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Are Carbon Nanotubes a Natural Solution? Applications in Biology and Medicine

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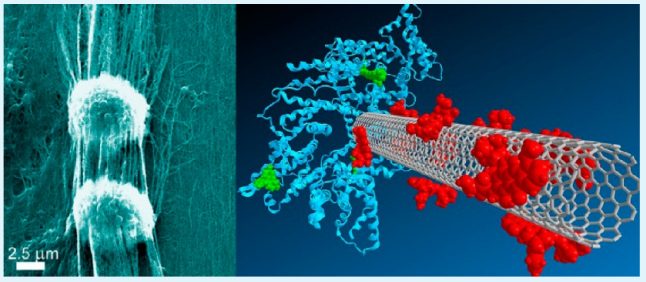
ABSTRACT: Carbon nanotubes and materials based on carbon nanotubes have many perceived applications in the field of biomedicine. Several highly promising examples have been highlighted in the literature, ranging from their use as growth substrates or tissue scaffolds to acting as intracellular transporters for various therapeutic and diagnostic agents. In addition, carbon nanotubes have a strong optical absorption in the near-infrared region (in which tissue is transparent), which enables their use for biological imaging applications and photothermal ablation of tumors. Although these advances are potentially game-changing, excitement must be tempered somewhat as several bottlenecks exist. Carbon nanotube-based technologies ultimately have to compete with and out-perform existing technologies in terms of performance and price. Moreover, issues have been highlighted relating to toxicity, which presents an obstacle for the transition from preclinical to clinical use. Although many studies have suggested that well-functionalized carbon nanotubes appear to be safe to the treated animals, mainly rodents, long-term toxicity issues remains to be elucidated. In this report, we systematically highlight some of the most promising biomedical application areas of carbon nanotubes and review the interaction of carbon nanotubes with cultured cells and living organisms with a particular focus on in vivo biodistribution and potential adverse health effects. To conclude, future challenges and prospects of carbon nanotubes for biomedical applications will be addressed.



KEYWORDS: *carbon nanotubes, biomedical applications, tissue engineering, drug delivery, bioimaging, toxicity,*

INTRODUCTION

Individual carbon nanotubes (CNTs) and materials based on CNTs have been widely investigated for a diverse array of applications.[1] In recent years, focus on CNTs has grown to include their possible biological applications, such as drug delivery transporters, selective cell destruction agents, biosensors, cellular growth substrates, and prosthetic implant materials (Figure 1). Several physical properties,[2] including high surface area, nanoscopic dimensions, a rigid platform structure, good electrical conductivity, and excellent as well as tunable mechanical properties, make them appealing for such diversified biological end goals. In addition to these intrinsic properties, CNTs can be derivatized with various chemical or biological molecules to further tailor them for specific aims. These derivatization schemes can be accomplished through covalent chemical functionalization or through physical interactions with hydrophobic and aromatic regions of biological molecules.[3−5] Furthermore, CNTs have demonstrated the ability to transverse cellular membranes at high efficiencies while carrying an assortment of cargos that retain their biological activities without incurring acute toxicity to most cell types, rendering them highly applicable for intracellular sensing and delivery applications.

Despite this unquestioned potential, there are many issues that must be resolved. In particular, there are contradictory opinions in the literature relating to internalization mechanisms and toxicity. These contradictions undoubtedly arise as a result of variations in synthesis and preparation methods used. Thus, as will be highlighted in this report, it is absolutely essential for

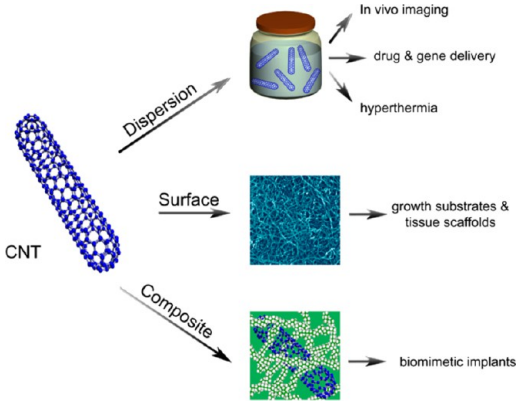


Figure 1. Different forms of carbon nanotubes and related biomedical applications.

Received: November 29, 2012
Accepted: February 21, 2013
Published: February 21, 2013

researchers to pay close attention to preparative methods. Second, many of the most popular methods to produce CNTs involve the use of nanosized catalyst particles during the production process which themselves may have detrimental effects on natural systems. Other problems also exist. Pristine CNTs are hydrophobic in nature and are found in mesoscopic aggregates as a result of intertube van der Waals attraction. As a result, the solubility in an aqueous environment is low and processing is thus cumbersome. However, these problems may be overcome through direct chemical functionalization or through physical interaction with an amphiphilic agent. However, chemical functionalization often affects the electrical and optical properties of CNTs by destroying/disrupting sp^2 conjugation of the carbon lattice. Additionally, CNTs when synthesized and prepared by various methods contain defect sites or possess biopersistent lengths, which trigger toxic responses somewhat complicating their application in biological environments. These shortcomings should not be viewed as death nail for bioapplications, however, as several processing remedies exist for these shortcomings. In this report, we highlight some of the application areas where CNTs have shown real promise. These are wide and varied, ranging from tissue scaffolds to drug delivery vehicles. From a materials science perspective, there is much cause to be optimistic. There are several examples in the literature where nanotubes offer unique and competitive alternatives to present technologies. However, optimism must be tempered as there are several issues that must be dealt with, not least, the issue of toxicity.

CARBON NANOTUBES AS CELLULAR GROWTH SUBSTRATES AND TISSUE SCAFFOLDS

Cell−substratum interactions rely heavily upon topological and chemical cues, and although it is well-established that microscale topographies and patterns influence cellular behaviors[6,7] only recently have nanoscale features and their effects been examined.[8] To date, variations in nanoscopic surface characteristics such as roughness,[9] architecture,[10] and elastic modulus[11] demonstrate alterations to a variety of cellular behaviors.[12] New insights and control over the nanoscale topography of cellular substrates will optimize medical implantation devices and will facilitate new investigations into biological processes, including embryogenesis, angiogenesis, and pathological conditions.[13] Nanotextured surfaces can be created by an assortment of methods, including, but not limited to, electron beam lithography,[14] ion beam lithography,[15] electrochemical etching,[16] electrospinning,[17] nanoparticle adhesion,[18] laser ablation,[19] molecular self-assembly,[20] and polymer phase separation.[21] Macromolecules and nanoparticles are inherently suited for nanoscale surface modifications by simple incorporation within or deposition onto a material's surface.[22] Among these nanomaterials are CNTs, which have a fibrillar shape and an array of versatile optical, electrical, and mechanical characteristics, opening new dimensions for cellular substratum applications. The depth and complexity of cell−substratum interactions should not be underestimated, as explanations for several of these biological mechanisms remain elusive; however, fundamental understanding of cellular responses to artificially designed surfaces begins with a look into naturally occurring cellular growth matrices.

CELL−SUBSTRATUM INTERACTIONS

In vivo mammalian tissue consists not only of cells, but also of an interconnecting meshwork of macromolecules constituting the "extracellular matrix" (ECM). The two principal classes of extracellular macromolecules include polysaccharide chains, such as glycosaminoglycans, and fibrous proteins, such as collagen, elastin, fibronectin, and laminin.[23] These polysaccharides and proteins vary widely in their monomer composition, surface charge, structural orientation, size, and occupied volume, allowing different bodily tissues to adopt specific functions, for instance transparent shielding for the cornea, calcified robustness necessary for teeth and bones, and molecule-specific filtration for the kidneys. Generally, these macromolecules possess molecular weights ranging from tens of thousands to up to several million Daltons and can adopt fibrillar, globular, or gelatinous phase structures. They interact with adjoining cells in a multitude of ways and are by and large, depending upon the tissue, neither a simple structural support for cell anchorage dependence nor a static fixture.[24]

In several types of tissues, interactions between cells and the ECM sway if not completely govern a series of cellular behaviors and attributes, including survival, adhesion, proliferation, migration, differentiation, development, metabolism, morphology, and orientation.[25,26] Elegant mechanisms for the interplay between cells and their surrounding ECM come into play and highlight the importance of selecting a substratum material both adapted and tailored for a multitude of cellular interactions. For instance, certain cell types employ contractile, cytoskeletal actin and myosin filaments in conjunction with bound integrin transmembrane adhesion proteins to exert tensile stress upon fibronectin fibrils attached to the exterior of the cellular membrane.[27] The exerted stress along the fibronectin axes aligns the fibronectin fibrils with the cytoskeletal filaments and exposes periodically spaced binding sites for other fibronectin molecules, ECM proteins, and cells. Thus, the intracellular cytoskeleton can influence the assembly, organization, and alignment of the surrounding ECM;[28] however, the relationship between extra- and intracellular organizational proteins is a two-way street, as the ECM can in turn orientate cellular morphology in an anisotropic manner.[29,30] In terms of applications, certain implantation grafts, such as cardiac, muscular, or vascular tissues, must perform under load or fluid shear stress, as well as match the elasticity of surrounding tissue, and alignment of cells by an underlying anisotropic architecture has increased the ultimate tensile stress of such an implant several-fold by imparting the necessary mechanical strength.[31] Any material tailored for a specific cellular response by offering a patterned surface—parallel arrays in this example—must nonetheless accommodate several other requisites associated with viable cellular substrata. Any material that adds only one piece to the jigsaw of medical implantation device requirements will never see preclinical trials, but CNTs are not restricted to their as-produced form and are easily adapted to remedy one or many obstacles by functionalization with molecular accessories. Underlying these adaptations remains a rigid starting platform with a high aspect ratio and fibrillar shape scaling with the physical dimensions of several of the basic biological components naturally occurring within the ECM.

UNREFINED CARBON NANOTUBES AS CELL SUBSTRATES

CNTs provide an additional, fundamental advantage for replicating cellular environments owing to their ability to form a porous meshwork with adjustable pore diameter distributions.[32] Many reports have successfully demonstrated proof-of-principle experiments emphasizing CNT utility for cellular growth surfaces to provide structural reinforcement or confer novel properties.[33] For example, a variety of cell phenotypes were reported to have high binding affinities for CNT surfaces, indicating the range of tissue implantation devices or novel substrata for which CNTs may prove advantageous. As a first example, thin films of CNTs increased adherence, proliferation, and improved cell−cell communications of mouse fibroblast cells compared to cell culture plates, polyurethane, or carbon fibers.[34] Second, mouse fibroblasts grown on top of vertically aligned multiwall carbon nanotube (MWNT) arrays showed a 20% increase than those grown on titanium.[35] Third, a CNT coating overlaid upon silicone promoted adherence and proliferation of Saos-2 cells (osteoblast-like cells), which was virtually nonexistent on bare silicon,[36] and last but not least, another study reported massive Saos-2 cell spreading and proliferation in conjunction with numerous filopodia attachments to such an extent that standard enzymatic treatment with trypsin-EDTA could not detach the cells from the CNT surface.[37] Neuronal cells also exhibited preferential adherence to CNTs by migrating off the surrounding SiO_2 surface onto the CNT fabricated islands.[38,39] A study by Sorkin and co-workers showed the neuronal processes entangled themselves within the pores of the same CNT fabricated islands, suggesting that additional mechanism may play a role in cell adhesion onto CNT surfaces.[40] Tutak and co-workers have proposed an adhesion mechanism whereby cells partially absorb the single-wall carbon nanotubes (SWNTs) of the substrate in a manner similar to endocytosis, which results in acute toxicity resulting in a boost to ECM production by surviving cells.[41] Ryoo et al. discovered that fibroblasts grown on supported thin films of graphene and CNTs allow for enhanced genetic transfection of the cells at low cell densities, although the detailed mechanism remains unknown.[42] As reported in the above studies, typically CNT surfaces received little postsynthesis processing and no advanced modifications to enhance cellular adherence, underscoring the innate suitability of CNTs for supporting cellular growth.

