FUNCTIONALIZED CARBON NANOTUBES FOR BIOLOGICAL APPLICATIONS

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FUNCTIONALIZED CARBON NANOTUBES FOR BIOLOGICAL APPLICATIONS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Chemistry

by
Lingrong Gu
August 2008

Accepted by:
Dr. Ya-Ping Sun, Committee Chair
Dr. Brian Dominy
Dr. Karl Dieter
Dr. Lesly Ann Temesvari
ABSTRACT

Carbon nanotubes with their unique pseudo-one-dimensional nanostructures and related electronic, optical, and mechanical properties have been attracting much attention for potential biological applications, such as biosensors, bio-delivery, bioimaging and so on. In this dissertation, recent studies and advances toward bioapplications of carbon nanotubes are critically reviewed, followed by detailed reports on my research projects concerning the use of carbon nanotubes as unique scaffolds for multivalent display of sugars and the relevant results from bio-evaluation of these novel nanomaterials.

Single-walled (SWNTs) and multiple-walled carbon nanotubes (MWNTs) were functionalized with amine-derivatived monosaccharides (galactoses and mannoses) and their dendritic constructs. These sugar-functionalized carbon nanotubes were readily water-soluble and biocompatible, and the intrinsic properties of the nanotubes were largely preserved post-functionalization. The bio-evaluation of the functionalized carbon nanotubes included experiments with pathogenic Escherichia coli O157:H7, in which specific interactions of the galactose (or its dendritic construct)-functionalized SWNTs with the E. coli cells to result in significant cell agglutination were observed. Similarly, the mannose-functionalized SWNTs were found to bind effectively to Bacillus subtilis (commonly used simulant for Bacillus anthracies or anthrax) spores for the formation of large aggregates. Also interesting was the finding that there were unique carbohydrate-carbohydrate interactions between SWNT-bound monosaccharides. All of the results suggest that carbon nanotubes are indeed unique scaffolds for displaying multiple copies
of sugars either individually or in pairs or quartets and that these unique nanomaterials offer valuable biologically significant functions.
DEDICATION

This dissertation is dedicated to my parents, for their love and support.
ACKNOWLEDGMENTS

I would like to thank my advisor, Professor Ya-Ping Sun, for his guidance and patience during my graduate study in Clemson University. I have been greatly benefited from his knowledge, experience, and philosophy in science and life, and shall certainly very much appreciate for my future career.

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CHAPTER I
INTRODUCTION-ADVANCES IN BIOAPPLICATIONS OF CARBON NANOTUBES

Carbon nanotubes are pseudo-one-dimensional carbon allotropes of high aspect ratio, high surface area, and excellent materials properties such as ultimate electrical and thermal conductivities and mechanical strength.\textsuperscript{1-3} While these all-carbon hollow graphitic nanomaterials may conceptually be viewed as “rolled-up” structures of one or more layers of graphene sheets for single-walled (SWNT) or multiple-walled (MWNT) carbon nanotubes, respectively, their formation is generally spontaneous in various production methods. There have been major advances in the availability of carbon nanotubes, both in quality and quantity, which has in turn stimulated the worldwide pursuit of carbon nanotubes for technological applications.\textsuperscript{3,4} Nevertheless, carbon nanotubes (especially SWNTs) are still relatively expensive, so that their uses in higher-value but lower-quantity technologies are probably better justified in the near term. As one of such technological areas of enormous potentials, bioapplications of carbon nanotubes have attracted much attention, with significant recent progress generating much excitement in the research community.

The unique properties of carbon nanotubes offer a wide range of opportunities and application potentials in biology and medicine. For example, the rich electronic properties of carbon nanotubes have been explored for the development of highly sensitive and specific nanoscale biosensors.\textsuperscript{5,6} Promising results have been produced on the use of carbon nanotubes in various electroanalytical nanotube devices,\textsuperscript{7} and as
electromechanical actuators for artificial muscles. The optical absorption of carbon nanotubes in the near-infrared has been used for the laser heating cancer therapy. In recent years, the rapid development toward maturation of methodologies for the chemical modification and functionalization of carbon nanotubes has helped to address many biocompatibility-related issues, opening up an even wider range of bioapplication opportunities in areas such as drug delivery, bioconjugation and specific recognition, including interestingly the use of sugar-functionalized carbon nanotubes to bind and aggregate anthrax spores. The biological fate and consequences of carbon nanotubes in vitro and in vivo have also been investigated for an understanding of the important issues on environmental impact and toxicity.

Here we report the recent advances in the chemical modification of carbon nanotubes targeting specifically their bioapplications, review various approaches and developments toward the use of carbon nanotubes in biology and medicine, and provide some perspectives on further investigations and emerging opportunities.

1.1 Functionalization of Carbon Nanotubes

There has been significant recent progress in the development and implementation of various covalent and non-covalent functionalization methods for chemical modification and solubilization of carbon nanotubes. The maturation that has been achieved in the functionalization and especially the aqueous solubilization of carbon nanotubes is particularly beneficial to the biocompatibility of the nanotubes toward
bioapplications. Among now widely used covalent functionalization approaches are addition reactions (Scheme 1.1) derived from those traditionally for graphite surfaces or established for fullerenes, and coupling reactions targeting oxidized defect sites on the carbon nanotube surface, commonly the surface defect-derived carboxylic acid moieties to react with amines and alcohols for amide and ester linkages, respectively (Scheme 1.2). For some functional groups such as amines, the covalent amidation functionalization is likely accompanied by significant non-covalent interactions or the adsorption of the functional groups on the nanotube surface, thus substantially improving some properties of the functionalized nanotube samples (such as excellent aqueous solubility for a hydrophilic functionalization agent\textsuperscript{16}).

Non-covalent approaches can usually preserve the structures and properties of carbon nanotubes after functionalization,\textsuperscript{17} thus are equally important to the bio-compatibilization and bio-applications of the nanotubes.\textsuperscript{15} Among commonly used schemes are surfactant dispersion,\textsuperscript{18} $\pi-\pi$ stacking with aromatic compounds,\textsuperscript{19} and polymer wrapping.\textsuperscript{20}

Besides synthetic polymers and small molecules, biological or bioactive species are used in the functionalization of carbon nanotubes for not only aqueous solubility but also enhanced bio-compatibilities and bio-recognition capabilities. Various proteins, DNAs, and carbohydrates have been covalently or noncovalently functionalized with carbon nanotubes, producing highly aqueous stable and biocompatible bio-carbon nanotube systems (Scheme 1.3). Some of the recent advances in this area are highlighted below.
Scheme 1.1 Covalent addition reactions on the sidewall of carbon nanotubes.
Scheme 1.2 Covalent reactions targeting carboxylic acids (derived from nanotube surface defects)
Scheme 1.3 Noncovalent biofunctionalized carbon nanotubes.
(From Ref. [21], [41], [53])
1.1.1 Proteins and Peptides

Non-specific adsorption is a commonly used method for preparing protein (and/or peptide)-carbon nanotube conjugates. For example, Nepal and Geckeler reported the dispersion and debundling of SWNTs with different proteins such as lysozyme, histone, hemoglobin, myoglobin, ovalbumin, bovine serum albumin, trypsin, and glucose oxidase. The dispersion ability of the proteins depends on various factors including their primary structure and pH. Proteins (histone and lysozyme) with higher basic residues contents were found to disperse carbon nanotubes better. The higher cationic (or anionic) charge density at a pH below (or above) isoelectric point may contribute to the repulsion of protein-stabilized SWNTs, thus less aggregation and better dispersion.

Hydrophobin (HFBI) is an amphiphilic protein and can self-assemble into monomolecular film at the air-water interface. With the aid of sonication, Kurppa et al. solubilized as-produced SWNTs with HFBI. The nanotubes were found to be embedded in the HFBI film. By engineering HFBI protein with gold nanoparticle, these same authors also prepared an interesting hybrid 1-D nanostructure of gold nanoparticles attached onto nanotube sidewalls in 2.6 nm spacing.

Mechanistically, electrostatic interactions as well as hydrophobic interactions (especially π-stacking) are recognized to play critical roles in protein-carbon nanotube interactions. For example, it has been suggested that the electron density of the aromatic residue on the hydrophobic face of the amphiphilic helical peptides can affect their efficiency in nanotube dispersion. Yan and coworkers studied interactions between functionalized MWNTs (in different diameters and surface functionalities) and various
proteins. Their results showed that the larger-diameter MWNTs were favored in the binding with proteins, and the binding was enhanced by the negative charges associated with nanotube functionalities and also affected by the surface electrostatic and stereochemical properties of the proteins.

Honek’s group recently identified a SWNT-binding motif (the tryptophan residue in a peptide called UW-1) through phage-displayed peptide library screening. Their study suggested that hydrophobic as well as π-stacking interactions between the tryptophan residue in UW-1 and the SWNT sidewall may dictate the high binding affinity of UW-1 to SWNTs. In a follow-up study, the tryptophan residue was altered and the effect of the alteration to the binding to SWNTs was evaluated. The experimental results combining with computational modeling indicated that the highest occupied molecular orbital of tryptophan residue interacts with the lowest unoccupied molecular orbital of SWNT.

Although the non-covalent binding is a versatile way to functionalize carbon nanotubes with proteins, the interaction is rather weak in comparison to covalent binding. For example, it has been reported that the nanotube-adsorbed proteins were largely removed via dialysis against water at room temperature, while covalently attached ones were stable under the same treatment.

As a classic example in covalent functionalization of carbon nanotubes with proteins, Sun and coworkers reported that carbon nanotubes could be functionalized with bovine serum albumin (BSA) or horse spleen ferritin via carbodiimide-activated amidation reaction. The BSA-functionalized SWNTs (BSA-SWNT) were then...
conjugated with goat anti-*Escherichia coli* O157 (Ab1) to form immuno-SWNT under physiologically compatible conditions. Compared to BSA-SWNT, the immuno-SWNT were capable of recognizing pathogenic *E. coli* O157:H7 cells through specific antibody-antigen interactions, as confirmed by SEM imaging results. The bacterial recovery determined by colony enumeration was as high as 80-90% when a larger amount of immno-SWNT and/or a high initial bacterial count were used.

The same platform was used to prepare BSA-functionalized MWNTs. After magnetic separation, those nanotubes with iron or iron oxide encapsulation were isolated and coated with anti-*E. coli* O157. The resulting “immunomagnetic MWNTs” were evaluated in immunomagnetic separation of *E. coli* O157:H7 cells in pure and mixed (with *Salmonella typhimurium*) cultures. The immunomagnetic MWNTs were able to efficiently capture and precipitate *E. coli* in aqueous media in a commercial magnetic separator within 5 min (Figure 1.1). Such capture was sensitive to approximately 40 bacterial counts per 100 µL aqueous buffer, and highly selective with no bound background bacteria even at a relatively high interfering concentration (e.g. selective toward *E. coli* O157:H7 at 800 CFU/100 µL with background flora of *S. typhimurium* DT104 cells at ~3,000 CFU/100 µL).

The same amidation reaction was employed by Kane and coworkers to covalently functionalize MWNTs with a specific peptide sequence, which specifically binds to the heptameric receptor-binding subunit of anthrax toxin. These nanotube-peptide conjugates were found to selectively destroy anthrax toxin with the reactive oxygen species generated by the nanotubes in near-IR radiation.
In addition to the direct amidation, proteins were also covalently attached to carbon nanotubes through a secondary bond which is usually cleavable under controlled condition. You et al. recently reported a BSA-conjugated MWNTs via disulfide linkage which can be cleaved at a glutathione concentration of 20 mM thus to release BSA. In their procedure, acid-treated MWNTs were first covalently bound with pyridyldithio groups through traditional defect site amidation reaction, and the pyridyldithio-MWNT complex was then conjugated with BSA in disulfide-exchange reaction.

1.1.2 Carbohydrates

Carbohydrates are highly hydrophilic biomolecules and extensively used in the functionalization of carbon nanotubes through either defect site covalent bonding or non-covalent wrapping. In addition to their high aqueous solubility and biocompatibility, carbohydrates-functionalized carbon nanotubes represent a unique class of pseudo one-dimensional sugar arrays of unusual properties. Many of these properties are not available to multivalent sugar ligands displayed on traditional scaffolds such as polymers, dendrimers, and proteins. For example, Sun and coworkers demonstrated that the monosaccharide (galactose or mannose)-functionalized SWNTs could effectively bind and aggregate anthrax (Bacillus anthracis) spores in the presence of a divalent cation like Ca$^{2+}$ (Figure 1.2). The overall effectiveness in the aggregation of B. anthracis spores was assessed as up to 97.7% CFU reduction in laboratory setting. The binding was found to be reversible by adding ethylene diamine tetraacetic acid (EDTA), which is a chelating agent for Ca$^{2+}$. The results suggested that the binding was likely due to Ca$^{2+}$-mediated
Figure 1.1 (a) A scheme on the formation of immunomagnetic-MWNT and their interaction with *E. coli* cells; (b) a high magnification TEM image of the magnetic-MWNT-BSA sample; (c) SEM images of the immunomagnetic separation sample showing *E. coli* cells bound with immunomagnetic-MWNT species. (From Ref. [32])
Figure 1.2 (a) SEM (scale bar = 500 nm) and (b) optical microscopy (scale bar = 20 µm) of the Ca\textsuperscript{2+}-mediated aggregation of anthrax spores with sugar functionalized SWNTs; (c) A schematic illustration of such aggregation. (From Ref. [11])
Figure 1.3 (top) The chemical structures of schizophyllan and curdlan. (a) TEM image of as-grown-SWNT/s-SPG composite, and (b,c) its magnified picture. (d) The original image of (c) was Fourier filtered to enhance the contrast of the composite. (From Ref. [41])
carbohydrate-carbohydrate interactions between the SWNT-displayed multivalent monosaccharides and the spore surface sugars. Interestingly, the experiments with same sugars-functionalized polymeric nanoparticles (~120 nm in diameter polystyrene beads) did not show any spore aggregation under same experimental conditions, suggesting that the observed binding and spore aggregation were unique to multivalent sugar ligands displayed on SWNTs.11

According to an earlier report from the same research group, the galactose-functionalized SWNTs (Gal-SWNT) were used to bind and agglutinate pathogenic *E. coli* O157:H7 cells.35 The binding required no divalent cations and was specific to β-D-galactose- but not α-D-mannose- or BSA-functionalized nanotubes, indicating that the multivalent interactions between the nanotube-bound galactoses and the *E. coli* cell surface galactose-binding proteins probably dictate the Gal-SWNT mediated aggregation of *E. coli* cells.

