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Review

Functionalized Carbon Nanotubes for Probing and Modulating Molecular Functions

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Carbon nanotubes (CNTs) entered the domain of biological research a few years ago, creating a significant amount of interest due to their extraordinary physicochemical properties. The integration of CNT-based strategies with biology necessitates a multidisciplinary approach that requires competences in the diverse fields of chemistry, physics, and life sciences. In the biomedical domain CNTs are extensively explored as novel drug delivery systems for therapy and diagnosis. Additionally, CNTs can also be designed as new tools for modulation of molecular functions, by directly affecting various biological processes or by interaction with bioactive molecules. The aim of this review is to discuss how CNTs can be exploited as new probes for molecular functions. The different sections illustrate various applications of CNTs, including gene silencing, surface cell interactions via glycoproteins, biosensing, intracellular drug delivery using an atomic force microscopy tip-based nanoinjector, modulation of antibody/antigen interaction and enzyme activity, and blocking of ion channels.

Introduction

Carbon nanotubes (CNTs) were discovered in the 1950s/60s (Monthioux and Kuznetsov, 2006; Bacon, 1960; Oberlin et al., 1976) and described as carbon filaments or carbon whiskers. Nonetheless, it was not until the early 1990s that their structure was described at the atomic level by S. Iijima (Iijima, 1991). Structurally speaking, CNTs are composed of carbon atoms arranged in a graphene sheet and rolled up to form a cylinder. CNTs can be constituted either of a single sheet of graphene, in which case they are referred to as single-walled carbon nanotubes (SWCNTs), or of multiple concentric layers to form structures known as multi-walled carbon nanotubes (MWCNTs). Most commonly, SWCNTs have a diameter from 0.4 to 3.0 nm and a length that spans from a few nanometers to a few micrometers, while MWCNTs are larger with a diameter reaching 100 nm and a length that varies from one to several micrometers and can go up to several millimeters (Figure 1). CNTs are largely exploited in materials science for their mechanical, electronic, optical, and magnetic properties (Jorio et al., 2008). In the field of biological and biomedical applications, interests in investigating possibilities of CNTs as new therapeutics and diagnostics and as probes for imaging have been attracting the interest of a rising number of research groups. Some of these topics have been discussed in recently published reviews (Bianco et al., 2008; Harrison and Atala, 2007; Kostarelos et al., 2009; Liu et al., 2009; Lu et al., 2009; Prato et al., 2008).

As produced, CNTs are highly insoluble in almost all organic solvents as well as aqueous solutions, which makes their manipulation extremely difficult and limits their use in life sciences. However, several strategies are currently available to enable application of CNTs under physiological conditions. The two main approaches, developed in the last few years, are based on noncovalent and covalent functionalization of CNTs (Tasis et al., 2006). Both approaches give rise to CNTs modified with different classes of molecules (i.e., surfactants, block-copolymers, peptides, proteins, nucleic acids, or small drugs), which results in improved solubility and dispersibility. In terms of differences between SWCNTs and MWCNTs, particularly in the field of biomedical applications, it is still not evident if one system presents more advantages than the other. They are certainly both attractive because they have shown the capacity of cellular uptake. Both functionalized SWCNTs and MWCNTs have displayed the possibility of effectively crossing biological barriers, which would allow their use in the delivery of therapeutically active molecules (Bhirde et al., 2009; Kostarelos et al., 2007, 2009; Liu et al., 2009). In addition, individual semiconducting SWCNTs exhibit intrinsic near-infrared (NIR) photoluminescent properties (Bachilo et al., 2002) and can be consequently developed for diagnostic purposes and in vivo detection (Welsher et al., 2009). In contrast, MWCNTs have a wide internal diameter that can be exploited for encapsulation of therapeutic molecules and a higher external surface available that offers more possibilities of conjugation or interaction with active molecules than SWCNTs (Ren and Pastorin, 2008). In general, one of the key advantages offered by functionalized CNTs in life science research is related to their capacity to easily translocate through cell membranes, displaying remarkably reduced cytotoxic effects (Kostarelos et al., 2007).

This review will discuss a series of examples to illustrate ways in which CNTs can be exploited as new probes for molecular functions. Separate sections will describe successively different applications of CNTs, including gene silencing, surface cell interactions, biosensing, and atomic force microscopy (AFM) tip nanoinjectors based on CNTs. A discussion of the capacity of CNTs to modulate antibody/antigen recognition, ion fluxes, and enzymatic activity will also be presented.