FUNCTIONALIZED CARBON NANOTUBES AS CELL SUBSTRATES

CNT surfaces can be functionalized with a variety of molecular accessories to enhance cellular viability or function. Neurons require substrata highly permissive to axon and neurite extension, and these neural processes can be enhanced or diminished depending upon the underlying surface chemistry. Simply coating the CNT sidewall by physisorption with the bioactive molecule 4-hydroxynonenal more than doubled average neurite length, number of neurites per cell, and branches per neurite compared to the uncoated CNT surface.[43] Chemical treatment of MWNTs prepared in one study produced MWNTs with carboxylic groups, poly(aminobenzene sulfonic acid) (PABS), or ethylenediamine imparting negative, zwitterionic, and positive surface charges, respectively. Neurons grown on the positively charged CNT surface possessed longer neurites and more branching than neurons grown on negatively or neutrally charged surfaces.[44] Surfaces fabricated from SWNTs wrapped with various polysaccharides were used to study the overall the effects that charge and functional groups and found that positively charged surfaces with hydroxyl groups significantly improve cell growth.[45] Modern bone and dental implants are often coated with hydroxyapatite to facilitate structural and functional connections between the tissue and load-bearing implant. In one study, CNTs functionalized with different types of negatively charged organic groups induced hydroxyapatite crystallization in varying amounts with one type of functionalization aligning the platelike hydroxyapatite crystals along the CNT axes.[46] In addition to forming sufficient amounts of hydroxyapatite for artificial bone materials, the study shows that CNTs can be functionalized in multiple steps and fashions before use in application. Besides creating scaffolds for specific cell types, attempts have also been made to create CNT composites that mimick the ECM. Liao and co-workers have fabricated MWNT-incorporated polyvinylalcohol (PVA)/chitosan nanofibers with improved protein adsorption ability, which significantly promoted cell proliferation of mouse fibroblasts,[47] while Lin et al. produced a biodegradable poly(lactic-co-glycolic acid)-MWNT composite to grow mesenchymal stem cells.[48] Similarly, Abarrategi et al. created a MWNT/chitosan scaffold with a well-defined microchannel porous structure as a biocompatible and biodegradable support for stem cell tissue engineering purposes.[49]

TWO-DIMENSIONALLY (2D) ALIGNED CARBON NANOTUBES AS CELL SUBTRATES

CNTs lying within a plane can be produced in highly or semialigned arrays to afford an anisotropic surface. Surfaces made from highly aligned MWNT sheets drawn from MWNT growth forests following Chemical Vapor Deposition (CVD) synthesis have supported long-term growth on assorted cell types including fibroblasts (shown in Figure 2), Schwann cells,

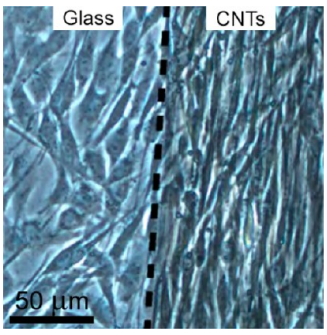


Figure 2. Human skin fibroblasts growing isotropically on glass and aligned in one direction on well-aligned MWNT arrays. Reprinted with permission from ref 50. Copyright 2007 Brill.

and primary neural cells.[50] The parallel-lying MWNTs induced cytoskeletal orientation and increased motility of fibroblast cells, promoted adhesion and axon extension of dorsal root ganglia cells, increased axonal length, and directed the outgrowth of the neuronal growth cone responsible for steering the direction of axonal growth. Highly aligned yarns drawn from these sheets also demonstrated similar cellular effects giving rise to potential implant devices for ligamentous skeletal or muscular tissue. Longer term application targets would be to utilize CNT based actuators (artificial muscles) for prosthetic

devices.[51,52] Demonstrated performance for actuators, using polymer-free nanotube fibres obtained by thermally annealing coagulation spun fiber composites, includes a force generation capability that is a hundred times that of the same size natural muscle, and twice the response rate.

In our own lab, we have grown Chinese hamster ovary (CHO) cells on isotropic, aligned, and patterned MWNT substrates and showed that the cells not only grew on all substrates, but also aligned strongly with the axis of the MWNT bundles on the aligned substrate.[53] Whereas our study featured uncoated MWNTs, Namgung and co-workers used fibronectin-CNT composites to demonstrate controlled adhesion and growth of varios cells, including selective filopodial growth of human mesenchymal stem cells (hMSCs).[54]

THREE-DIMENSIONALLY (3D) SHAPED CARBON NANOTUBES AS CELL SUBTRATES

CNTs substrata need not be thought of in only 2D terms, as different methods have created variations in the microscale roughness of CNT surfaces. Convex and concave structures can be formed by acid treatment of perpendicularly aligned MWNT forests. The concave structures formed pits, which varied in diameter depending upon the length to which the MWNTs were grown. These substrates acted as a cell-seeding device with adjustable dimensions that promoted extensive growth, spreading, and adhesion of mouse fibroblasts.[55] Correa-Duarte et al. coated polystyrene particles with functionalized MWNTs imparting a nanotextured surface to microscale polymer beads, which could be produced with different diameters.[56] Firkowska and co-workers were able to create free-standing 3D hexagonally shaped networks by CNT incorporation within the polystyrene interstitial sites and subsequent removal of the polystyrene by dissolution in tetrahydrofuran (THF) and a reactive ion etching process.[57] Dionigi and co-workers expanded on this 3D hexagonal structure by applying a voltage across the substrate to increase neuronal adhesion.[58] The porosity of these hexagonal structures could be easily tuned by the adjusting the diameter of the polystyrene beads. Smooth muscle cells were incorporated into a 3D collagen-CNT matrix gel, whose overall mechanical properties were slightly improved by the CNTs by delaying gel compaction.[59] Da Silva et al. fabricated a nanostructured 3D collagen/nanotube composite for future bone regeneration scaffolds, which was bioresorbable and biodegradable, had the desired mechanical rigidity, and induced mineralization of hydroxyapatite crystals in vitro.[60] Zhang et al. demonstrated that MWNTs can provide nanotopagraphy on polyethylene terephthalate (PET) fibrous matrices and significantly improve their performance as 3D tissue scaffolds resulting in enhanced neuronal differentiation of mouse embryonic stem (mES) cells.[61] These instances demonstrate that CNTs and their advantages for 3D cell substrata may act in combination with their mechanical strength, flexibility, and low density to simultaneously resolve many drawbacks commonly encountered with implantation devices.

CARBON NANOTUBES AS NEURAL INTERFACES

Specialized cell types, such as neurons, have seen novel uses of CNT substrata or have specific requirements setting them apart from generic cellular applications. Several papers have reported CNTs to be conducive to neuronal adhesion and permissive to neural processes outgrowth, suggesting that CNT are fundamentally biocompatible with neurons. The innate suitability of CNTs proposed by Cellot and co-workers is hypothesized by CNTs providing a shortcut for electrical signaling between tight junctions adhered onto the nanotube surface at proximal and distal portions of the neuron.[62] Neural compatibility in combination with their electrical and mechanical properties constitutes CNTs as a candidate for neural prosthesis and interfacing. The mere presence of CNTs below neuronal cells has induced higher frequencies of synaptic transmissions compared to glass coverslips suggesting that CNTs boost neuronal signaling,[63] and layer-by-layer assembled films of a SWNT−poly(ethyleneimine) complex demonstrated equal viability with neural stem cells to poly-L-ornithine, one of the most commonly used growth substrates for neural stem cells.[64] These layer-by-layer films also promoted neural stem cell differentiation into mature neurons, astrocytes, and oligodendrocytes. Mature neuronal cells have also been derived directly from hESCs using polymer-grafted CNT thin film scaffolds.[65] Malarkey and co-workers were able to modulate neuron morphology, neurite outgrowth, and the number of growth cones by modulating the thickness and subsequently the conductivity of the film.[66] Although neuronal interfacing may have no immediate clinical benefits, improvements in this area may elucidate biological mechanisms and neural interactions relevant to injury and disease. Preferential adhesion of neural cells for CNTs allows for the study of axonal outgrowth and connection between neural clusters spaced distances apart on patterned CNT islands[67] as well as directed growth and migration along CNT surface architectures.[68]

More relevant toward the clinic is the prevention and repair of nerve injuries, such as spinal cord injury or stroke. Lee and co-workers have pretreated rats with amine-modified SWNTs to protect neurons and enhance the recovery of behavioral functions in rats with induced stroke through reduced apoptosis, inflammation and glial cell activation.[69] The amination, positive charge, and high surface-energy of the amine-modified SWNTs also may have contributed to a favorable environment for neurons. Roman et al. investigated whether administration of PEGylated SWNTs after traumatic neural cord injury could promote regeneration of axons into the lesion cavity and functional recovery of the hindlimbs.[70] They found that neurofilament-positive fibers and corticospinal tract fibers grew back into the lesion without increasing reactive gliosis. Furthermore, a modest improvement in hindlimb locomotor recovery was observed.

CARBON NANOTUBES AS BONE PROSTHETICS

Neurons are not the only cells for which CNTs are fundamentally suited, as CNTs are envisioned materials for permanent bone or dental implanted prosthetics. Compared to titanium, a leading material for bone prosthetics, CNTs are stronger,[71] lighter,[72] and have excellent flexibility, as well as the potential to form surfaces that naturally mimic an ECM morphology with the capability to facilitate hydroxyapatite crystallization. Additionally, bone cell proliferation and viability have been reported for several CNT surfaces fabricated through varying methods.[73] Increased Young's modulus of human osteoblasts grown on patterned MWNT suggest tight junctions between the osteoblasts and MWNTs indicative of enhanced osteoblast compatibility.[74] Saos-2 cells demonstrated higher metabolic activity on small-diameter CNT surfaces compared to the control surfaces (highly ordered pyrolytic graphite) or large-diameter CNT surfaces, although cells on any of the CNT

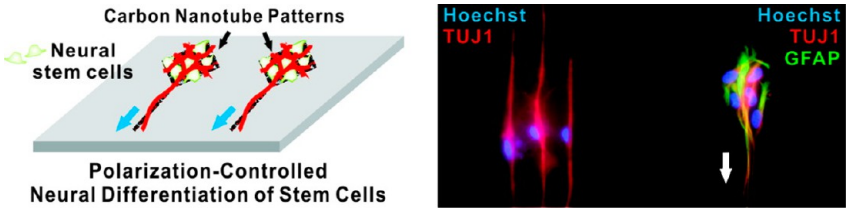


Figure 3. Control of neural stem cell (NSC) orientation and differentiation using line shape CNT patterns. Left: Schematic diagram showing CNT monolayer patters fabricated on a substrate with laminin was absorbed selectively on the CNT-coated regions. This induced preferential adhesion of NSCs, finally achieving structural polarization-controlled neuronal differentiation as shown in the immunofluorescence images of the differentiated cells on the right (Astroglial cells (GFAP) in green, differentiated neuronal cells (TUJ1) in red, Hoechst nuclear stain in blue). Reprinted with permission from ref 84. Copyright 2011 American Chemical Society.

surfaces displayed a disorganized cytoskeleton.[75] This negative correlation between CNT diameter and metabolic activity may be due to smaller-diameter tubes having dimensions closer to naturally occurring Type I collagen fibrils. As with other cell types, patterned CNT lanes coated with fibronectin demonstrated orientational alignment of osteoblast-like cells, which may significantly increase the overall mechanical strength of a bone implantation prosthetic.[76] Electrical stimulation of osteoblast cells may not seem intuitive for any practical advantage, but exposure to AC currents increased bone cell proliferation and extracellular calcium production of osteoblasts grown on CNT-polylactic acid composites, demonstrating application for accelerated bone repair.[77] The use of CNTs in these in vitro experiments of osteoblast and Saos-2 cells exemplifies novel and alternative strategies arising from a newly investigated material for the tissue engineering field.