The multivalency of carbohydrate ligands should in principle affect significantly the overall affinity of multivalent ligand-receptor interactions.36 Thus, a series of dendritic galactopyranosides and mannopyranosides were synthesized for their functionalization of SWNTs to increase the valency of the sugar ligands displayed on each nanotube as well as to improve the aqueous solubility of the functionalized nanotubes.37,38 According to the bio-evaluation of those sugar dendron-functionalized carbon nanotubes by using established binding assays with *E. coli* cells, the β-D-galactose dendron-functionalized SWNTs (Gal₂-SWNT), compared to simple β-D-galactose-functionalized SWNTs (Gal-SWNT), had more effective CFU reduction in the agglutina-
tion of *E. coli* cells.\textsuperscript{38}

The non-covalent wrapping with polysaccharides is another commonly used approach in the carbohydrate functionalization and solubilization of carbon nanotubes.\textsuperscript{39,40-42} For example, Liu et al. used alginic acid (AA), a natural polysaccharide constituted with repeated β-D-mannuronic acid (M) and α-L-guluronic acid (G) segments, to solubilize raw MWNTs by sonication.\textsuperscript{40} The TEM and NMR studies of MWNT-AA complex showed that MWNT was partially wrapped by AA in possibly a way of M segments lying on the nanotube sidewall while part of G segments standing up on it. The addition of divalent or trivalent metal cations, such as Zn\textsuperscript{2+}, Ni\textsuperscript{2+}, Co\textsuperscript{2+}, Pb\textsuperscript{2+}, La\textsuperscript{3+} and Eu\textsuperscript{3+}, resulted in precipitation of the MWNT-AA complex. The precipitation could be re-dispersed in water by chelating the metal cations with EDTA.\textsuperscript{40}

Schizophyllan (SPG) is a polysaccharide with natural triple helix (t-SPG) structure which can be dissociated to single chain (s-SPG) in DMSO. The triple helix structure (t-SPG) can be recovered in a simple exchange of DMSO with water. Similar to that in cyclodextrin, the inside hollow of helical column in SPG is considered as hydrophobic, thus could entrap SWNTs through hydrophobic interactions. Shinkai and coworkers prepared SPG- and curdlan- (an analogue of SPG containing the main chain but not the side chain) wrapped SWNTs through first mixing/sonicating in DMSO and then exchanging with water.\textsuperscript{39,41,42} The microscopy characterization showed a unique periodical stripe structure in SWNT/s-SPG complex with two s-SPG chains right-handed helically wrapped on one nanotube. This was the first visual image that clearly demonstrated a patterned structure of water-soluble polymer wrapped SWNTs (Figure
1.3). The similar patterned structure was observed later in the case of Amylose-wrapped SWNTs.

1.1.3 DNAs/RNAs

DNA- and RNA-functionalized carbon nanotubes hold interesting prospects in various fields including biosensors and gene delivery. Similar to proteins and carbohydrates, DNA can be conjugated to carbon nanotubes through either non-covalent adsorption or covalent functionalizations in terms of direct amidation or the use of a bifunctional linker. For example, Hamers and coworkers used the bifunctional linker approach to functionalize carbon nanotubes with single-strand DNAs (ssDNA). The procedure included several steps, first the sidewall functionalization of SWNTs with 4-nitrobenzenediazonium salt, then the electrochemical reduction of -NO₂ to -NH₂ for reaction with a heterobifunctional cross-linker to introduce a maleimide group, followed by coupling with 5’-thiol-modified ssDNA (Scheme 1.4). The nanotube-bound ssDNAs were able to recognize their appropriate complementary sequence with a high degree of selectivity.

The specific interaction between DNA sequences on the carbon nanotube surface facilitates the assembly of nanotubes into architecture necessary for electrical circuits and molecular sensing applications. The DNA-directed multicomponent self-assembly of carbon nanotubes and gold nanoparticles has been demonstrated. For example, Mao et al. grafted ssDNA onto nanotube surface via non-covalent wrapping. The ssDNA-SWNT complex then hybridized with complimentary DNA-coated gold nanoparticles
(prepared through thiol-gold interaction). Interestingly, the DNA-directed assembly was found to be a reversible process, the formation at low temperature and dissociation at high temperature. Similarly, Deng and coworkers prepared a thiolated DNA strand d(GT)$_{29}$SH to wrap SWNT, which was later used as a rigid template for the assembly of gold nanoparticles.

The functionalization of carbon nanotubes with DNAs allows a precise control of their interfacial properties. Dai and coworkers non-covalently adsorbed poly-T DNA onto SWNT sidewalls, which were well dispersed and used for ultrathin atomic layer deposition of HfO$_2$ for high $k$ dielectrics. With the ~3 nm HfO$_2$ coating, high performance SWNT field effect transistors (FETs) free of gate-leakage were obtained. The same group also grafted short interfering RNA (siRNA) onto the SWNT surface. In their strategy, SWNTs were first non-covalently coated with amine-terminated phospholipid-polyethylene glycol (PL-PEG), and the amine attached to the nanotube was then covalently coupled with thiol-modified siRNA. The resulting siRNA-SWNT conjugates exhibited efficient silencing effects (50-60% knockdown) on both CD4 receptor and CXCR4 co-receptor (required for HIV entry into human T cells and infection).

The DNAs attached to carbon nanotubes were shown to mostly preserve their biological activity. For example, luciferase DNAs containing T7 promoter sequence required for in vitro transcription were adsorbed onto SWNT bucky papers. Compared to free DNAs, ~36% of the nanotube-bound DNAs remained functional in terms of RNA polymerase-catalyzed transcription/translation reaction. Strano et al. found that the
double-stranded DNA non-covalently wrapping SWNTs preserved its property of the conformation transition from right-handed B form to the left-handed Z form upon the addition of divalent cations such as Hg$^{2+}$, which causing a detectable red shift in band-gap emission of SWNTs. Thus, these DNA-wrapped nanotubes were used as a detecting agent for the ion in whole blood and tissue and within living mammalian cells (Figure 1.4).53 However, in some cases, the bounded carbon nanotubes can interfere with the normal DNA activities. For instance, a recent study by Qu and coworkers also found that the attachment of SWNTs to human telomeric i-motif DNA dramatically changed its kinetics of S1 cleavage by increasing the turnover number 22-fold over that of free DNA alone.54

The interaction between DNA and carbon nanotubes is stable enough to allow for separation of the dispersed nanotubes into well-defined sub-populations.46,55,57 Recently, ssDNA-wrapped SWNTs were separated into multiple fractions with different length distributions, and each fraction was examined for cellular toxicity to various cell lines.55 As demonstrated experimentally, while the longer SWNTs (>200 nm) were excluded by cells, the shorter ones were found to internalize into the cytoplasm, causing significantly decreased metabolic activity.55 Therefore, shorter SWNTs may be more toxic to cells than longer ones.

The DNA-carbon nanotube interaction is a complicated and dynamic process. Many studies on this subject have been pursued through a series of techniques including molecular dynamic simulation, microscopy, circular dichroism (CD), and optical spectroscopy.57-61 Although the detailed mechanism is not fully understood at present,
Scheme 1.4 Schematic illustration of addressable biomolecular functionalization of carbon nanotubes. (From Ref. [51])
Figure 1.4 (A) Concentration-dependent fluorescence response of the DNA-encapsulated (6, 5) nanotube to divalent chloride counterions. The inset shows the (6, 5) fluorescence band at starting (blue) and final (pink) concentrations of Hg$^{2+}$. (B) Fluorescence energy of DNA-SWNTs inside a dialysis membrane upon removal of Hg$^{2+}$ during a period of 7 hours by dialysis. (C) Circular dichroism spectra of unbound d(GT)$_{15}$ DNA at various concentrations of Hg$^{2+}$. (D) DNA-SWNT emission energy plotted versus Hg$^{2+}$ concentration (red curve) and the ellipticity of the 285-nm peak obtained via circular dichroism measurements upon addition of mercuric chloride to the same oligonucleotide (black curve). Arrows point to the axis used for the corresponding curve. (E) Illustration of DNA undergoing a conformational transition from the B form (top) to the Z form (bottom) on a carbon nanotube. (From Ref. [53])
several physical factors have been proposed to be driving DNA-carbon nanotube interactions,\textsuperscript{46,59-61} such as entropy loss due to confinement of the DNA backbone, van der Waals and hydrophobic (\(\pi\)-stacking) interactions, electronic interactions between DNA and carbon nanotubes, and nanotube deformation. A recent UV optical spectroscopy study of the ssDNA-SWNT system demonstrated experimentally that \(\pi\)-stacking was a force behind the DNA-SWNT binding, and the bound nucleotides were probably oriented along the nanotube’s long axis.\textsuperscript{60} This was later confirmed by Golovchenko \textit{et al.} in their study based on the time-dependent density functional theory (TDDFT) and optical spectroscopy measurements of the DNA-SWNT complex.\textsuperscript{61} Their results suggested that the DNA bases were orientated relative to the nanotube axis, and the orientation mainly depended on the base identity instead of the diameter and chirality of the nanotubes.\textsuperscript{61}

Generally, DNA-nanotube conjugates are widely pursued for their interesting and promising prospects in a number of areas, including especially nanoscale devices, biosensors, electronic sequencing, and therapeutic delivery, taking advantage of the unique pseudo-one-dimensional cylindrical structure of carbon nanotubes and the sequence-specific pairing interaction and conformational flexibility of DNA.

1.2 Bioapplications of Carbon Nanotubes

As reviewed above, there has been significant progress in addressing some fundamental and technical issues in the functionalization and solubilization of carbon
nanotubes with various biomolecules and bioactive species. It has stimulated the exploration of carbon nanotubes for applications in a variety of biological and biomedical systems and devices. Here some interesting recent studies and achievements for the use of carbon nanotubes in biosensors, bio-delivery, bioimaging, tissue engineering, and related results on toxicity and bio-distribution are highlighted and discussed.

1.2.1 Biosensors

Carbon nanotubes, especially SWNTs, with their fascinating electrical properties, dimensional proximity to biomacromolecules (e.g. DNA of 1 nm in size), and high sensitivity to surrounding environments, are ideal components in biosensors not only as electrodes for signal transmission but also detectors for sensing biomolecules and bio-species. In terms of configuration and detection mechanism, biosensors based on carbon nanotubes may be divided into two categories: electrochemical sensors and field effect transistor (FET) sensors. Since a number of recent reviews on the former have been published, our focus here is mostly on FET sensors.

Generally, a carbon nanotube FET device is constructed by a substrate (gate), two micro-electrodes (source and drain), and bridging material between the electrodes, which is typically an individual SWNT or a SWNT network. A SWNT FET is usually fabricated by casting a dispersion of bulk SWNTs or directly growing nanotubes on the substrate by CVD either before or after the electrodes are patterned. Due to the diffusive electron transport properties of semiconducting SWNTs, the current flow in SWNT FET is extremely sensitive to the substance adsorption or other related events on
which the sensing is based.

A wide variety of applications for SWNT FET have been explored, especially for the detection of proteins, antibody-antigen interactions, carbohydrates, DNAs and their hybridization, and single nucleotide polymorphism. The detection limit for the sensing of proteins or protein-protein interactions has generally been in the range of 100 pM to 100 nM. In a recent study, however, Byon and Choi improved the sensitivity to 1 pM for both nonspecific and specific protein bindings by modifying the geometry of the SWNT FET device. The substantially higher sensitivity (a factor of 10,000) was accredited mainly to the larger Schottky contact area on electrodes, which was achieved by angled deposition of electrode metals. This work might contribute to an acceleration in the progress toward the realization of nanoscale and label-free electronic biosensor systems.

Although antibodies are often used as specific targeting agents in FET devices for highly selective biosensing, they are typically not very stable and their large sizes (10-15 nm) may sacrifice the detect sensitivity in some cases. Alternatively, synthetic oligonucleotides (i.e. aptamers) have been demonstrated for their advantages (stability, small size, etc.) in the specific detection of amino acids, drugs, and proteins. For example, Maehashi et al. immobilized 5’-amino-modified aptamer onto the nanotube surface with the aid of a linker molecule, 1-pyrenebutanoic acid succinimidyl ester. The corresponding FET device exhibited a sharp decrease in the source-drain current in the presence of immunoglobulin E (IgE) target, with the detection limit down to 250 pM. Compared to the IgE-mAb (monoclonal antibody against IgE)-modified carbon nanotube FET devices,
The aptamer-modified ones were better performing in the detection of IgE under similar experimental conditions.\textsuperscript{68}

Star \textit{et al.} recently reported the synthetic oligonucleotide-immobilized carbon nanotube FETs as selective detectors for DNA immobilization and hybridization.\textsuperscript{69} Their device could specifically recognize target DNA sequences, including H63D single-nucleotide polymorphism discrimination in \textit{HFE} gene (responsible for hereditary hemochromatosis).\textsuperscript{69} The addition of divalent cation Mg\textsuperscript{2+} dramatically increased sensitivity by three orders of magnitude to reach the detection limit of 1 pM.\textsuperscript{69} As reported by Li and coworkers, synthetic oligonucleotide-immobilized SWNT networks could also be fabricated with two different metal contacts (Au and Cr) for electrical detection of DNA hybridization.\textsuperscript{70} Upon DNA hybridization, the drain current ($I_d$) increased in Cr-contacted FET device while decreased in Au-contacted one, indicating that the electrode-SWNT interface played an important role in the electrical signal transmission.\textsuperscript{70}

While it is generally acknowledged that SWNT devices are operated in terms of the Schottky barrier modulation effect,\textsuperscript{65,71} as well as the chemical gating effect,\textsuperscript{72,73} the sensing mechanism for nanotube-based FETs still remains a subject of debate. According to a recent report, experimentally the effect of protein adsorption on the relationship ($I$-$V$ curve) between source-drain current and the liquid gate potential could be used as a tool to identify the sensing mechanism in the SWNT transistor (Figure 1.5).\textsuperscript{74} It was found that the sensing was indeed due to a combination of Schottky barrier and electrostatic gating effects. The finding was consistent with another recent study of DNA immobiliza-
Figure 1.5 Experimental layout and results of a typical biosensing experiment. (a) Atomic force microscopy topology image of a SWNT between Cr/Au contacts on an insulating SiO$_2$ substrate. (b) Measurement setup, where a source-drain bias potential is applied and the device is gated through an Ag/AgCl reference electrode inserted in the electrolyte. The electrolyte is contained in a home-built flow cell (not depicted). (c) Band diagram for a holedoped SWNT showing electron conduction through thermally activated carriers into conductance band and hole conduction through tunneling through the Schottky barrier. $E_F$ is the Fermi energy, $E_C$ and $E_V$ are the energies of SWNT conductance and valence band edges respectively. The liquid gate potential changes the doping level in the bulk of the SWNT as indicated by the red arrows. (d) Results of a typical biosensing experiment. Current versus liquid gate potential curves acquired before (black line) and after (red line) adsorption of 1 µM HHCC in PB buffer ($V_{sd} = 10$ mV). The inset shows the real-time drop in conductance when HHCC is flushed over the device, while the gate potential is held constant at -50 mV vs Ag/AgCl, (as indicated by the blue dashed line). (From Ref. [74])
-tion on back-gated SWNT network. While earlier reports suggested that the sensitive region was limited to the nanoscale contacts, these new results indicated that it could also extend to the bulk channel section of the SWNT.