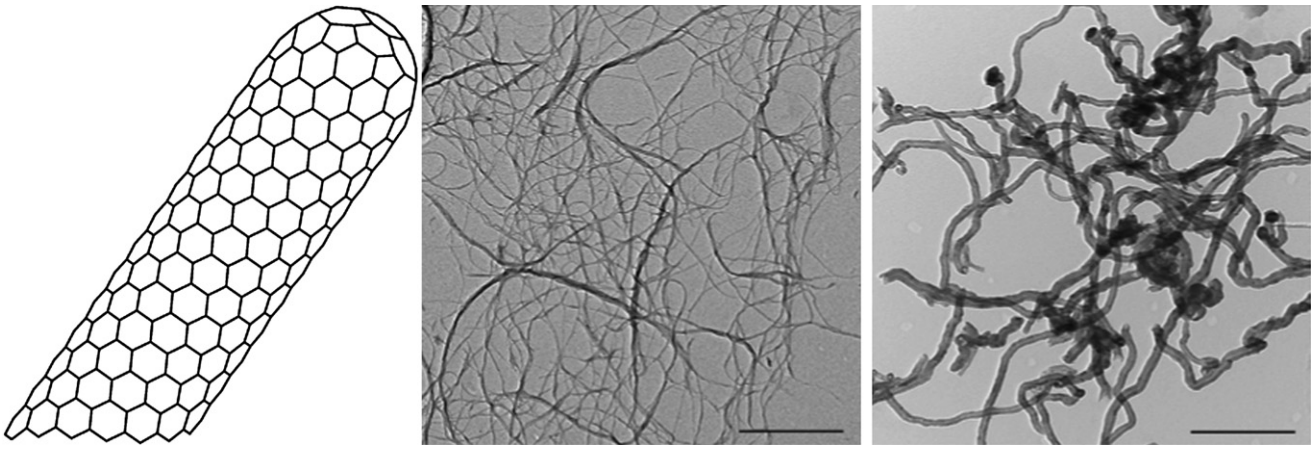

Figure 1. Molecular Structure and TEM Images of CNTs
Schematic representation of carbon atom organization within a CNT (left), TEM of SWCNTs (center), and TEM of MWCNTs (right). The two TEM images were taken with the same magnification so that the relative sizes of SWCNTs and MWCNTs can be directly compared. The scale bars correspond to 500 nm.

Functionalized Carbon Nanotubes for siRNA Silencing

One of the most promising areas where CNTs are used to influence and modulate molecular functions is as tools to achieve gene silencing (Wang et al., 2009a). Silencing a gene using a small interfering RNA (siRNA) sequence is becoming a clinically relevant option in therapy of different diseases (i.e., cancer, immune diseases, etc.) (Nguyen et al., 2008). Effective gene silencing in vivo has been achieved by forming supramolecular complexes between short RNA oligomers and cationic NH_3^+-functionalized MWCNTs or SWCNTs (Podesta et al., 2009; Zhang et al., 2006). We believe that in designing modalities for genetic intervention, the efficient capacity of CNTs to penetrate into the cells (Kostarelos et al., 2007) and release siRNA into the cytoplasm (the required site of activity) are two crucial requirements that have been achieved as reported in the examples described below. An additional advantage of CNTs is that they seem to be able to protect siRNA from cellular nuclease degradation, as recently demonstrated for DNA sequences (Wu et al., 2008b). Different types of CNTs functionalized with ammonium groups by amidation of oxidized nanotubes or via cycloaddition reactions have been used to form complexes with siRNA stabilized by positive/negative charge interactions. For example, ammonium-functionalized SWCNTs were used to complex siRNA encoding for knockdown of telomerase reverse transcriptase (TERT). Specific silencing of TERT expression was assessed by the effect of the complexes on the proliferation and growth of tumor using a mouse model (Zhang et al., 2006). The same functionalized nanotubes were complexed to an siRNA specifically targeting human cyclin A_2, which plays a critical role in DNA replication, transcription, and regulation of cell cycle. The complexes promoted apoptosis and inhibited proliferation of chronic myelogenous leukemia K562 cells in vitro (Wang et al., 2008, 2009b). In another study, SWCNTs modified with hexamethylene diamine were wrapped with a positively charged polymer [poly(diallyldimethylammonium)chloride] to improve the efficacy of the siRNA complexed sequence. Extracellular signal-regulated kinase siRNA silenced almost completely the expression of the target protein in primary cardiomyocytes (Krajcik et al., 2008).

In an alternative approach, SWCNTs were coated with lipopolyethylene glycol chains terminated with ammonium groups, which could be exploited to link SWCNTs and siRNA covalently or to form noncovalent complexes between functionalized SWCNTs and different siRNA sequences via electrostatic interactions. The proof of principle of this strategy was demonstrated using an siRNA known to silence the gene encoding for laminin A/C protein present inside the nuclear lamina (Kam et al., 2005). Similarly, complexation with an siRNA able to block CXCR4 and CD4 receptors on human T cells and peripheral blood mononuclear cells was shown to modulate HIV viral cell entry and lead to reduction of infection (Liu et al., 2007). Knockdown of transient receptor potential 3 channel, a possible target for the treatment of insulin-resistant conditions (Lanner et al., 2009), using the same type of functionalized SWCNTs complexed with siRNA resulted in pronounced decreases in induced Ca^{2+} influx and insulin-mediated glucose uptake in adult skeletal muscle cells.