CARBON NANOTUBES FOR STEM CELL DIFFERENTIATION

Aligned and nonaligned CNT substrata have further relevance in stem cell technology, as slight variations in growing adult and embryonic stem cells can have profound effects over self-renewal and differentiation behaviors[78−80] Sridharan and co-workers aligned Type I collagen and collagen-dispersed CNTs along a plastic surface by dip-coating slides into solutions and studied the differentiation responses of cultured human embryonic stem cells.[81] While cells on the aligned collagen and collagen-CNTs matrices displayed phenotypic morphologies of ectodermal differentiated lineages in contrast to the control, remarkably higher levels of an early neural progenitor marker were expressed on the collagen-CNT matrix alone. Semialigned patterns of CNTs have also been constructed by deposition between patterned self-assembled monolayers of a masking agent. Mesenchymal stem cells grown on these SWNT monolayers of aligned SWNTs demonstrated orientation, stretching, and directed growth.[82] Nayak et al. demonstrated how a thin film of nonaligned, PEGylated MWNTs influenced proliferation and morphology of hMSCs and led to their final differentiation into osteoblasts even in the absence of biochemical inducing agents.[83] Similarly, Park and co-workers achieved selective growth and structural polarization-controlled differentiation of human neural stem cells (hNSCs) into neurons using CNT network patterns (Figure 3).[84] However, carboxylated CNTs have also been reported to inhibit proliferation, oseoteogenic/adipogenic differentiation, and mineralization of multipotent MSCs.[85]

Overall, CNTs and CNT composite materials are likely to gain importance as in vivo or ex vivo tissue scaffolds or cell substrates in the near future due to their superior characteristics over other biomaterials. Nonetheless, this requires their effects on cells and living tissue to be fully understood; an area where many questions are still unanswered.

CARBON NANOTUBES AS A DELIVERY SYSTEM FOR BIOLOGICAL AND BIOMEDICAL CARGOS

As soon as it became apparent that CNTs are able to transverse cellular membranes, a new area of application was launched: the use of CNTs as intracellular transporters. CNTs can immobilize biological or biomedical molecules on their surface or in the hollow cavity and have therefore been applied in numerous studies to transport a broad spectrum of molecules into mammalian cells, including peptides, proteins, DNA, immunogenic molecules, and drugs.[86−90] Early studies have loaded CNTs with peptides and proteins as model cargos, whereas more recently a trend toward targeted gene and drug delivery seems to emerge. The next sections will discuss the interaction of CNTs with mammalian cells with a special focus on uptake mechanism and intracellular distribution and will then highlight key studies on CNT-mediated gene and drug delivery.

INTERACTION OF CARBON NANOTUBES WITH CELLS: UPTAKE MECHANISM AND INTRACELLULAR DISTRIBUTION

The application of CNTs as intracellular transporters requires a good understanding of their interaction with mammalian cells, especially with regard to uptake mechanism, intracellular distribution, elimination from cells and possible adverse effects. The first study undertaken in this direction, which was also the first to demonstrate the translocation of CNTs across cellular membranes, was published in 2004 by Pantarotto and co-workers.[86] Therein, water-soluble, amino-functionalized SWNTs were conjugated to a fluorescent dye via a short organic linker or a peptide. Both conjugates were internalized by two different cell lines—however, the peptide-SWNTs were found to accumulate in the nuclei of the cells, whereas the directly labeled SWNTs distributed in the cytoplasm. Because internalization was not affected by temperature or the presence of endocytosis inhibitors, the authors claimed that the uptake mechanism was endocytosis-independent and hypothesized that the cylindrical shape and high aspect ratio of functionalized CNTs allowed their penetration through the plasma membrane similar to a "nanosyringe". A study carried out shortly afterward by Kam et al. also demonstrated cellular uptake of SWNTs, this time functionalized with a fluorescent dye or a fluorescently labeled protein.[91] In contrast to the previous study, their results suggested an endocytic uptake mechanism, since no uptake was observed at low temperatures inhibiting endocytosis and nanotubes were found to colocalize with red-stained endo-

somes. Furthermore, both types of nanotubes were only observed in the cytoplasm, but not in the nucleus.

These two studies started the controversy about the internalization mechanism of CNTs and their fate inside cells, which has still not been fully resolved to date. Intracellular distribution and uptake mechanism of applied CNT formulations differ widely from one study to the next. This indicates that the interaction of CNTs with mammalian cells very likely depends on the physical and chemical properties of the nanotubes, as well as on the type of functionalization. Particularly in the case of noncovalent functionalization methods, possible interactions of the attached surfactants or biomolecules with plasma proteins in cellular growth media might alter the interaction with cells significantly. Since the uptake mechanism determines the fate of drug or gene-loaded CNT conjugates inside cells, it is hence of utmost importance for delivery applications. In the case of an endocytic uptake pathway, CNT conjugates are initially enclosed inside intracellular vesicles, so-called endosomes, which are located in the periphery of the cells and have a mildly acidic pH. In the next step of this pathway, fusion of the endosomes with lysosomes causes a drop in the endosomal pH to approximately 5.5 and incorporation of hydrolytic enzymes into the vesicles. These conditions can degrade drugs and nucleic acids and therefore, the CNT conjugates need to escape the lysosomes in order to maintain the effectiveness of the therapeutic entity. This is often achieved by pH-dependent binding of the cargo to CNTs and will be explained in more detail in a later section of this article. The nonendocytotic, needle-like uptake pathway, however, transports the cargo directly into the cytoplasm and hence avoids lysosomal degradation. This is a clear advantage for delivery applications; however, the nanotubes might be able to pierce other intracellular membranes in a similar way, e.g. those of mitochondria, and hence, their fate after delivery of the cargo and the mechanism of elimination needs to be well understood. All in all, much more work is needed to understand the interaction of CNTs with mammalian cells, particularly taking into account the different shapes, surface properties, and functionalization schemes of the applied CNT vectors, and the effect of the type of cells used. Some recent studies have succeeded in shedding a little more light upon these issues. Mu et al.[92] investigated the cellular uptake and fate of MWNTs by transmission electron microscopy (TEM) and developed a model, in which the nanotubes are divided into two classes, clusters and single tubes. According to their findings, clusters are taken up by cells through energy-dependent endocytotic processes, whereas single nanotubes enter cells though direct membrane permeation. Furthermore, nanotube bundles trapped in endosomes were observed to release single nanotubes, which thereupon escaped the endosomes by penetrating the endosomal membrance and entered the cytoplasm (Figure 4). Most nanotubes, however, eventually ended up in lysosomes for excretion. A study by Raffa and co-workers supports these findings.[93] Mu et al. also evaluated the effect of surface charge on cell uptake by comparing carboxylated (−) with amine-terminated (+) nanotubes. Interestingly, no difference in cellular interactions was observed due heavy protein coating on both types of tubes, which led to compensation of the surface charges. This is supported by theoretical calculations by Pogodin et al., which suggest that the coating of "naked" CNTs by biomolecules commonly present in cell culture supernatants enhances the possibility of transduction through cell membranes.[94] These findings indicate

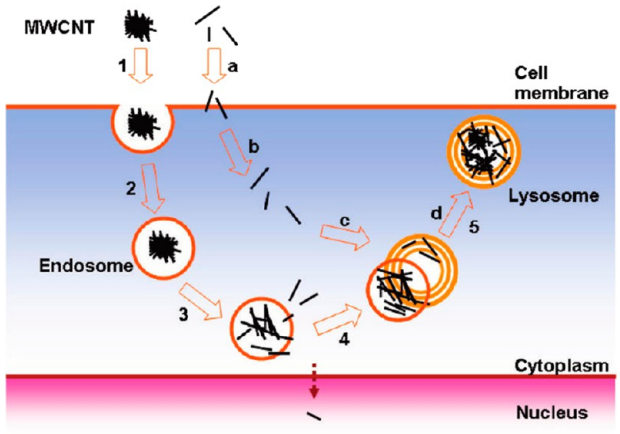


Figure 4. Model for cell uptake of MWNTs. Bundled MWNTs bind to cell membrane (1) and are subsequently internalized into cells by endocytosis (2). Inside endosomes bundles release single MWNTs, which penetrate the endosomal membrane and enter the cytoplasm (3). Both residual bundled MWNTs in endosomes and free MWNTs in the cytoplasm are recruited into lysosomes for excretion (4, 5). Single MWNTs enter cells through direct membrane permeation (a) and enter the cytoplasm (b), followed by excretion via lysosomes (c, d). Short MWNTs are also able to enter the nucleus. Reprinted with permission from ref 92. Copyright 2009 American Chemical Society.

that an appropriate functionalization scheme that reduces nonspecific bindings of biomolecules to CNTs and concomitantly ensures a good dispersion stability is crucial for controlling the interactions of nanotubes with cells, as also demonstrated in a recent study in our lab.[95]

Antonelli et al. investigated whether the uptake mechanism of CNTs is dependent on length.[96] They used functionalized SWNTs with phospholipids that mimick the cell surface to increase interaction with cellular membranes, which is an interesting approach considering that unfunctionalized SWNTs would otherwise be attracted by the hydrophobic cores of the phospholipid bilayer, thus hindering their translocation by simple thermal motion.[97] They found that uptake by phagocytotic cells occurs via an endocytotic mechanism for constructs larger than 400 nm, whereas constructs up to 400 nm diffuse through the cellular membrane and localize in the cytoplasm. This is an interesting result and in reasonable conformity with a study by Becker et al., who observed an uptake threshold of about 190 nm for DNA-wrapped SWNTs and fibroblast cells.[98] However, phagocytes greatly differ from other types of mammalian cells in that they are designed to digest harmful foreign particles. Indeed, Zhou et al. recently showed that PL-PEG functionalized SWNTs localized in lysosomes of macrophages after having been phagocytosed, whereas they exclusively localized in the mitochondria of four other types of mammalian cells after uptake by a non-endocytotic process.[99] Interestingly, the attachment of a large molecule, in this case the protein bovine serum albumin (BSA), redirected the uptake route of these cells toward endocytosis, as also observed by Wang and co-workers.[100] This demonstrates that the influence of the functionalization chemistry on cellular uptake pathways is still not fully understood. Overall, it seems that the use of targeting ligands, such as folate or integrins, will become a necessity to guarantee specific, endocytotic cell uptake, whereas the ligation of low-molecular-weight molecules may favor nonspecific internalization.[101]

Besides understanding the mechanism by which CNTs enter cells, it is just as important to investigate what happens afterward—where do they go and how are they released? The studies described above have already shown that CNTs have been observed in a variety of subcellular compartments, such as endosomes,[100] mitochondria,[99,102] the perinnuclear region,[103] the nucleus itself,[104] the cytoplasm,[101] or a combination of these,[105−107] again depending on their physicochemical properties and functionalization. The first conclusive evidence for CNT exocytosis was given by Jin and co-workers in 2008, who showed by single-particle tracking that the exocytosis rate of DNA-wrapped SWNTs closely matched the endocytosis rate with a slight temporal offset.[108] A study in our lab has investigated the uptake and release of oxidized, RNA-wrapped double-walled CNTs (DWNTs), which increasingly accumulated in Hela and PC3 cells within 3 h and were subsequently released from the cells over a 24 h time period.[109] The Raman mapping experiments also indicated a modification of the DWNTs' outer wall during their passage through the cell, although the reasons for this are still unclear. Experiments by various groups suggest that in particular oxidized CNTs may degraded by certain enzymes[110−112] and conditions mimicking the phagolysosomal fluid.[113] In summary, both exocytosis and the degradation of CNTs in physiological environments support their safe use in biomedical settings for therapeutic or diagnostic purposes, such as gene and drug delivery or imaging of cells and tissues. The next subsections will focus on the use of CNTs in these areas of application.

CARBON NANOTUBE-MEDIATED DELIVERY OF GENES AND OTHER TYPES OF FUNCTIONAL NUCLEIC ACIDS

Genes are segments of nucleic acids, which can be applied to treat a disease by correcting a genetic deficiency. The biggest challenge in gene therapy lies in the effective transfer of the genetic material into human cells, which has to not only be introduced safely into a sufficiently large population of cells but also produce a therapeutic effect for a sustained period of time. Traditional gene transfer vectors are often based on replication-defective viruses, because of their capability to enter a host, pass the physical tissue barriers, infect cells by active mechanisms, and deliver their genetic cargo directly to the nucleus of a cell. However, viral vectors can elicit strong immune responses in the host and some viruses are genetically unstable and can rapidly rearrange their genomes. Therefore, much research is focusing on the development of nonviral gene delivery vectors as a safer option. Materials that have been investigated for gene delivery include liposomes, polymers, dendrimers, nanoparticles, peptides, and among these, CNTs.

