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Chemistry of carbon nanotubes in biomedical applications

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Carbon nanotubes (CNTs) have attracted great interdisciplinary interest due to their peculiar structural, mechanical and electronic properties. Applications of CNTs in biomedical research are being actively explored by many scientists worldwide. However, manipulation of CNTs is impeded by several problems, such as 1) formation of complex and entangled bundles; 2) very low solubility of CNTs in organic solvents and water; 3) inert properties of pristine CNTs under many chemical reaction conditions, *etc*. Chemical modification of CNTs has partly solved the above issues and is still one of the most effective means of manipulating and processing CNTs. Many bioapplications of CNTs rely on successful outer/inner surface functionalizations. This Feature Article is comprised of two main parts. In the first part, we briefly review the covalent surface chemistry for the CNT functionalization; in the second part, we focus on the biomedical applications of surface chemistry for CNTs, in particular, the chemistry for controlling biomedical functions and meanwhile lowering nanotoxicity of CNTs. We also analyze the underlying factors that led to the controversy in the previous experimental data of safety studies of CNTs.

1. Introduction

Since their discovery in 1991,[1] carbon nanotubes (CNTs) have attracted immense interest from a wide variety of disciplines such as chemistry, physics, biology, medicine and engineering.[2] CNTs are allotropes of carbon with a nanostructure of cylindrical shape that can be viewed as seamless rolls of graphitic sheets. They are capped at the ends by one half of a fullerene-like molecule. CNTs have extraordinarily high aspect ratio. Typical diameters of CNTs are in the range of 1 nm to 100 nm while the length can be up to several centimeters.[3] CNTs are categorized as multi-walled nanotubes (MWNTs) and single-walled nanotubes (SWNTs). A MWNT is composed of an array of concentric cylinders; a SWNT possesses the simplest single-layered geometry with a diameter between 0.4 nm and 3 nm. Depending on the orientation of the hexagon rings along the tubular surface, CNTs can be metallic or semiconducting.[4–6] Due to their extraordinary structural, mechanical and electronic properties, CNTs show strong application potential in electronics, scanning probe microscopy,

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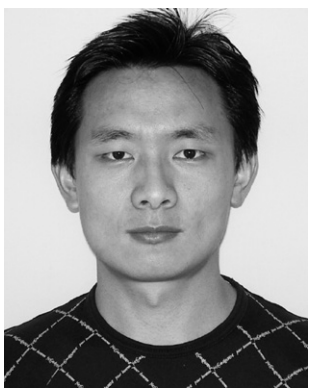

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chemical and biological sensing, medicinal chemistry, reinforced composite materials, and in many other areas.[7–10]

However, there are several obstacles on the way to controlling and manipulating CNTs. First, CNTs form bundles, which are composed of up to hundreds of single nanotubes and entangled together in the solid state giving rise to a highly complex network. The bundles can be dispersed in some solvents by sonication but precipitation occurs immediately when the process is interrupted. Second, as-produced CNTs have very low solubility in all organic solvents and aqueous solutions. The lack of solubility and the difficult manipulation in any solvents have imposed great limitations on any practical use of CNTs. Third, in the early stage of development, CNTs were found to be inert under many chemical reaction conditions.

Despite the above-mentioned difficulties, continuous efforts have been made towards solubilising and functionalizing CNTs since their discovery.[11] Significant advances have been achieved in this new area in less than two decades.[11,12] The main approaches for the modification of CNTs can be grouped into three categories including (i) the covalent attachment of chemical groups onto π-conjugated skeleton of CNTs; (ii) the noncovalent adsorption or wrapping with various functional molecules; and (iii) the endohedral filling of their inner empty cavity.

Many practical uses depend on chemical modifications of CNTs, especially, in biomedical applications. Carbon itself has good biocompatibility, however, when it forms the nanostructure of CNT, it shows profound toxicity.[13] The property of water-insolubility is another factor that confines the use of CNTs in the biomedical field. Furthermore, CNTs are considered as ideal carriers for drug delivery, which requires proper chemical functionalization. Accordingly, chemistry of CNTs in biomedical fields has multiple purposes, such as water-solubilization, enhancement of functions, and lowering the toxicity of CNTs, *etc*.

2. Covalent surface chemistry of CNTs: method and strategy

The chemical modification of CNTs is an emerging area in materials science. Many applications of CNTs rely on successful surface functionalization.[14] Here we first summarize the reported covalent surface chemistry of CNTs in Fig. 1, and then briefly discuss chemical processes of CNTs, only detailing those reactions that lead to biomedical applications.

2.1. Carboxylation of the terminal carbons and defect sites of CNTs

During the early stage of manipulating CNTs, acid cutting with nitric acid or a mixture of concentrated sulfuric acid and nitric acid was found effective to decap[15] or shorten CNTs.[16] It was speculated at that time that the terminal carbons and the carbons at the defect sites were converted into carboxylic acids. Indeed, this was soon proven by the Haddon group using the acid moieties for attaching long alkyl chains to SWNTs *via* amide linkages[17] or carboxylate-ammonium salt ionic interactions.[18] Sun *et al* showed that the esterification of the carboxylic acids can also be applied to functionalize and solubilise CNTs of any length.[19,20] These solubilised CNTs allow solution-based characterizations and investigations. There is now ample experimental evidence that a variety of different functional groups can be loaded onto CNTs *via* reactions with these nanotube-bound carboxylic acids.[21] This has led to an active branch of applications of CNTs in biomedical studies (Fig. 1).

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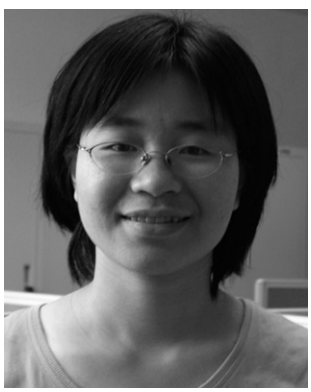

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2.2. Halogenation

Being the strongest element, fluorine can fluorinate the sidewalls of CNTs between room temperature and 600 °C[22–26] (Fig. 1). Fluorinated CNTs have been extensively characterized by transmission electron microscopy (TEM),[24] scanning tunneling microscopy (STM),[27] electron energy loss spectroscopy (EELS),[28] and X-ray photoemission spectroscopy (XPS),[29,30] whereas thermodynamic data were obtained using theoretical calculations.[31,32] The sidewall carbons on which fluorine atoms attached adopt sp^3 hybridization and possess tetrahedral configuration. This destroys the electronic band structure of metallic or semiconducting CNTs and generates an insulating material.[33] Although there is controversy regarding the favorable pattern of F addition onto the sidewall of CNTs being either 1,2-addition or 1,4-addition, DFT calculations suggested an energetic gain of 4 kcal/mol in favor of the 1,2-addition pattern.[32] This result implies that both addition patterns might coexist. The highest degree of functionalization was estimated to be C_2F by elemental analysis.[24] Fluorinated CNTs were reported to have a moderate solubility (~1 mg/mL) in alcoholic solvents.[34]

The fluorination reaction is very useful because further substitution of F can introduce useful functional groups.[26] The fluorine atom can be replaced with alkyl groups using Grignard[35] or organolithium reagents.[36] The alkylated CNTs are well dispersed in organic solvents and can be completely dealkylated upon heating at 500 °C in inert atmosphere, thus recovering pristine CNTs. Several diamines[37] and diols[38] were also reported to substitute fluorine atoms on fluorinated CNTs. Infrared (IR) spectroscopy was used to confirm the disappearance of the C–F bond stretching at 1225 cm^{-1} as the indication of F-substitution on CNTs. These reacted CNTs, such as aminoalkylated CNTs, can be further modified on the free amino groups to introduce more sophisticated functionalities which may bind biomolecules for biological applications.

Chlorination or bromination reactions to CNTs were also accomplished through electrochemical means but the products contain significant amounts of carboxyl or hydroxyl groups.[39]

2.3. Cycloadditions

In Fig. 2 we summarized the cycloaddition reactions of CNTs. Carbenes and nitrenes both react with CNTs to afford cycloaddition products. Dichlorocarbene generated *in situ* using a chloroform/sodium hydroxide or a phenyl(bromodichloromethyl)mercury reagent was first employed by the Haddon group.[17,40] The added CCl_2 moiety to the CNTs was confirmed by XPS, far-infrared spectra and chemical analysis. In the Bingel [2 + 1] cyclopropanation reaction, diethylbromomalonate served as a formal precursor of carbene and reacted with CNTs dispersed in 1,8-diazobicyclo[5,4,0]undecene (DBU).[41] The carboxylate groups attached on the CNTs can be further derivatized for other applications. A recent example of Bingel cyclopropanation was for the functionalization of ultrashort nanotubes (20–80 nm lengths) with malonic acid bis(3-*tert*-butoxycarbonylaminopropyl) ester in CBr_4/DBU to yield 4–5 adducts nm^{-1}.[42] The covalent attachment and tight wrapping of adduct arms around CNTs were confirmed by thermogravimetric analysis (TGA) and solid-state NMR spectroscopy.

