R.; Chang, Y.; Chen, C. The Triggering of Apoptosis in Macrophages by Pristine Graphene through the MAPK and TGF-Beta Signaling Pathways. *Biomaterials* **2012**, *33*, 402–411.

8 Zhou, H.; Zhao, K.; Li, W.; Yang, N.; Liu, Y.; Chen, C.; Wei, T. The Interactions between Pristine Graphene and Macrophages and the Production of Cytokines/Chemokines via TLR- and NF-kB-Related Signaling Pathways. *Biomaterials* **2012**, *33*, 6933–6942.

9 Lao, F.; Chen, L.; Li, W.; Ge, C.; Qu, Y.; Sun, Q.; Zhao, Y.; Han, D.; Chen, C. Y. Fullerene Nanoparticles Selectively Enter Oxidation-Damaged Cerebral Microvessel Endothelial Cells and Inhibit JNK-Related Apoptosis. *ACS Nano* **2009**, *3*, 3358–3368.

10 Lao, F.; Li, W.; Han, D.; Qu, Y.; Liu, Y.; Zhao, Y.; Chen, C. Fullerene Derivatives Protect Endothelial Cells against NO-Induced Damage. *Nanotechnology* **2009**, *20*, No. 225103.

11 Fubini, B; Ghiazza, M; Fenoglio, I. Physico-chemical Features of Engineered Nanoparticles Relevant to Their Toxicity. *Nanotoxicology* **2010**, *4*, 347–363.

12 Fubini, B; Fenoglio, I; Tomatis, M; Turci, F. Effect of Chemical Composition and State of the Surface on the Toxic Response to High Aspect Ratio Nanomaterials. *Nanomedicine* **2011**, *6*, 899–920.

13 Zhao, Y.; Xing, G.; Chai, Z. Nanotoxicology: Are Carbon Nanotubes safe? *Nat. Nanotechnol.* **2008**, *3*, 191–192.

14 Chen, Z.; Meng, H.; Xing, G.; Chen, C.; Zhao, Y. Toxicological and Biological Effects of Nanomaterials. *Int. J. Nanotechnol.* **2007**, *4*, 179–196.

15 Pumera, M. Carbon Nanotubes Contain Residual Metal Catalyst Nanoparticles even after Washing with Nitric Acid at Elevated Temperature because These Metal Nanoparticles Are Sheathed by Several Graphene Sheets. *Langmuir* **2007**, *23*, 6453–6458.

16 Liu, X.; Gurel, V.; Morris, D.; Murray, D.; Zhitkovich, A.; Kane, A.; Hurt, R. Bioavailability of Nickel in Single-Wall Carbon Nanotubes. *Adv. Mater.* **2007**, *19*, 2790–2796.

17 Pulskamp, K.; Diabate, S.; Krug, H. Carbon Nanotubes Show No Sign of Acute Toxicity but Induce Intracellular Reactive Oxygen Species in Dependence on Contaminants. *Toxicol. Lett.* **2007**, *168*, 58–74.

18 Kagan, V.; Konduru, N.; Feng, W.; Allen, B.; Conroy, J.; Volkov, Y.; Vlasova, I. I.; Belikova, N.; Yanamala, N.; Kapralov, A.; Tyurina, Y.; Shi, J.; Kisin, E.; Murray, A.; Franks, J.; Stolz, D.; Gou, P.; Klein-Seetharaman, J.; Fadeel, B.; Star, A.; Shvedova, A. Carbon Nanotubes Degraded by Neutrophil Myeloperoxidase Induce Less Pulmonary Inflammation. *Nat. Nanotechnol.* **2010**, *5*, 354–359.

19 Lam, C.; James, J.; McCluskey, R.; Hunter, R. Pulmonary Toxicity of Single-Wall Carbon Nanotubes in Mice 7 and 90 Days after Intratracheal Instillation. *Toxicol. Sci.* **2004**, *77*, 126–134.

20 Gao, Y.; Chen, C.; Chai, Z. Advanced Nuclear Analytical Techniques for Metalloproteomics. *J. Anal. At. Spectrom.* **2007**, *22*, 856–866.

21 Wang, H.; Wang, J.; Deng, X.; Sun, H.; Shi, Z.; Gu, Z.; Liu, Y.; Zhao, Y. Biodistribution of Carbon Single-Wall Carbon Nanotubes in Mice. *J. Nanosci. Nanotechnol.* **2004**, *4*, 1019–1024.