Carbon Nanotube-Mediated Delivery of DNA to Mammalian Cells. To be expressed in the nucleus of a cell, an extrinsically introduced gene needs to cross the cellular and nuclear membrane. However, same as nucleic acids, cellular membranes are negatively charged, resulting in electrostatic repulsion. Thus, nonviral gene delivery vectors are often composed of cationic materials to facilitate cellular entry. Pantarotto and co-workers have applied this concept to create functionalized, positively charged CNTs, which can bind negatively charged nucleic acids via electrostatic interactions.[114] Nucleic acids may also be attached to CNTs via self-assembly based on $\pi-\pi$ interactions between the DNA/RNA bases and the nanotubes' sidewalls,[115] or via covalent attachment of nucleic acids to functionalized CNTs.[116] The transfection efficiencies of most DNA-nanotube constructs in the literature exceed that of naked DNA, but are often much lower than commercially available transfection agents, such as Lipofectamine or FuGENE.[88,114] This is mainly due to enzymatic DNA degradation inside lysosomes or ineffective translocation through the cellular and nuclear membrane. However, most commercially available transfection agents are toxic and cannot be used in in vivo scenarios, indicating the need for an efficient but safe DNA delivery system.

The first study to achieve transfection efficiencies comparable to commercial agents was carried out by Liu and co-workers, who prepared polyethylenimine (PEI)-grafted MWNTs for delivery of the luciferase gene to three different types of mammalian cells.[117] PEI is among the most efficient and versatile nonviral vectors available owing to its "proton sponge effect", which causes rupture of endosomes and thus allows the vector to escape from the degradative lysosomal trafficking pathway.[118] Its transfection efficiency could be slightly enhanced by grafting PEI onto MWNTs. Similar to this, Nunes et al. grafted PEI, polyallylamine (PAA) or a mixture of the two polymers onto carboxylated MWNTs for delivery of pCMV-βGal plasmid DNA, with PEI/PAA-MWNTs achieving greater gene expression than PEI-MWNTs.[119] However, in this study free PEI/PAA and PEI alone showed higher levels of gene expression compared to those achieved with polymer-MWNTs. Another study by Ahmed et al. applied SWNTs functionalized with a cationic glycopolymer for the delivery of a plasmid encoding green fluorescent protein.[120] By varying the DNA:CNT-polymer weight ratio, the researchers tried to find a compromise between maximal transfection efficiency and minimal cytotoxicity caused by the cationic polymer. At a ratio of 1:43.5, their system achieved 40% transfection efficiency accompanied by 60% cell viability. As a comparison, Lipofectamine yielded a transfection efficiency of 55% at a cell viability of 25%. This shows that the polymer-CNTs are comparable to a commercially available agent in terms of transfection efficiency, while featuring a better cytotoxicity profile. Nevertheless, 40% cell death is too high for a gene delivery system to be considered as a potential candidate for in vivo studies. Qin and co-workers used PAMAM dendrimer-functionalized MWNTs to deliver plasmid DNA encoding the GFP gene to Hela cells and achieved 8% transfection efficiency (compared to 4% with dendrimers alone and 27% transfection efficiency with Lipofectamine), while cell viability for a 50 μg/mL MWNT preparation was 60% (compared to 25% with Lipofectamine). Richard et al. functionalized SWNTs and MWNTs with two cationic amphiphiles (lipid RPR12035 and pyrenyl polyamine) for the delivery of plasmid DNA containing the luciferase transporter gene.[121] SWNT−lipid complexes were found to be much more efficient than MWNT−lipid complexes and yielded a higher transfection efficiency than the lipid alone. These results do seem very promising for the use of CNTs as a gene delivery system; however, no comparison with the commercially available Lipofectamine was made and cytotoxicity was not investigated in this study. In summary, it appears that gene delivery with CNTs requires their functionalization with positively charged molecules, which possess an inherent cytotoxicity. In most cases, these complexes feature better transfection efficiencies than the cationic molecules on their own. However, it would seem desirable to develop a CNT-based gene delivery system, which does not rely on the use of these cationic cytotoxic substances. CNTs functionalized with dendrons have recently emerged as a

potential candidate,[122,123] especially for the delivery of siRNA; the next few years will likely show whether this is a feasible option.

Carbon Nanotube-Mediated Delivery of Functional RNA to Mammalian Cells. As introduced in the last paragraph, the traditional idea of gene therapy was based on the delivery of functional genes to cells and their insertion into the genome in order to replace non- or malfunctioning DNA. A rather novel area of gene therapy is based on RNA interference (RNAi), i.e., gene silencing at a posttranscriptional level[124] in the cytoplasm of mammalian cells, where the translation of so-called "messenger RNA" (mRNA) into proteins takes place. Different types of molecules have been investigated to date to inhibit gene expression by sequence-specific targeting of mRNA in the hope of creating therapeutic agents, the three major ones being siRNA, ribozymes, and antisense oligodeoxyribonucleotides (ODNs).[125]

The first study demonstrating siRNA delivery via CNTs was carried out by Hongjie Dai's group in 2005.[126] In their approach, siRNA was attached to PEGylated phospholipids via cleavable disulfide bonds attached to the surface of SWNTs. siRNA-mediated gene silencing was demonstrated using two nonpathogenic genes (lamin A/C and luciferase) as a model system. The achieved silencing efficiency exceeded that of LipofectamineTM by a factor of 2. Shortly afterward, Zhang and co-workers succeeded in silencing a pathogenic gene (telomerase transverse transcriptase, or "TERT") by CNT-mediated delivery of siRNA both in vitro and in vivo.[127] Because TERT is critical for the development and growth of tumors, the effect of siRNA could be monitored via its effect on tumor cell proliferation and tumor growth in mice. Indeed, tumor growth was found to be inhibited after treatment with the CNT−siRNA complexes and the average tumor weight was reduced significantly compared to untreated animals. A study by the Dai group in 2007 attempted silencing the expression of a HIV-specific cell surface coreceptor in order to block HIV virus entry. They reported a silencing effect superior over conventional liposome-based nonviral agents.[128] The applied functionalization scheme was again based on cleavable disulfide bonds, same as in their previous study. Pan and co-workers followed a different approach by developing a composite of CNTs and positively charged PAMAM dendrimers, which efficiently bind nucleic acids via electrostatic interactions. In two in vitro/in vitro studies published in 2005 and 2009, they demonstrated the delivery of two different antisense oligonucleotides (antisurvivin and anti-c-myc) involved in the development and growth of cancer to MCF-7 breast cancer cells.[129,130] In both cases, the nanotube−dendrimer−oligonucleotide complexes inhibited cancer cell growth to a higher extent than CNT-oligonucleotide or dendrimer−oligonucleotide constructs. These examples and other in vitro studies published more recently[131−133] already show that the delivery of functional RNA via CNTs seems to be much more successful than the delivery of DNA, which is further confirmed by three promising in vivo studies. The first by Podesta and co-workers used amino-functionalized MWNTs to deliver a proprietary, toxic siRNA sequence to a human lung tumor xenograph model after intratumoral application.[134] The research group demonstrated that siRNA delivered by amino-MWNTs lead to tumor growth inhibition and prolonged survival of tumor-bearing animals. The therapeutic efficiency of this system was higher in comparison to one of the most widely used cationic liposome delivery systems, DOTAP:cholesterol. In the second study, McCaroll and co-workers used SWNTs functionalized with lipids and natural amino acid-based dendrimers to complex siRNA.[135] The targeted gene was an endogenous gene for apolipoprotein B (ApoB), which is involved in cholesterol metabolism. Injection of only 0.96 mg/kg of the CNT-dendrimer/lipid-siRNA complexes silenced ApoB mRNA, decreased ApoB plasma levels, and lowered total plasma cholesterol. Moreover, the treatment was nontoxic, did not elicit an immune response and most of the siRNA was cleared from the body 48h after treatment. The results of these two studies are already very promising for in vivo siRNA drug delivery; however, preparation of the delivery vehicles appears complex and cumbersome. The last study to be discussed in this framework conducted by Bartholomeusz et al. features an extraordinary simple functionalization strategy: siRNA-CNT complexes were prepared by simple sonication of the two compounds in an aqueous environment.[136] The siRNA in these complexes retained its biological activity and readily entered cells even in the presence of serum. The drawback of this method might be that large amounts of siRNA are needed, although the researchers did not state this as a problem. When the therapeutic activity of the complexes was tested on tumor-bearing mice, the activity of the targeted transcription factor HIF-1α (Hypoxia-inducible factor 1 alpha) was found to be significantly reduced. The simplicity of the functionalization method, combined with a clear in vitro and in vivo response, demonstrates the enormous potential of this approach.

In summary, the development of CNT-based gene delivery systems is still in its infancy, being actively researched for only about six years now. In comparison, other nonviral gene delivery vectors, such as liposomes, polymers, dendrimers, polypeptides, or nanoparticles, have already being investigated since decades and lipid- and polymer-mediated vectors have been used to target genetic diseases and cancer in clinical trials. Obviously, CNTs as gene delivery vectors still have a long way to go until reaching this stage; nevertheless, their unique shape and chemical composition offer major advantages over other nonviral vectors, which will very likely show their true potential in the coming years.

CARBON NANOTUBE-MEDIATED DELIVERY OF DRUGS

One of the first studies on the use of CNTs for drug delivery has been carried out by Wu and co-workers in 2005, who conjugated MWNTs to the antibiotic amphotericin B, a drug considered problematic due to its narrow therapeutic index and poor aqueous solubility.[137] Both issues could be resolved by conjugating to MWNTs, which reduced the toxicity of the drug toward mammalian cells while preserving its antifungal activity. In the following years, many more studies have been conducted, almost solely focusing on the delivery of anticancer drugs. The first studies undertaken in this area mainly focused on functionalization issues, whereas subsequent studies investigated the therapeutic activity of the systems in vitro. Current studies are trying to tackle the challenge of in vivo settings in order to move a step further toward the clinic and investigate the fate of the nanotubes in living systems after fulfilling their task.