Alkoxycarbonylnitrene was prepared through thermal decomposition of an organic azide. The resultant nitrene was added to the sidewalls of CNTs, affording alkoxycarbonylaziridino-CNT.[43,44] A variety of different functional groups have been attached onto CNTs by using different azides.[45,46] The attached functional groups can be used in molecular recognition, chelating metal ions and cross-linking *etc*. Derivatized CNTs are soluble in dimethyl sulfoxide or 1,2-dichlorobenzene and can be characterized by ^{1}H NMR, XPS, UV-vis and IR spectroscopies. Similarly, nitrenes obtained by irradiation of photoactive azides

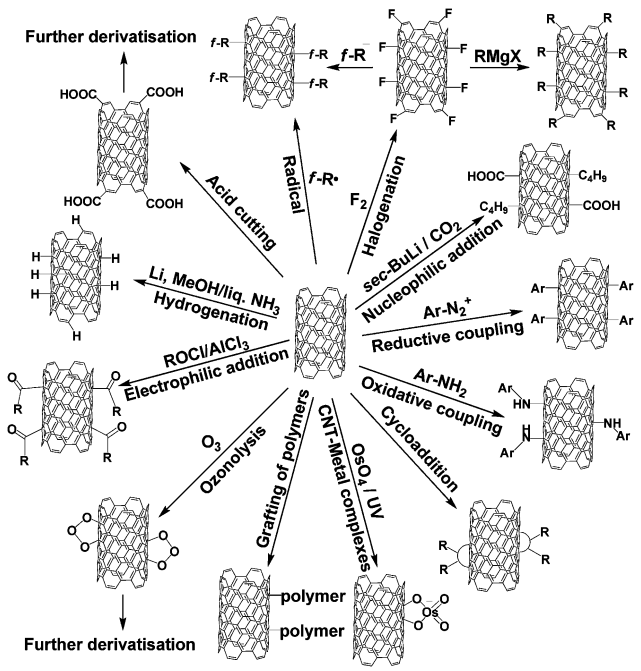


Fig. 1 Surface functionalization of carbon nanotubes.

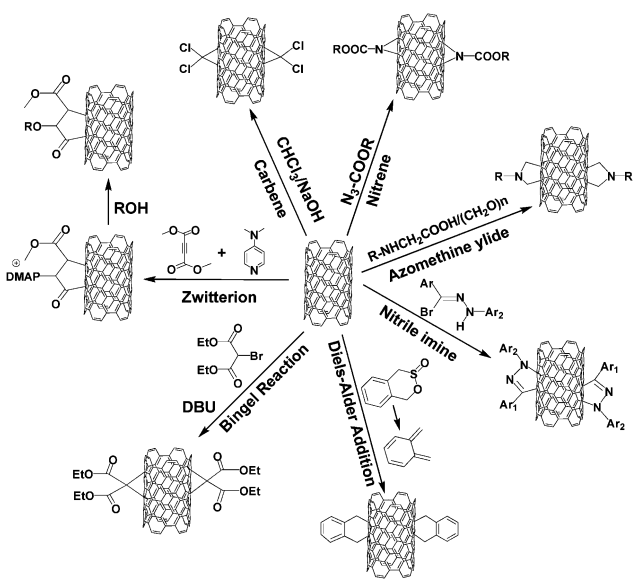


Fig. 2 Functionalization of carbon nanotubes by cycloaddition reactions.

have also been reported to couple to CNTs and form aziridine adducts.[47,48]

In a different approach, azomethine ylides, thermally generated *in situ* by condensation of an α-amino acid and an aldehyde, were successfully added to the CNT surface *via* a 1,3-dipolar cycloaddition reaction, forming pyrrolidine fused rings.[49,50] This method is versatile for preparing a wide variety of functionalized CNTs. Various applications of this chemistry have been demonstrated in the fields of medicinal chemistry, solar energy conversion, and selective recognition of chemical species, *etc*.[51–56]

Alvaro *et al.* reported another method for CNT modification by thermal 1,3-dipolar cycloaddition of nitrile imines under microwave conditions.[57] The pyrazoline-modified tubes were characterized by UV-vis, NMR and FT-IR spectroscopies. The applicability of the 1,3-dipolar cycloadditions onto the sidewalls of CNTs was also supported by theoretical calculations.[58] Other cycloaddition reactions have also been reported for the functionalization of CNTs. A Diels–Alder cycloaddition was performed on the sidewalls of SWNTs using *o*-quinodimethane (generated *in situ* from 4,5-benzo-1,2-oxathiin-2-oxide) by microwave irradiation.[59] A molecular modeling approach predicted this cycloaddition by observing the possible aromaticity stabilization at the corresponding transition states and products.[60] A more recent example of the cycloaddition approach was reported by the Swager group using zwitterions, a class of dipolar species resulting from the addition of nucleophiles to activated electrophiles.[61] The reaction was initiated by the attack of nucleophilic 4-dimethylaminopyridine (DMAP) in the presence of an electrophile, dimethyl acetylenedicarboxylate (DMAD). In the final step, the DMAP moiety is replaced with other nucleophiles, such as alcohols, to yield the desired functional groups.

2.4. Radical additions

Molecular dynamics simulations showed that there is a great probability of reaction of radicals on the sidewalls of CNTs.[62] Experimentally the covalent sidewall functionalization *via* radical addition was achieved with diazonium salts (Fig. 1). Electrochemical reduction of substituted aryl diazonium salts in organic media generates an aryl radical *in situ* which covalently attaches to the surface of CNTs.[63,64] The formation of aryl radicals was triggered by electron transfer between CNT and the aryl diazonium salts in a self-catalyzed reaction. A similar reaction was later reported, utilizing water-soluble diazonium salts, which have been shown to react selectively with metallic CNTs.[65,66] These diazonium salt functionalized CNTs are well-dispersed in DMF or aqueous solutions.

In situ electrochemical modification of individual CNTs was demonstrated by attachment of substituted phenyl groups.[67–69] Two types of coupling reactions were proposed, namely the reductive coupling of aryl diazonium salts and the oxidative coupling of aromatic amines. In both cases a radical species is produced on the surface of the nanotube, which attacks the carbon lattice to form a covalent bond. In the former case, the reaction resulted in a C–C bond formation at the graphitic surface, whereas in the latter, amines were directly attached to CNTs.

Reductive alkylation of SWNTs with lithium metal in liquid ammonia followed by the addition of either alkyl iodides/sulfides or aryl iodides/sulfides is also proposed to be a radical process.[70,71] The reductive intercalation of lithium ion onto the nanotube surface in ammonia or in polar aprotic solvents has been observed by TEM and AFM. The negatively charged tubes were found to exchange electrons with long chain aryl/alkyliodides, resulting in transient aryl/alkyl radicals. The latter were covalently added to the graphitic surface of the nanotubes to afford modified products. A similar reaction was reported for the functionalization of CNTs *via* one-electron reduction of benzophenone by potassium.[72] A radical anion is generated from the reaction of a potassium atom with a benzophenone molecule that results in transferring one electron from the potassium to the benzophenone. The radical anion adds readily to sidewalls to yield diphenylcarbinol-functionalized SWNTs.[72]

Thermal and photochemical methods have also been applied to the successful covalent functionalization of CNTs with radicals. Alkyl or aryl peroxides were decomposed thermally and the resulting radicals added to the graphitic network.[73,74] The reaction of CNTs with succinic or glutaric acid acyl peroxides resulted in the addition of carboxyalkyl radicals onto the sidewalls. These acid-functionalized CNTs can be converted to materials with new functions. Addition of perfluoroalkyl radicals to CNTs was obtained by photoinduced reactions.[48,75] The precursor, an alkyl iodide, underwent a homolytic cleavage upon illumination to produce the corresponding radical. A silylation protocol developed by the Wong group for the addition of silanes, *e.g.* trimethoxysilane and hexaphenyldisilane, onto SWNTs in the presence of UV irradiation is also believed to be a photoinduced radical process.[76]

2.5. Electrophilic additions

Prato *et al.* reported electrophilic addition of chloroform to SWNTs in the presence of Lewis acid followed by hydrolysis resulting in the addition of hydroxyl groups to the surface of CNTs.[77] Further esterification with propionyl chloride led to the corresponding ester derivatives, which greatly increased the solubility of SWNTs and allowed better characterization of their structure. A more versatile electronic addition to SWNTs was carried out by Balaban *et al.* under Friedel–Crafts conditions to afford polyacylate nanotubes.[78] This renders CNTs highly soluble/dispersible in standard organic solvents. The generality of this method allows efficient insertion not only of normal acyl chains but also of perfluorinated acyl residues or cross-linking possibilities by the use of α,ω-diacyl dichlorides (Fig. 1).

2.6. Nucleophilic additions

Hirsch and coworkers first reported nucleophilic addition of carbenes to CNTs.[43] The zwitterionic adducts were found to be the addition product rather than cyclopropane systems (Fig. 3). The derivatized CNTs are quite soluble in DMSO and can be characterized by mass spectrometry. Nucleophilic addition of octadecylamine to CNTs was reported by Basiuk *et al.* in a solvent-free amination of closed caps of MWNTs.[79] It was suggested that the addition takes place only on five-membered rings of the graphitic network of CNTs and the benzene rings are

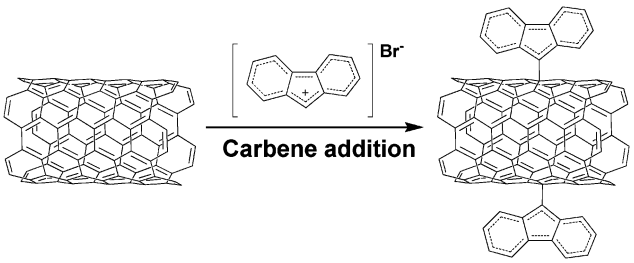


Fig. 3 Carbene addition to carbon nanotubes.

inert to the direct amination. The real nanotube sidewalls must contain numerous reactive five-membered rings as defects and thus can be derivatized with octadecylamine to a relatively high extent. Chen *et al.* reported another example of nucleophilic addition treating pristine CNTs with *sec*-BuLi and subsequently with carbon dioxide.[80] CNTs were found to be modified with both alkyl and carboxylic groups. The resulting modified CNTs can be individually dispersed in water at the concentration of 0.5 mg/mL.