Recently, it was reported that interfering with gene expression using an siRNA-MWCNT conjugate is more efficacious in cancer treatment in small model animals than a well-established delivery system based on liposomes (Podesta et al., 2009). In that study, cationic MWCNTs, obtained by 1,3-dipolar cycloaddition reaction of azomethine ylides (Georgakilas et al., 2002; Pantarotto et al., 2004), mediated siRNA silencing in a human lung xenograft model, triggering a significant antitumoral activity and a prolonged animal survival. In all of the strategies discussed above, CNTs intervene in modulation of cell functions at the cytoplasm level indirectly, by improving the biological activity of siRNA sequences they carry.

Functionalized Carbon Nanotube Interactions with Glycoproteins

Carbohydrates, in the forms of glycans, proteoglycans, glycoproteins, or glycolipids, are major components of the cell membrane and major determinants of molecular recognition processes on the cell surface. They are involved in diverse biological processes such as cell growth and development, cell-cell communication and interaction, cell trafficking, endocytosis,

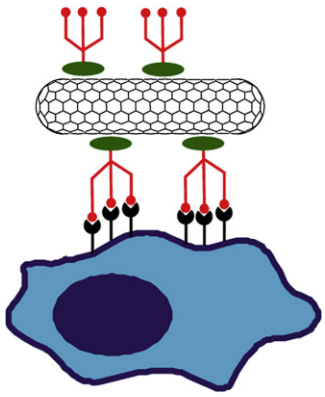

Figure 2. Molecular Recognition Interactions between CNTs Displaying Multivalent Glycoligands Covalently Attached to Pyrene Moieties and Carbohydrate Receptors on the Cell Membrane
Glycoligands are represented in red, pyrene in green, and carbohydrate receptors in black.

modulation of cell signaling, pathogen binding, inflammation, tumor cell metastasis, immune responses, signal transduction, and mediating cell adhesion through carbohydrate-carbohydrate or carbohydrate-receptor (lectin) interactions (Bertozzi and Kiessling, 2001; Bucior and Burger, 2004; Collins and Paulson, 2004; Dwek, 1996; Lee and Lee, 1995; Ohtsubo and Marth, 2006; Spillmann and Burger, 1996).

Abnormal glycosylation has been associated with many diseases such as cancers, autoimmune diseases (e.g., rheumatoid arthritis), and viral and bacterial infections. Therefore, characterization of the carbohydrate expression on the cell surface is crucial to understand their role in disease development as well as to provide basis for development of diagnostic tools. Due to their high surface area, CNTs have the ability to carry multiple copies of molecules grafted on their sidewall (Figure 2). This property is particularly interesting for recognition processes that require multivalency interactions between ligands and receptors (Mammen et al., 1998). Thus, over the past several years, the interface between CNTs and cells has been carefully tailored to reflect physiological interactions at the cell surface.

Understanding and mimicking specific interactions in bacterial adhesion is a challenging task that could lead to improvements in pathogen detection and inhibition of bacterial infections. In this respect, CNTs displaying multiple copies of carbohydrate moieties have been evaluated as inhibitors of bacterial infections, where they exert their activity by direct binding to pathogenic cells via specific adhesion-receptor interactions. Sun and coworkers demonstrated that SWCNTs are able to display multivalent ligands for capturing pathogens (Gu et al., 2005). SWCNTs functionalized with galactose were able to bind to pathogenic *Escherichia coli* O157:H7 cells specifically under physiological conditions, due to the presence of periplasmic galactose-binding proteins on the *E. coli* cell surface, leading to strong interactions and significant cell agglutination. In this case, some SWCNTs bound to a single *E. coli* cell, while others interacted with adjacent cells. Here, the high aspect ratio and surface area of SWCNTs enable the display of a large number of galactose residues while the linear and semiflexible nature of SWCNTs likely facilitates the binding with *E. coli* cells. In another study, Wang et al. (2006) reported that SWCNTs functionalized with galactose (Gal-SWCNTs) or mannose (Man-SWCNTs) were able to bind and aggregate anthrax (*Bacillus anthracis*) spores in the presence of calcium due to the expression of various carbohydrates on the surface of *B. anthracis* spores (Fox et al., 2003). *B. anthracis* spores were found to be aggregated and covered by SWCNTs. The agglutination was associated with practically complete (97.7%) reduction in colony forming units when Man-SWCNTs were used. The binding was found unique to the nanotube-displayed carbohydrates as the use of 120 nm diameter polystyrene beads functionalized with the same carbohydrates did not exhibit similar effects. The presence of Ca^{2+} ions was crucial, as the aggregation of *B. anthracis* spores was found to be reversible when Ca^{2+}-chelating agent was added. This result suggested an aggregation mechanism based on divalent cation-mediated carbohydrate-carbohydrate interactions between SWCNTs displaying multivalent monosaccharides and the sugar moieties on the *B. anthracis* spore surface. Thus, SWCNTs displaying carbohydrates have potential applications in the development of potent inhibitors or effectors of specific cellular responses for anti-bioterrorism, detection, and decontamination.