Drug Loading. CNTs can be loaded with drugs in two main ways: attachment of the drug to the outer walls or filling of the inner cavity. The chosen strategy plays a crucial role for the therapeutic success of the drug delivery system and the fate of the different components upon administration. In terms of

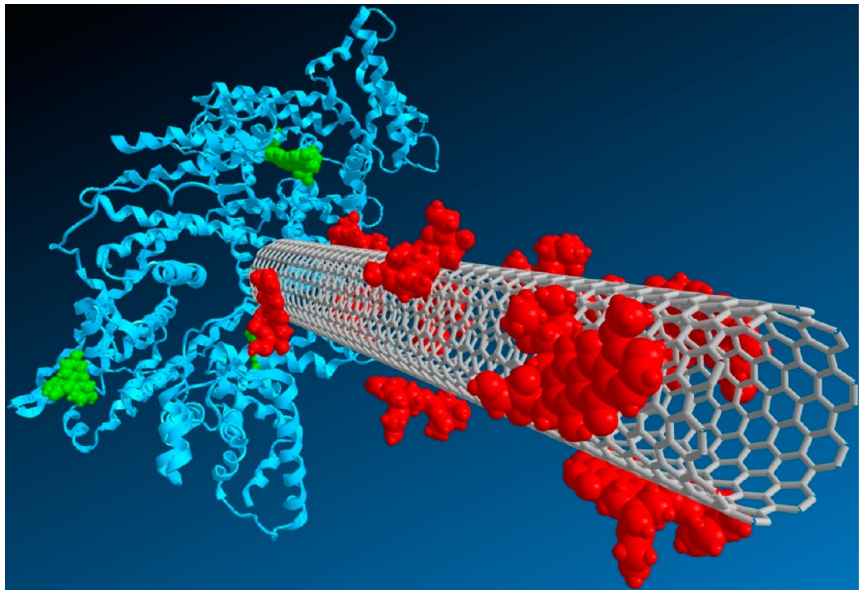


Figure 5. Schematic illustration of a multifunctional drug delivery vector employing a multifunctional carrier (a carbon nanotube), a therapeutic payload (here the anticancer drug doxorubicin, in red), and a fluorescent marker (in green) linked to the CNT through a spacer (here the protein BSA, in blue). Based on previous work in our group.[138]

sidewall functionalization, the drug binding approach can be noncovalent or covalent; each having its benefits and shortcoming. Noncovalent binding usually relies on intermolecular forces, such as electrostatic interactions, hydrophilic interactions, hydrogen bonding, or π-stacking. Experimentally, this is generally achieved by simple mixing of the drug with the nanotubes. Noncovalent binding, however, is susceptible to environmental factors, such as pH and salt concentration, and in general less stable than a covalent bond. This can be disadvantageous for efficient and durable attachment of the drug, but beneficial for its release at the target location. pH-dependent drug release can for example be achieved if a drug–CNT complex is taken up by endocytosis and encounters the slightly acidic microenvironment within endosomes and lysosomes. Furthermore, the attached drug molecule needs to be a base and form a salt at these conditions. A prominent and widely used example[138−144] in CNT drug delivery is the anticancer drug doxorubicin, which binds to CNTs via $\pi-\pi$ interactions. At higher pH (>8), the amino group in the sugar moiety of doxorubicin is deprotonated, promoting strong hydrophobic interactions with the nanotubes' sidewalls and a low solubility in water. At lower pH, however, the amino group becomes protonated and thus charged, which increases the molecule's solubility in water and facilitiates its release from the nanotubes. Other drugs that have been noncovalently bound to CNTs include paclitaxel[145,146] and camphotecin.[147]

Conversely, covalent binding strategies provide strong and stable chemical bonds. The underlying chemistry can be complicated and laborious, but offers higher control and flexibility. Drug release often requires the integration of bonds that are cleavable at intracellular conditions, such as the reductive environment in the cytoplasm or the lower pH environment inside endosomes. Examples for cleavable bonds are disulfide bonds,[126,148] ester bonds,[149,150] carbamate bonds,[151] or prodrugs.[152,153] Besides, covalent coupling chemistry often uses spacers between CNTs and the drug molecule to increase aqueous solubility and biocompatibility. The most prominent example by far is poly (ethylene glycol) (PEG) with the first approved PEGylated products being on the market for 20 years,[154] but other linear polymers, such as poly (vinyl alcohol) are also being used.[155] In recent years, branched polymers have become increasingly popular, among these dendrimers[156] and dendrons,[150] and the encapsulation of CNTs in polymer shells.[141,144]

Besides sidewall functionalization, drugs can be incorporated into the inner cavity of CNTs.[157] This approach is still in its infancy, although it potentially allows for the protection of unstable drugs and controlled drug release at the desired site of action depending on the tube diameter. Hilder and co-workers attempted to calculate the optimal diameter of SWNTs for filling with small-molecule anticancer drugs, such as cisplatin, paclitaxel, or doxorubicin to take advantage of the maximum suction energy.[158,159] They found that a CNT should have a diameter of about 1 nm to entrap cisplatin, between 1.83 and 2.54 nm for paclitaxel and between 1.77 and 2.10 nm for doxorubicin. Hampel and co-workers have been one of the first to test this apporach experimentally by filling MWNTs with the anticancer drug carboplatin using a wet chemical approach.[160] The complexes exhibited a concentration-dependent cytotoxic effect on human bladder cancer cells. This work was followed up by Arlt et al., who incorporated carboplatin not only into CNTs, but also carbon nanofibers.[161] The platinum release greatly depended on the carrier material: CNTs showed time-dependent release of carboplatin, whereas the nanofibers did not significantly release the drug. Curiously, they still reduced the growth of cancer cells more effectively than free carboplatin, which indicates that carboplatin encapsulation provided a protective environment for the drug and therefore prevented its degradation in the course of the experiment.

Targeting. In many cases, the attachment of drugs to CNTs helps to overcome various administration problems, such as insolubility, inefficient distribution, and inability of drugs to cross cellular barriers. This is a first major step toward an effective drug delivery system; however, it does not guarantee that the drug will reach its target location upon administration. Drug targeting is therefore a crucial aspect to increase the

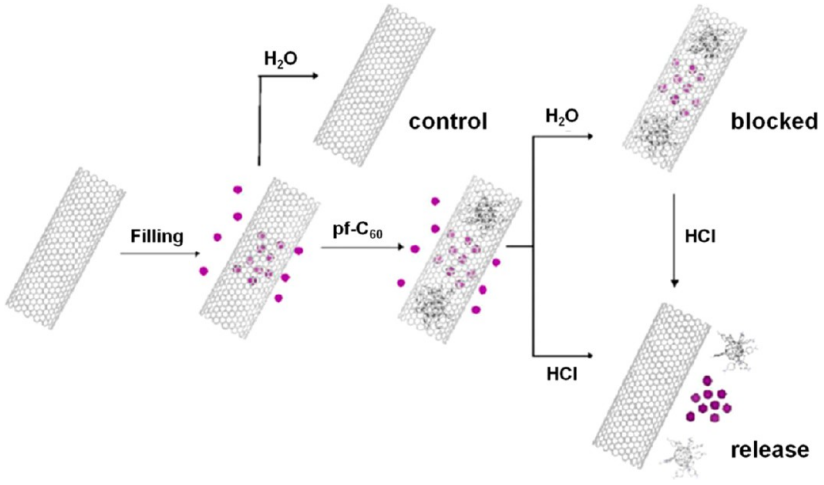


Figure 6. Schematic representation of the process for the encapsulation, "corking" and release of material from SWCNTs. Reprinted with permission from ref 174. Copyright 2010 Elsevier.

specificity of the therapeutic devices. In general, targeting strategies can be divided into vector-based and nonvector-based approaches. Vector-based targeting strategies generally employ a multifunctional carrier, such as CNTs, consisting of a core constituent material, a therapeutic payload, and a targeting agent (Figure 5).[162] This multifunctional concept allows for the delivery of large amounts of therapeutic agents per targeted site of action, which is a major clinical improvement oversimple immuno-targeted drugs. Vector-based targeting strategies can be divided into active and passive approaches.

Passive targeting is often based on the size and physical properties of the nanovector. A typical example is the enhanced permeation and retention (EPR) effect, which originates from the leaky vasculature of tumor-associated blood vessels and can be exploited by particle-mediated drug delivery systems to increase drug concentrations at tumor sites.[163] This concept has for example been followed up by Liu and co-workers,[90] but is generally more popular for spherically shaped nanovectors, such as liposomes. Another passive targeting approach is based on the application of prodrugs, which are therapeutically inactive molecules that are transformed into the active form at the target location, as demonstrated by various research groups.[148,152,153] Active targeting, on the other hand, employs targeting agents, such as antibodies, aptamers, or ligands to cell surface receptors. A widely employed example in the latter category is the attachment of cyclic arginine-glycine-aspartic acid (RGD) peptides to CNTs.[140,164] RGD peptides impart a recognition moiety for integrin $\alpha_v\beta_3$ receptors; a class of transmembrane cell adhesion receptors that are up-regulated in a variety of solid tumors. A similar strategy exploits the interaction of folate and its receptor, which is a common, though relatively unspecific tumor marker expressed at high levels by a broad spectrum of human cancers.[165] Binding of folate to its receptor facilitates cellular internalization of folate-conjugated SWNTs by receptor-mediated endocytosis and has become a widely used targeting strategy.[144,153,166] Similarly, epidermal growth factor (EGF) has been used as an active targeting agent as a ligand to the epidermal growth factor receptor (EGFR) to selectively target squamous cancer cells.[167,168] Antigen−antibody interactions are the second of the above-mentioned active targeting strategies in relation to CNT-mediated drug delivery. McDevitt and co-workers were one of the first to design a SWNT-antibody construct to target the CD20 epitope on human Burkitt lymphoma cells. They showed that covalent attachment of antibodies to the nanotube conjugates altered their pharmacokinetics and biodistribution dramatically when comparing tumor bearing and nontumor bearing mice.[169] In the same year, Shao and co-workers reported that SWNTs functionalized with HER2- and IGFR1-specific antibodies showed selective attachment to breast cancer cells in contrast to SWNTs functionalized with nonspecific antibodies.[170]

Monoclonal antibodies are overall quite effective targeting agents, but come with a number of innate problems, such as insufficient tumor penetration, interaction with the immune system, circulating free antigen, changes in the antigen over time, and their general bulkiness. During the hunt for alternative active targeting options, aptamers have emerged as a feasible alternative. They are synthetic, single-stranded nucleic acid molecules able to fold up into unique 3D structures, which give them molecular recognition properties similar to antibodies. Aptamers are readily producible by chemical methods for a variety of targets and elicit little or no immunogenicity in therapeutic applications. They do, however, suffer from inefficient cell uptake and are therefore often used for extracellular targets. Van der Bossche and co-workers have shown that aptamers grafted onto CNTs translocated into the cytosol of different cell types independent of receptor-mediated uptake.[101] Taghdisi et al. used an aptamer for the biomarker protein tyrosin kinase-7 noncovalently bound to SWNTs to achieve targeted delivery of the anticancer drug daunorubicin to acute lymphoblastic leukemia T-cells.[171] Li and co-workers went even further and designed a dual-targeted drug delivery system consisting of iron-filled MWNTs conjugated to folate in order to guide the complex to the target location using an external magnetic field and then achieve selective internalization by cancer cells overexpressing the folate receptor.[143] Overall, these studies show promise for the use of aptamers as targeting agents; nevertheless, it is still too early to predict their future role for drug delivery applications.

Controlled Drug Release. Besides possessing a target selectivity, ideal cancer treatments should also allow for controlled release of the therapeutic entity, which provides prolonged delivery of a drug while maintaining its blood concentration within therapeutic limits. Stimuli-responsive drug release has been achieved by several research groups by means

of near-IR laser irradiation.[143,172,173] A more complex method to achieve sustained drug release has been developed by Zhang et al., who wrapped SWNTs with cationic or anionic polysaccharides.[141] Using these either on their own or in combination enabled them to manipulate the surface potential of the modified nanotubes and thus not only to control the amount of drug loading, but also the release rate of the associated drug inside cells. Another interesting concept for controlled drug release is the use of removable "corks" at the openings of filled CNTs (Figure 6). Luksirikul et al. have used C60 fullerenes to close uranyl acetate-filled SWNTs and demonstrated pH-triggered release of the encapsulated material.[174] In a similar fashion, Chen et al. attached silica nanospheres to the openings of fluorescein-filled SWNTs, which enabled controlled release of the encapsulated material by exposure to a reducing agent or at elevated temperatures.[175] The feasibility of this approach for a biological environment remains to be tested.

Overcoming Multidrug Resistance. Multidrug resistance is considered a common problem in cancer drug administration and can be caused by a number of reasons, including increased drug efflux, enzymatic deactivation, decreased permeability of the cell membrane, altered binding sites, and alternate metabolic pathways. Increased drug efflux is often due to up-regulation of p-glycoprotein (P-gp), a trans-membrane, ATP-dependent efflux pump capable to transport a broad variety of substrates out of cells. Li et al. have attempted to address this problem by conjugating doxorubicin-loaded SWNTs to a P-gp antibody in order to selectively target drug-resistant cells.[173] Additionally, drug release was triggered by NIR irradiation upon cellular uptake. The CNT-based delivery system achieved a 2.4-fold higher cytotoxicity in multidrug resistant cells than the free drug. Another study by Cheng et al. has followed a similar route using doxorubicin-loaded, PEGylated MWNTs, albeit without using a targeting agent.[176] They found that their complexes accumulated in the drug-resistant cancer cells as efficiently as in the sensitive cancer cells. It remains to be clarified whether this is simply due to the nanotubes' transmembrane delivery potential or to modulation of Pgp efflux.