As discussed earlier, semiconducting SWNTs are the ones useful in nanotube-based FET devices. With rare exceptions, however, as-produced SWNTs are mixtures of semiconducting and metallic nanotubes (in a ratio close to the statistical limit of 2-to-1). Therefore, the selective removal of metallic SWNTs for purer semiconducting ones, such as by improving the post-fabrication electrical breaking down technique or taking advantage of the recent advances in post-production separation of semiconducting SWNTs from the as-produced mixtures, may prove highly beneficial.

1.2.2 Bio-Delivery

The current cancer and gene therapy requires the internalization of desired drugs and genes into the target cells. However, most anticancer drugs and genes suffer from low cellular uptake. Many delivery systems, such as liposomes, polymers, dendrimers, and a variety of nanomaterials, are developed for the need. Carbon nanotubes for their unique ability of penetrating cell membranes without the need for any external transporter system are particularly interesting as potential delivery vehicles. In the literature, nanotubes have been demonstrated as effective carriers for shuttling and delivering various peptides, proteins, nucleic acids, and small-molecule drugs into living cells.

Methotrexate (MTX) is a widely used anticancer drug, though it has a very low
cellular uptake. Prato, Bianco and coworkers employed a “double functionalization” strategy to attach both fluorescein isothiocyanate (FITC, a fluorescent probe) and MTX onto the sidewall of MWNTs (1,3-cycloaddition of azomethine ylides). In their procedure, carbon nanotubes were first co-functionalized with two orthogonally protected amino groups that were selectively de-protected and subsequently derivatized with FITC and MTX. As demonstrated in the in vitro study with Jurkat cells, the nanotube-bound MTXs were rapidly internalized into cells and accumulated in the cell cytoplasm. The same group of researchers prepared MWNT-doxorubicin (DOX, a popular anticancer drug) supramolecular complex through presumably the π-stacking interactions between the nanotube sidewall (MWNTs suspended by pluronic copolymer) and the DOX aromatic hydroxyl-anthraquinonic rings. The fluorescence intensity of the MWNT-bound DOX was suppressed due to the nanotube quenching effect. The toxicological assay with MCF-7 human breast cancer cells suggested a significant enhancement in cytotoxicity of the MWNT-DOX complex as compared to the free DOX and DOX/pluronic mixture. Since pluronic copolymer-dispersed MWNTs did not exhibit any toxicity, the enhanced cytotoxicity of the MWNT-DOX complex was attributed to an effective delivery of DOX into MCF-7 cells via MWNTs.

Dai and coworkers have been extremely active in studies of using carbon nanotube as delivery vehicles. According to their recent report on the cellular delivery of a large amount of DOX via SWNTs, two aqueous soluble SWNT samples were prepared, with one (PL-SWNT) from the non-covalent suspension with phospholipid-polyethylene glycol (PL–PEG, ~120 PEG units) and the other (PEG-SWNT)
from covalent functionalization with PEG (220 units). These samples were used for the non-covalent attachment of DOX through π-stacking. The binding of DOX to and their dissociation from SWNT were found to be dependent on pH and the nanotube diameter, generally lower pH and smaller diameter in favor of DOX dissociation from the nanotube. The *in vitro* toxicity study with U87 cancer cell showed significant cell death and cell apoptosis induced by the DOX-attached SWNTs (PL-SWNT-DOX), while PL-SWNT (no DOX loading) exhibited no toxic effect on cells. The IC₅₀ (half-maximum inhibitory concentration) value for PL-SWNT-DOX was estimated to be ~8 µM, higher than that of the free DOX (~2 µM). For specifically targeting U87 cancer cells, a cyclic arginine-glycine-aspartic acid peptide (RGD, targeting integrin αvβ3 receptors up-regulated in many solid tumors) was conjugated to the terminal amine groups in PL-SWNT. The bio evaluation on the effectiveness of RGD-conjugated PL-SWNT-DOX showed a lower IC₅₀ value of ~3 µM for RGD-positive U87 cancer cells, but no improvement for MCF-7 cells (short of corresponding receptors, negative control). The enhanced toxicity of RGD-conjugated PL-SWNT-DOX was attributed to the targeted intracellular delivery of DOX (Figure 1.6).

Similarly, Dai, Lippard, and coworkers covalently tethered PL-PEG (45 PEG units) with a platinum (IV) predrug complex to be used for the dispersion of SWNTs through hydrophobic interactions. The resulting platinum (IV) complex-SWNT conjugates [SWNT-Pt(IV)] showed dramatic enhancement of cytotoxicity to Ntera-2 cancer cells, while the free platinum (IV) complex was nearly non-toxic. The cellular platinum concentration of SWNT-Pt(IV) was six times of that of the free platinum (IV)
Figure 1.6 RGD peptide selectively enhances doxorubicin delivery by SWNTs and toxicity to integrin $\alpha_v\beta_3$-positive cells. (a) Schematic structure of PL-SWNT-RGD-DOX, i.e., SWNT functionalized with RGD at the termini of PEG and loaded with doxorubicin on the sidewall by $\pi$-stacking. (b) Confocal fluorescence images of integrin $\alpha_v\beta_3$-U87MG cells (top) and negative MCF-7 cells (bottom) treated with either PL-SWNT-DOX (right) or PL-SWNT-RGD-DOX (left). The concentration of DOX was 2 $\mu$M in all experiments. The U87MG cells incubated with PL-SWNT-RGD-DOX showed stronger DOX fluorescence in the cells than in the other three cases. (c,d) Concentration dependent survival curves of U87MG cells (c) and MCF-7 cells (d) treated by various samples, as indicated. The viable cell percentage was measured by the MTS assay. PL-SWNT-DOX had a lower toxic effect than free DOX on both types of cells, while PL-SWNT-RGD-DOX exhibited increased toxicity to U87MG cells but not to MCF-7 cells. (From Ref. [83])
Figure 1.7 Selective targeting and killing of cancer cells. (a) Chemical structure of PL-PEG-FA and PL-PEG-FITC, for solubilizing individual SWNTs. (b) (Upper) Schematic of selective internalization of PL-PEG-FA-SWNTs into folateoverexpressing (FR⁺) cells via receptor binding and then NIR 808-nm laser radiation. (Lower) Image showing death of FR⁺ cells with rounded cell morphology after the process in Upper (808-nm laser radiation at 1.4 W/cm² for 2 min). (Inset) Higher magnification image shows details of the killed cells. (c) (Upper) Schematic of no internalization of PL-PEG-FA-SWNTs into normal cells without available FRs. (Lower) Image showing normal cells with no internalized SWNTs are unharmed by the same laser radiation condition as in b. (Inset) Higher magnification image shows a live normal cell in stretched shape. (d) Confocal image of FR⁺ cells after incubation in a solution of SWNTs with two cargoes (PL-PEG-FA and PL-PEG-FITC). (e) The same as d for normal cells without abundant FRs on cell surfaces. (Magnifications: ×20.) (From Ref. [9])
complex alone, suggesting the effective delivery of platinum (IV) via SWNTs.\textsuperscript{84}

Beyond small-molecule drugs, other bioactive species such as nucleic acids were also successfully delivered into the cell by using carbon nanotubes.\textsuperscript{45,85} Zhang \textit{et al.} functionalized SWNTs with $\text{–CONH-(CH}_2\text{)}_6\text{-NH}_3^+\text{Cl}^-$ to mediate the conjugation of siRNA with specifically targeted murine telomerase reverse transcriptase (mTERT) expression to form the mTERT siRNA:SWNT+ complex.\textsuperscript{85} These specifically biofunctionalized SWNTs successfully entered tumor cell lines, silencing the expression of the targeted gene, inhibiting cell proliferation and promoting cell senescence \textit{in vitro}, as well as suppressing tumor \textit{in vivo}.\textsuperscript{85} Similarly, siRNA was grafted onto carbon nanotubes for delivery into human T cells and primary cells, which exhibited superior silencing effects over conventional liposome-based non-viral agents.\textsuperscript{45}

As mentioned earlier, the exceptional cell membrane-penetrating ability combined with the near-infrared (NIR) absorption characteristics of carbon nanotubes make them suitable materials in hyperthermia cancer therapy.\textsuperscript{9} Kam \textit{et al.} conjugated Cy3-labeled single-stranded DNA with SWNTs (DNA-SWNT), which were internalized into HeLa cells and cumulated in the cytoplasm region. It was found that the DNA was dissociated from SWNTs and entered into the cell nucleus upon six 10-second on-and-off pulses of 1.4 W/cm\textsuperscript{2} laser radiation at 808 nm, and the continued exposure under the same radiation for 2 min induced cell death. This was most likely due to the extensive local heating generated by the NIR absorption of SWNTs inside the cells.\textsuperscript{9} To selectively target and kill cancer cells, the folic acid (FA)-terminated phospholipid-polyethylene glycol (PL-PEG)-functionalized SWNTs (PL-PEG-FA-SWNTs) were synthesized and incubated
with folate-overexpressing (FR\(^+\), receptors for FA ligands) HeLa cells and also normal
cells before laser radiation. Extensive death in the FR\(^+\) cells was observed, while the
normal cells maintained normal proliferations (negative control, Figure 1.7).\(^9\) The direct
injection of SWNTs into tumor cells to induce cell death by NIR radiation represent an
attractive alternative to endocytosis for applications in cancer therapeutics.\(^9\)

For eventual \textit{in vivo} applications of the widely pursued carbon nanotube delivery
systems, a critical challenge is to be able to keep the nanotubes in the bloodstream long
enough for their intended functions.\(^{86}\) In a recent study by Yang \textit{et al.}, the oligomeric
poly(ethylene glycol) functionalization (PEGylation) of SWNTs was found to be
remarkably effective in achieving prolonged blood circulation (half-time on the order of
20 hours for the PEGylated nanotubes).\(^{87}\) Experimentally, skeleton \(^{13}\)C-enriched SWNTs
were functionalized with the oligomeric PEGs by targeting the nanotube surface defects
in well-established reactions. The plasma pharmacokinetic study was performed by
injecting male KM mice intravenously with a solution of the PEGylated SWNTs, and by
quantifying the nanotube concentrations in the blood post-exposure at different time
intervals in terms of the \(^{13}\)C isotopic abundance determination (isotope ratio mass
spectrometry). At one day post-exposure, for example, about 30\% injected dose (%ID)
remained in blood circulation,\(^{87}\) compared to only 0.2\%ID for pristine SWNTs at the
same time post-exposure.\(^{88}\) In a similar study by Dai and coworkers, a comparable blood
circulation time was obtained for SWNTs non-covalently functionalized with branched
PEGs.\(^{89}\) There were also preliminary results\(^{87}\) suggesting that the tumor uptake of the
PEGylated SWNTs benefited from their prolonged blood circulation. For the EMF6
model (breast cancer in BABL/c mice) and the Lewis model (lung cancer in C57BL mice), the tumor uptakes of intravenously administered PEGylated SWNTs were 8 %ID/g and 9 %ID/g, respectively, considerably higher than those for SWNTs without covalent PEGylation (in the absence of any specific targeting moieties).

Despite the experimental demonstration on the efficiency of carbon nanotube-enabled or associated delivery, the cell uptake mechanism and pathway of such delivery are still being debated. On the basis of the observed temperature dependent cell uptake of carbon nanotubes, Dai and coworkers suggested an endocytosis mechanism. For example, they used non-covalently DNA- and protein-functionalized SWNTs to study the uptake by HeLa (adherent) and HL60 (non-adherent) cells. These functionalized nanotubes were generally short (50-200 nm in length), individualized or in small bundles. The in vitro experiments with both cell lines were consistent with the energy-dependent endocytosis mechanism for the internalization of these short nanotubes. The internalization pathway could be mainly through clathrin-coated pits instead of caveolae or lipid rafts. They also studied the uptake of large DNA-SWNT aggregates (200-2,000 nm long and up to 15 nm wide), their results were similarly consistent with endocytosis being the uptake mechanism. However, because of the low solubility of these micro-scale nanotube aggregates, the uptake pathway was largely unclear.

The group of Kostarelos, Prato, and others disagreed with the endocytosis mechanism and proposed instead an energy-independent non-endocytotic mechanism involving insertion and diffusion of nanotubes through the lipid bilayer of the cell membrane. In their mechanism, the large biomolecules used in the solubilization of
nanotubes might alter the interactions with cells and affect the intracellular transport kinetics of the nanotubes.\textsuperscript{94,95} To elucidate such a possibility and to study the key steps in the cell uptake process, this group functionalized SWNTs and MWNTs on the sidewall with a wide range of functionalities including ammonium, small fluorescent probes, anticancer drugs, and antibiotics.\textsuperscript{95} All of these functionalized nanotubes were taken up by a wide variety of cells, and intracellularly trafficked through different cellular barriers into the perinuclear region, even under endocytosis-inhibiting conditions (Figure 1.8).\textsuperscript{95} These experimental results were consistent with those from molecular simulation, supporting the hypothesis of carbon nanotubes acting as ‘nanoneedles’ to pierce or penetrate the plasma membrane.\textsuperscript{96}

The apparent disagreements on the nanotube uptake mechanism could be due in part to differences in the nanotube constructs and the experimental procedures.\textsuperscript{95} More specifically, the non-endocytic internalization was found for small molecules covalently attached to carbon nanotubes, while translocation via endocytosis was found for large bio- or aqueous soluble macromolecules-conjugated SWNTs.\textsuperscript{95} Nevertheless, mechanistic debate is healthy and stimulating, and an improved mechanistic understanding is critical to facilitate future development of carbon nanotube transporters for bio-delivery applications.