A different approach, where carbohydrate-decorated CNTs were designed to bind lectins, was investigated by Bertozzi and coworkers. Lectins are sugar-binding proteins that play a role in biological recognition involving cells and proteins and possess high specificity for their cognate sugar moieties. In a report by Chen et al. (2004), synthetic glycosylated polymers that mimic mucins, a family of heavily glycosylated proteins that cover the surface of many cells, present epitopes for receptor-mediated cell-cell recognition, and protect against biofouling (nonspecific protein binding), were used to coat SWCNTs as well as MWCNTs and to promote their cell binding through receptor-ligand interactions. Besides being soluble in water, which in itself is a desired property, CNTs with mucin mimics adsorbed on their surface displayed galactosamine residues that were recognized by a lectin, the carbohydrate receptor *Helix pomatia* agglutinin (HPA). Through this interaction, CNTs coated with mucin mimetics exerted the dual role of specific molecular recognition with protein receptors and resistance to biofouling. In addition, the complex of HPA with mucin mimic-coated CNTs possessed sufficient available HPA binding sites for further complex formation with glycoproteins from CHO cell surface (Chen et al., 2006). After binding to HPA, the cell surface glycans still had available binding sites for galactosamine residues on mucin mimic-coated CNTs.

Alternatively, SWCNTs were functionalized with glycodendrimers that contained peripheral carbohydrate units and a pyrene tail designed to adsorb on the nanotube surface through π-π interactions (Wu et al., 2008a). Glycodendrimers have been explored as mimics of cell-surface glycans due to their branched architectures and high density of peripheral functional groups (Cloninger, 2002). In addition, their geometry resembles the multiantenna N-linked glycans that populate eukaryotic cell surfaces. Small bundles and individual SWCNTs appeared to be coated with a thin homogeneous layer of glycodendrimers, in comparison to the thick nonuniform coating observed with glycopolymer-coated CNTs (Chen et al., 2004). Different glycodendrimers exhibiting α-mannose (Man-SWCNTs), lactose

(Lac-SWCNTs), or β-galactose (Gal-SWCNTs) residues were used to cover SWCNTs. The capacity for specific binding to *Canavalia ensiformis* agglutinin (Con A), *Arachis hypogaea* (peanut) agglutinin (PNA), and *Psophocarpus tetragonolobus* agglutinin (PTA), which recognize α-mannose, lactose, and β-galactose, respectively, was investigated. Man-SWCNTs and Gal-SWCNTs bound specifically to Con A and PTA, respectively. Lac-SWCNTs bound to PNA, as expected, and also to PTA as the non-reducing terminal monosaccharide in lactose is galactose. SWCNTs were also coated at different ratios with two glycodendrimers bearing mannose and lactose units. They were capable of binding to both Con A and PNA, thus demonstrating that SWCNTs displaying multiple glycan epitopes can bind simultaneously to different agglutins. This work opens new opportunities for probing biological processes as ligands other than carbohydrates could be introduced on the nanotube surface to modulate interactions with the corresponding receptors.

CNTs have been developed as molecular platforms for multivalent display of saccharide epitopes using a similar self-assembly process as the one developed by the Bertozzi group. In these studies, SWCNTs and MWCNTs were noncovalently functionalized with glycolipids and able to engage in specific ligand-lectin interactions, similar to glycoconjugates on the cell surface (Assali et al., 2009). Pyrene-polyethylene glycol-lactose was adsorbed on the surface of SWCNTs and MWCNTs via π-π interactions. The CNTs coated with the glycoconjugate exhibiting lactose residues were soluble in water and selectively recognized by a lactose-specific receptor such as PNA. In an alternative approach, SWCNTs were noncovalently functionalized with polymerized polydiacetylene-based glyconanorings (Khiar et al., 2009). This strategy was based on self-organization of charged anionic surfactants around the nanotube surface and on photopolymerization of diacetylene moieties. The nanotube surface was fully covered by striations of hemi-micelles. The glyconanoring-coated SWCNTs exhibited a large number of lactose residues, similarly to glycocalyx on the cell membrane and were recognized by PNA. These results confirm the capacity of such sugar epitope-bearing CNTs to engage in specific interactions with protein receptors.