Novel Innovative Drug Delivery Strategies. In most in vitro drug delivery studies, mammalian cells are incubated with drug-loaded CNTs, which thereupon make their way into the cell by mechanical insertion or endocytotic uptake. Some research groups, however, have developed novel and more innovative delivery methods. In 2005, for example, Cai et al. drove nickel-embedded nanotubes through the membranes of lymphoma cells, primary B cells, and neurons by magnetic force.[177] This technique, named "nanotube spearing", was also useful for efficient gene transfer into these cells. Park and co-workers developed a "carbon nanosyringe array", which enables simultaneous delivery of material into a number of cells without the need to apply an external force.[178] Using this array, they were able to demonstrate successful delivery of plasmid DNA and quantum dots into the cytoplasm of cancer cells and human mesenchymal stem cells. A year later Wu et al. designed a CNT membrane for transdermal delivery of nicotin as a candidate for programmable nicotin cessation treatment.[179] The unique physical properties of this device allowed highly efficient electrophoretic pumping, which created a steady-state flux of nicotin at a controllable delivery rate. Zhang and co-workers had the idea to create alginate microspheres as drug carriers and use CNTs for mechanical reinforcement in order to prevent rapid swelling and high biokmolecule leakage.[180] In a similar fashion, Fujigaya et al. embedded CNTs in a gel matrix to serve as a scaffold for adsorption of small molecules, such as the anticancer drug doxorubicin, and triggered drug release by lowering the pH or NIR irradiation.[172] It will be interesting to see if these innovative drug delivery methods will gain acceptance and will eventually be tested in animal models.

In vivo Drug Delivery. Successful targeting and sufficient therapeutic efficiency in vitro are crucial requirements for testing drug delivery systems in vivo. In 2008, Liu and co-workers conducted the first in vivo study using PEGylated SWNTs conjugated to the anticancer drug paclitaxel.[90] The efficacy of the complexes in vitro was similar to that of free paclitaxel but higher than the free drug in vivo in a murine 4T1 breast cancer model due to prolonged blood circulation and 10-fold higher tumor uptake. In 2009, Bhirde and co-workers functionalized SWNTs with the anticancer drug cisplatin, epidermal growth factor (EGF) as a targeting agent, and quantum dots as imaging agents for the treatment of head and neck squamous carcinoma tumors.[167] Mice treated with the targeted conjugates showed a rapid decrease of tumor size, whereas mice treated with a nontargeted nanotube-cisplatin conjugate did not show tumor regression. A follow-up study further examined the biodistribution and clearance of the compelxes in mice.[168] In the same year, Wu et al. published a study, in which MWNTs functionalized with the anticancer agent 10-hydroxycamptothecin (HCPT) inhibited the growth of hepatic tumors much more effectively than HCPT injection alone.[181] Liu et al. were also able to successfully apply their previously developed system, consisting of PEGylated SWNTs loaded with the anticancer drug doxorubicin, in an in vivo scenario.[139] Experiments on mice bearing lymphoma xenografts showed that the CNT-based drug delivery system exhibited a marked reduction in toxicity compared with free doxorubicin or a commercially available product. Similar to this, Chaudhuri et al. conjugated SWNTs to doxorubicin via an enzymatically cleavable carbamate bond that allowed for sustained release of the active drug in cell lysate.[151] Efficient reduction of tumor growth without the systemic toxicity of free doxorubicin could be demonstrated in a mouse B16−F10 melanoma model. Last but not least, a study by Yang and co-workers used SWNTs to deliver acetylcholine into the brain of mice for treatment of experimentally induced Alzheimer's disease. In contrast to the previously discussed studies, drug-loaded SWNTs were administered into the gastrointestinal tract by gastrogavage rather than injected into the bloodstream.[107] They were then absorbed by the cells in the mucous membrane and found their way into the brain, where they had significant effects on the learning and memory capabilities of the Alzheimer mice.

The promising results of these studies are a milestone for the development of CNTs as clinically successful therapeutic agents, although toxicological studies need to be run alongside to obtain a clear picture of both the beneficial and adverse health effects of CNT-based drug delivery systems. In comparison to other nanovectors, such as liposomes or nanoparticles, CNTs generally have a higher surface area and therefore a higher drug loading capacity, which minimizes the amount of excipient required in therapeutic formulations. Furthermore, they able to pierce through cellular membranes and thus allow for cytoplasmatic delivery of drugs, which avoids the degradative lysosomal pathway. Last but not least, the option of incorporating drugs in the inner cavity of CNTs provides a protective environment for drugs of poor stability

and potentially allows for diameter-dependent, controlled drug release. On the negative side, CNTs are nonbiodegradable and hence their elimination from the body after i.v. or intratumoral application needs to be well understood and controlled. In addition, nonfunctionalized CNTs are associated with a certain toxicity (discussed later in more detail) and are potentially taken up by the reticuloendothelial system (RES).[182] However, these issues can be overcome by choosing an appropriate functionalization scheme. In terms of in vivo applications, more work needs to be done to improve the pharmacological properties of CNT-drug formulations. For example, short and well-functionalized CNTs were shown to be eliminated via the kidneys, but feature a very short blood circulation time (~30 min),[183] which is unfavorable for the treatment of chronic diseases. On the contrary, PEGylation of CNTs increases their blood half-life and decreases RES uptake, but causes the nanotubes to accumulate in tissues[184] and persist in the body for months.[185] Nevertheless, drug delivery is overall clearly one of the most promising bioapplications of CNTs.

CARBON NANOTUBES AS SELECTIVE CELL DESTRUCTION AGENTS

Apart from destroying diseased or dysfunctional cells by delivering drugs and therapeutic nucleic acids, CNTs can also be applied for selective cancer cell destruction by acting as near-infrared (NIR) heating devices. This technology is based on the fact that biological systems are highly transparent to NIR light in contrast to CNTs, which strongly absorb light within this spectral window. Thus, treating tissue or cell layers after internalization of CNTs with NIR irradiation can cause cell death due to excessive local heating. The first study to demonstrate this effect was published by Kam and co-workers in 2004.[186] Therein, SWNTs functionalized with phospholipid-PEG and folate as a targeting moiety were incubated for 12−18 h with HeLa cervival carcinoma cells overexpressing the folate receptor. After abundant washing, the cells were irradiated continuously by a 808 nm laser (1.4 W/cm^2) for 2 min, causing drastic morphology changes and extensive cell death. HeLa cells that did not overexpress the folate receptor, however, did not internalize the nanotubes and were thus not affected by the laser treatment.

Since this pilot study, a number of other groups have reported the destruction of cancer cells in vitro using CNTs.[170,187−194] Panchapakesan and co-workers, for example, have grown cells on a bucky paper and were able to create cell-killing explosions, which were caused by SWNTs heating up to more than 100 °C and thus vaporizing the water molecules present in the nanopores between CNT bundles.[195] Shao et al. used dispersed SWNTs functionalized with cancer-specific antibodies (anti-IGFR1 and anti-HER2) to achieve a more selective cell killing effect.[170] In a similar fashion, Chakravarty et al. have demonstrated thermal ablation of human Burkitt's lymphoma cells (CD22+CD25-) and peripheral blood mononuclear cells (CD22-CD25+) after targeting them with anti-CD22 and antiCD25-targeted SWNT constructs.[187] While in this study antibodies were attached to the CNTs in a noncovalent manner, a follow-up study by the same group applied a covalent functionalization scheme in order to prevent disassociation of the targeting moiety from the nanotubes.[188] Further studies employing antibody-functionalized CNTs for selective cell-killing after NIR irradiation have been carried out by Wang et al. and Xiao and co-workers.[189,190] In the first case, it was shown that the CNT-antibody complexes were internalized by cells, whereas in the second case they remained attached to the cell membrane. According to Marches et al., however, the treatment is more efficient if the CNT-antibody complexes are internalized.[196] So far SWNTs have been used as a mixture of metallic and semiconducting species. Very recently, Diao et al. developed a gel filtration method to selectively sort and isolate semiconducting (12,1) and (11,3) SWNTs that are strongly in resonance with 808 nm excitation and exhibit spectrally confined NIR-II emission near ~1200 nm that could potentially be used for deep organ imaging and registration at much lower dosage than needed for unsorted SWNTs.[194]

Gannon and co-workers have pursued a different approach to heat up nanotubes by using a radiofrequency field (13.56 MHz), which affords deeper tissue penetration than NIR irradiation.[197] They were able to show successful cell killing not only in vitro, but also in vivo on rabbits bearing hepatic VX2 tumors after direct intratumoral injection of SWNTs. Irradiation of all SWNT-treated tumors lead to complete necrosis after 48 h, whereas control tumors without SWNTs remained viable. Whereas all former studies have used SWNTs, Burke et al. and Gosh and co-workers used MWNTs functionalized with Pluronic or DNA.[198,199] In contrast to SWNTs, MWNTs feature broad absorption spectra, rendering them amenable to stimulation by a range of NIR energy sources. In addition, they can absorb more NIR radiation than SWNTs due to the higher number of available electrons and the higher percentage of metallic tubes (per weight), which reduces the amount of irradiation needed to treat embedded cancers. Both studies showed complete and durable irradiation of the treated tumors, dependent on the concentration of MWNTs injected. DNA-wrapping was furthermore shown to enhance the efficiency of heat production, possibly due to decreased agglomeration. A study published in 2009 by Kang and co-workers also used NIR light at 1064 nm, but in millisecond pulses rather than continuous irradiation.[200] This caused the selective destruction of cancer cells via "firecracker-like explosions" at the nanoscale. The temperature did not increase by of more than 3 °C during the treatment, which suggests that the destructive effect of CNTs on cancer cells was mainly due to mechanical damage induced by the shockwave generated during the photoacoustic explosion, rather than thermal damage caused by high temperatures. The group further employed a targeting scheme based on the use of folic acid as a targeting agent, which increased the treatment efficiency from 10% to 85%. Moon and co-workers applied PEGylated SWNTs to destroy solid malignant tumors in vivo after intratumoral injection of PEG-SWNTs and NIR irradiation at 808 nm for 2 min (laser power 5 W/cm^2).[201] The photothermally treated mice displayed complete destruction of the tumor without recurrence for over six months (Figure 7), whereas the tumors treated in the control groups grew continuously. Most SWNTs were excreted within 2 months through the biliary or renal pathway. A study by Liu et al., who used a laser power of 1 W/cm^2 for 2 min (also at 808 nm) and an intravenous application route, yielded similar results and additionally showed that heavy PEGylation led to accumulation of the nanotubes in the skin, whereas an optimized PEGylation protocol afforded an optimal blood circulation half-life of 12−13 h, relatively low RES accumulation, high tumor uptake, and low skin retention.[184] Huang and co-workers, who also used PEgylated SWNTs, attempted to optimize their protocol by testing various SWNT concentrations and a range of different laser powers, exposure

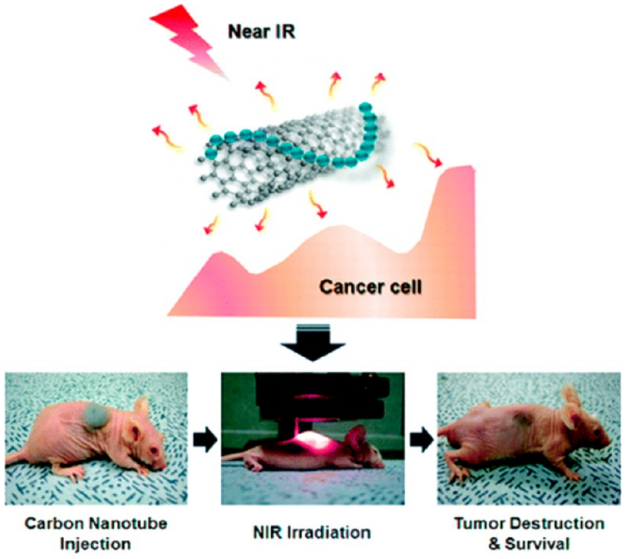


Figure 7. Photothermal treatment for in vivo tumor ablation in mice using PEGylated SWNTs: Schematic view of the procedure and suppressed tumor growth (bottom right image) after 60 days of treatment. Reprinted with permission from ref 201. Copyright 2009 American Chemical Society.

times, and energy densities using a large population of mice (n = 100) and successfully achieved tumor eradication at a much lower light power density (200 mW/cm^2) than other published studies.[202]

In summary, the use of CNTs for selective cancer cell destruction has made much progress in recent years and can be considered as one of the most promising biomedical applications of CNTs alongside drug delivery. The NIR heating effect of CNTs is a unique property of this material, its only competitor thus far being graphene,[203−205] and although the treatment is restricted to surface-localized tumors, as NIR light is only capable of passing through several centimeters of tissue, it appears to be a quite efficient and targetable option for both in vitro and in vivo applications.