Beyond the traditional delivery that relays on the cellular uptake of carbon nanotubes, Chen \textit{et al.} recently developed a MWNT-based cell nano-injector which delivered quantum dots (QDs) into cells through physical insertion into the cell membrane.\textsuperscript{97} Experimentally, a biotin-tethered pyrene (via a disulfide linkage) was
Figure 1.8 (top) A scheme of SWNT–NH$_3^+$ (f-CNT 1). And the intracellular trafficking and perinuclear localization of f-CNT 1. Triple-channel confocal image of A549 cells incubated for 2 h (a) in the absence (control) and (b) with 25 µg of f-CNT 1 (green). Plasma membrane stained with dye WGA-TRITC (red) and nucleus counterstained with dye TO-PRO3 (blue). The scale bars corresponds to 20 µm. (From Ref. [95])
Figure 1.9 (top) Schematic of the nanoinjection procedure. And nanoinjection of QDot streptavidin conjugates into a target HeLa cell. (A) Fluorescence image of the cells before nanoinjection. (B) Combined bright-field and fluorescence image of the cells before nanoinjection. The inserted arrow indicates the target cell. The dark shape in the lower left corner is the AFM cantilever. (C) Fluorescence image of the cells after the nanoinjection, showing fluorescent QDot streptavidin conjugates released inside the target cell. (D) Combined bright-field and fluorescence image of the cells after the nanoinjection. The QDot streptavidin conjugates are shown in red. The dark shape in the upper left corner is the retracted AFM cantilever. In all cases, fluorescence images were acquired with $\lambda_{ex} = 415$ nm and data collection with a 655-nm filter. Images are $70 \times 70 \mu m$. (From Ref. [97])
synthesized and attached to MWNT-AFM tips through \(\pi\)-stacking. The biotin-functionalized MWNT-AFM tips were then conjugated with steptavidin-coated QDs at a ratio of 5-50 QDs per nanotube. The QDs were delivered into a specific cell via nano-injection and released due to the cleavage of the disulfide bond. A major advantage suggested for this technology was that the delivery-release process can be repeated many times without cell damage (Figure 1.9).97

1.2.3 Bioimaging

Due to the exceptional capability of carbon nanotubes to penetrate cell membranes as well as their other unique physical and chemical properties, there is an increasing interest in their uses for cellular and sub-cellular imaging.98-105 There are two major types of bioimaging with carbon nanotubes, one in the direct imaging through the use of either the near-infrared band-gap fluorescence in semiconducting SWNTs18 or the UV/visible emission due to passivated surface defects in functionalized SWNTs and MWNTs;106 and the other in the indirect imaging based on associated fluorescent or radioactive agents.

In the absence of harsh chemical treatment or alteration, the well-dispersed semiconducting SWNTs exhibit band-gap fluorescence emissions in the near-IR spectral region (typically 900-1,600 nm), and the fluorescent spectra are characteristic of the chiral indices \((n,m)\) of the specific nanotubes.18 Since most natural biomolecules are relatively transparent and non-emissive in this spectral region, the sharp emission spectra of SWNTs can be detected even in a complex biological environment. The band-gap
fluorescence is quenched by surface defects in the carbon nanotubes. However, upon oxidation and chemical functionalization of carbon nanotubes at surface defect sites, both SWNTs and MWNTs exhibit strong photoluminescence in the visible and near-IR spectral regions.\textsuperscript{106,107} Therefore, the well-functionalyzed carbon nanotubes are also amenable to optical bioimaging applications.

Dai and coworkers used antibody-conjugated semiconducting SWNTs as near-IR fluorescent tags for selective probing of cell surface receptors and cell imaging.\textsuperscript{105} The SWNTs were non-covalently dispersed by phospholipid-polyethylene glycol-amine (PL-PEG-NH\textsubscript{2}). The functionalized SWNTs bearing amine groups were then conjugated with thiolated antibodies (Rituxan and Herceptin) specific to different cell surface receptors. In solution, these antibody-conjugated SWNTs emitted in the 1,000-1,600 nm spectral region when excited at 785 nm, suggesting that the electronic properties of the SWNTs were preserved after the antibody conjugation.\textsuperscript{105} The \textit{in vitro} cell experiments and near-IR imaging showed specific binding of the nanotube-attached antibodies to the host cells. The binding specificity was evaluated in terms of the fluorescence intensity ratio between host cells and no-host cells, and the results suggested a high selectivity of 55-20:1 for these antibody-conjugated SWNTs.\textsuperscript{105}

Weisman and coworkers employed pluronic copolymer-suspended pristine SWNTs (~1.0 nm average diameter, and ~1 µm average length) for the intracellular near-IR imaging of mouse peritoneal macrophage-like cells.\textsuperscript{98} It was found that the nanotubes were continually uptaken by the cells (up to 7.3 µg/mL in SWNT concentration) without any toxic effect, and that the uptake was temperature, time, and SWNT concentration
dependent. Macrophage samples with internalized SWNTs exhibited characteristic nanotube fluorescence spectra (Figure 1.10). The fluorescence intensities increased smoothly with incubation time and external nanotube concentration. The macrophage-like cells maintained their normal behavior at a nanotube concentration of ~4 µg/mL. In another recent study of Weisman, Beckingham, and coworkers, *Drosophila melanogaster* (fruit flies) were fed with bovine serum albumin (BSA)-dispersed SWNTs. The organisms and biological tissues were studied by using the intrinsic near-IR fluorescence imaging of SWNTs. It was reported that only a very small fraction (~10⁻⁸) of the ingested nanotubes became incorporated into the organs of the larvae.

The visible photoluminescence (extending to the near-IR) in functionalized carbon nanotubes is much brighter, amenable to optical bioimaging applications. A representative example is the characteristic photoluminescence of ammonia salts-functionalized SWNTs (SWNT-NH₃⁺), with the emission peak at 485 nm (corresponding to 395 nm excitation). The photoluminescence was used by Lacerda et al. to visualize the internalization and perinuclear localization of SWNT-NH₃⁺ in human caucasian lung carcinoma A549 cells. There was no cell plasma membrane damage even at a nanotube dose up to 500 µg/mL and 24 h post-incubation. This was the first report on the UV/vis florescence imaging of SWNTs in cells without the need of attaching a large fluorescent label. By minimizing the possible alternation on nanotube surface, the method could offer sharper images in comparison with those obtained with large fluorophore-attached SWNTs.

For the indirect imaging with fluorescent molecules-labeled SWNTs, a represent-
ative example was due to Dai and coworkers.\textsuperscript{102} They solubilized as-produced SWNTs via sonication with fluorescein-modified polyethylene glycol (Fluor-PEG, 114 PEG units). The absorption peak of the nanotube-attached Fluor-PEG was red shifted ~3 nm from that of free fluorescein, and the fluorescence intensity was quenched about 67% in phosphate buffer solution (pH 7.4). In the bio-evaluation with BT474 breast cancer cells, substantial intracellular fluorescence was observed, suggesting cellular uptake of the Fluor-PEG-functionalized SWNTs.\textsuperscript{102}

In addition to fluorescence, radioisotope tracing (see also section 1.2.5 for more details) and physical methods (\textit{e.g.} microscopy) are also effective techniques for the imaging of carbon nanotubes \textit{in vitro} and \textit{in vivo}.\textsuperscript{100,103} For example, Porter \textit{et al.} directly visualized individual SWNTs in cell through a new technique called low-loss energy-filtered transmission electron microscopy (EFTEM), which enabled the direct determination of SWNT distribution in both stained and unstained cells.\textsuperscript{103} The nanotubes were seen to enter the cytoplasm and localize within the cell nucleus, causing cell mortality in a dose dependent manner.\textsuperscript{103}

1.2.4 Tissue Engineering

As a result of the extended carbon-carbon sp\textsuperscript{2} network structure, carbon nanotubes are of high mechanical strength and chemical stability.\textsuperscript{108} The Young’s modulus of SWNTs is close to 1 TPa, and the maximum tensile strength is about 30 GPa, which make them suitable substrates for tissue engineering.\textsuperscript{109} For example, the as-grown MWNTs on quartz substrate from the CVD production method were used as constructs
Figure 1.10 (top) SWNT emission spectra in an aqueous Pluronic F108 suspension (blue trace) and in macrophage cells incubated in SWNT suspension and then washed (red trace). Samples were excited at 660 nm. Intensities have been scaled to aid comparison. (bottom) Fluorescence image of one macrophage-like cell incubated with SWNTs, showing emission detected from 1125 to 1600 nm with excitation at 660 nm. Intensities are coded with false color, and the image was obtained from a z-axis series by deconvolution processing. Intensity along the yellow vertical line is plotted on the graph to the right, showing high image contrast and localized emission sources. (From Ref. [98])
Figure 1.11 Picture (a) shows the surgery implantation of rhBMP-2 adsorbed MWNT/CHI scaffolds into mouse subcutaneous muscular pocket. Optical microscope micrograph (b) shows regenerated bone tissue and a minor fraction of remaining MWNT/CHI scaffold. Optical micrograph (c) shows a detail of regenerated bone tissue (collagen expressing cells, blue–green colored) after major disassembly of the MWNT/CHI scaffold, surrounded by muscle tissue (pink colored). It is remarkable the well-limited interface between adjacent tissues (see black dash line). The remaining MWNT/CHI scaffold (black colored) is pointed by black arrow. Optical micrograph (d) shows a detail of remaining scaffold plenty of fibroblasts (purple colored), prior to its disassembly and colonization by collagen expressing cells (blue–green colored). (From Ref. [112])
for culturing human osteoblastic cells. The cells were found to be successfully attached to and grown on the MWNT constructs with higher metabolic activities than those cultured on the control surface (highly ordered pyrolytic graphite or HOPG). Compared to the well-spread cells cultured on HOPG, those grown on MWNTs exhibited a spherical morphology and a disorganized cytoskeleton.

Neural stem cells (NSC) are known for their sensitivity to the environment. Kotov and Jan reported the differentiation of mouse embryonic neural stem cells on SWNT-polyelectrolyte multilayer thin films (PEI/SWNT, 6 layers), which were assembled layer-by-layer (LBL). The cells were successfully differentiated to neurons, astrocytes, and oligodendrocytes with a clear formation of neurites. Compared to the widely used poly-L-ornithine (PLO) substrates, the 6-layer PEI/SWNT thin films exhibited similar properties in terms of biocompatibility, neurites outgrowth, and the expression of neural markers.

Abarrategi et al. prepared a well-defined microchannel porous substrate constructed by acid-treated MWNTs (up to 89 wt%) and chitosan (CHI). The in vitro cell growth results showed that MWNT/CHI scaffolds were biocompatible for adhesion, spreading, proliferation, and viability of C2Cl2 (myoblastic mouse) cells. To study the in vivo ectopic bone formation at non-skeletal site, the rhBMP-2 (recombinant human bone morphogenetic protein-2)-adsorbed MWNT/CHI scaffolds were implanted into the mouse muscle tissue. After 3 weeks, the bone tissue regeneration was observed with significant MWNT/CHI scaffold degradation and replacement by cells (Figure 1.11). The biocompatibility of the MWNT/CHI scaffold was confirmed by the absence of chro-
nic inflammation during the whole implantation period.

1.2.5 Toxicity and \textit{in vivo} Biodistribution

With the rapid advances in the development of carbon nanotube-based biomaterials and technologies, there is an urgent need for the understanding of their toxicological and pharmacological properties. In the current knowledge, the toxicity of carbon nanotubes depends on many factors including dosage, physical form, and chemical modifications.\textsuperscript{12,113} For example, Cui and coworkers studied \textit{in vitro} toxicity of as-produced SWNTs in human HEK293 kidney cells.\textsuperscript{114} They found that SWNTs could inhibit cell proliferation and at the same time decrease the cell’s ability to adhere in a dose and time-dependent manner. However, the observed toxicity of the pristine SWNTs might be due to the lack of water solubility and the metal contamination, as debated in different reports.\textsuperscript{115,116} For an elucidation of these issues, Isobe \textit{et al.} prepared a metal-free and water-soluble carbon nanohorn aggregate (NHA) to simulate the covalently bonded class of self-aggregated carbon nanotubes.\textsuperscript{116} The cytotoxicity study of the NHA on 3T3 and HeLa cell lines showed a very low cytotoxicity, even lower than that of the widely used quartz microparticles, though there were still doubts on the reliability of the results because of some experimental problems.\textsuperscript{116}

In general, carbon nanotubes upon chemical modification exhibit much less toxicity or non-toxicity to living cell lines that have been investigated so far.\textsuperscript{117,118} For instance, Dumortier \textit{et al.} conducted \textit{in vitro} cell uptake study of the functionalized SWNTs with B and T lymphocytes and macrophages.\textsuperscript{117} Two types of functionalized
SWNTs were used, one prepared via 1,3-dipolar cycloaddition reaction and the other obtained through oxidation/amidation treatment. Both types of the functionalized nanotubes were rapidly uptaken by lymphocytes and macrophages without affecting the overall cell viability, and the one functionalized through the cycloaddition (thus higher water solubility) was found not affecting the functional activity of the different types of immunoregulatory cells.\textsuperscript{117}

Carbon nanotubes may enter the body via the route of intravenous, dermal, subcutaneous, inhalational, intraperitoneal, or oral.\textsuperscript{119} The potential \textit{in vivo} toxicity of carbon nanotubes has been reported and discussed.\textsuperscript{12} Largely preliminary \textit{in vivo} toxicity studies on respiratory and skin exposure to pristine carbon nanotubes did show some harmful effects.\textsuperscript{120,121} In a recent study reported by Donaldson and coworkers,\textsuperscript{122} pristine and BSA-dispersed MWNTs were intraperitoneal injected into mice at a dose of 50 µg per mouse. Only the long pristine MWNTs (more than 20 µm in length) showed some pathogenic effects similar to the foreign body inflammatory response caused by long asbestos (Figure 1.12).\textsuperscript{122} Conclusive results on the long-term exposure to these commercially supplied long MWNTs are still needed.

Gambhir and coworkers also conducted a pilot toxicologic study of the covalently and non-covalently PEG-functionalized SWNTs in mice.\textsuperscript{123} Their results suggested that the functionalized SWNTs persisted within liver and spleen macrophages for 4 months without apparent toxicity.\textsuperscript{123}

In the \textit{in vivo} experiments on pristine and the PEGylated SWNTs in mice, Yang \textit{et al.}\textsuperscript{87,88} did not find any of the animals exhibiting any signs of acute toxicity response
during the experimental period, even at a high exposure of 80 mg pristine or 24 mg PEGylated SWNTs (nanotube equivalent, both skeleton $^{13}$C-enriched) per kilogram body weight, consistent with other reports.