SWCNTs coated with polysaccharides could also be used as mimics of the nanofibrous extracellular matrix. For this purpose, noncovalent wrapping of SWCNTs with natural polysaccharides, such as amylose, sodium alginate, and chitosan, afforded different SWCNT biomimetic nanofibrous scaffolds (Zhang et al., 2009a). Compared to non-coated SWCNTs, the polysaccharide-wrapped SWCNTs significantly enhanced cell adhesion and proliferation. The relationship between surface properties of the SWCNT scaffolds and cell behavior was investigated. The surface properties of the functionalized SWCNTs, such as functional groups, charge, and hydrophilicity, could directly influence the protein adsorption and lead to changes in cellular focal adhesion kinase (FAK) expression, thus affecting the mammalian cell morphology, migration, and proliferation as FAK is a protein tyrosine kinase that is recruited at an early stage to focal adhesions and regulates cell cytoskeletal organization, adhesion, migration, survival, and proliferation. Indeed, FAK was found to aggregate at the periphery of cells grown on non-functionalized SWCNTs, while FAK was homogeneously distributed in the whole body of cells grown on polysaccharide-modified SWCNTs, suggesting a strong cell adhesion. Among the different polysaccharide-wrapped SWCNTs, the amylose-coated SWCNT scaffold (i.e., bearing hydroxyl groups), with higher zeta potential and lower water contact angles, showed the highest cell adhesion strength and cell viability. Indeed, cells grown on the amylose-coated SWCNT scaffold appeared well spread with extensive network of cell lamellopodia and filopodia. This result can be partially explained by the charge of the cellular membranes, which is negative and favors attachment and growth of cells on positively charged surfaces. These examples illustrate the potential of CNTs coated with glycoprotein mimics to modulate ligand-receptor surface processes following their interfacing will cells.

Carbon Nanotube Biosensors

As probes for molecular functions, biosensors based on SWCNTs are certainly of extreme interest. Their electrical characteristics and their sensitivity to changes in the surrounding environment have made SWCNTs promising biosensors, such as electrodes for signal transmission and detectors for sensing chemical and biological molecules. In the field-effect transistors, the measurement of the electronic conductivity of SWCNTs allows the identification of the electronic state of the molecules immobilized on the nanotube surface. CNT-based field-effect transistor devices have been used, with success, for the detection of proteins, aptamers, and DNA hybridization, in antibody-antigen assays, and in enzymatic reactions involving glucose. In particular, many CNT-based glucose sensors have been developed where the electrochemical glucose detection relies on enzymatic glucose oxidation and subsequent hydrogen peroxide detection on the CNT electrodes (Allen et al., 2007).

In addition to electronic detection, the optical properties of SWCNTs can also be exploited for detection of biomolecules. SWCNTs have a tunable NIR emission that responds to changes in the local dielectric properties. Molecular adsorption can be transduced into an optical signal by perturbing the electronic structure of the nanotubes. SWCNT band gap fluorescence has been investigated as a methodology for NIR imaging of both in vitro and in vivo biological systems (Cherukuri et al., 2004; Leeuw et al., 2007; Welsher et al., 2008). The nanotube photoluminescence can be detected down to single-molecule level and it is more photostable than other common fluorophores such as organic dyes and semiconductor quantum dots. Human tissues and biological fluids are relatively transparent to NIR light, allowing deep tissue penetration of light up to several centimeters in thick tissue or blood media. Moreover, background cellular fluorescence is low in the NIR range, while most conventional fluorophores emit in the visible range where tissue and biological media have a strong background signal due to absorption in this wavelength range. Therefore, molecular detection using NIR light is promising for in vivo biomedical applications, even if optically based biosensors currently have limitations such as a slow response or unsuitability for in vivo use.

The Strano group has developed a CNT-based optical sensor for long-term glucose detection, where SWCNT emission was modulated in response to glucose adsorption (Barone et al., 2004). Their approach is based on the use of band gap modulation and charge transfer effects via photoluminescence for quantification of β-D-glucose. In another study, a glucose affinity