CARBON NANOTUBES AS DIRECT LABELS FOR IN VITRO AND IN VIVO IMAGING

The extraordinary structure of CNTs results in optical properties that enable their use as labels for imaging applications. Semiconducting SWNTs, for example, feature band gap photoluminescence in the NIR region,[206] whereas surface-defective SWNTs and MWNTs exhibit strong photoluminescence in the visible range of the spectrum upon chemical functionalization.[207] CNTs also possess a characteristic Raman scattering signal, which is easily distinguishable from other organic carbon in living systems and is not subjected to photobleaching.[208] Apart from this, CNTs produce thermal and acoustic impulse responses when excited by a short-pulsed laser beam. This section will review in vitro and in vivo imaging applications of CNTs in each of these areas.

IMAGING APPLICATIONS OF CARBON NANOTUBES BASED ON PHOTOLUMINESCENCE

In 2004, Cherukuri and co-workers employed the intrinsic NIR photoluminescence properties of CNTs to image CNTs in macrophage-like cells.[209] After ingestion of the nanotubes, cells were excited by light from a 660 nm diode laser and emission was detected between 1125 and 1600 nm using an adapted confocal microscope. It was demonstrated that the ingested CNTs remained fluorescent inside the cells and could be imaged with high contrast due to the low levels of endogenous fluorescence of the biological material in this spectral region. A follow-up study by the same group applied this technique to visualize CNTs in living organisms.[210] SWNTs were intravenously administered to rabbits and CNT biodistribution examined by performing NIR fluorescence spectroscopy on tissue specimen of various organs. The next step, direct in vivo imaging, was achieved a year later in *Drosophila* (fruit fly) larvae, which were raised on food containing 10 ppm of disaggregated SWNTs.[211] Two years later, Welsher and co-workers were able to demonstrate whole animal in vivo imaging on mice.[212] They used a specialized functionalization scheme, which improve the quantum yield by more than 1 order of magnitude and afforded high image contrast with resolution of small blood vessels inside tumors at a relatively low dose of administered CNTs (17 μg/mL). In their latest studies they even followed the path of SWNTs through the mouse anatomy in real-time by dynamic contrast-enhanced imaging in the

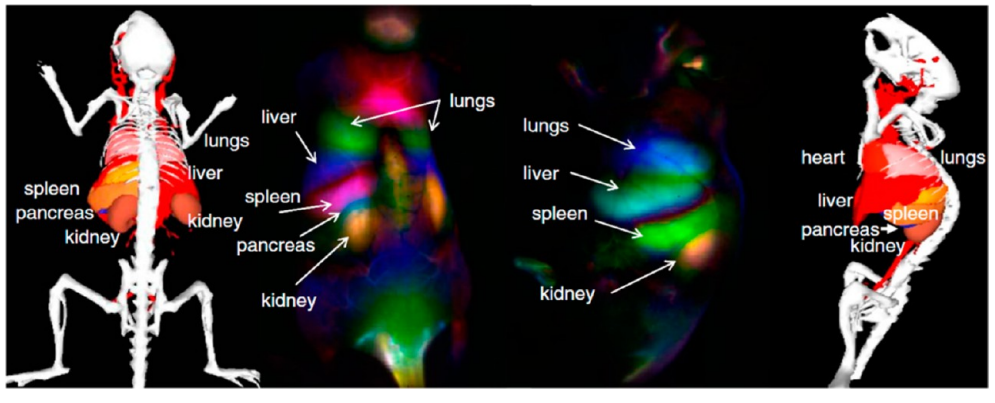


Figure 8. Dynamic contrast-enhanced imaging with SWNTs as NIR II fluorescent agents through principal component analysis (PCA). Images were taken over the first 130 s following intravenous injection by taking 150 evenly speaced frames out of the 2,000-frame data set. Major features observed belong to the lungs, liver, kidney, and spleen, as well as the pancreas in the interstitial space between kidney and spleen. Reprinted with permission from ref 213. Copyright 2011 National Academy of Sciences, USA.

second NIR window from 1000 to 1400 nm (Figure 8)[213] and accomplished the first dual application of intravenously injected SWNTs as photoluminescent agents for in vivo/in vivo tumor imaging and NIR absorbers and heaters for photothermal tumor elimination.[214] Studies by other groups have also applied the NIR properties of CNTs for imaging purposes, for example for single particle tracking and endocytosis/exocytosis of CNTs in cells[108,215] and selective probing of cell surface receptors with antibody-tagged SWNTs.[216]

Apart from the intrinsic NIR fluorescence properties of semiconducting SWNTs, the photoluminescent properties of chemically functionalized, defective SWNTs and MWNTs have also been applied for intracellular imaging. Lacerda et al. used the UV−vis luminescence properties of aqueous dispersions of amino-functionalized SWNTs to monitor their intracellular trafficking.[103] This approach offers the advantage that no special NIR lasers are required to excite the sample in confocal microscopy imaging. In addition, it was demonstrated that protocols of multiple fluorescent staining of cellular compartments can be used without crosstalk between the different fluorophores.

IMAGING APPLICATIONS OF CARBON NANOTUBES BASED ON RAMAN SCATTERING

SWNTs exhibit strong and distinct Raman scattering features, which can be distinguished from other carbon-based materials and structures in cells and organisms.[217] This property has since been utilized in several in vitro[109,208] and in vivo imaging studies,[182,185,218,219] which mainly investigated the biodistribution of CNTs. Liu and co-workers were the first to image SWNTs ex vivo in mouse tissue by means of their Raman signature.[185,218] The SWNT concentrations in various tissue samples were calculated and compared to data obtained by microPET (positron emission topography), showing reasonable agreement. Similar to these studies, Yang et al. studied the long-term accumulation of CNTs in mice after i.v. administration by Raman spectroscopy and TEM.[182] A major step forward was achieved by Zavaleta and colleagues, who showed demonstrated noninvasive deep tissue Raman imaging in living mice with a special optimized Raman microscope.[219] Last but not least, an entirely different imaging application based on the Raman fingerprint of CNTs has been envisioned by Liu and co-workers, who used SWNTs with different isotope compositions as multicolour contrast agents for multiplexed Raman imaging due to shifting of the G-band. This concept has been applied for multiplexed two-color protein detection in protein microarrays[220] as well as for three-color recognition of cancer-specific cell surface receptors on live cells.[221] A follow-up study published recently even demonstrated five-color multiplexed imaging of ex vivo tumor tissues.[222]

IMAGING APPLICATIONS OF CARBON NANOTUBES BASED ON PHOTOACOUSTIC EFFECTS

Photoacoustic imaging of cells and tissues with CNTs has only emerged within the last years. In general, photoacoustic imaging techniques offer a higher spatial resolution and afford deeper tissue penetration than other optical imaging techniques. To our best knowledge, de la Zerda and co-workers were the first to use CNTs as photoacoustic contrast agents. In their study, they achieved a high photoacoustic signal in U87MG tumor xenografts implanted in mice after intravenous administration.[223] When an RGD peptide was conjugated to the CNTs as a targeting agent, signal intensity was 8 times higher than that of the nontargeted CNTs. Pramanik et al. have followed a similar approach in order to identify sentinel lymph nodes in a rat model with a high signal-to-noise ratio and a resolution of about 500 μm.[224] In comparison to the previous study, the amount of injected CNTs was 10-times higher (500 μg/mL vs 50 μg/mL), which might give cause for toxicity concerns; however, the researchers state that concentrations down to 100 μg/mL can be used, as shown in a parallel study.[225] In order to further increase the concentration of CNTs as photoacoustic contrast agents, Kim et al. had the idea to deposit a thin layer of gold around their CNTs to enhance NIR absorption, which allowed for the use of CNT concentrations in the femtomolar range.[226] After conjugation to an antibody targeted at the lymphatic endothelial hyaluronan receptor-1 (LYVE-1), the golden nanotubes were able to target lymphatic vessels with high spatial resolution. Another study that has employed antibody-targeted CNTs for targeted photoacoustic imaging has been carried out by Xiang and co-workers for the purpose of early tumor detection.[227]

IMAGING APPLICATIONS OF CARBON NANOTUBES FUNCTIONALIZED WITH IMAGING AGENTS

Besides making use of their intrinsic physicochemical properties, researchers have also functionalized CNTs with imaging agents, such as quantum dots or metallic nanopartcles, to facilitate imaging via conventional techniques. Chen and co-workers have coupled superparamagnetic iron oxide nanoparticles (SPIO) and NIR fluorescent CdTe quantum dots to the surface of CNTs.[228] These nanohybrids showed an enhanced MRI signal as contrast agent for detecting 293T cells in comparison with pure SPIO. At the same time, their intracellular fluorescence was significantly improved in comparison with SPIO-CdTe due to ability of CNTs to penetrate into cells. Al-Faraj et al. and Vittorio et al. also used magnetic resonance imaging (MRI) for visualizing CNTs in cells[229] or living organisms,[230,231] based on present iron oxide impurities. Hong and co-workers recently followed a different approach and filled CNTs with metal halides serving as radioprobes to afford in vivo imaging in mice after intravenous injection by single-photon emission computed tomography (CT).[232] This prevented leakage of the radionuclide to high affinity-organs, such as thyroid and stomach, and led to specific tissue accumulation (here in the lung).

All in all, CNTs as optical labels hold several advantages over conventional organic fluorophores. Due to their unique properties, they allow for various imaging modes, giving rise to optical, photoacoustic, and Raman signals that are not limited by photobleaching effects. This allows for the repeated investigation of labeled specimens over long time periods. The main competitor of CNTs for imaging applications are possibly quantum dots, which are highly luminescent semiconducting nanoparticles featuring size-tunable emission and simultaneous excitation, thus allowing for multicolor optical coding in biological applications.[233] Yet, quantum dots show signal attenuation under prolonged excitation and have a limited lifetime in aqueous solutions,[208] which is a clear disadvantage in comparison to CNTs. Besides, their cores are often composed of cadmium selenide, a cytotoxic compound that can be difficult to shield from the cellular medium.[234] In summary, both

materials are of great value for in vitro and in vivo imaging applications, limited mainly by toxicity issues, which are currently highly researched and will be discussed in the next subsection.

TOXICITY

Much attention has been given to the versatile and potential uses of CNTs in biological and nonbiological applications; yet relatively few studies in comparison to the whole of CNT literature have investigated the health hazards that might arise from this novel material. This trend of lacking toxicological investigations has begun to reverse over the years, with several in vitro and in vivo studies showing detrimental effects on cell cultures and bodily tissues; the most alarming studies demonstrating asbestos-like behavior by extremely long CNTs (which are unlikely to be suitable for clinical or medicinal applications).[235,236] However, CNTs come in a variety of forms spanning differences in their contaminants, surface chemistries, processing methods, agglomerate states, lengths, diameters, and more. Each of these variations in a given CNT batch can singularly or synergistically with other parameters render CNTs benign or toxic. Toxicity assays are further complicated by effects arising from the large CNT surface area and hydrophobic nature, allowing CNTs to interact with commonly used toxicity assay reagents and giving rise to false results.[237−240] The specific properties of CNTs and relative effects these properties pose to mammalian health will be assessed below.