A systematic and quantitative analysis of metabolic process for carbon nanotubes in living body, such as their degradation, distribution, clearance, and bioaccumulation, is mandatory in order to fully understand their *in vivo* toxicity. Again according to the studies by Yang *et al.*, the $^{13}$C-enriched pristine SWNTs were cleared from the bloodstream quickly and distributed throughout most of the organs within 24 h (Figure 1.13). The nanotubes primarily accumulated in the lungs, liver, and spleen and retained at the relatively high accumulation levels over 28 days. Most noticeably and interestingly, there was around 1-3%ID accumulation of SWNTs in the brain, suggesting that the nanotubes could overcome the blood brain barrier. While the accumulation level of SWNTs in liver was relatively constant, there was a gradual decrease in the lungs from 15%ID to 9.4%ID in the monitoring period, for which the secretion by alveolar macrophage as mucus through mucociliary transport and the translocation through lymph nodes were suggested as pathways for the clearance of the SWNTs from lungs. Differ from their modified/functionalized counterparts, these pristine SWNTs could hardly be detected in urine and feces by either $^{13}$C isotope ratio measurements or transmission electron microscopy (TEM) analyses, suggesting that the nanotube morphology along with surface functionalities might significantly affect the nanotube *in vivo* behaviors. The PEGylated SWNTs were found to have the similar biodistribution profile to that of the pristine ones, with a rapid distribution time frame of ~ 1 h and also significant accumu-
Figure 1.12 Effect of the fibre on diaphragms after 7 days. a, TEM images of the test samples, NT_{tang2}: low-aspect-ratio tangled MWNT aggregates, LFA: long-fibre amosite, and NT_{long1}: MWNTs with a substantial portion longer than 20 µm. Female C57Bl/6 mice were injected i.p. with 50 µg of sample, killed after 7 days, and the diaphragms excised and prepared for visualization. b,c, SEM images (b) and haematoxylin and eosin histology sections (n = 3) (c) of the diaphragms show the presence of granulomatous inflammation (GI) in mice exposed to LFA, NT_{long1}. A small granuloma response in one of the three mice treated with NT_{tang2} was observed. The muscular portion of the peritoneal diaphragm (PD) and the mesothelial layer (ML) are aligned to show granulomatous inflammation at the peritoneal aspect of the diaphragm surface. Scale bars in b: 200 µm. Scale bars in c: 50 µm. (From Ref. [122])
Figure 1.13 The biodistribution of pristine SWNTs (skeleton $^{13}$C-enriched) in mice at different time points post-exposure. Data are presented as the mean ± SD (n = 3). (From Ref. [88])
ulation in liver and spleen.\textsuperscript{87} At day 7 post exposure, for example, \textasciitilde 25\%ID and \textasciitilde 3\%ID of the PEGylated SWNTs remained in liver and spleen, respectively. Nevertheless, the uptake of the PEGylated SWNTs by reticuloendothelial system (RES) was significantly reduced (28\%ID)\textsuperscript{87} in comparison with the pristine SWNTs (37\%ID).\textsuperscript{88}

Radiotracing is an efficient and broadly adopted method for \textit{in vivo} visualization of functionalized carbon nanotubes. The radio-labeling surface functionalities with \textsuperscript{\textit{125}}I,\textsuperscript{124} \textsuperscript{\textit{111}}In,\textsuperscript{100,104} \textsuperscript{\textit{64}}Cu,\textsuperscript{90} \textsuperscript{99m}Tc,\textsuperscript{125} and \textsuperscript{\textit{14}}C,\textsuperscript{126} has made it possible to quantitatively map the location of carbon nanotubes inside animals at different doses and time points. In a study by Singh \textit{et al.}, SWNTs were sidewall functionalized with \textsuperscript{\textit{111}}In chelated diethylentriaminepentaacetic ([\textsuperscript{\textit{111}}In]DTPA) and were intravenously administered into mice.\textsuperscript{100} The conjugates were rapidly cleared from systemic blood circulation with a half-life of \textasciitilde 3 h through renal excretion route, and no retaining was found in any of the RES organs (liver and spleen).\textsuperscript{100} The same functionalization strategy was later transferred to the preparation of [\textsuperscript{\textit{111}}In]DTPA-labeled MWNTs.\textsuperscript{104} After the injection into the mice through tail vein, the dynamic radioactivity tracking of the [\textsuperscript{\textit{111}}In]DTPA-MWNT showed that the nanotubes quickly entered systemic blood circulation and started their accumulation in kidney and bladder within 60 seconds. At 24 h, almost all MWNTs were eliminated from the body, and only a trace amount of radioactivity was detected in the kidney (Figure 1.14).\textsuperscript{104} The bio-distributions of DTPA-functionalized MWNTs (DTPA-MWNT) and serum-coated purified MWNTs were also compared by using the traditional histological examination of the major organs 24 h after the mice were intravenously administrated. The purified MWNTs exhibited accumulation in lung and liver, while no
such accumulation was found for DTPA-MWNT, consistent with the dynamic radioactivity tracking results obtained with $^{[111]}$InDTPA-MWNT.$^{104}$

Similarly, Dai and coworkers investigated the biodistribution and tumor targeting ability of HiPco-produced SWNTs wrapped with radio-labeled ($^{64}$Cu) phospholipid polyethylene-glycol copolymers (PL-PEG) in mice by in vivo positron emission tomography (PET), ex vivo biodistribution analysis, and Raman spectroscopy.$^{90}$ They found that the PL-PEG-functionalized SWNTs were surprisingly stable in vivo, exhibiting relatively long blood circulation time and low RES uptake. Efficient targeting of integrin positive tumor in mice was also demonstrated by tethering PL-PEG chains with RGD peptides (recognizing integrin positive tumors).$^{90}$

Radioactive elements have been conjugated to carbon nanotubes usually through chelation with surface functionalities. Such conjugates could suffer from decreasing or even losing activity over time due to gradual decay or dissociation of radiolabel from carbon nanotubes. Therefore, direct detection techniques based on the intrinsic physical properties of carbon nanotubes, such as Raman and near-IR fluorescence, might be more advantageous in the monitoring of their in vivo behaviors in both short and long terms. For example, the non-covalently PL-PEG (both linear and branched)-functionalized SWNTs were intravenously injected into mice for the study of their blood circulation and long-term fate in terms of the nanotubes’ intrinsic Raman signatures (Figure 1.15).$^{89}$ Effective PEG coating (longer chain and branched structure) on SWNTs afforded longer blood circulation, lower RES uptake, and near-complete clearance from main organs via biliary and renal pathways in ~2 months, which was likely due to their improved
Figure 1.14 (top) A scheme of $^{[111\text{In}]\text{DTPA-MWNT}}$. Normal rat distribution of $^{[111\text{In}]\text{DTPA-MWNT}}$. a) Dynamic anterior planar images of whole body distribution of $^{[111\text{In}]\text{DTPA-MWNT}}$ within 5 min after intravenous administration in rats. Color scale for radioactivity levels shown in arbitrary units. b) Static anterior planar images of whole body distribution of $^{[111\text{In}]\text{DTPA-MWNT}}$ in rats after 5 min, 30 min, 6 h, and 24 h post-injection (difference between a, 0–299 s image and b, 5 min image is due to lag-time in camera setup). c) % ID radioactivity per gram tissue at 24 h after intravenous administration of $^{[111\text{In}]\text{DTPA-MWNT}}$ quantified by gamma counting ($n = 3$ and error bars for standard deviation). (From Ref. [104])
Figure 1.15 (top) A scheme of functionalized SWNTs by various phospholipid-PEGs. And SWNTs in mice tissues probed by *ex vivo* Raman spectroscopy after injection into mice. (a) At 1 day p.i., biodistribution of SWNT-*l*-2kPEG, SWNT-*l*-5kPEG, and SWNT-*br*-7kPEG samples. (b and c) Evolution of the concentrations of SWNTs retained in the liver and spleen of mice over a period of 3 months. SWNT-*l*-2kPEG exhibited highest concentrations of retained SWNTs. (d) Raman mapping images of liver slices from mice treated with SWNT-*l*-2kPEG (*Left*), SWNT-*l*-5kPEG (*Center*), and SWNT-*br*-7kPEG (*Right*) at 3 months p.i. Again, highest SWNT Raman signals were observed in the SWNT-*l*-2kPEG-treated mouse sample under the same Raman imaging conditions (laser power, beam size, etc.). The error bars in a–c were based on three to four mice per group. Note that the injected SWNT solutions had a concentration of 0.1 mg/mL (optical density was 4.6 at 808 nm for 1 cm path). (From Ref. [89])
Similarly, Cherukuri et al. used the near-IR fluorescence of individualized semiconducting SWNTs to study their blood elimination kinetics and biodistribution in rabbits. The pluronic copolymer-coated SWNTs were intravenously administered into rabbits at a dose of ~20 µg SWNT per kg body mass. The pluronic coating was found to be displaced by blood protein within seconds, as indicated by changes in the near-IR emission spectra. The nanotube concentration in the blood serum decreased exponentially with a half-life of 1 h, and no adverse effects were observed in terms of the rabbit behavior and the pathological examination. At 24 h post administration, significant concentrations of nanotubes were found only in the liver.

It seems that based on the available in vivo biodistribution and toxicity studies there is no good evidence suggesting any significant acute toxicity or negative health effects of carbon nanotubes on the animals involved in the experiments. Obviously, further investigations are necessary and are likely already in progress in many laboratories to better address the toxicity and related issues.

1.3 Conclusions and Perspectives

The gradually maturing research topic concerning the chemical modification and functionalization of carbon nanotubes has stimulated the proliferation of studies on their potential bioapplications. Here we have reviewed the recent advances in the modification and functionalization of carbon nanotubes by biomolecules (e.g. proteins, DNAs, and
carbohydrates). Upon the modification and/or functionalization, the toxicological impacts of carbon nanotubes are significantly reduced, offering great opportunities to the use of carbon nanotubes for diagnostic and therapeutic purposes. Many experimental investigations have demonstrated that carbon nanotubes are ideal scaffolds to carry/display multiple biomolecules or bioactive species for applications such as capturing pathogens, substance delivery, bioimaging, and high-performance biosensors.

Carbon nanotubes currently represent one of the most promising platforms for novel biomaterials, though many technological challenges have to be overcome in order to realize their full potentials. For example, the performance of carbon nanotube-based biosensors depends strongly on the nanotube structural and electrical properties, so that the patterned growth and controlled deposition of nanotubes at desired sites with defined structures are necessary for more effective biosensing. The establishment of more accurate mechanisms for the interactions of carbon nanotubes with cells and other biological species is fundamentally important to the development of carbon nanotube-based bio-nanotechnologies. Along the same line, in-depth and systematic studies of pharmacokinetic profiles and long-term in vivo toxicity of carbon nanotubes are critical to their eventual clinical applications and therefore must be vigorously and carefully pursued. Nevertheless, the obviously rapid advances in this field have led us to conclude that there is a bright future for carbon nanotubes in biology and medicine.
References


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CHAPTER II

SUGAR-FUNCTIONALIZED CARBON NANOTUBES
AND BIOAPPLICATIONS

2.1 Overview

Single-walled and multiple-walled carbon nanotubes (SWNTs and MWNTs, respectively) have attracted much attention for their exceptional physical and chemical properties, and ever since their discovery for potential biological and biomedical applications. As reviewed in chapter I, recent advances in these applications include the use of carbon nanotubes as highly sensitive nanoscale biosensors, and as bio-carriers for drug or gene delivery and specific cell recognition. In this chapter, we report our contribution to this field, specifically the use of carbon nanotube as unique scaffold to display multivalent sugars in solution for various biological applications.

We functionalized SWNTs with tethered galactoses for aqueous solubility and for the nanotube-bound multivalent carbohydrate ligands to bind specifically to pathogenic Escherichia coli in solution to result in the significant cell agglutination (section 2.2). We extended our study to use dendritic molecules with paired galactoses to functionalize MWNTs, which offer advantages such as more economical production, higher purity, and larger inner tubular cavity (section 2.3). We then explored more systematically the use of carbon nanotubes to display multiple sugar moieties in pairs and in quartets, including the synthesis of a series of dendritic galactopyranosides and mannopyranosides with a terminal amino group for their functionalization of carbon nanotubes (section 2.4). These
sugar dendron-functionalized nanotube samples were thoroughly characterized and evaluated in biological assays such as the binding with pathogenic *E. coli* and with *Bacillus subtilis* (a nonvirulent simulant for *Bacillus anthracis* or anthrax) spores (section 2.4).
2.2 Single-Walled Carbon Nanotubes Displaying Multivalent Carbohydrate Ligands for Capturing Pathogens

2.2.1 Introduction

There have been considerable scientific interests in the understanding and mimicking of bacterial adhesin-specific interactions for various purposes, such as pathogen detection and the inhibition of bacterial infections via the chemotactic responses of the bacteria toward the corresponding ligands.\textsuperscript{1,2} Both natural and synthetic multivalent inhibitors have been evaluated.\textsuperscript{1} The latter includes the use of linear and branched polymers,\textsuperscript{3} dendrimers,\textsuperscript{4} proteins,\textsuperscript{5} polymeric and other nanoparticles,\textsuperscript{6,7} etc. to display multiple copies of sugar moieties.

Single-walled carbon nanotubes (SWNTs) represent a unique class of one-dimensional nanostructures, which offer many properties that are not available in traditional polymeric materials and nanoparticles.\textsuperscript{8} Potential biological applications of carbon nanotubes have been discussed and explored.\textsuperscript{9,10} Among widely investigated have been the uses of the nanotubes in nanoscale biosensors.\textsuperscript{10-12} Most of these applications require chemical modifications or functionalization of the nanotubes to impart aqueous solubility and/or to introduce biofunctionalities.\textsuperscript{10} In fact, carbon nanotubes have been functionalized with a variety of bioactive groups,\textsuperscript{9-16} and the functionalized carbon nanotubes have allowed the studies of their interactions with biological species.\textsuperscript{17,18} These studies are not only important fundamentally but also critical to the development of practical biosensors.

Because of the versatile chemical modification and solubilization, the one-
dimensional nanostructure of a SWNT, with the high surface area-to-weight ratio and some structural flexibility, may be exploited as a platform for multivalent array of carbohydrates in solution under physiological conditions. Here we report that SWNTs can be solubilized via the functionalization with derivatized galactoses and that the nanotube-bound galactoses could serve as polyvalent ligands and thus strongly interact with receptors on pathogenic *E. coli*, resulting in significant cell agglutination (Scheme 2.2.1).

2.2.2 Experimental Section

2.2.2.1 Materials

Palladium (10 wt% on activated carbon), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDAC), and amberlite IR-120 (plus) ion-exchange resin were purchased from Sigma-Aldrich. 18-crown-6, β-D-galactose-pentaacetate, α-D-mannose, pyridine, and anthrone were obtained from Acros. Sodium azide (99%) were from Alfa Aesar, tetrabutylammonium bromide (98%) was from Lancaster, and ethanol, chloroform, methylene chloride, THF, DMF, and diethyl ether were from Fisher. Solvent grade THF was dried and distilled over molecular sieves and then distilled over sodium before use. Other solvents were either spectrophotometry/HPLC grade or purified via simple distillation. Deuterated NMR solvents were obtained from Cambridge Isotope Laboratories.