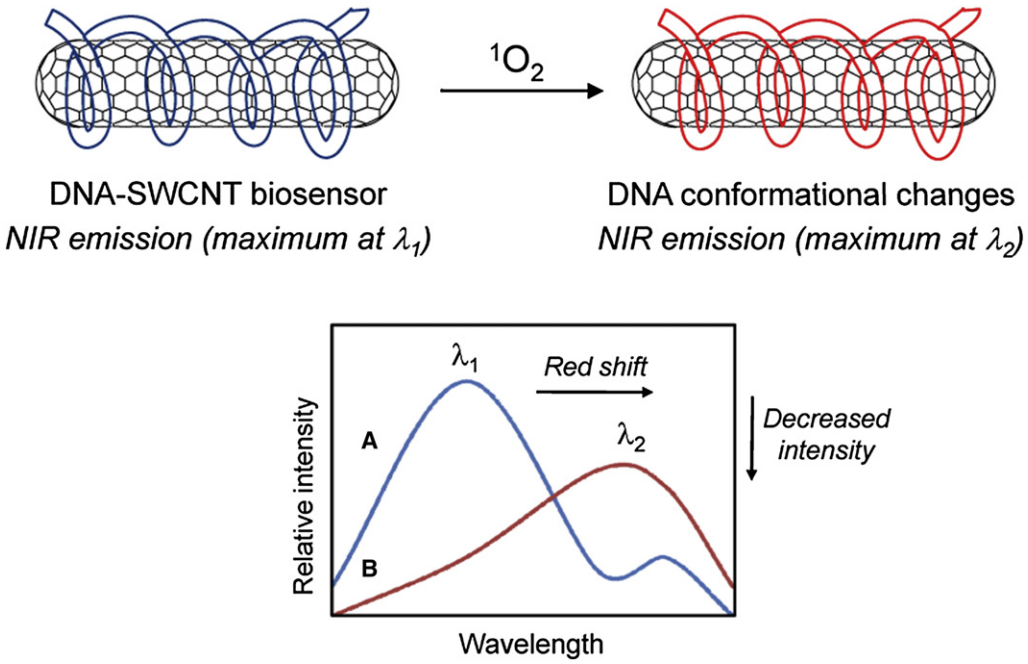

Figure 3. Red Shift and Decreased Intensity of Photoluminescence of DNA-SWCNT Biosensor
Red shift and decreased intensity after exposure to singlet oxygen leading to DNA conformational changes (B) in comparison with photoluminescence before exposure (A).

sensor was developed based on the control of SWCNT aggregation (Barone and Strano, 2006). Here, SWCNTs were first coated with dextran to form stable colloidal suspensions. The addition of lectin Con A induced aggregation of the SWCNTs and decreased their photoluminescence. Subsequent additions of glucose caused nanotube disaggregation, which restored photoluminescence.

The same group also investigated DNA polymorphism by using a SWCNT-based optical sensor (Heller et al., 2006). The conformational rearrangement of DNA was transduced directly via a SWCNT sensor. Indeed, hybridization of DNA immobilized on SWCNTs with its complementary strand induced a shift of the NIR emission, whereas non-complementary DNA did not cause any significant shift. In addition, the change of double stranded DNA conformation from the right handed B form to the left handed Z form induced a shift of the SWCNT emission. This biosensor is promising for Hg^{2+} detection, for instance in blood, as Hg^{2+} can specifically generate this conformational transition.

Reversible fluorescence quenching of SWCNTs was also used for biosensing purposes (Satishkumar et al., 2007). The strategy relied on immobilization of a dye-ligand conjugate, such as biotinylated anthracene, that adsorbed on the nanotube surface, resulting in quenching of the nanotube fluorescence. Target analytes, bearing an avidin receptor, induced the recovery of fluorescence by removing the dye-ligand conjugate from the nanotube surface due to the high affinity interaction between avidin and biotin. The reversibility of this approach allows sensitive and selective detection of selected analytes at the nanomolar level. In addition, high versatility is easily achievable by changing ligand-receptor groups for various analytes such as proteins and antibodies.

Recently, a multiplexed optical biosensor based on different types of SWCNTs was proposed. The variation of the emission wavelength of semiconducting SWCNTs was exploited to detect different biological analytes at the same time. This system allowed rapid and real-time identification and detection of multiple toxic analytes inside a living cell (Heller et al., 2009). Four genotoxins were detected and identified spectroscopically as distinct spectral responses using a DNA-SWCNT biosensor. Both alkylating agent and reactive oxygen species activity were detected in real time in living cells due to changes in the wavelength and emission band intensity of the NIR photoluminescence of SWCNTs induced by the genotoxic molecules that damage DNA (Figure 3). Active alkylating drugs and reactive oxygen species are important biological analytes and are difficult to measure in vivo and in real time due to fast degradation in the body. The signal transduction was attributed to charge transfer interactions between damaged DNA and SWCNTs and to solvatochromic shifts, which modify photoluminescence of the SWCNTs. The effect of hydrogen peroxide perfusion on photoluminescence of the SWCNTs was found reversible, most likely because of the degradation of H_2O_2 by intracellular enzymes. This work could lead to the development of optical sensors able to identify multiple biological processes arising from separate reaction pathways inside living organisms. A major breakthrough of the sensor developed by Strano and coworkers is its rapid and real-time identification of biological analytes with no need for additional sample processing (“label-free” sensor). It is indeed almost impossible to perform the amplification steps involved in many biological sensing schemes inside cells (Krauss, 2009). However, the technical challenge here is an existing difficulty in isolating macroscopic samples of a single type of

SWCNT. Moreover, an uncertainty lies in the probable large number of responses of other unique SWCNTs to the many other biological analytes found in vivo.

Sensor devices comprising glycosylated SWCNTs have been interfaced with living cells to electronically detect dynamic secretion of biomolecules (Sudibya et al., 2009). Ultra-thin networks comprising small SWCNT bundles were noncovalently functionalized with N-acetyl-D-glucosamine, D-glucose, or D-mannose. This coating conferred biocompatibility to SWCNTs without modifying their electronic properties. The monosaccharide-bound SWCNTs were capable of binding lectins, HPA, or Con A. The glycosylated SWCNT network supported the adhesion and growth of PC12 cells. The device was then used for the real-time monitoring of exocytosis. In this example, catecholamine release from PC12 cells during exocytosis induced significant changes in nanotube current. The glycosylated SWCNT-network offers the possibility to probe dynamic cellular activities, such as the release of biomolecules from living cells.

In summary, the SWCNT-based electrical and optical sensors demonstrate new opportunities for detection and quantification of biomolecules in samples relevant to biology and medicine, under physiological conditions and in real time.