2.7. Ozonolysis

Raw HiPco SWNTs in methanol have been subjected to ozonolysis at −78 °C[81,82] and room temperature,[83] followed by controlled modification. In independent runs, treatments on SWNT-ozonides with various reagents including H_2O_2, DMS and $NaBH_4$ generate a high proportion of carboxylic acid/ester, ketone/aldhehyde, and alcohol groups on the nanotube surface (Fig. 1). These active moieties can be further derivatized to afford functionalized CNTs. Banerjee *et al.* found that the chemical reactivity of the sidewall ozonation is dependent on the diameter of the nanotubes.[84] Smaller diameter CNTs have greater strain energy per carbon atom due to increased curvature and higher rehybridization energy and thus exhibit higher reactivity in the solution-phase ozonolysis.

2.8. Grafting of polymers

The non-covalent interaction between polymers and CNTs has been extensively studied by different groups. Many discoveries about the non-covalent macromolecule-CNT complexes have greatly advanced the research of CNTs.[85–91] On the other hand, the covalent reaction of CNTs with polymers is equally important because the long polymer chains help to solubilize the nanotubes into a wide range of solvents even at a low degree of functionalization. There are two main methods for the covalent attachment of polymers to the graphitic surface of CNTs, which are defined as "grafting to" and "grafting from" methods (Fig. 1).

The "grafting to" method relies on the synthesis of a polymer with reactive functional groups and subsequent covalent attachment of this polymer chain to the surface of CNTs *via* coupling reactions. For example, poly(methyl methacrylate) was reported to react with CNTs in monochlorobenzene by using ultrasonication.[92,93] Wu *et al.* treated poly(vinylcarbozole) or poly(butadiene) with sodium hydride or butyllithium to yield the polymeric anions and then grafted them to the surface of CNTs.[94] Alternatively, the Blau group functionalized CNTs with *n*-butyllithium and subsequently coupled them with halogenated polymers.[95,96] Other reactions, such as cycloaddition[97] and radical coupling[98] reactions, have also been used to graft polymer chains to CNTs.

The "grafting from" method is based on the covalent immobilization of the polymer precursors on the surface of the CNTs and subsequent polymer chain elongation in the presence of monomeric species. By this method, polymers will grow on the surface of CNTs.[99–103] For instance, Qin *et al.* reported the grafting of polystyrenesulfonate to the CNTs by *in situ* radical polymerization.[102] Viswanathan *et al.* used a similar strategy to attach polystyrene chains to full-length pristine CNTs using *in situ* anionic polymerization.[104] The raw CNT was treated with *sec*-butyllithium, which introduces a carbanionic species on the graphitic surface and causes exfoliation of the bundles. When monomers are added, the nanotube carbanions initiate polymerization to result in covalent grafting of the polystyrene chains.

Another approach for grafting polymers to CNTs involves a technique called atomic transfer radical polymerization (ATRP).[105,106] In ATRP, initiators are covalently immobilized on the surface of CNTs and the CNT surface plays the role of grafting agent. The initiators used by Adronov and coworkers were found to be active in the polymerization of methyl methacrylate and *tert*-butyl acrylate from the surface of the nanotubes. However, the polymerizations were not controlled and resulted in the production of high molecular weight polymers. The authors suggested as the polymerization time lengthens, the elevation in sample mass results from an increased number of nanotube-bound initiators promoting rapid, uncontrolled growth of polymer chains, rather than any controlled polymer molecular weight increase over time.

The preparation of nanotube-polyaniline composites[107–110] and *in situ* Ziegler–Natta polymerization of ethylene[111] on SWNTs were also reported. However, the exact mechanism of nanotube-polymer interaction remains unclear.

2.9. Miscellaneous

A few other covalent reactions on CNTs are also listed in Fig. 1. For instance, thinning and opening CNTs with carbon dioxide was the first chemical reaction on CNTs reported in the literature.[112] Hydrogenation of CNTs by Birch reduction with Li metal and methanol dissolved in liquid ammonia affords CNTs with a formula of $C_{11}H$.[113] Formation of nanotube-metal complexes is essential for employing CNTs as catalysts or reusable catalyst supports.[114–119] Because these reactions are not directly relevant to biomedical applications of CNTs, here we omit the details of these covalent functionalizations on CNTs.

3. Functionalized CNTs for biomedical applications

CNTs hold great promise in biomedical applications, as it was found that CNTs can cross membrane barriers.[120] The translocation and biodistribution of pristine CNTs in mice and plants have been studied by several groups. However, contradictory data were obtained on the toxic effects of SWNTs.[121–125] This strongly suggests a need for a standardized framework to understand the bioactivities of CNTs and assess their toxicities

in vivo.[126] Despite the uncertainty of the hazard of CNTs, scientists have begun to explore their applicability in biomedical research. Nevertheless, several issues have to be resolved before their mass implementation. Firstly, legitimate concerns about the nanotoxicity and biocompatibility of CNTs are being addressed, and so far there is no consensus on the harmful effects of CNTs on human health and the environment. Secondly, from a viewpoint of materials chemistry, the key scientific questions of CNTs are batch-to-batch irreproducibility, poor processability, and inherent variability and broadness in the length, diameter and chirality distribution of these tubes. Covalent surface chemistry on CNTs has partly solved the problems of pristine CNT bundle formation, low solubility in organic solvents and water, and removal of catalyst metal impurities and other carbonaceous species. Apparently, for the applications of CNTs in biomedical research, chemical functionalization is still one of the most effective means for manipulating and processing CNTs.

3.1. Control of biocompatibility and nanotoxicity of CNTs by surface chemistry

3.1.1. *In vivo* biodistribution and biocompatibility. As CNTs are being proposed and employed in a wide variety of areas in biomedical fields, it becomes imperative to acquire data on the increasing biosafety concerns of biocompatibility and bioavailability of CNTs *in vivo*. Several studies have been conducted with aims toward systematic and quantitative *in vivo* analyses of CNTs, addressing issues of distribution, metabolism, degradation, clearance, and bioaccumulation of CNTs. It has been experimentally challenging to trace pristine CNTs in biological systems due to the lack of quantitative detection methods.

In a pioneering study, Wang *et al.* developed the method for quantifying CNTs *in vivo* with ^{125}I-labeling techniques.[127] The hydroxylated SWNTs were prepared and then labeled with iodine-125 by the chloramine-T (N-chloro-p-toluenesulfonamide) method and a simple separation process using a Sephadex G-25 column.[128] Synchrotron XPS studies demonstrated the covalent bonding between iodine and carbon atoms of SWNTs and good biostability of ^{125}I-SWNTols *in vivo*. The high sensitivity of radiolabeling allows very few exotic atoms per single CNT, which generally maintains the intrinsic properties of CNTs.[129]

CNTs consist of only carbon atoms, so the ideal isotope-labeling technique for the quantification of CNTs is ^{13}C-labeling or ^{14}C-labeling. Yang *et al.* successfully used skeleton ^{13}C-enriched pristine SWNTs with isotope ratio mass spectrometry for quantification of the biodistribution of SWNTs at different time intervals post-exposure.[130] For functionalized CNTs, the generally adopted strategy is to incorporate radiolabels onto the nanotubes. So far, a number of radioisotopes, such as ^{111}In,[131] ^{14}C,[132] ^{64}Cu,[133] ^{99m}Tc[134] and ^{125}I[135] *etc.*, have been used as radiotracing agents. For example, Singh *et al.* modified SWNTs with the chelating molecule diethylenetriaminepentaacetate (DTPA) and labeled with indium (^{111}In) for imaging purposes.[131] Intravenous administration of these functionalized SWNTs followed by radioactivity tracing using gamma scintigraphy indicated that the nanotubes are rapidly cleared from systemic blood circulation (half life ~3 h) through the renal excretion route, without being retained in any of the reticuloendothelial system (RES) organs (liver or spleen). Similarly, no accumulation was shown for intravenously administrated DTPA-modified MWNTs, while purified MWNTs with serum protein coatings were accumulated in liver and lungs. This example demonstrated that surface functionalization can significantly alter the *in vivo* biodistribution of CNTs. In another example, Liu *et al.* radiolabeled HiPco SWNTs wrapped in the surfactant PL-PEG with ^{64}Cu for investigation of the biodistribution of functionalized nanotubes in mice by using *in vivo* positron emission tomography (PET), *ex vivo* biodistribution and Raman spectroscopy.[133] It is found that PL-PEG modified SWNTs are surprisingly stable *in vivo* and effectively PEGylated SWNTs exhibit relatively long blood circulation times and low uptake by the RES. A comparable study was conducted by Yang *et al.* using isotope ratio mass spectrometry to determine the *in vivo* biodistribution of skeleton ^{13}C-enriched SWNTs functionalized with diamine-terminated PEGs.[135] These PEGylated SWNTs were intravenously administrated into mice and distributed throughout most organs within one hour. The accumulation of PEG-SWNTs in liver and spleen is similar to that of pristine SWNTs. However, uptake of PEG-SWNTs by RES was significantly reduced in comparison with pristine SWNTs at day 7 post-exposure. These results again show that the surface functionality of CNTs does have significant biological consequences.

Radiolabeling of organic molecules which can be readily attached onto CNT surfaces but may suffer from decreasing or even losing activity over time due to decay or dissociation of the label. CNTs possess many unique and intrinsic physical and chemical properties; therefore it is advantageous to establish direct detection and quantification based on these properties. Liu *et al.* used the intrinsic Raman signature to probe the blood circulation and long-term fate of SWNTs noncovalently functionalized with PL-PEGs in mice.[136] In another study, Cherukuri *et al.* used near-IR fluorescence of individual SWNTs to determine the blood elimination kinetics and biodistribution of the CNTs in rabbits.[137]

3.1.2. Toxicity of CNTs. The nanotoxicity of CNTs has been a heated debate in recent several years. Research in determining the toxicity of CNTs has been conducted by several groups using different strategies.[121–125] However, preliminary results are fragmentary and contradictive to one another. This is mainly because parameters such as structure, size distribution, agglomeration state, surface functionalization as well as purity of the sample, all have considerable impact on the activities of CNTs *in vivo*. Therefore, detailed characterization of the CNT materials being assessed for cytotoxicity is critical for evaluating different results obtained in CNT cytotoxicity studies.