The SWNT sample was produced in Prof. A. M. Rao’s laboratory (Physics Department, Clemson University) by using the arc-discharge method. It was purified by
Scheme 2.2.1 A schematic illustration for Gal-SWNT caused *E. coli* cell agglutination.

$= \text{D-galactose-binding protein}$
oxidative acid treatment. In a typical experiment, a nanotube sample (1 g) was added to an aqueous HNO$_3$ solution (2.6 M), and the mixture was refluxed for 48 h. Upon centrifuging at 1380g (Fisher scientific, centric 228 centrifuge) to discard the supernatant, the remaining solids were washed with deionized water until neutral pH and then dried under vacuum.

2.2.2.2 Measurements

NMR measurements were carried on a JEOL Eclipse +500 NMR spectrometer and a Bruker Advance 500 spectrometer that is equipped with a high-resolution magic-angle-spinning (HR-MAS) probe designed specifically for gel-phase NMR. The residual proton and carbon resonance of the deuterium solvents were used as internal standards. MALDI-TOF MS was performed on a Bruker AutoFlex system, with 2,5-dihydroxybenzoic acid as the sample matrix. Optical absorption spectra were recorded on a Shimadzu UV3600 UV-VIS-NIR spectrophotometer. Thermo gravimetric analysis (TGA) was carried out on a Mettler-Toledo TGA/SDTA851e system. Raman spectra were obtained on a John Yvon T64000 spectrometer equipped with a Melles-Griot 35mW He: Ne laser source for 632.8nm excitation, a triple monochromator, a research grade Olympus BX-41 microscopy and a liquid nitrogen-cooled symphony detector. Scanning electron microscopy (SEM) images were obtained on a Hitachi S4700 field-emission SEM system. Atomic force microscopy (AFM) analysis was conducted on a Molecular Imaging PicoPlus system equipped with a multipurpose scanner. The height profile analyses were assisted by using the SPIP software distributed by Image
Metrology.

The spectrophotometric method with the use of anthrone reagent was used to determine sugar contents. \(^\text{19}\) In a typical experiment, a solution of sugar-functionalized SWNTs (50 µL) was mixed with deionized water (50 µL), HCl (37%, 1 mL), formic acid (0.1 mL) and anthrone reagent (8 mL, 0.2 mg/mL in 80% H₂SO₄). The solution mixture was kept in a boiling water bath for 12 min, and then rapidly cooled in an ice bath for 30 min. The UV/vis absorption of the mixture was measured and corrected. Separately, the standard sugar (β-D-galactose or α-D-mannose) solutions of known concentration (1.0 mg/mL) were prepared. Various aliquots of the standard solutions (0, 25, 50, 75, and 100 µL) were tested in terms of the same procedure above to establish standard curves, from which the sugar content in the functionalized nanotube sample was obtained.

2.2.2.3 Synthesis of Sugar Monomers

The sugar monomers (Gal- and Man-) were synthesized in terms of procedures \(^\text{20}\) shown in Scheme 2.2.2 and 2.2.3.

2-Chloroethyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (1). To a cold solution (0 °C) of β-D-galactose-pentaacetate (7.8 g, 20 mmol) and 2-chloroethanol (1.93 g, 24 mmol) in dry CH₂Cl₂ was added BF₃.OEt₂ (48% Et₂O solution, 15 mL, 120 mmol) dropwise. Reaction mixture was gradually warmed to rt and stirred for 12 h before washed with cold water (10 mL × 3), aqueous sodium bicarbonate (5%, 10 mL), and cold water (10 mL). The organic phase was dried over anhydrous MgSO₄. Filtrate was condensed on a rotary evaporator. Crude product was purification by column
chromatography on silica gel with eluent of ethyl acetate : hexane (1:1) to afford 1 as a colorless solid (7.5 g, 91%): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 5.39 (d, $J = 3.65$ Hz, 1H), 5.22 (q, 1H), 5.02 (dd, 1H), 4.53 (d, $J = 8.25$ Hz, 1H), 4.20-4.16 (m, 1H), 4.15-4.09 (m, 2H), 3.91 (t, 1H), 3.78-3.73 (m, 1H), 3.64-3.61 (t, 2H), 2.15 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 1.98 (s, 3H); $^{13}$C NMR (125.7 MHz, CDCl$_3$) $\delta$ 170.5, 170.3, 170.3, 169.7, 101.7, 70.9, 70.8, 70.1, 68.6, 67.0, 61.3, 42.6, 20.9, 20.8, 20.8, 20.7 ppm. MALDI-TOF MS (M+Na)$^+$ : 433.88.

2-Azidoethyl-2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (2). Sodium azide (5.6 g, 80 mmol) was added to a mixture of 1 (7.5 g, 18 mmol) and 18-crown-6 (100 mg, 0.37 mmol) in DMF (30 mL). The system was stired at 65 ºC for 24 h. Water (60 mL) and toluene (120 mL) were added, organic layer was dried, condensed, and purified by column chromatography on silica gel with the eluent of ethyl acetate : hexane (1:1.5) to yield 2 (6.5 g, 86%): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 5.37 (dd, 1H), 5.23-5.19 (t, 1H), 5.01-4.99 (dd, 1H), 4.53 (d, $J = 7.75$ Hz, 1H), 4.19-4.09 (m, 2H), 4.04-4.00 (m, 1H), 3.92-3.88 (m, 1H), 3.70-3.64 (m, 1H), 3.51-3.45 (m, 1H), 3.30-3.25 (m, 1H), 2.13 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.95 (s, 3H); $^{13}$C NMR (125.7 MHz, CDCl$_3$) $\delta$ 170.5, 170.3, 170.2, 169.6, 101.2, 70.9, 70.1, 68.6, 67.0, 61.3, 50.6, 20.8, 20.7 (2C), 20.6 ppm. MALDI-TOF MS (M+Na)$^+$ : 438.85.

2-Azidoethyl-β-D-galactopyranoside (Gal-N$_3$). 2 (2.085 g, 5 mmol) was dissolved in 200 mL NaOCH$_3$ (0.1 M) and stirred at room temperature for 24 h. After concentration, the resulting residue was dissolved in water (50 mL) and then neutralized by Amberlite resin. The solvent was removed on a rotary evaporator to give Gal-N$_3$.
quantitatively: $^1$H NMR (500 MHz, MeOH-$d_4$)  δ 4.27-4.25 (dd, 1H), 4.04-3.99 (m, 1H), 3.83-3.81 (dd, 1H), 3.78-3.69 (m, 3H), 3.52 (m, 2H), 3.47 (m, 3H); $^{13}$C NMR (125.7 MHz, MeOH-$d_4$) δ 105.2, 76.8, 75.0, 72.5, 70.3, 69.3, 62.5, 52.1 ppm. MALDI-TOF MS (M+Na)$^+$: 272.73.

1,2,3,4,6-Penta-O-$\text{D}$-acetyl mannopyranoside (3). A solution of D-mannose (15.0 g, 83.3 mmol) in anhydrous pyridine (70 mL) was placed in an ice bath, followed by dropwise addition of acetic anhydride (100 mL, 1058.8 mmol). The mixture was stirred at 0 °C for 1 h and then at room temperature for 12 h. Pyridine and excess acetic anhydride were removed on a rotary evaporator, and chloroform (70 mL) was added. The mixture was washed with cold water (10 mL each for 3 times), aqueous sodium hydrogen carbonate (5%, 10 mL), and again cold water (10 mL). The organic fraction was dried over anhydrous MgSO$_4$. After filtration, the solvent was removed on a rotary evaporator to yield 3 (29.2 g, 90% yield): $^1$H NMR (500 MHz, CDCl$_3$) δ 6.08 (d, $J = 1.85$ Hz, 1H), 5.1-5.5 (m, 3H), 4.2-4.4 (m, 1H), 4.0-4.2 (m, 2H), 2.0-2.3 (m, 15H); $^{13}$C NMR (125.7 MHz, CDCl$_3$) δ 170.5, 169.9, 169.7, 169.6, 168.0, 90.5, 70.5, 68.7, 68.3, 65.4, 62.0, 20.0-22.0(m) ppm.

2-Chloroethyl-2,3,4,6-tetra-O-acetyl-$\alpha$-$\text{D}$-mannopyranoside (4). To a cooled (ice-water), solution of III (13.5 g, 34.6 mmol) and 2-chloroethanol (4.18 g, 51.9 mmol) in anhydrous dichloromethane (60 mL) was added (with stirring) BF$_3$-etherate (22 mL, 173 mmol) dropwise. The mixture was stirred at 0 °C for 1 h, and then at room temperature for 12 h. The same procedure described above for 3 was used in the workup to yield 4 (12.7 g, 90% yield): $^1$H NMR (300 MHz, CDCl$_3$) δ 5.22-5.37 (m, 3H), 4.89 (d,
Scheme 2.2.2 Synthesis of Gal-N₃.
Scheme 2.2.3 Synthesis of Man-N₃.
$J = 1.42 \text{ Hz, 1H), 4.25-4.35 (m, 1H), 4.00-4.16 (m, 2H), 3.80-3.97 (m, 2H), 3.70 (t, J = 5.54 \text{ Hz, 2H), 2.0-2.3 (m, 12H);^{13}C NMR (75.4 MHz, CDCl}_3; \delta 170.5, 169.9, 169.8, 169.7, 97.7, 69.3, 68.9, 68.5, 67.1, 65.9, 62.3, 42.4, 20.0-22.0 (m) ppm.}$

2-Azidoethyl-2,3,4,6-tetra-O-acetyl-α-D-mannopyranoside (5). To a solution of 4 (12.7 g, 30.9 mmol) and tetrabutylammonium bromide (0.2 g, 0.6 mmol) in DMF (50 mL) was added sodium azide (20 g, 309 mmol). The mixture was kept at 65 °C for 12 h. DMF was removed on a rotary evaporator, and the sample was re-dissolved in chloroform (50 mL). The organic phase was washed by water (10 mL each for 3 times) and dried over anhydrous MgSO$_4$. After filtration, the solvent was removed to yield 5 (12.6 g, 98% yield): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 5.28-5.40 (m, 3H), 4.90 (d, $J = 1.41 \text{ Hz, 1H), 4.30 (m, 1H), 4.10 (m, 2H), 3.88 (m, 1H), 3.70 (m, 1H), 3.47 (m, 2H), 1.9-2.3 (m, 12H);^{13}C NMR (125.7 MHz, CDCl}_3; \delta 170.7, 170.0, 169.9, 169.8, 97.7, 69.4, 68.9, 68.8, 67.1, 66.0, 62.5, 50.4, 20.0-22.0 (m) ppm.$

2-Azidoethyl-α-D-mannopyranoside (Man-N$_3$). 5 (5.0 g, 12 mmol) was dissolved in CH$_3$ONa/CH$_3$OH (0.1 M, 500 mL) and stirred at room temperature for 24 h. After being concentrated, the resulting residue was dissolved in water (100 mL) and then neutralized by Amberlite resin. The solvent was removed on a rotary evaporator to yield Man-N$_3$ quantitatively: $^1$H NMR (500 MHz, D$_2$O) $\delta$ 4.89 (d, $J = 1.85 \text{ Hz, 1H), 3.95 (m, 1H), 3.84-3.93 (m, 2H), 3.80-3.83 (m, 1H), 3.60-3.78 (m, 4H), 3.40-3.56 (m, 2H);^{13}C NMR (125.7 MHz, D$_2$O) $\delta$ 100.0, 73.1, 70.5, 70.1, 66.8, 66.5, 61.1, 50.3 ppm.$

2.2.2.4 Nanotube Functionalization
Before the functionalization reaction, Gal-N\textsubscript{3} and Man-N\textsubscript{3} were reduced to Gal-NH\textsubscript{2} and Man-NH\textsubscript{2} by the classical palladium-catalyzed hydrogenation. At 0 °C, to a solution of Gal-N\textsubscript{3} or Man-N\textsubscript{3} (2.085 g, 4 mmol) in methanol (10 mL) was added Pd/C (10 wt% palladium on activated carbon, 200 mg). The reaction mixture was gradually warmed to room temperature and stirred with the purging of hydrogen gas for 4 h. The Pd/C was removed by filtration, and the filtrate was evaporated to obtain Gal-NH\textsubscript{2} or Man-NH\textsubscript{2}.

The functionalization of SWNTs was based on the carbodiimide-activated amidation of the galactose-tethered amino groups with the nanotube-bound carboxylic acids.\textsuperscript{21} In a typical reaction, a purified SWNT sample (45 mg) was mixed with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, 112 mg, 60 mmol) in aqueous KH\textsubscript{2}PO\textsubscript{4} buffer (45 mL, pH = 7.4). Upon activation via sonication for 2 h, Gal-NH\textsubscript{2} or Man-NH\textsubscript{2} (450 mg, 2 mmol) was added. After sonication for another 36 h, the reaction mixture was loaded into a membrane tubing (cutoff molecular weight ~ 12,000) for dialysis against fresh deionized water for 3 days. The resulting suspension was centrifuged (1,380 g) for 30 min, yielding a dark-colored homogeneous aqueous solution of the galactose functionalized SWNTs (Gal-SWNT) or mannose-functionalized SWNTs (Man-SWNT).

**Gal-SWNT:** $^{1}$H NMR (500 MHz, D\textsubscript{2}O) $\delta$ 4.1-4.0 (broad), 4.0-3.8 (broad), 3.8-3.6 (broad), 3.6-3.5 (broad) ppm.