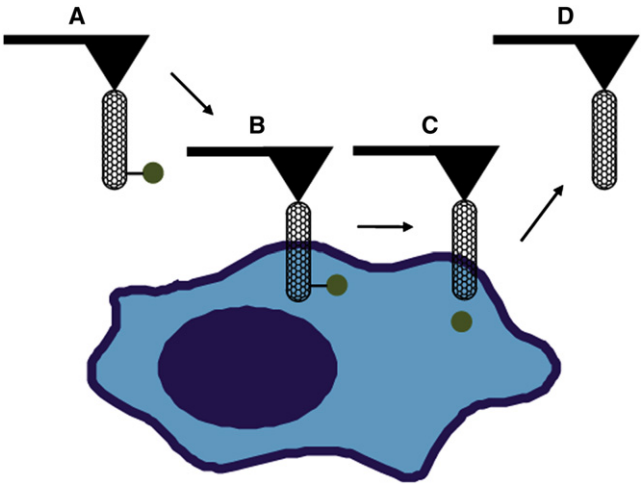

Figure 4. Nanoinjection Process
(A) Cargo attached to CNT AFM tip.
(B) Penetration of the cell membrane.
(C) Release of the cargo in the cytosol.
(D) Retraction of the nanoneedle.

Functionalized Carbon Nanotube AFM Tips as Nanoinjectors

The development of AFM over the past twenty years had a major impact on materials science, surface science, and various areas of biology, in particular the elucidation of structure and function of biomolecules. The challenge in developing AFM tips still lies with the production of smaller tips that have a long lifetime and are mechanically noninvasive. The size of the tip defines the resolution of the image, and the tip can additionally be used to detect and influence functional processes at the molecular level. CNTs have considerable potential as AFM tips due to their small diameter, high aspect ratio, and exceptional mechanical and electrical properties, in particular high Young's modulus (Treacy et al., 1996), toughness, and electrical conductivity. CNTs have the ability to reversibly buckle rather than break when they are subjected to axial compression. However, they are still not widely adopted due to their expensive and time-consuming fabrication (Wilson and Macpherson, 2009).

Recent developments highlighted the possibility of using CNTs as AFM tips to insert molecules inside cells at a precise site of action. Contrary to spontaneous molecular delivery using CNTs, recent experimental studies have successfully used AFM tips to deliver protein-coated quantum dots (Chen et al., 2007) and nanoparticles to cells (Vakarelski et al., 2007). The development of technologies to insert molecules into living cells offers an exciting opportunity to probe physical properties and biochemical interactions that govern cell functions. The crossing of the plasma membrane can be achieved via different techniques like increasing permeabilization of the membrane using lipids, electric currents, or pore-forming toxins (Stephens and Pepperkok, 2001). Microinsertion techniques, such as the microinjection method based on the physical penetration with a micropipette, have also been widely used (Tsulaia et al., 2003). However, the use of these techniques is hampered by the limited access to intracellular organelles that they provide and by the physical damage to the cell membrane they induce, which often leads to cell death. Recently, nanoinsertion techniques using an AFM tip have been developed and CNTs were described as possible nanoinjectors for the introduction of therapeutic agents into cells with minimal invasiveness.

A nanoscale cell injection system using a MWCNT-based AFM tip has been reported (Chen et al., 2007). The described MWCNT-based nanoinjector was able to penetrate inside the cells without cell damage and deliver a particular cargo into cells with capability of controllable release. The cell nanoinjector was constructed by attaching a single MWCNT to an AFM tip that served as the nanomanipulator. The system could be controlled at the nanometer scale due to the diameter of the MWCNT needle and to the nanoscale resolution of the AFM, allowing precise positioning of the nanoneedle as well as high sensitivity monitoring of the membrane piercing. As the MWCNT diameter was comparable to the dimension of some proteins such as calcium ion channels, the piercing induced by the AFM tip was probably healed by lipid diffusion without perturbing the cytoskeleton. The MWCNT AFM tip was functionalized with protein-coated quantum dots via a cleavable linker to trigger the release of the protein conjugate within the cell cytosol before retracting the needle by AFM control (Figure 4).

Similarly, a study has shown the use of MWCNTs as nanoinjectors to perform surgical procedures on living cells (Vakarelski et al., 2007). The robust nanoneedles consisted of an extra long MWCNT-terminated AFM tip (1 to 5 μm). The MWCNT AFM probe was fortified with carbon layers to improve resiliency to mechanical perturbations, which increased the force necessary to extend the buckling limit of the nanotube. The carbon layer also enhances the capacity of the MWCNT AFM tip to withstand capillary forces occurring during transfer to and from aqueous media. The MWCNT AFM tip was then coated with a layer of gold several nanometers in thickness on top of the carbon coating to enhance chemical versatility, as gold surfaces can be conjugated with various chemical and biological molecules. The decreased diameter of the MWCNT AFM tip in comparison

to conventional silicon nanoneedles (200–300 nm diameter) allowed a better manipulation with nanoscale resolution. The MWCNT AFM tip was able to penetrate through the plasma membrane with minimal cell deformation. Therefore, the MWCNT AFM tip with enhanced mechanical stabilization and possibility of chemical conjugation affords a robust and versatile injection system for surgical operation of single cells at the nanoscale.