Jia *et al.* reported different cytotoxicity of SWNTs, MWNT10 (diameter range 8from10 nm to 20 nm) and C_{60} observed in alveolar macrophage (AM) after a 6 h exposure *in vitro*.[13] The authors found that the cytotoxicity follows a sequence order on a mass basis: SWNTs > MWNT10 > C_{60} (Fig. 4). This is consistent with other *in vivo* studies that SWNTs and MWNTs produced by different methods all showed pulmonary toxicity in mice after intratracheal instillation.[138,139] A recent study led by Porter also showed that SWNTs can enter human cells and accumulate in the cytoplasm, causing cell death.[140] More recently, Donaldson and coworkers reported that exposure of

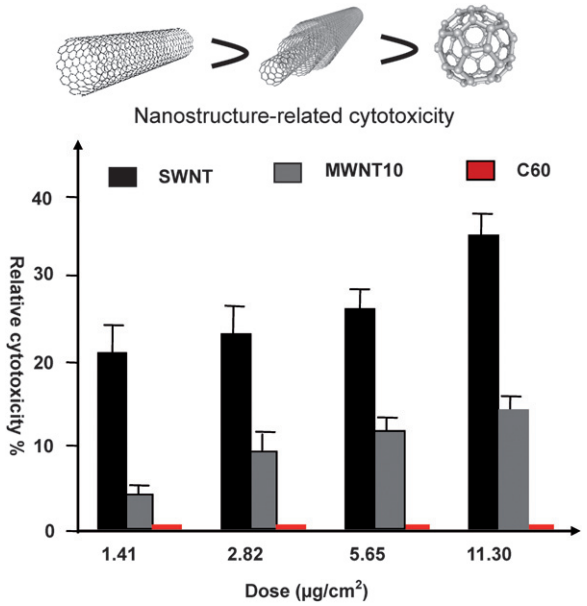


Fig. 4 The cytotoxicities of carbon nanomaterials in alveolar macrophage show a nanostructural dependent feature. The cytotoxicity follows a sequence order on a mass basis: SWNTs > MWNT10 > C_{60}. MWNT10: diameter range from 10 nm to 20 nm.

the mesothelial lining of the body cavity of mice to MWNTs results in asbestos-like, length-dependent, pathogenic behaviour.[141] A few other reports also claimed to find cardiovascular effects of pulmonary exposure to SWNTs[142] and clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells.[143] However, the overly simple characterization of nanotube materials in these studies makes it difficult to evaluate the conclusions. In contrast to the above reports, Cherukuri *et al.* did not observe acute toxicity of surfactant-dispersed (Pluronic F108, a nonionic poloaxamer surfactant) pristine SWNTs intravenously administered to rabbits.[137] This highlights the poor comparability between CNT cytotoxicity studies under different conditions.

At present, it is difficult to isolate the size effect in CNT toxicity studies due to the lack of systematic investigation of cytotoxicity of different size CNTs. Particle agglomeration has also been suggested to be an important factor in SWNT cytotoxicity.[144] However, conflicting results were obtained by two groups regarding the influence of agglomeration state on CNT cytotoxicity using two different cell lines, *i.e.* asbestos-induced lung-cancer cells *versus* keratinocytes.[145,146] In contrast, no dose-dependent effect of cytotoxicity was observed for C_{60} when the dose was changed in a range of 0.38–226.0 μg/cm^2 in alveolar macrophage,[13] given the fact that aggregation of C_{60} might occur at high concentrations in the cell culture medium. Apparently, further systematic research under comparable experimental conditions is required to resolve these issues.

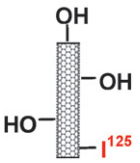
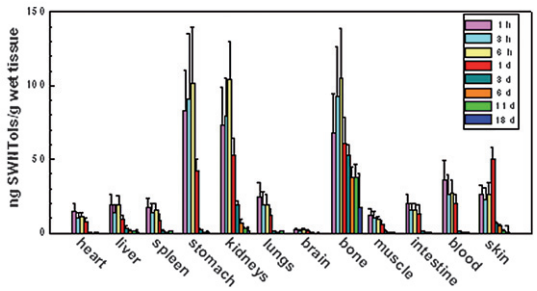
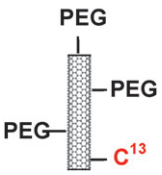
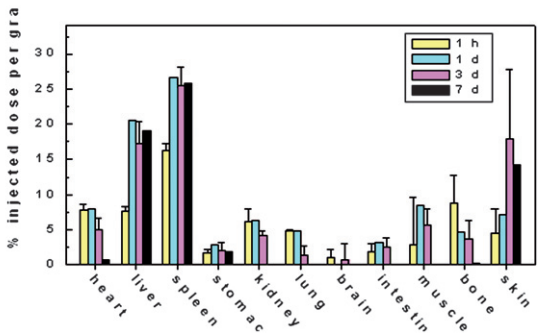
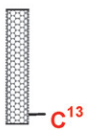
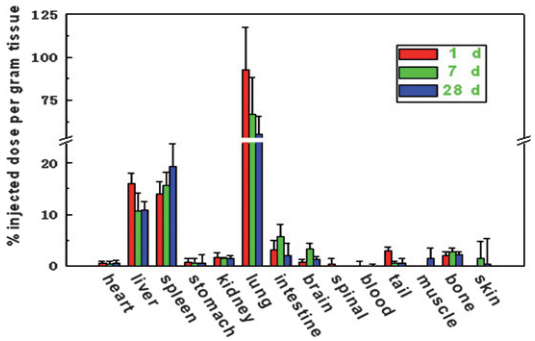
It is demonstrated by Sayes *et al.* that surface functionalization has a profound effect on the cytotoxicity of SWNTs.[125] The authors found that as the degree of sidewall functionalization increases, the SWNT sample becomes less cytotoxic. Furthermore, sidewall functionalized SWNT samples are substantially less cytotoxic than surfactant stabilized SWNTs. Dumortier *et al.* also found that functionalized CNTs could be taken up rapidly by B and T lymphocytes as well as macrophages without affecting the overall cell viability.[147] This conclusion was further supported by later studies that non-covalently PEGylated SWNTs induced no toxic side effects when injected intravenously in mice[136] and polyethyleneimine-functionalization of MWNTs altered their surface charges and cytotoxicity, thereby significantly improving the biocompatibility of the MWNTs for biomedical applications.[148] Gambhir and coworkers also reported the absence of acute and chronic toxicity of covalently/non-covalently functionalized SWNTs when injected into the bloodstream of mice. Nevertheless, this study used a small number of mice and the information regarding the detailed characterization of functionalized SWNTs was limited.[149] Interestingly, with a different functionalization approach, Carrero-Sanchez *et al.* found that N-doped multiwalled carbon nanotubes (CN_x) were far more tolerated by mice compared to MWNTs.[150] All routes of administration of CN_x did not induce signs of distress or tissue changes on any treated mouse except that intratracheal administration induced granulomatous inflammatory responses, while MWNTs could kill mice by dyspnea depending on the dose. The authors proposed that the CN_x nanotubes are less harmful than CNTs and might be more advantageous for bioapplications.

On the other hand, studies of biodistribution of the pristine and functionalized CNTs *in vivo* have shown that the surface modification can greatly alter the target organs. Wang *et al.* reported that the ^{125}I-labeled multiple hydroxylated SWNTs (^{125}I-SWNTols) accumulated mainly in bone, kidney and stomach (Table 1a).[127] In two other related studies, Yang *et al.* found that pristine ^{13}C-enriched SWNTs accumulated mostly in lung, spleen and liver, whereas the PEGylated ^{13}C-SWNTs were found mainly in liver, spleen, heart and skin (Table 1b and c).[130,151] In all the cases, the authors found no signs of acute toxicity responses during the experimental period even at a relatively high dose (80 mg pristine or 24 mg PEGylated SWNTs per kilogram body weight).

Metal impurities in CNT samples are also believed to contribute to the observed cytotoxicity. Two very recent studies confirmed that impurities have significant influence on the toxicity of CNTs.[152,153] For instance, the subcutaneously implanted CNTs with impurities clearly induced immunological toxicity and localized alopecia in mice, while extremely pure implanted tubes showed good biocompatibility. This seems contrary to an earlier *in vitro* investigation that the presence of metal impurities did not influence the toxicity of CNTs in A549 cells.[154] Again, systematic investigations are required to determine the role of impurities in CNT cytotoxicity. A protocol recently reported by Ge *et al.* used ICP-MS spectroscopy for quantitative analysis of metal impurities in CNTs.[155] This practically useful method may help evaluate sample purity for future CNT cytotoxicity studies.

A new theory regarding the toxicity of MWNTs during pulmonary exposure was proposed by Lison and coworkers in 2008.[156] The authors found that the acute pulmonary toxicity and genotoxicity of CNTs was reduced upon heating at 2400 °C (annealing of structural defects) but was restored upon grinding (introduction of structural defects). Thus, they proposed that the intrinsic toxicity of CNT is mainly mediated by the presence of defective sites in their carbon framework. But this proposal needs to be validated by more experiments.