**Man-SWNT:** $^{1}$H NMR (500 MHz, D\textsubscript{2}O) $\delta$ 4.1-4.0 (broad), 4.0-3.7 (broad), 3.7-3.5 (broad) ppm.
2.2.2.5 Assay for \textit{E. coli}

The \textit{E. coli} O157:H7 strain C7927 was kindly provided by Prof. Michael P. Doyle, University of Georgia. After growth, repeated washing, and separation via centrifuging, the \textit{E. coli} cells were suspended in sterile saline solution (0.85\% NaCl) to an optical density of 0.5 (~5 x 10^8 CFU/mL according to McFarland standard #2). An aliquot of the suspension (200 µL) was added to an aqueous solution of the Gal-SWNT (0.32 mg/mL, 0.1 mL), and the mixture was rotated (40 rpm) at room temperature for 1 h. Upon centrifuging at 14,000 g (Eppendorf 5417R), the supernatant was discarded and the pellet was washed with phosphate buffered saline (PBS) via the same centrifuging-suspending procedure twice. The final pellet was suspended in a gluteraldehyde solution (1 mL 2.5\% gluteraldehyde in 0.2 M sodium cacodylate - hydrochloric acid buffer, pH 7.5) for fixing at room temperature for 30 min. The suspension was collected on a polycarbonate filter (Whatman nucleopore 0.2 µm) and allowed to fix for another 30 min. The filter with the specimen was rinsed with the sodium cacodylate - hydrochloric acid buffer (shaking for 5 min) 3 times, followed by post-fixing with freshly prepared osmium oxide solution (1\%, enough to cover the filter) for 1 h. Upon repeated rinsing with double-distilled water (shaking for 5 min each time), the specimen was dehydrated with graded ethanol and shaken: 50\% for 30 min, 75\%, 85\%, and 95\% each for 10 min, and 100\% for 10 min twice. The specimen was subjected to critical point drying to remove the ethanol completely, and was then mounted onto an aluminum stub with double-sided carbon tape for platinum coating before the electron microscopy analysis.
2.2.3 Results and Discussion

The Gal-SWNT sample was characterized by a series of instrumental techniques including solution-phase NMR, scanning and transmission electron microscopy (SEM and TEM), Raman, and near-IR optical absorption. The NMR results suggested significant effect on the galactoses from the nanotube attachment, consistent with the expected covalent functionalization. The SEM and TEM results showed that the nanotubes were exfoliated and well-dispersed (Figure 2.2.1). The Raman and near-IR absorption spectra were also typical of solubilized SWNTs. For example, the $S_{11}$ (1,828 nm) and $S_{22}$ (1,035 nm) absorption bands associated with the van Hove singularity pairs were preserved in the Gal-SWNTs (Figure 2.2.2). The nanotube content in the sample was estimated to be about 30% (wt/wt) in terms of the thermogravimetric analysis (TGA), in which the galactose functionalities were thermally defunctionalized and removed from the nanotube surface. On the other hand, the Gal-SWNTs in aqueous buffer was tested for total sugar content in terms of the classical spectrophotometry with the anthrone reagent. The result thus obtained (65 wt% sugar, with the rest being primarily nanotubes) in reference to the galactopyranoside before nanotube functionalization is in reasonable agreement with the TGA estimate of nanotube content.

Shown in Figure 2.2.3 are typical SEM images of the specimen of Gal-SWNT interact with *E. coli* O157:H7. There are apparently strong interactions between Gal-SWNTs and the pathogenic *E. coli* cells, with multiple nanotubes binding to one cell and some nanotubes “bridging” adjacent cells to result in significant agglutination.

The observed binding in Figure 2.2.3 is specific to the Gal-SWNTs. In the control
experiments, SWNTs covalently functionalized with either α-D-mannose or bovine serum albumin (BSA) protein\textsuperscript{16} and thus similarly soluble in aqueous PBS buffer were used to replace Gal-SWNTs under otherwise essentially the same experimental conditions, but no apparent binding was observed (Figure 2.2.4). This suggests that the nanotube-bound galactoses in Gal-SWNTs were responsible for the binding and cell agglutination shown in Figure 2.2.3. This is consistent with the report that there are periplasmic galactose-binding proteins on the \textit{E. coli} cell surface to couple with galactose ligands\textsuperscript{24}.

Multivalent ligands carried on polymers, dendrimers, or proteins are known to be more potent than their monovalent counterparts in cell adhesion\textsuperscript{25}. For the Gal-SWNTs in this study, the high aspect ratio and large surface area of the nanotubes enable the display of abundant sugar arrays, which are excellent polyvalent ligands toward the specific receptors on the cell surface. The semi-flexible nature of the nanotube scaffold may also facilitate the binding of multiple galactose ligands with the \textit{E. coli} cell.

2.2.4 Summary

In summary, the results reported here suggest that SWNT could serve as a unique carrier for multiple carbohydrate ligands and that the Gal-SWNTs are highly efficient in the capturing of pathogenic \textit{E. coli} in physiological solutions. The same materials may be applied to other pathogens bearing galactose receptors. In addition, the aqueous soluble SWNTs displaying galactoses and other bioactive ligands may also be developed as potent inhibitors or effectors for specific cellular responses.
Figure 2.2.1 (a) SEM image and (b) HR-TEM image (scale bar = 10 nm) of Gal-SWNT.
Figure 2.2.2 Near-IR absorption spectrum of Gal-SWNT.
Figure 2.2.3 SEM images for Gal-SWNT capturing pathogenic \textit{E. coli} cells. (Courtesy of T. Elkin)
Figure 2.2.4 SEM images from the control experiments of *E. coli* O157:H7 with (a) BSA-functionalized SWNTs and (b) Man-SWNT. Other experimental procedure and conditions were kept the same. (Courtesy of T. Elkin)
Reference


2.3 Carbon Nanotube as Scaffold to Display Paired Sugars in Solution

2.3.1 Introduction

Single-walled (SWNT) and multiple-walled (MWNT) carbon nanotubes have been studied extensively for their interesting and often unique properties.\(^1\) Recently, their potential biological and biomedical applications have been attracting increasing attention.\(^2\) For example, Kam, \textit{et al.} found that protein-SWNT conjugates could be internalized in various types of mammalian cells;\(^3\) and according to Hasegawa, \textit{et al.}, schizophyllan (a natural \(\beta\)-1, 3-glucan) could non-covalently wrap around SWNTs, with the pendant lactoside functional groups on schizophyllan still available for specific lectin recognition.\(^4\) It has also been shown that the one-dimensional nanostructure of a nanotube could be used for displaying multiple copies of bioactive species for their specific interactions with bacterial cells,\(^5,6\) such as the use of the bovine serum albumin-functionalized SWNTs in conjugation with \textit{Escherichia coli}-specific antibody for capturing the pathogen in physiological solution.\(^5\) In another example, Gu, \textit{et al.} solubilized SWNTs via covalent functionalization with the derivatized galactose 2'-aminoethyl-\(\beta\)-D-galactopyranoside in likely the amidation of nanotube-bound carboxylic acids.\(^6\) These galactose-functionalized nanotubes (Gal-SWNT), each displaying multiple copies of the sugar, were found to have adhesion to \textit{E. coli} O157:H7 to result in significant cell agglutination.\(^6\) It is also desirable to use MWNTs for the same purpose, not only for their more economical production and generally better purity but also for their unique properties and related opportunities, such as their larger inner tubular
cavities allowing easier access and encapsulation of various species. However, the functionalization of MWNTs with 2’-aminoethyl-β-D-galactopyranoside resulted in the Gal-MWNT sample of poor aqueous solubility, hardly useful to the cell adhesion and other biological applications in solution. An obvious difference between MWNT and SWNT is the former being heavier, thus requiring larger and/or more extended functional groups in the solubilization (Scheme 2.3.1). In fact, the necessary aqueous solubility could be achieved by functionalizing MWNTs with the compound containing a galactose pair, β-aminophthaloyl-N,N'-bis[11-O-(β-D-galactopyranosyl)-ethyl]-diamide (2, Scheme 2.3.2).

2.3.2 Experimental Section

2.3.2.1 Materials

Palladium (10 wt% on activated carbon), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDAC), triethylamine, 5-nitroisophthaloyl dichloride, and amberlite IR-120 (plus) ion-exchange resin were purchased from Sigma-Aldrich. 18-crown-6, β-D-galactose-pentaacetate, and anthrone were obtained from Acros. Sodium azide were from Alfa Aesar. Ethanol, chloroform, methylene chloride, THF, DMF, and diethyl ether were from Fisher. Solvent grade THF was dried and distilled over molecular sieves and then distilled over sodium before use. Other solvents were either spectrophotometry/HPLC grade or purified via simple distillation. Deuterated NMR solvents were obtained from Cambridge Isotope Laboratories.

The sample of MWNTs was supplied by Nanostructured & Amorphous Materials,
Scheme 2.3.1 A schematic illustration for Gal-MWNT and Gal$_2$-MWNT.
Inc. In a typical purification procedure, the nanotube sample (1 g) was suspended and refluxed in aqueous nitric acid solution (14%, 500 mL) for 48 h. The mixture was cooled to room temperature and centrifuged (1,380 g, 15 min). The supernatant was discarded, and the remaining solids were collected and washed repeatedly with deionized water until neutral, followed by the removal of water and then drying in vacuum to obtain the purified MWNT sample.

2.3.2.2 Measurements

NMR measurements were carried on a JEOL Eclipse +500 NMR spectrometer and a Bruker Advance 500 spectrometer that is equipped with a high-resolution magic-angle-spinning (HR-MAS) probe designed specifically for gel-phase NMR. The residual proton and carbon resonance of the deuterium solvents were used as internal standards. MALDI-TOF MS was performed on a Bruker AutoFlex system, with 2, 5-dihydroxybenzoic acid as the sample matrix. Optical absorption spectra were recorded on a Shimadzu UV3600 UV-VIS-NIR spectrophotometer. Thermo gravimetric analysis (TGA) was carried out on a Mettler-Toledo TGA/SDTA851e system. Raman spectra were obtained on a Jobin Yvon T64000 spectrometer equipped with a Melles-Griot 35mW He: Ne laser source for 632.8nm excitation, a triple monochromator, a research grade Olympus BX-41 microscopy and a liquid nitrogen-cooled symphony detector. Scanning electron microscopy (SEM) images were obtained on a Hitachi S4700 field-emission SEM system. Atomic force microscopy (AFM) analysis was conducted on a Molecular Imaging PicoPlus system equipped with a multipurpose scanner. The height
profile analyses were assisted by using the SPIP software distributed by Image Metrology.

The spectrophotometric method with the use of anthrone reagent was used to determine sugar contents. 7 Similar to that described in chapter 2.2, a small aliquot (50 µL) of the Gal2-MWNT solution (0.3 mg/mL) was mixed with deionized water (550 µL), HCl (1 mL), formic acid (0.1 mL), and 80% anthrone/H$_2$SO$_4$ solution (8 mL). The mixture was kept at 100 °C for 12 min, then rapidly cooled in an ice-bath for 30 min. The corrected absorption spectrum of the mixture was obtained, and the absorbance at 625.5 nm was used against a standard curve (prepared separately under the same experimental conditions) to determine the sugar content.

2.3.2.3 Synthesis of Galactose Dendron

The synthesis of 1 was available in chapter 2.2. The 2 (Gal$_2$) was synthesized in terms of procedures shown in Scheme 2.3.2.

5-Nitroisophthaloyl Dichloride: At 0 °C, the thionyl chloride (10 mL, excess) and DMF (10 droplet, catalytic) were added to a suspension of 5-Nitroisophthalic acid (2.111 g, 10 mmol) in dry CH$_2$Cl$_2$ (15 mL). The reaction mixture was warmed to the rt. and refluxed until a homogenous solution was developed (around 4 hrs). After refluxing for another hour, the yellowish solution was concentrated and dried in vacuum oven for 2 hrs. The product showed up as a yellowish solid (quitative) which can be used directly for next step without further purification. $^1$H NMR (500 MHz, CDCl$_3$) δ 9.20 (s, 2H), 9.09 (s, 1H); $^{13}$C NMR (125.7 MHz, CDCl$_3$): 165.6, 148.9, 137.4, 136.0, 130.9 ppm.
5-Nitroisophthaloyl-N,N’-bis[2’-O-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-ethyl]-diamide (I): At r.t., in a solution of CH₂Cl₂ (30 mL) containing 10% Pd/C (208 mg), 2’-azidoethyl-2,3,4,6-tetra-O-acetyl-galactopyranoside (2.085 g, 5 mmol) (see chapter 2.2) was added and stirred for 4 hrs with the H₂ purged in and out. The Pd/C was filtered out and the filtrate was added triethylamine (4.5 mL) and 5-nitroisophthaloyl dichloride (0.6 g, 2.4 mmol) at 0 °C. The mixture was stirred under nitrogen atmosphere at 0 °C for 2hrs and further 8 hrs at r.t., then washed with 1M HCl (30mL), saturated NaHCO₃ (30 mL), and water (30 mL). The organic layer was dried and concentrated, followed by column chromatography on silica gel with the EtOAc as the eluent to give I as a white solid (2.04 g, 88.8%): ¹H NMR (500 MHz, CDCl₃) δ 8.81 (d, J = 1.35 Hz, 2H), 8.58-8.57 (t, J = 1.375 Hz, 1H), 5.40-5.36 (d, J = 3.2 Hz, 2H), 5.20-5.16 (dd, J = 7.8, 7.3 Hz, 2H), 5.01-4.98 (dd, J = 3.2, 3.25 Hz, 2H), 4.50-4.49 (d, J = 7.75 Hz, 2H), 4.12-4.03 (m, 4H), 3.97-3.90 (m, 4H), 3.87-3.83 (m, 2H), 3.74-3.68 (m, 2H), 3.65-3.59 (m, 2H), 2.16 (s, 6H), 2.96 (s, 6H), 1.95 (s, 6H), 1.93 (s, 6H); ¹³C NMR (125.7 MHz, CDCl₃) δ 170.4, 170.3, 170.1, 164.2, 148.5, 136.3, 131.1, 124.8, 101.3, 71.0, 70.7, 69.0, 68.3, 67.0, 61.4, 40.1, 20.8, 20.7, 20.6, 20.5 ppm. MALDI-TOF-MS (M+Na⁺): 981.98.