The effect of the AFM tip on the cell membrane has been recently described theoretically using simulation studies on the penetration of a MWCNT with a diameter of 30–40 nm through a phospholipid bilayer (Wallace and Sansom, 2008). An intricate interaction between the nanotube and the lipid molecules from the plasma membrane was described with no apparent effect on the integrity of the lipid bilayer after MWCNT penetration, indicating that the bilayer was able to self-seal. This work highlighted the complexity of the interaction mechanism between CNT nanoinjectors and biological membranes.

Development of AFM tips functionalized with CNTs has opened the prospect to analyze processes at the single-molecule level and to sense and elucidate individual molecular interactions. Until now CNT tips have been used to measure the binding forces between protein-ligand complexes (Wong et al., 1998). We can certainly envision that the possibility to modulate and influence other fundamental biological processes, including molecular recognition between receptor-ligand, antibody-antigen, and complementary DNA strands, will become a more practical reality in the near future. In addition, to precisely insert a cargo into subcellular compartments of a single cell or extract materials from this cell with minimal impact on cellular function, the nanoinjector could also probe the physical properties and biochemical interactions that govern the cell behavior, in conjunction with organelle-specific optical probes.

Functionalized Carbon Nanotubes for Antibody/Antigen, Enzyme, and Ion Channel Activity Modulation

The biochemical functionalization of SWCNTs was used for the preparation of anti-CD3 antibody-nanotube conjugates for T cell activation by interaction with antigen receptors (Fadel et al., 2008). This work demonstrated that anti-CD3 adsorbed onto SWCNTs stimulates cells more efficiently than soluble anti-CD3. T cell responses were dependent on the concentration of SWCNTs. Efficient activation of lymphocytes was likely attributed to the high local concentration of antibody stimuli induced by SWCNTs (avidity of interaction to the antigen-presenting surface). Indeed, due to their high surface area, SWCNTs can present multiple copies of the antibody. This can be extremely useful in immunotherapy as ex vivo therapeutic stimulation of T cells requires, for example, efficient expansion by a robust stimulus. In another approach, MWCNTs were interfaced with enzyme to modulate enzymatic activity (Zhang et al., 2009b). α-Chymotrypsin specifically binds to functionalized MWCNTs via its catalytic site. These nanotubes were shown to recognize the enzyme active pocket and inhibit enzymatic function in a competitive manner. Although many studies have observed nonspecific interactions between proteins and CNTs, it is possible to design molecularly diverse functionalized CNTs with site-specific enzyme recognition for targeted enzyme inhibition and molecular sensing applications. Alternatively, SWCNTs were also able to affect the kinetics of S1 nuclease, an enzyme widely used as an analytical tool for the determination of the structure of nucleic acids (Peng et al., 2007). SWCNTs were complexed to human telomeric i-motif DNA and significantly accelerated the S1 enzyme cleavage rate by increasing the turnover number more than 22-fold. SWCNTs likely stabilize i-motif structure more efficiently for a better collision with the enzyme molecule. This approach may be promising for the modulation of human telomeric DNA structures in drug delivery and cancer therapy.

Finally, SWCNTs can directly modulate calcium and potassium movement by direct interaction with ion channels. Pristine and purified SWNTs were able to block K^+ channel subunits in a dose-dependent manner. The mechanism seems to be governed by geometrical factors, as SWCNTs having an average diameter of 0.9 nm blocked the channel better than those with a diameter of 1.3 nm (Park et al., 2003). More recently, carboxyl-functionalized MWCNTs were found to be able to suppress the current densities of three types of potassium channel in a time-dependent and irreversible way. This has important implications on the electrical signaling of excitable cells such as neurons and muscles (Xu et al., 2009). Alternatively, MWCNTs were shown to activate blood platelets by inducing extracellular calcium efflux (Semberova et al., 2009). These results have significance in the understanding of the mechanism of prothrombotic and other blood and vascular toxic effects of CNTs relevant to their development as biocompatible material for clinical use.

Conclusions

Functionalized carbon nanotubes entered the realm of biological research only few years ago. Nevertheless, they are showing a great deal of promise particularly when used in developing cancer therapeutics. Their biological activity can be associated with their capacity to interfere with molecular processes and functions. In this review, we have discussed some representative examples to illustrate how CNTs can influence molecular or cellular functions, directly or indirectly. These examples include siRNA-based therapy, biosensing, and enzyme activity modulation. Although the current state of the art on CNTs as probes for molecular interactions is still at its infancy, as the activities and capabilities of this type of nanomaterial to interact with biological matter is expanding, many new possibilities are anticipated in the near future.

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