Table 1 Comparisons between biodistribution of functionalized and pristine CNTs in mice. (a) Biodistribution histogram of ^{125}I-SWNTols in mice at eight different time intervals; (b) time-dependent biodistribution of PEG-SWNTs in mice post-exposure at a dose of 2.4 mg SWNT-equivalent/kg body weight; (c) biodistribution of pristine SWNTs (^{13}C-SWNT) in mice at different time points post-exposure

CNT	Target Organs	Reference
Hydroxyl-Functionalized CNTs	OH-^{125}I-SWNT  (a) 	127
PEG-Functionalized CNTs	PEG-^{13}C-SWNT  (b) 	141
Pristine CNTs	^{13}C-SWNT  (c) 	130

The above discussions clearly indicate that further systematic investigations on cytotoxicity of CNTs are highly necessary and such researches are probably ongoing in many laboratories in the world.

3.2. Delivery of bioactive molecules

A drug delivery system is generally designed to improve the pharmacological and therapeutic profile of a drug molecule. It is aimed to solve problems associated with the administration of free drugs, such as limited solubility, poor bioavailability, unfavorable pharmacokinetics, lack of selectivity, and healthy issue damage *etc*. Among the newly developed approaches, CNTs have been pursued for their potentially high loading capacity and the ability to penetrate into cells without the need for any external transporter system. There has been significant recent progress in using CNTs as effective carriers for delivering various peptides, nucleic acids, antigens, and small molecular drugs into living cells.[54,157–163]

3.2.1. *In vivo* tumor targeting and drug delivery. One major problem in cancer chemotherapy is the non-selective killing of healthy cells that divide rapidly under normal circumstances. This results in the most common side-effects of chemotherapy—myelosuppression (decreased production of blood cells), mucositis (inflammation of the lining of the digestive tract) and alopecia (hair loss). To deliver the drug molecules precisely to the tumor target with minimal side-effects on normal cells is the

objective of many researches. Functionalized CNTs have been actively explored as drug carriers and designed to be tumor-targetable.

Dai and coworkers investigated the biodistribution of radio-labelled SWNTs in mice by *in vivo* positron emission tomography (PET), *ex vivo* biodistribution and Raman spectroscopy (Fig. 5).[133] The efficient targeting of integrin $\alpha_v\beta_3$-positive U87MG tumours was achieved *via* RGD-functionalization (RGD: a cyclic arginine–glycine–aspartic acid peptide) of SWNT-PEG_{5400} and specific RGD-integrin $\alpha_v\beta_3$ recognition. SWNT-PEG_{5400}-RGD exhibited a high tumour uptake of ~10–15% injected dose (ID) g^{-1}, a significant increase from ~3–4% ID g^{-1} for SWNT-PEG_{5400} free of RGD. The same group also complexed SWNTs with a large amount of doxorubicin (DOX, a popular anticancer drug), where the complexation was non-covalent attachment *via* π–π stacking of the DOX aromatic hydroxyl-anthraquinonic rings on the nanotube surface (Fig. 6).[164] According to the *in vitro* toxicity experiments, the DOX-loaded phospholipids (PL)-SWNT induced significant U87 cancer cell death and cell apoptosis compared with PL-SWNT, though the IC50 (half-maximum inhibitory concentration) value for the nanotube-bound DOX (~8 mM) was higher than that of free DOX (~2 mM). For specifically targeting U87 cancer cells, RGD was conjugated to the terminal groups in PL-SWNT. The effectiveness of the targeted delivery of the nanotube-bound DOX was reflected in the lower IC50 value (~3 mM) for the RGD positive U87 cancer cells.

In a similar study, Villa *et al.* synthesized different SWNT constructs using regioselective chemistries to confer capabilities of selective targeting using RGD ligands, radiotracing using radiometal chelates, and self-assembly using oligonucleotides.[165] The SWNT-oligonucleotide conjugate annealed with a complementary oligonucleotide sequence had a melting temperature of 54 °C. Biodistribution in mice was quantified using radiolabeled SWNT-oligonucleotide conjugates. To verify specific antigen targeting of the nanotube-oligonucleotide conjugates (SWNT-RGD-ODNFAM), an $\alpha_v\beta_3$ positive human coronary artery endothelial cell line was used as a model in a flow cytometric assay for binding specificity. The analysis demonstrated a significant increase in median fluorescence intensity of cells treated with the SWNT-RGD-ODNFAM over the isotype control SWNT-RAD-ODNFAM. This result demonstrated that

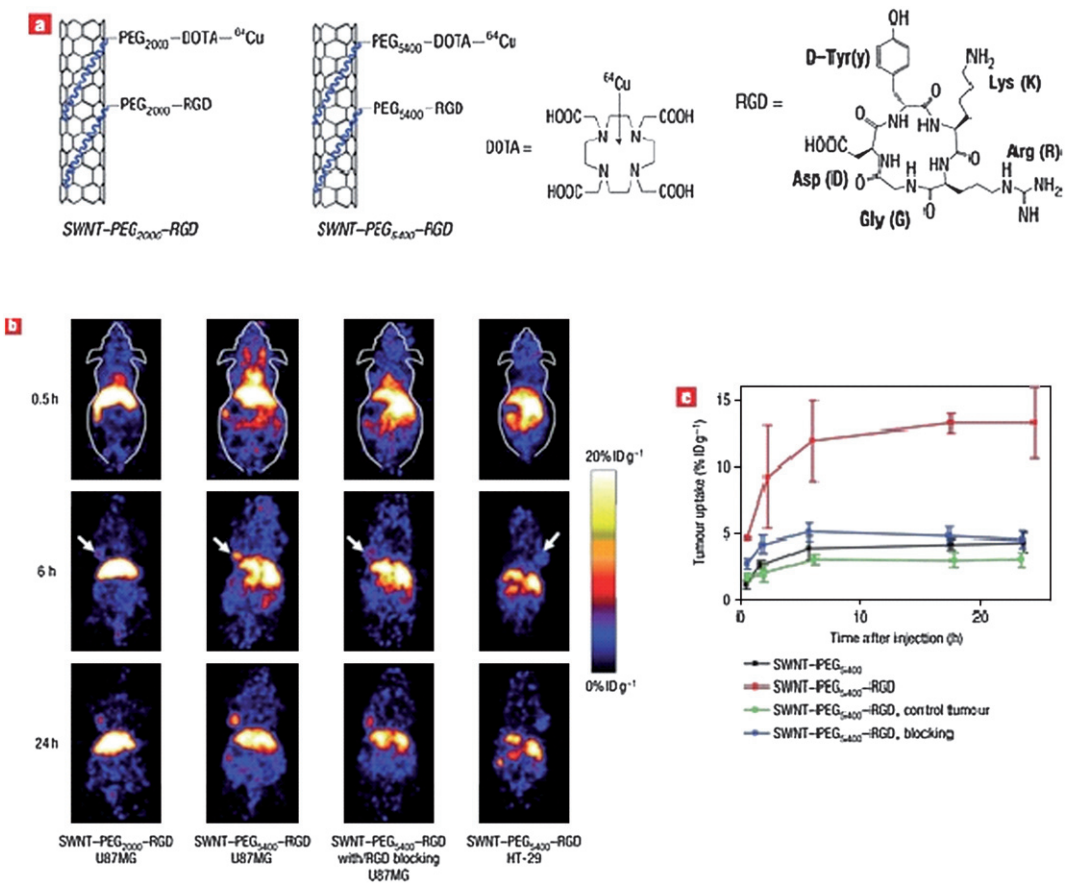


Fig. 5 Water-soluble carbon nanotubes functionalized with PEG, radio-labels and RGD peptide and targeting of integrin avb3-positive U87MG tumour in mice by RGD-functionalized SWNTs. (a) Schematic drawings of non-covalently functionalized SWNT-PEG2000-RGD, SWNT-PEG5400-RGD with DOTA-64Cu. (b) MicroPET images of mice. The arrows point to the tumours. High tumour uptake (>15% ID g21) of SWNT-PEG5400-RGD is observed in the U87MG tumour (second column), in contrast to the low tumour uptake (first column) of SWNT-PEG2000-RGD. The third column is a control experiment showing blocking of SWNT-PEG5400-RGD tumour uptake by co-injection of free c(RGDyK). The fourth column is a control experiment showing low uptake of SWNT-PEG5400-RGD in an integrin avb3-negative HT-29 tumour. (c) U87MG tumour uptake curves for mice injected with SWNT-PEG5400, with and without RGD. All data shown represent three mice per group (reproduced with permission from ref. 133).

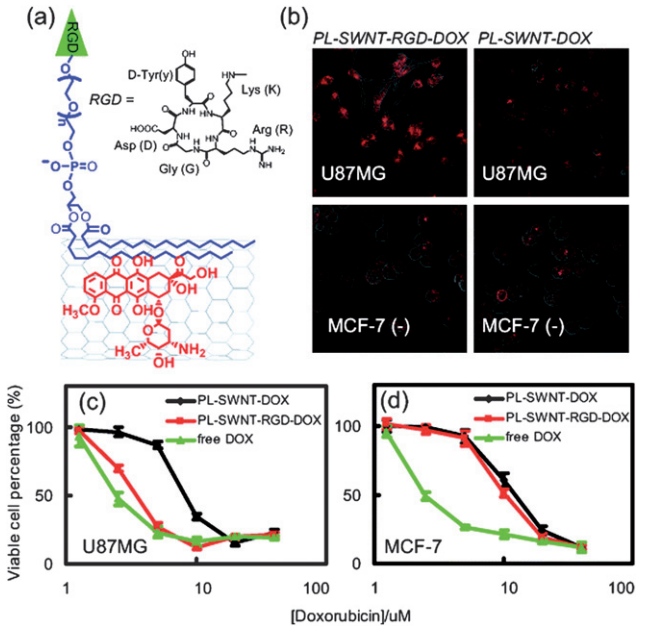


Fig. 6 RGD peptide selectively enhances doxorubicin delivery by SWNTs and toxicity to integrin $\alpha_v\beta_3$-positive cells. (a) Schematic structure of PLSWNT-RGD-DOX, *i.e.*, SWNTs functionalized with RGD at the termini of PEG and loaded with doxorubicin on the sidewall by π-stacking. (b) Confocal fluorescence images of integrin $\alpha_v\beta_3$-positive U87MG cells (top) and negative MCF-7 cells (bottom) treated with either PL-SWNT-DOX (right) or PL-SWNTRGD-DOX (left). The concentration of DOX was 2 μM in all experiments. The U87MG cells incubated with PL-SWNT-RGD-DOX showed stronger DOX fluorescence in the cells than in the other three cases. (c,d) Concentration-dependent survival curves of U87MG cells (c) and MCF-7 cells (d) treated by various samples, as indicated. The viable cell percentage was measured by the MTS assay. PL-SWNT-DOX had a lower toxic effect than free DOX on both types of cells, while PL-SWNT-RGD-DOX exhibited increased toxicity to U87MG cells but not to MCF-7 cells (reproduced with permission from ref. 152).

addition of the RGD targeting moiety to the SWNT-oligonucleotide construct allowed for specific tumor targeting, and should allow the SWNT-RGD-ODNFAM to serve as an anchor construct in a neovasculature targeted self-assembly approach.