22 Deng, X.; Jia, G.; Wang, H.; Sun, H.; Wang, X.; Yang, S.; Wang, T.; Liu, Y. Translocation and Fate of Multi-Walled Carbon Nanotubes in vivo. *Carbon* **2007**, *45*, 1419–1424.

23 Singh, R.; Pantarotto, D.; Lacerda, L.; Pastorin, G.; Klumpp, C.; Prato, M.; Bianco, A.; Kostarelos, K. Tissue Biodistribution and Blood Clearance Rates of Intravenously Administered Carbon Nanotube Radiotracers. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 3357–3362.

24 Lopez, C.; Nielsen, S.; Moore, P.; Klein, M. Understanding Nature's Design for a Nanosyringe. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4431–4434.

25 Porter, A.; Gass, M.; Bendall, J.; Muller, K.; Goode, A; Skepper, J.; Midgley, P.; Welland, M. Uptake of Noncytotoxic Acid-Treated Single-Walled Carbon Nanotubes into the Cytoplasm of Human Macrophage Cells. *ACS Nano* **2009**, *3*, 1485–1492.

26 Jaurand, M.; Renier, A.; Daubriac, J. Mesothelioma: Do Asbestos and Carbon Nanotubes Pose the Same Health Risk? *Part. Fibre Toxicol.* **2009**, *6*, 16–29.

27 Poland, C.; Duffin, R.; Kinloch, I.; Maynard, A.; Wallace, W.; Seaton, A.; Stone, V.; Brown, S.; Macnee, W.; Donaldson, K. Carbon Nanotubes Introduced into the Abdominal Cavity of Mice Show Asbestos-Like Pathogenicity in a Pilot Study. *Nat. Nanotechnol.* **2008**, *3*, 423–428.

28 Palomäki, J.; Välimäki, E.; Sund, J.; Vippola, M.; Clausen, P.; Jensen, K.; Savolainen, K.; Matikainen, S.; Alenius, H. Long, Needle-Like Carbon Nanotubes and Asbestos Activate the NLRP3 Inflammasome through a Similar Mechanism. *ACS Nano* **2011**, *5*, 6861–6870.

29 Sato, Y.; Yokoyama, A.; Shibata, K.; Akimoto, Y.; Ogino, S.; Nodasaka, Y.; Kohgo, T.; Tamura, K.; Akasaka, T.; Uo, M.; Motomiya, K.; Jeyadevan, B.; Ishiguro, M.; Hatakeyama, R.; Watari, F.; Tohji, K. Influence of length on cytotoxicity of multi-walled carbon nanotubes against human acute monocytic leukemia cell line THP-1 in vitro and subcutaneous tissue of rats in vivo. *Mol. Biosyst.* **2005**, *1*, 176–182.

30 Yamashita, K.; Yoshioka, Y.; Higashisaka, K.; Morishita, Y.; Yoshida, T.; Fujimura, M.; Kayamuro, H.; Nabeshi, H.; Yamashita, T.; Nagano, K.; Abe, Y.; Kamada, H.; Kawai, Y.; Mayumi, T.; Yoshikawa, T.; Itoh, N.; Tsunoda, S.; Tsutsumi, Y. Carbon Nanotubes Elicit DNA Damage and Inflammatory Response Relative to Their Size and Shape. *Inflammation* **2010**, *33*, 276–280.

31 Belyanskaya, L.; Weigel, S.; Hirsch, C.; Tobler, U.; Krug, H.; Wick, P. Effects of Carbon Nanotubes on Primary Neurons and Glial Cells. *Neurotoxicology* **2009**, *30*, 702–711.

32 Wick, P.; Manser, P.; Limbach, L.; Dettlaff-Weglikowska, U.; Krumeich, F.; Roth, S.; Stark, W.; Bruinink, A. The Degree and Kind of Agglomeration Affect Carbon Nanotube Cytotoxicity. *Toxicol. Lett.* **2007**, *168*, 121–131.

33 Jia, G.; Wang, H.; Yan, L.; Wang, X.; Pei, R.; Yan, T.; Zhao, Y.; Guo, X. Cytotoxicity of Carbon Nanomaterials: Single-Wall Nanotube, Multi-Wall Nanotube, and Fullerene. *Environ. Sci. Technol.* **2005**, *39*, 1378–1383.

34 Nagai, H.; Okazaki, Y.; Chew, S.; Misawa, N.; Yamashita, Y.; Akatsuka, S.; Ishihara, T.; Yamashita, K.; Yoshikawa, Y.; Yasui, H.; Jiang, L.; Ohara, H.; Takahashi, T.; Ichihara, G.; Kostarelos, K.; Miyata, Y.; Shinohara, H.; Toyokuni, S. Diameter and Rigidity of Multiwalled Carbon Nanotubes Are Critical Factors in Mesothelial Injury and Carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, E1330–1338.