A BRIEF OVERVIEW OF CNT TOXICITY STUDIES

Before understanding how individual CNT characteristics influence toxicity issues, a quick overview of CNT toxicity studies and their methods will be described. In vitro studies have investigated the effects on CNT exposure mainly to lung, skin, and immune system cell cultures, because these tissues are the most likely to be exposed to CNTs within a workplace environment or are responsible for clearance and detection of foreign materials in the body. In terms of methods, in vitro studies generally cover the basics of population viability in terms of proliferation, changes in cell morphology, viability, and changes in expression levels of signature enzymes. Most of these generally report decreases in proliferation with various results in terms of increased cell death.[241−245] Decreases in proliferations may CNTs hindering adhesion mechanisms,[246] inducing down-regulation of extracellular matrix proteins,[247] increasing oxidative stress,[248,249] reorganizing actin structures,[250] causing membrane damage,[251] or a combination of these effects. Other in vitro studies have investigated genotoxic effects and reported increases in mutation frequency,[252] DNA or chromosome damage,[243,249,253] and changes in gene expression[247,254,255] following CNT treatment.

The majority of in vivo studies have explored the effects arising from CNTs after pulmonary exposure and concluded that CNTs induce mild to severe toxic responses including scarring,[256] oxidative stress,[248] formation of fibrous tissue,[257−259] inflammatory responses,[258,260,261] granuloma formation,[257,259] artherosclerotic plaque formation,[262] and genotoxicity.[253] Potential toxic effects after intravenous injection are also being widely investigated.[182,263−265] Besides examining direct toxic effects of CNTs in vivo, the biodistribution and clearance of CNTs is a focus of many studies as a factor indirectly influencing CNT toxicity. Generally, CNTs can accumulate in a number of organs[266] depending on their functionalization[185,263] and can be excreted via the biliary or renal pathway.[185,267,268]

EFFECTS OF CNT PURITY ON TOXICITY

Mechanisms and origins of CNT toxicity are not fully elucidated and observed toxic effects may arise from poorly characterized CNT samples. The simplest and most straightforward contribution to CNT toxicity is the presence of large amounts residual CNT catalysts such as Ni, Co, and Fe, which in their elemental forms are capable of generating reactive oxygen species (ROS) in cellular environments. ROS in turn give rise to inflammatory symptoms including mitochondrial membrane degradation, depletion of antioxidant agents, rise in inflammatory biomarkers, and in great amounts decreases to cell viability. An early study demonstrated that SWNTs containing 30% iron were able to generate free radicals within 15 min of exposure to epidermal keratinocytes in the presence of DMPO,[269] whereas later investigations showed that higher amounts of embedded catalysts generate greater amounts of free radicals and increase inflammatory responses.[248,270,271] Several methods can be employed to remove residual catalyst including centrifugation,[272] high-temperature annealing,[273] and oxidation treatment by acid reflux.[274] Acid treatment results in the development of defect sites along the CNT surface and may be prone to redeposition of the catalyst particles on the CNT surface, which will remain bioactive for the generation of free radicals.[275]Acid treatment of CNTs is also commonly used as a starting or end point in functionalization schemes, but acid-treated CNTs have resulted in augmented toxic effects compared their untreated counterparts.[276−278] A likely explanation for these observed toxic effects associated with acid-treated CNTs is attributable to an increase in the number of defect sites along the CNT surface and therefore an increase in surface reactivity. Muller and co-workers varied the number of defect sites on MWNTs by mechanical grinding and high temperature annealing methods and demonstrated that acute pulmonary toxicity and genotoxicity increased for intratracheally administered MWNTs with a larger number of defect sites.[279] However, it has also recently been shown by Kagan et al. that SWNTs can be biodegraded by the enzyme myeloperoxidase found in neutrophils and macrophages, likely by interaction of the enzyme with carboxylic sites on the nanotubes surface,[112] which suggests that in the long run, oxidized CNTs may be more biocompatible than pristine CNTs.

EFFECTS OF CNT FUNCTIONALIZATION ON TOXICITY

Other covalent functionalization schemes have succeeded in reducing toxic effects by employing different functional groups. Sayes and co-workers reported decreases in cellular viability with oxidized SWNTs compared to phenylated SWNTs; this observed effect might be on account that this phenyl group sterically hinders access to adjacent defect sites.[280] Dumortier and co-workers report that functionalized CNTs proceeded by cycloaddition chemistry, which leaves no dangling bonds, did not affect cellular viability or activation of primary immune cells.[281] However, CNT toxicity is not only influenced by functional groups or conjugated molecules, but also by unintended interactions with biomolecules due to their high surface area per unit volume of the CNT and hydrophobic

nature, giving rise to further complications. Numerous examples of adsorption through dispersive forces or aromatic-stacking interactions with proteins,[282,283] RNA,[284,285] DNA,[286] and enzymes[287] have been shown for CNTs. Dutta and co-workers reported that bovine or human serum albumin adsorbed onto the CNT surface resulted in scavenger receptor binding and inflammatory responses upon uptake by macrophage cells that normally only occurs when albumin adopts a structurally altered or damaged state.[283] These effects, however, could be suppressed by first coating the CNT surface with Pluronic 127 surfactant to prevent albumin adsorption. Salvador-Morales and co-workers demonstrated another effect of CNT−protein adsorption resulting in immunotoxicity: activation of the complement system through the classical and alternate pathway.[282] The complement system recognizes and clears foreign material or altered host cells; activation of the classical or alternate pathways begins by receptor recognition of a pathogen via charge and hydrophobic interactions. Precoating the CNT surface with 0.5% Triton X-100 successfully prevented complement activation, highlighting the need for an effective dispersant for avoid binding to complement activation receptors. Ling et al. recently reported binding of the complement recognition protein C1g to SWNTs, although this did not activate the C1 complex (and therefore the classical pathway).[288] Casey and co-workers have proposed an indirect cytotoxic mechanism for in vitro trials resulting from media depletion of components adsorbed onto SWNTs[289] after observing significant color changes of the media.[239] These studies taken together demonstrate that an exposed CNT sidewall can elicit cytotoxic effects preventable by effectively coating the nanotube surface with a dispersing agent.

EFFECTS OF CNT AGGLOMERATION STATE ON TOXICITY

Several noncovalent functionalization schemes exist to achieve biocompatible dispersions of CNTs, as well as uniform suspensions of individualized CNTs for maximized exposed surface area and solubility. Intuitively, CNT dispersing agents that are themselves toxic, such as sodium dodecyl sulfate, sodium dodecylbenzene sulfonate, or Triton X-100, will also render CNT dispersions toxic,[290] whereas biocompatible surfactants used to disperse CNTs, such as sodium cholate, single-stranded DNA, gum arabic, hydroxypropylcellulose, and polyvinyl pyrrolidone, will not affect cell viability or proliferation.[291] Effective dispersal and exfoliation will be critical for in vivo bioapplications, as CNT agglomerates will evade bioclearance mechanisms, giving rise to toxic effects. Elgrabli and co-workers observed no pulmonary fibrosis, granulomas, and inflammation after intratracheal installation as observed in other studies by effective reduction of agglomerate size through dispersal with albumin,[292] and Mutlu and co-workers demonstrated macrophage clearance after effective dispersion and exfoliation of SWNTs through dispersal with Pluronic F108NF.[293] Such functionalization schemes with the ability to isolate CNTs will be necessary to avoid devastating effects that arise from ineffective bioclearance.

EFFECTS OF CNT LENGTH ON TOXICITY

Similar to the agglomerate state of a CNT dispersion, the lengths of CNTs within a sample also factor heavily into bioclearance. A CNT's length can span from nanometer to millimeter length scales depending upon the synthesis method, but the comparatively small diameter of a typical CNT in comparison to its length often draws a comparison between CNTs and other carcinogenic materials, such as synthetic vitreous fibers and asbestos. Exposure to fiber-derived free radical generation often induces hazardous amounts of DNA damage and genetic mutations over periods of exposure, causing a highly malignant form of cancer, mesothelioma. Common symptoms of these biopersistent fibers include alveolar lesions termed "granulomas", signs of oxidative stress, and excessive fibrous tissue within the examined specimen.[235] Macrophage cells are responsible for the removal of foreign materials from their host, but as shown in Figure 9, high aspect

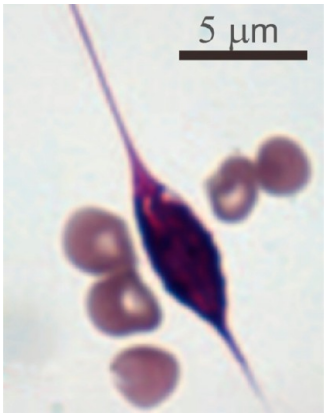


Figure 9. Incomplete internalization of long MWCNTs leading to frustrated phagocytosis and impeded bioclearance. Reprinted with permission from ref 235. Copyright 2008 Nature Publishing Group.

ratio fibers with lengths exceeding 20 μm encumber complete phagocytosis,[294] making them extremely resistant to bioclearance mechanisms.[295] Regarding CNTs, Poland et al. demonstrated that CNTs instilled as spherical or stellate shaped agglomerates with entire populations less than 20 μm produced no statically significant adverse reactions compared to samples with individually suspended MWNTs, agglomerates, and ropes of MWCNTs with lengths exceeding 20 μm.[235] Takagi et. al demonstrated actual mesothelioma formation in mice genetically susceptible to cancer and with treatments containing excessive amounts of MWNTs possessing significantly large lengths (between 10 and 20 μm).[236] Bioapplications that CNTs may be well-suited for will generally not require such lengths, especially because long CNT transporters are entirely unpractical for drug delivery, whereas shortening CNT samples batches is easily achieved by altering synthesis conditions or processing by extended sonication in combination with centrifugation.[296,297]

Several of the properties that make CNTs beneficial for bioapplications also render them toxic, but numerous viable solutions exist to mitigate or eliminate adverse effects arising from these individual problems. In summary, CNTs should be free of metal catalysts, appropriately functionalized for the intended purpose with minimal surface oxidation, coated effectively to shield biointeractions, and rendered short to avoid long bioretention times.

CONCLUSION

Despite some negative effects attributed to CNTs in vitro and in vivo, CNTs will still likely find many prominent and useful roles in biological applications.[298] Modification to CNT

physical properties in combination with functionalization schemes and administrative routes should circumvent most toxicological problems reported in the literature for the relevant application. For example, Dai et al demonstrated that the circulation lifetime of intravenously (IV) administered SWNTs depended on the branching extent of the associated polyethylene glycol polymer and that no SWNT accumulation was found in the spleen, liver, or brain but excretion from the body was observed through the urinary and renal pathways. Other authors have reported similar excretory pathways for IV administered CNTs. Association with PEG and other water-soluble nonfouling polymers prevents any detrimental effects arising from the CNT surface with the surrounding biological environment. Prospects for CNTs in terms of novel in vitro growth substrates and prosthetic implants remain promising as CNT incorporation with a biocompatible adhesive or fabrication of tightly interwoven CNT mats will ensure minimal CNT escape from cellular growth devices. In vivo CNT biosensors are easily rendered safe by placing the CNTs in a cage constructed of porous membranes with appropriate molecular weight cut off ranges. Thus, safe-handling and appropriate CNT modification for specific application eliminates most hazards reported in the current literature.

Despite this potential, there is no doubt that very large strides will have to be made until CNT-based systems and materials will be competitive with existing technologies. Moreover, regardless of whether such applications areas highlighted here approach realization, the issues with nanotube toxicity and nanoparticles toxicity more generally will give to the fore as a result of the widespread use of nanoparticles for consumer and industrial products. The extent of future exposure to nanoparticles associated with these new products is still unknown. So far only limited data is available regarding CNT toxicity. As a result, still not much is known about their impact on biological systems. Discussions regarding the potential risks of their widespread use, as well as their possible positive impact, are just beginning to take place.

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Author Contributions

The manuscript was written through contributions of all authors. Dr. E. Heister and Dr. E. W. Brunner equally contributed to this work. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CCR2, CC chemokine receptor 2
CCL2, CC chemokine ligand 2
CCR5, CC chemokine receptor 5
TLC, thin layer chromatography

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