In other cases, drug-carrying CNTs without covalent/noncovalent tumor targeting ligands also exhibit superiority to conventional drug delivery vehicles. Feazell *et al.* used amine-functionalized soluble SWNTs to deliver multiple cisplatin prodrug centers for their internalization.[166] The platinum(IV) complex, *c*,*c*,*t*-[$Pt(NH_3)_2Cl_2(OEt)(O_2CCH_2CH_2CO_2H)$], by itself was nearly nontoxic to testicular cancer cells, but its conjugate with SWNT exhibited a dramatic enhancement in cytotoxicity, along with a significant increase (six times) in the cellular platinum concentration. These results were consistent with an effective delivery of platinum(IV) with SWNTs. Similarly, Ali-Boucetta *et al.* employed pluronic copolymer-dispersed MWNTs to complex with doxorubicin (DOX) *via* π–π stacking.[167] According to the toxicological assay with MCF-7 human breast cancer cells, there was a significant enhancement in cytotoxic activity with the DOX-nanotube complexes. The enhancement was attributed to a more effective delivery of DOX with the aid of MWNTs.

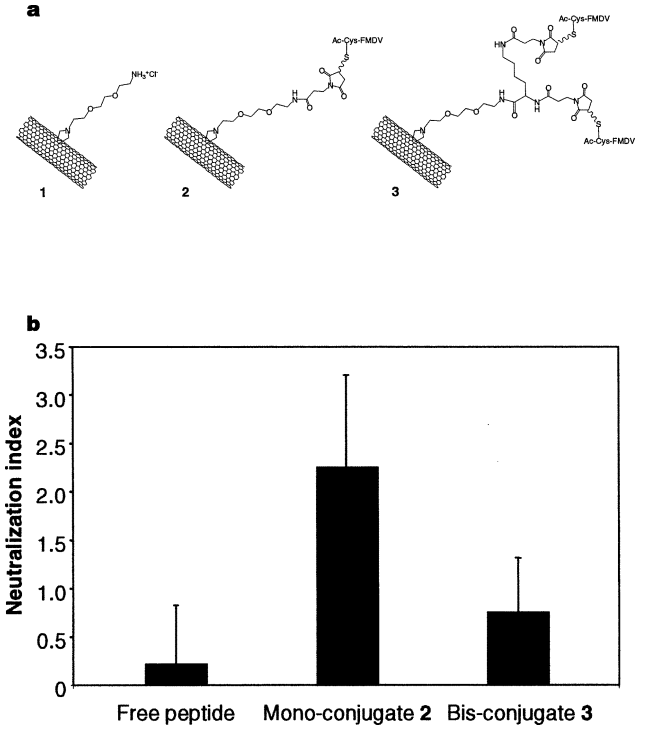


Fig. 7 Molecular structures of the CNT derivative and mono- and bis-conjugates (a) and neutralization indices of serum samples of immunized mice (b) (reproduced with permission from ref. 54).

3.2.2. Delivery of functional macrobiomolecules. Apart from small molecule drugs, large functional biomolecules can also be delivered into cells with the aid of functionalized CNTs. Pantarotto *et al.* covalently linked a neutralizing B cell epitope from the foot-and-mouth disease virus (FMDV) to mono- and bis-derivatized CNTs (Fig. 7).[53] Immunological characterization of these conjugates revealed that the epitope was appropriately presented after conjugation to CNTs for recognition by antibodies as measured by BIAcore technology. Moreover, peptide-CNTs elicited strong anti-peptide antibody responses in mice with no detectable cross-reactivity to the CNTs. However, only the mono-derivitized CNT conjugate induced high levels of virus-neutralizing antibodies. These findings demonstrated for the first time the potential of CNTs to present biologically important epitopes in an appropriate conformation both *in vitro* and *in vivo*. In another study, Zhang *et al.* conjugated siRNA with SWNTs and specifically targeted murine telomerase reverse transcriptase (mTERT) expression to form the mTERT-siRNA:SWNT+ complex.[168] These functionalized SWNTs successfully entered three cultured murine tumor cell lines, silenced the expression of the targeted gene, inhibited cell proliferation and promoted cell senescence *in vitro*, and also suppressed tumor *in vivo*. Similarly, Dai and coworkers employed SWNTs as molecular transporters to deliver siRNA into human T cells and primary cells.[169] It was found that SWNT conjugates have superior silencing effects over conventional liposome-based nonviral agents. Functionalized SWNTs ready for specific conjugation might represent a new class of molecular transporters for applications in gene therapeutics and investigations of cell functions in cell culture, with potential extensions to *in vivo* applications.

3.2.3. Cellular uptake mechanism of functionalized CNTs. Although experimental demonstrations have shown the efficiency of CNT-enabled delivery, the mechanism of cellular uptake of CNTs is still being debated. The major issue is the entry mechanism that regulated the cellular internalization of SWNTs and their carried cargos. Dai and coworkers proposed an endocytosis uptake mechanism based on the observed temperature dependence in cellular uptake of CNTs (Fig. 8).[161] They functionalized shortened SWNTs (50–200 nm in length) with DNAs and proteins noncovalently to study their uptake by HeLa (adherent) and HL60 (nonadherent) cells. According to their results, these short SWNTs transported the bioactive cargos into living cells in an energy-dependent fashion. Therefore they suggested that the endocytosis pathway for these well-dispersed, short SWNTs with bioconjugation was through clathrin-coated pits rather than caveolae or lipid rafts. The same group also studied larger aggregates of DNA-functionalized SWNTs (200 nm–2 mm long and up to 15 nm in diameter) for their uptake by HeLa cells and the results were similar to those of the shortened SWNTs, consistent with endocytosis.

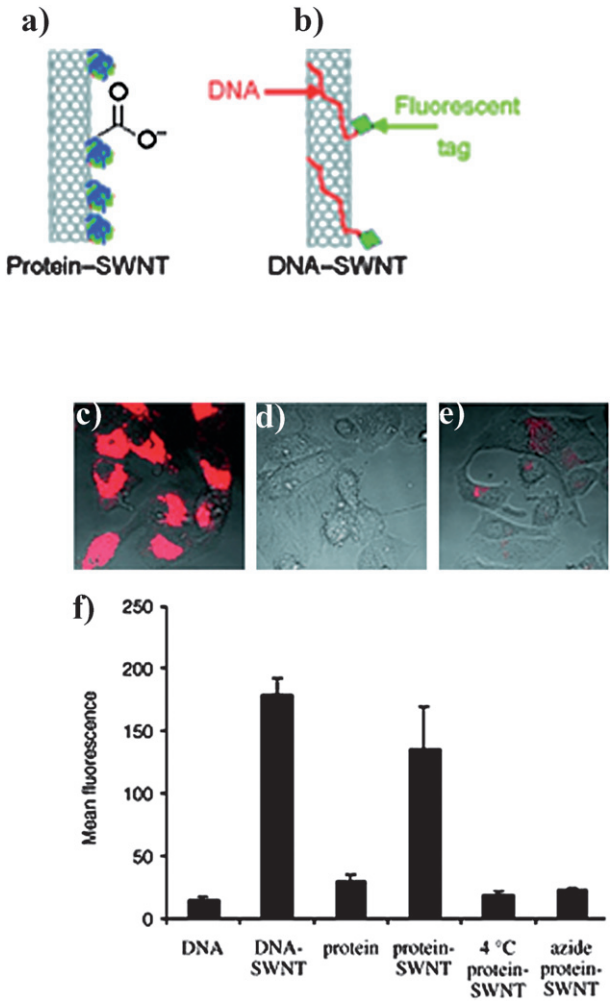


Fig. 8 Schematic representation of (a) protein-SWNT and (b) DNA-SWNT and confocal microscopy images of HeLa cells after incubation in fluorescently labeled DNA-SWNT at (c) 37 °C, (d) 4 °C, and (e) after pretreatment with NaN_3. (f) Flow cell cytometry data for HL60 cells that were incubated in fluorescently labeled pure DNA or protein solutions without nanotubes, DNA-SWNT and SA-SWNT at 37 °C, and for protein-SWNT at 37 °C in cells incubated at 48C or pretreated with sodium azide (reproduced with permission from ref. 149).