35 Herzog, E.; Casey, A.; Lyng, F.; Chambers, G.; Byrne, H.; Davoren, M. A New Approach to the Toxicity Testing of Carbon-Based Nanomaterials-the Clonogenic Assay. *Toxicol. Lett.* **2007**, *174*, 49–60.

36 Tutak, W.; Park, K.; Vasilov, A.; Starovoytov, V.; Fanchini, G.; Cai, S.; Partridge, N.; Sesti, F.; Chhowalla, M. Toxicity Induced Enhanced Extracellular Matrix Production in Osteoblastic Cells Cultured on Single-Walled Carbon Nanotube Networks. *Nanotechnology* **2009**, *20*, 255101–255108.

37 Casey, A.; Herzog, E.; Lyng, F.; Byrne, H.; Chambers, G.; Davoren, M. Single Walled Carbon Nanotubes Induce Indirect Cytotoxicity by Medium Depletion in A549 Lung Cells. *Toxicol. Lett.* **2008**, *179*, 78–84.

38 Johansson, L.; Chen, C.; Thorell, J.; Fredriksson, A.; Stone-Elander, S.; Gafvelin, G.; Arner, E. Exploiting the 21st Amino Acid-Purifying and Labeling Proteins by Selenolate Targeting. *Nat. Methods* **2004**, *1*, 61–66.

39 Monteiro-Riviere, N.; Inman, A.; Zhang, L. Limitations and Relative Utility of Screening Assays to Assess Engineered Nanoparticle Toxicity in a Human Cell Line. *Toxicol. Appl. Pharmacol.* **2009**, *234*, 222–235.

40 Tsukahara, T.; Haniu, H. Cellular Cytotoxic Response Induced by Highly Purified Multi-Wall Carbon Nanotube in Human Lung Cells. *Mol. Cell. Biochem.* **2011**, *352*, 57–63.

41 Pacurari, M.; Yin, X.; Zhao, J.; Ding, M.; Leonard, S.; Schwegler-Berry, D.; Ducatman, B.; Sbarra, D.; Hoover, M.; Castranova, V.; Vallyathan, V. Raw Single-Wall Carbon Nanotubes Induce Oxidative Stress and Activate Mapks, AP-1, NF-Kappa b, and Akt in Normal and Malignant Human Mesothelial Cells. *Environ. Health Perspect.* **2008**, *116*, 1211–1217.

42 Watts, P.; Fearon, P.; Hsu, W.; Billingham, N.; Kroto, H.; Walton, D. Carbon Nanotubes as Polymer Antioxidants. *J. Mater. Chem.* **2003**, *13*, 491–495.

43 Fenoglio, I.; Tomatis, M.; Lison, D.; Muller, J.; Fonseca, A.; Nagy, J.; Fubini, B. Reactivity of Carbon Nanotubes: Free Radical Generation or Scavenging Activity? *Free Radical Biol. Med.* **2006**, *40*, 1227–1233.

44 Shvedova, A.; Kisin, E.; Porter, D.; Schulte, P.; Kagan, V.; Fadeel, B.; Castranova, V. Mechanisms of Pulmonary Toxicity and Medical Applications of Carbon Nanotubes: Two Faces of Janus? *Pharmacol. Ther.* **2009**, *121*, 192–204.

45 Wang, L.; Luanpitpong, S.; Castranova, V.; Tse, W.; Lu, Y.; Pongrakhananon, V.; Rojanasakul, Y. Carbon Nanotubes Induce Malignant Transformation and Tumorigenesis of Human Lung Epithelial Cells. *Nano Lett.* **2011**, *11*, 2796–2803.

46 Takagi, A.; Hirose, A.; Nishimura, T.; Fukumori, N.; Ogata, A.; Ohashi, N.; Kitajima, S.; Kanno, J. Induction of Mesothelioma in p53+/− Mouse by Intraperitoneal Application of Multi-wall Carbon Nanotube. *J. Toxicol. Sci.* **2008**, *33*, 105–116.

47 Zhu, L.; Chang, D.; Dai, L.; Hong, Y. DNA Damage Induced by Multiwalled Carbon Nanotubes in Mouse Embryonic Stem Cells. *Nano Lett.* **2007**, *7*, 3592–3597.