However, the groups of Kostarelos, Bianco, and Prato proposed a different translocation mechanism termed the *nanoneedle mechanism*.[54] In order to examine key steps in the cellular uptake process, Kostarelos *et al.* functionalized both SWNTs and MWNTs with a wide range of molecules and bioactive species, including ammonium, small molecule fluorescent probes, anticancer drugs, and antibiotics.[170] All of these functionalized nanotubes were found to be taken up by a wide variety of cells and intracellularly trafficked through different cellular barriers to the perinuclear region, even under endocytosis-inhibiting conditions (Fig. 9). They suggested that CNTs act as nanoneedles to passively pierce or penetrate membranes of many different types of cells. The molecular simulation results also seemed consistent with the hypothesis of the nanoneedle mechanism.[171]

It is possible that some of the debate on the cellular uptake mechanism might be due to different molecular loadings on the CNTs and/or different experimental procedures.[161,170] Dai's proposal is mainly based on the observation of large biomolecules solubilizing CNTs, while Kostarelos *et al.*'s suggestion is more suitable for CNTs covalently attached with small molecules. Nevertheless, an improved understanding of the translocation mechanism is critical for the further development of CNT-based delivery systems for applications *in vitro* and *in vivo*.

There has been another CNT-based delivery called "nano-injector", which went through the cell membrane *via* physical insertion.[172] For instance, for the delivery of fluorescent quantum dots, biotinylated pyrene (*via* a disulfide linker) was attached to MWNT as an AFM tip. The biotin moieties on the tip were readily conjugated with strepavidin-coated QDs. The nano-injection by the MWNT could deliver the QDs into a specific cell and release it *via* the cleavage of the disulfide linker. A major advantage of this approach is that the delivery-release process could be repeated many times without cell damage.

3.3. Biosensors—SWNT field effect transistor

The peculiar electrical properties of CNTs and their sensitivity to changes in the surrounding environment have made CNTs ideal components in chemo-/biosensors. Electrochemical sensors and field effect transistor (FET) based sensors have both been developed and fabricated in different configurations and mechanisms.[173–175] Here we focus on the FET-based sensors because various functionalized CNTs are used for different specific sensing functions.

In general, the FET is constructed by a substrate (gate), two microelectrodes (source and drain), and a SWNT (or SWNT network) bridging the electrodes (Fig. 10).[176] The functions of the transistors are associated with their diffusive electron transport properties. The current flow in a SWNT FET is extremely sensitive to the substance adsorption or other related events on which the sensing is based. For example, once biological macromolecules bind to the nanotube, a change in the charge state perturbs the current flow in the nanotube, thus producing detectable signals for sensing. The SWNT can be functionalized

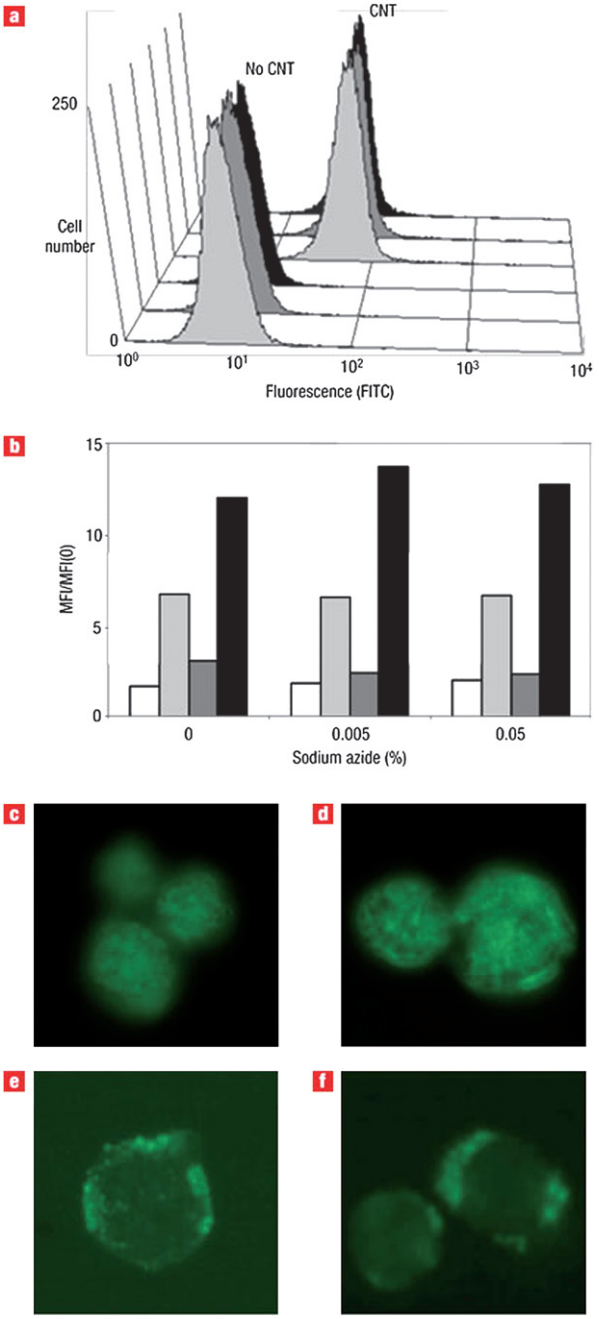


Fig. 9 Internalization under endocytosis-inhibiting conditions. (a) Flow cell cytometry histograms after interaction of Jurkat cells for 1.5 h at 37 °C with CNT 5 (5 μg ml^{-1}) following incubation of cells with different doses of NaN_3 in comparison to control cells treated with NaN_3 in the absence of CNTs. Light grey: 0% NaN_3; dark grey: 0.005% NaN_3; black: 0.05% NaN_3. (b) Flow cell cytometry data showing the ratios between mean fluorescent intensity (MFI) of cells with *f*-CNTs and MFI of control cells without *f*-CNTs, MFI(0) at different doses of NaN_3. White bar: *f*-CNT 5 at 0.5μg ml^{-1}; light grey bar: *f*-CNT 5 at 5μg ml^{-1}; dark grey bar: *f*-CNT 4 at 0.5μg ml^{-1}; black bar: *f*-CNT 4 at 5μg ml^{-1}. (c, d) Epifluorescence images of Jurkat cells incubated for 16 h at 37 °C (c) with *f*-CNT 4 (0.5μg ml^{-1}) and (d) with *f*-CNT 5 (5μg ml^{-1}) in the presence of NaN_3. (e, f) Epifluorescence images of Jurkat cells incubated for 1 h with *f*-CNT 6 (20μg ml^{-1}) (e) at 4 °C and (f) in the presence of NaN_3 (reproduced with permission from ref. 158).

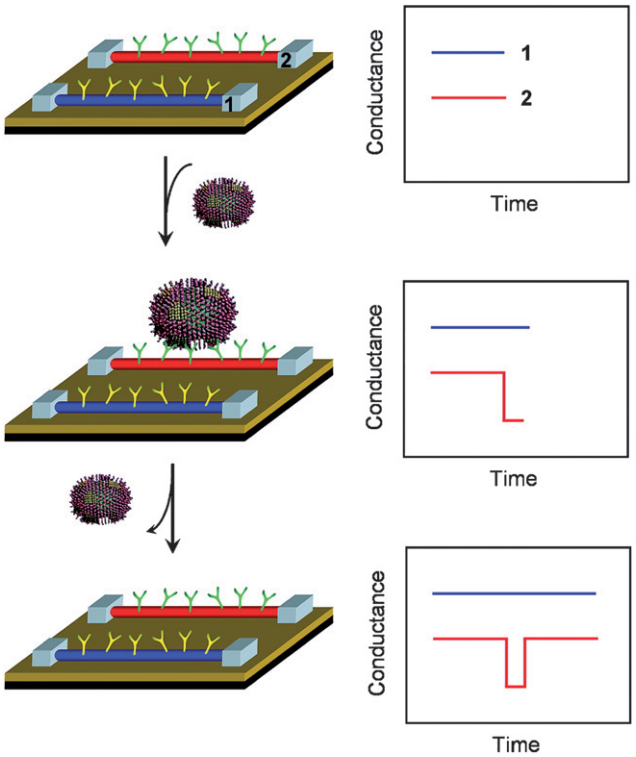


Fig. 10 Nanowire-based detection of single viruses. (Left) Schematic shows two nanowire devices, 1 and 2, where the nanowires are modified with different antibody receptors. Specific binding of a single virus to the receptors on nanowire 2 produces a conductance change (right) characteristic of the surface charge of the virus only in nanowire 2. When the virus unbinds from the surface the conductance returns to the baseline value (reproduced with permission from ref. 164, Copyright 2004 The National Academy of Sciences).

with different attachments for specific sensing. A wide variety of applications for SWNT-FET have been explored, including the detection of proteins, antibody-antigen interactions, DNA-DNA hybridization, and single nucleotide polymorphism.[174] The detection limit for the sensing of proteins or protein-protein interactions has generally been in the range of 100 pM to 100 nM.[177] Antibodies are often attached to SWNTs as specific targeting agents in FET for highly selective sensing.[178,179] Synthetic oligonucleotides, such as aptamers, have also been used in the specific detection of amino acids, proteins and small molecule drugs.[180] The detection limit is directly related to the binding efficiency between the attached sensing agents and the target molecules.

However, extraneous agents can also affect the detection limit. Star *et al.* fabricated SWNT-FETs as selective detectors for DNA hybridization.[181] The device with immobilized synthetic oligonucleotide specifically recognized the target DNA sequences, including H63D single nucleotide polymorphism discrimination in HFE gene (responsible for hereditary hemochromatosis). Upon addition of divalent cation Mg^{2+} there was a significant increase in the extent and overall efficiency of DNA hybridization on nanotubes, increasing the sensitivity by three orders of magnitude to push the detection limit down to 1 pM. In a similar approach, Gui *et al.* used two different metal contacts (Au and Cr) in SWNT-FET for electrical detection of DNA

hybridization.[182] The study of location-selective capping using photoresists provides comprehensive evidence that the sensing of DNA is dominated by the change in metal-SWCNT junctions rather than the channel conductance.

There are still unresolved issues in SWNT-FET for biosensing, especially with respect to localizing nanoscale contacts of SWNTs with bio-surfaces and improving the fabrication of devices with complex arrays of semiconducting SWNTs. Moreover, as-produced SWNTs are generally mixtures of metallic and semiconducting SWNTs. A good separation to remove metallic tubes and leave pure semiconducting nanotubes for FET is highly desirable. Finally, much remains to be explored on directly connecting these nanoelectronic devices to living cells for probing electronic responses in living systems.

3.4. Bioimaging with functionalized SWNTs

CNTs have attracted much attention in bioimaging due to their unique optical properties and the ability to cross cell membranes.[122,131,140,183–187] The majority of bioimaging studies with CNTs are categorized into two groups: the direct optical imaging relying on the intrinsic fluorescence of CNTs (including the band-gap emission of semiconducting SWNTs in the near-IR[188] and the visible emission[19] due to passivated surface defects in functionalized SWNTs and MWNTs), and indirect imaging based on nanotube-attached radioactive or fluorescent labels.

The band-gap fluorescence in individual semiconducting SWNTs was discovered by the group led by Weisman and Smalley.[188] For a defect-free SWNT with a diameter of ~1 nm, fluorescence emission could be observed in the near-IR spectral region of 900–1600 nm. Dai and coworkers used semiconducting SWNTs as near-IR fluorescent tags for selective probing of cell surface receptors and cell imaging.[187] The CNTs were dispersed noncovalently with the amine-terminated surfactant PL-PEG-NH_2, and the resulting nanotube-bound residual amine groups were conjugated with thiolated Rituxan (an antibody recognizing the CD20 cell surface receptor) and Herceptin (recognizing the HER2/neu receptor on certain breast cancer cells). In solution, emissions of these antibody-conjugated SWNTs were in the 1000–1600 nm spectral region (at 785 nm excitation). This demonstrates that the known near-IR fluorescence of semiconducting SWNTs was preserved after the noncovalent antibody conjugation. The fluorescence quantum yield was relatively low, but sufficient for the imaging experiments. Near-IR fluorescence was also used by other groups to study the uptake of pluronic surfactant-dispersed pristine SWNTs into macrophage-like cells and to image SWNTs in organisms and biological tissues of nanotube-fed *Drosophila* larvae. These studies demonstrated that near-IR fluorescent SWNTs could be used as effective probes for potential diagnostic applications.[137]

However, the band-gap fluorescence emission is sensitive to surface defects on the nanotubes, and might be quenched in oxidized or functionalized SWNTs.[184] Interestingly, both SWNTs and MWNTs with surface defects exhibit relatively strong photoluminescence upon chemical functionalization at the defect sites, and the emission is brighter with better functionalization.[189] The defect-derived photoluminescence is excitation wavelength dependent in the visible and extending into the near-IR region. Therefore, well functionalized carbon nanotubes

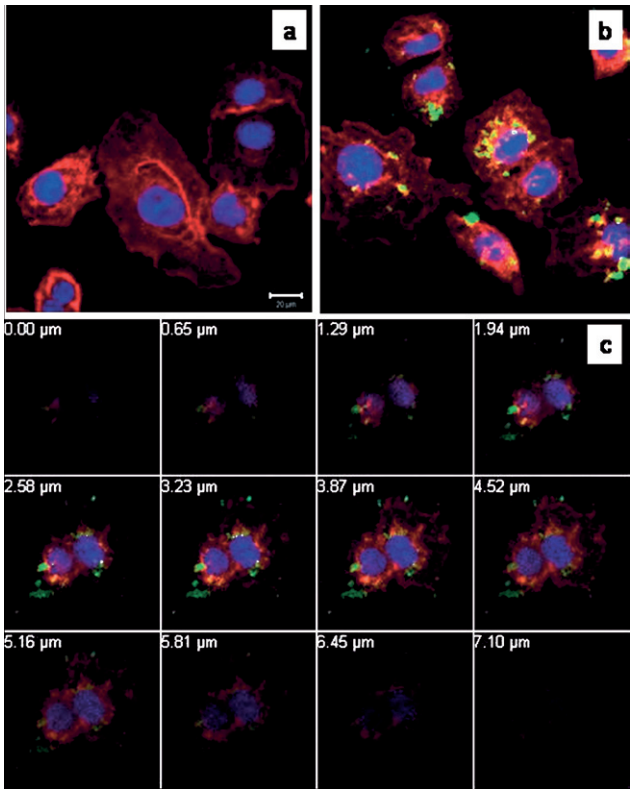


Fig. 11 Multiple stain confocal images of A549 cells (a) incubated for 2 h (37 °C, 5% CO_2) in the absence of SWNT-NH^{3+}s (control); (b) in the presence of 20 μg of SWNT-NH^{3+}s (scale bar: 20 μm); (c) *z*-stack imaging data obtained from the preparation where A549 cells were incubated with 20 μg of SWNT-NH3$^+$s. Cellular membranes are stained in red with WGA-TRITC and nuclei are counterstained in blue with TO-PRO 3 (reproduced with permission from ref. 170).

are also amenable to optical bioimaging applications. Lacerda *et al.* used SWNTs that were covalently linked with NH_3^+-terminated aliphatic appendages (SWNT-NH_3^+, emission peak at 485 nm with 395 nm excitation) in confocal laser scanning microscopy to visualize the interaction of SWNT-NH_3^+ with human caucasian lung carcinoma A549 cells (Fig. 11).[183] Intracellular and perinuclear localization of SWNT-NH_3^+ was observed, but there was no cell plasma membrane damage at a dose up to 500 μg mL^{-1} and 24 h post-incubation. This was the first report on the visible fluorescence imaging of SWNTs in cells without the need for large fluorescent labels attached to biological macromolecules. The imaging methodology may serve as a widely applicable tool for elucidating the intracellular transport mechanism of CNTs.

For indirect bioimaging with CNTs, CNTs are essentially carriers for the fluorescent labels. It is known that CNTs may quench fluorescence of the attached fluorophores. However, CNTs also serve as intracellular transporters and carry the fluorescent labels into cells, thus enabling the detection of fluorescence inside the cells.[185] Similarly, radioactive labels in combination with CNTs are equally effective in the studies of the translocation and distribution of CNT-conjugates. Porter *et al.* visualized individual SWNTs in cells through a new technique called low-loss energy-filtered transmission electron microscopy in combination with electron energy loss spectrum imaging.[140]

This technique made it possible to directly determine the distribution of SWNTs in both stained and unstained human cells. The cellular actions of the CNTs can be observed, such as their entering the cytoplasm, localizing within the cell nucleus, and causing cell mortality in a dose dependent manner.

4. Conclusion and perspectives

The past two decades have witnessed the birth and subsequent tremendous development of the chemistry of CNTs. This new branch of chemistry has produced continuous advances and many novel materials. We discussed several tens of chemical reactions that have been demonstrated for the surface functionalization of CNTs (Fig. 1, 2, and 3) and their biomedical applications. The applications of this new chemistry have generated far-reaching influences on biosensing, biodelivery and bioimaging. However, the controlled functionalization of CNTs is still well out of reach. Improved purification techniques and new characterization methods are urgently required. Besides, chemical modification of the inner wall of CNTs is still unattainable at present, while theoretical investigations indicate many intriguing functions with inner-wall modification of CNTs.[190,191] Molecular dynamics simulations have shown that modification of the inner wall is crucial for controlling the functions of CNT-based nanodevices,[190,191] although experimental realization of this strategy is a new challenge for chemists.

In biomedical applications, the safety issue is of highest importance. The nanotoxicity of CNTs is currently a topic of controversy. Further systematic researches under comparable experimental conditions (such as CNT length, diameters, mass/particle/concentration, defective/reactive sites, media, surface/functionalization/absorption, aggregation/agglomeration states, metallic impurities, cell lines, animals, administration routes, endpoint of the test, and experimental protocols, *etc*.) are required to resolve the safety controversy of CNTs. This makes things somewhat complicated, as there might be a large body of experimental work to do. Nevertheless, scientists are accumulating toxicological data of pristine and functionalized CNTs *in vivo* and trying to establish a standardized framework to assess the toxicity of various forms of CNTs. Metal impurities in CNT samples are believed to contribute to the differences in observed cytotoxicity and the contradictive results obtained from different experiments. Previously, there was no analytical method for quantifying the metal impurities in CNTs. Recently, the protocol developed by Ge *et al.* used the neutron activation analysis (NAA) technique as a nondestructive standard quantification method and inductively coupled plasma mass spectrometry (ICP-MS) as a practical approach was recommended as the standard method of metal impurity analysis in CNTs.[155] This practically useful method with proper sample pretreatment may help evaluate sample purity for future toxicity studies of CNTs and thus eliminate the contradictions caused by metal impurities.

Most biomedical uses of CNTs need outer- or inner-surface chemistry for the purposes of water-solublization, good biocompatibility, low toxicity, and the control of biomedical functions. With the continuing worldwide efforts being carried out in many laboratories for the development of CNT chemistry, we hope to achieve full control of size, shape, and functionalization of CNTs. On the other hand, new methodology for controlling and detecting the aggregation or de-aggregation states of CNTs in the environment of biological systems will be of particular importance for ultimately clarifying how CNTs or their functionalized forms work *in vivo*. In the meanwhile, toxicological studies are being conducted to clarify biosafety concerns of using many types of advanced CNT-based products in biological systems. These achievements together will certainly lead to safe and more precise and manipulable applications of functionalized CNTs in not only biomedical fields but also many other areas.

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