5-Nitroisophthaloyl-N,N’-bis[11-O-(β-D-galactopyranosyl)-ethyl]-diamide (II): At r.t., compound I (1 g, 1.04 mmol) was dissolved in 0.05 M NaOMe / HOMe (15 mL) solution and stirred for 12 hrs, the white product as precipitate was filtered out and washed with methanol. II was obtained after dried in vacuum oven (quantitative): ¹H NMR (500 MHz, D₂O) δ 8.80 (s, 2H), 8.53(s, 1H), 4.44 (d, J = 7.75 Hz, 2H), 4.13-4.08 (m, 2H), 3.93-3.89 (m, 4H), 3.74-3.68 (m, 10H), 3.65-3.63 (dd, J = 3.7, 3.2 Hz, 2H), 3.55
Scheme 2.3.2 Synthesis of $2$ (Gal$_2$).

a: Pd/C, H$_2$; 5-nitroisophthaloyl dichloride, Et$_3$N;  
b: NaOMe/MeOH; c: Pd/C, H$_2$
-3.51 (dd, \( J = 7.8, 7.75\text{Hz}, 2\text{H} \)); \(^{13}\text{C} \text{NMR (125.7 MHz, } \text{D}_2\text{O}) \delta 166.8, 147.7, 135.5, 131.9, 124.9, 103.0, 75.1, 72.7, 70.7, 68.6, 68.2, 60.9, 40.2 \text{ ppm. MALDI-TOF-MS (M+Na\textsuperscript{+}): 644.57.} 

5-Aminophthaloyl-N,N'-bis[11-O-(\(\beta\)-D-galactopyranosyl)-ethyl]-diamide (2, \text{Gal}_2): At r.t., 10\% Pd/C (60 mg) was added in a H\textsubscript{2}O/MeOH (3:1, 20 mL) solution of II (0.6 g, 1 mmol), the reaction mixture was stirred for 4 hrs with the hydrogen purged in and out. The Pd/C was been separated by filtering and the filtration was evaporated and dried under vacuum to give 2(\text{Gal}_2) as a colorless liquid (quantitative): \(^1\text{H} \text{NMR (500 MHz, } \text{D}_2\text{O}) \delta 7.47 (s, 1\text{H}), 7.29 (s, 2\text{H}), 4.41 (d, \( J = 8.25 \text{ Hz}, 2\text{H}), 4.09-4.04 (m, 2\text{H}), 3.92-3.87 (m, 4\text{H}), 3.73-3.62 (m, 12\text{H}), 3.54-3.50 (m, 2\text{H}); \(^{13}\text{C} \text{NMR (125.7 MHz, } \text{D}_2\text{O}) \delta 170.3, 147.3, 135.3, 117.5, 116.1, 103.1, 75.1, 72.7, 70.7, 68.6, 68.4, 60.9, 40.0 \text{ ppm. MALDI-TOF-MS (M+Na\textsuperscript{+}): 614.79.} 

2.3.2.4 Nanotube Functionalization

A purified MWNT sample (20 mg) was suspended in KH\textsubscript{2}PO\textsubscript{4} buffer (pH = 7.4, 20 mL), and to the suspension was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, 180 mg, 0.94 mmol). After sonication for 1 h, 2 (496 mg, 0.84 mmol) was added, followed by sonication for another 2 h. The mixture was stirred for 36 h, and then centrifuged (1,380 g) for 30 min. The supernatant was transferred to a membrane tubing (molecular weight cutoff \( \sim 12,000 \)) for dialysis against deionized water for 3 days. The solution from the dialysis tubing contained only a small amount of solid residue, which was removed via centrifuging at 1,380 g for 30 min. The resulting colored
but transparent supernatant was the solution of Gal$_2$-MWNT (Scheme 2.3.1).

2.3.3 Results and Discussion

It is well-established that carbon nanotubes contain defect-derived carboxylic acids after oxidative acid treatment (as a part of sample purification). These nanotube-bound acid groups were targeted in amidation reactions with 1(Gal) and 2(Gal$_2$) (Scheme 2.3.2). The functionalization of MWNTs with 2 resulted in a colored but transparent supernatant which was the solution of Gal$_2$-MWNT (Scheme 2.3.1).

The same reaction conditions were applied to the functionalization of MWNTs with 1, but the resulting Gal-MWNT could only form an unstable aqueous suspension. Shown in Figure 2.3.1 is a visual comparison of Gal$_2$-MWNT and Gal-MWNT for their different solubilities. For the comparison, the two samples in similar amounts were dispersed in water, followed by same centrifugation. The supernatant obtained from the Gal-MWNT dispersion is essentially colorless (Figure 2.3.1), suggesting that the sample is hardly soluble. For Gal$_2$-MWNT, on the other hand, the aqueous solubility was estimated gravimetrically (by drying a fix volume of the solution) to be ~0.3 mg/mL, which is lower than those of other water-soluble functionalized carbon nanotubes.$^{2a,10}$

The solution of Gal$_2$-MWNT in D$_2$O was used for $^1$H NMR measurement. The spectrum is compared with that of Gal$_2$ in Figure 2.3.2. The proton signals in the nanotube-bound Gal$_2$ are obviously much broader, consistent with effects associated with the low mobility of the nanotubes.$^{9b}$ In addition, the signals of aromatic protons in Gal$_2$ are suppressed in the spectrum of Gal$_2$-MWNT, which is likely due to the aromatic
moiety being close to the nanotube surface, as already observed and discussed in the literature on other functionalized carbon nanotubes.\textsuperscript{11,12}

The Gal\textsubscript{2}-MWNT sample was characterized by various microscopy techniques, including scanning (SEM) and transmission electron microscopy (TEM) and atomic force microscopy (AFM). As shown in Figure 2.3.3, there are apparently abundant MWNTs of different lengths, well-dispersed to individual nanotubes. In TEM and AFM at a high magnification, the coating on the nanotube surface could be observed, which might logically be attributed to the Gal\textsubscript{2} functionalities.

In order to determine the nanotube content in the Gal\textsubscript{2}-MWNT sample, thermogravimetric analysis (TGA) with inert gas (nitrogen) was tried to selectively remove the sugar functionalities from the nanotube surface (or thermal defunctionalization\textsuperscript{9b}). However, it was found that unlike Gal,\textsuperscript{6} Gal\textsubscript{2} carbonized at high temperatures, thus could not be evaporated under TGA conditions (Figure 2.3.4). There was no evidence suggesting that the carbonization of Gal\textsubscript{2} remained quantitatively the same before and after the attachment to nanotubes. Therefore, the TGA results (Figure 2.3.4) could not be used to provide a reasonable estimate of the nanotube content. On the other hand, the solubility of Gal\textsubscript{2}-MWNT allowed the classical sugar analysis in aqueous solution, which is based on spectrophotometry with anthrone as the coloring agent.\textsuperscript{7} According to the analysis, the sample solution contained 0.12 mg/mL of galactose (or 0.2 mg/mL Gal\textsubscript{2}). Thus, the Gal\textsubscript{2} content in the solid-state Gal\textsubscript{2}-MWNT sample was 67 % wt/wt (the remaining 33 % wt/wt being MWNTs).
Figure 2.3.1 A visual comparison of the supernatants from centrifuging the aqueous dispersions of Gal$_2$-MWNT (left) and Gal-MWNT (right).
Figure 2.3.2 $^1$H NMR spectra of Gal$_2$-MWNT (top) in comparison with that of Gal$_2$ (bottom), both in room-temperature D$_2$O.
Figure 2.3.3 Representative SEM (top left), TEM (dark-field, top right), and AFM (amplitude: bottom left, and topography: bottom right) images of the Gal$_2$-MWNT sample. The SEM specimen was from evaporation of a concentrated Gal$_2$-MWNT solution, while the AFM specimen was from spraying a more dilute sample solution onto a heated mica substrate to preserve nanotube dispersion.
Figure 2.3.4 TGA traces (N\textsubscript{2}, 10 °C/min) of Gal\textsubscript{2}-MWNT (---), the stable solid precursor of Gal\textsubscript{2} (with nitro head group instead of amine, ----), and the MWNT sample (-.-.-).
2.3.4 Summary

In the solubilization of carbon nanotubes via chemical functionalization, the size of the functional group relative to that of the nanotube should be a critical factor. The results presented here are rather striking, providing a useful piece of evidence for the expected effect. The Gal-SWNT is soluble, but not Gal-MWNT because MWNTs are considerably larger than SWNTs. It requires a larger functional group like Gal$_2$ to “drag” the nanotube into solution. The solubilization is obviously important to the purpose of these materials. The reported work shows that similar to SWNTs, MWNTs can also be used to display multiple copies of sugars in physiological solution, which thus makes it possible to exploit those properties only available to sugar-functionalized MWNTs. In addition, since Gal-SWNT is known to exhibit significant cell adhesion, the galactose pairs displayed on the nanotube scaffold may prove to be more efficient in the binding with cells. A comparative biological evaluation of these materials is discussed in later chapters.


2.4 Single-Walled Carbon Nanotube as Unique Scaffold for Multivalent Display of Sugars

2.4.1 Introduction

Multivalent carbohydrate ligands are known to be considerably more potent than their monovalent counterparts in biological ligand-receptor interactions.\(^1{,}^2\) Therefore, there have been growing recent interests in various multivalent ligands and their associated configurations for a wide range of applications,\(^3\) including the development of high-affinity inhibitors and drugs.\(^2\) Among more extensively studied scaffolds for the display of multivalent carbohydrate ligands are dendrimers,\(^4\) linear and polydisperse polymers,\(^5\) metal nanoparticles,\(^6\) polymeric nanospheres,\(^7,^8\) and so on. For example, Stoddart and coworkers synthesized highly branched carbohydrate dendrimer containing 3-36 peripheral α-D-mannose copies and evaluated their binding affinity to Con A lectin.\(^9\) Disney, et al. reported the use of a carbohydrate-conjugated fluorescent polymer, poly(p-phenylene ethynylene), to detect pathogenic *Escherichia coli*.\(^10\) For the binding with *E. coli*, Wu and coworkers attached D-mannose moieties to gold nanoparticles to target selectively the type 1 pili of the ORN178 strain.\(^6^a\) Qu, et al. developed sugar-coated polystyrene nanobeads for the binding and agglutination of *E. coli* cells.\(^7\)

Single-walled carbon nanotubes (SWNTs) have attracted much recent attention for potential biological applications,\(^11^-18\) including their functionalization with various bioactive species such as carbohydrates,\(^12\) DNA,\(^13\) proteins,\(^14\) and peptides.\(^15\) The unique pseudo-one-dimensional structure of SWNT has been exploited to serve as a linear and semi-flexible nanoscale carrier for displaying multiple copies of biomolecules in specific
interactions with cells. A representative example is due to Elkin, et al. who used the bovine serum albumin-functionalized SWNTs in conjugation with E. coli-specific antibody to capture the pathogen in physiological solution.\textsuperscript{16} In another example, Gu, et al. solubilized SWNTs via covalent functionalization with the derivatized 2-aminoethyl-\(\beta\)-D-galactopyranoside (Scheme 2.4.1) in likely the amidation of nanotube-bound carboxylic acids.\textsuperscript{17} These galactose-functionalized nanotubes (Gal-SWNT), each displaying multiple copies of the sugar, were found to have adhesion to pathogenic E. coli O157:H7 to result in significant cell agglutination (see chapter 2.2).\textsuperscript{17} Recently, Wang, et al. found that monosaccharides (\(\beta\)-D-galactoses or \(\alpha\)-D-mannoses)-functionalized SWNTs bind to and aggregate effectively \textit{Bacillus anthracis} (Sterne) spores in the presence of a divalent cation (such as Ca\textsuperscript{2+}) and that the binding is unique to the nanotube-displayed carbohydrates.\textsuperscript{18}

In the functionalization of nanotubes with tethered \(\beta\)-D-galactoses and \(\alpha\)-D-mannoses, the carboxylic acid moieties on the nanotubes, which are resulted from the oxidation of the surface defects, have been targeted.\textsuperscript{19} With limited defect sites on the nanotube surface, a higher population of multivalent ligands could be attained by using sugar dendrons (Scheme 2.4.1) in the nanotube functionalization. The resulting larger number of displayed ligands per nanotube also corresponds to significantly improved aqueous solubility of the functionalized nanotube samples,\textsuperscript{20} which not only enhances biocompatibility but also enables more quantitative characterization for a better understanding of the structural details on the nanotubes displaying multivalent carbohydrate ligands. In this chapter, we report the synthesis and characterization of
dendritic β-D-galactopyranosides and α-D-mannopyranosides for the functionalization of SWNTs (Scheme 2.4.1). As the functionality varies from mono-, bis-, to tetra-, the population of the sugar moieties in the functionalized nanotube sample increases, as are improved solubility and related properties. Results from the biological evaluation of these sugar-functionalized SWNTs in binding assays with pathogenic *E. coli* and with *Bacillus subtilis* (a nonvirulent simulant for *Bacillus anthracis* or anthrax) spores are presented and discussed.

2.4.2 Experimental Section

2.4.2.1 Materials

$t$-Butyl bromoacetate, $N$-hydroxy-succinimide, triphenylphosphine, trifluoroacetic acid (TFA), Palladium (10 wt% on activated carbon), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDAC), and amberlite IR-120 (plus) ion-exchange resin were purchased from Sigma-Aldrich. 3, 5-Dihydroxybenzyl alcohol, 18-crown-6, $N,N'$-dicyclohexyl-carbodiimide, β-D-galactose-pentaacetate, α-D-mannose, pyridine, and anthrone were obtained from Acros. $N$-Bromosuccinimide was supplied by ACOCADO Research Chemicals, Ltd. Sodium azide were from Alfa Aesar. Ethanol, chloroform, methylene chloride, THF, DMF, and diethyl ether were from Fisher. Solvent grade THF was dried and distilled over molecular sieves and then distilled over sodium before use. Other solvents were either spectrophotometry/HPLC grade or purified via simple distillation. Deuterated NMR solvents were obtained from Cambridge Isotope Laboratories.
Scheme 2.4.1 A schematic illustration for Gal-SWNT, Gal$_2$-SWNT, Gal$_4$-SWNT and Man-SWNT, Man$_2$-SWNT, Man$_4$-SWNT.
The sample of SWNT (arc-discharge method) was bought from Carbon Solutions, Inc. It was purified by using a combination of thermal oxidation and oxidative acid treatment. In a typical experiment, a nanotube sample (1 g) was thermally oxidized in air in a furnace at 300 °C for 30 min. After the thermal treatment, the remaining sample was added to an aqueous HNO₃ solution (2.6 M), and the mixture was refluxed for 48 h. Upon centrifuging at 1380g (Fisher scientific, centric 228 centrifuge) to discard the supernatant, the remaining solids were washed with deionized water until neutral pH and then dried under vacuum.

2.4.2.2 Measurements

NMR measurements were carried on a JEOL Eclipse +500 NMR spectrometer and a Bruker Advance 500 spectrometer that is equipped with a high-resolution magic-angle-spinning (HR-MAS) probe designed specifically for gel-phase NMR. The residual proton and carbon resonance of the deuterium solvents were used as internal standards. MALDI-TOF MS was performed on a Bruker AutoFlex system, with 2, 5-dihydroxybenzoic acid as the sample matrix. Optical absorption spectra were recorded on a Shimadzu UV3600 UV-VIS-NIR spectrophotometer. Thermo gravimetric analysis (TGA) was carried out on a Mettler-Toledo TGA/SDTA851e system. Raman spectra were obtained on a JoBin Yvon T64000 spectrometer equipped with a Melles-Griot 35mW He: Ne laser source for 632.8 nm excitation, a triple monochromator, a research grade Olympus BX-41 microscopy and a liquid nitrogen-cooled symphony detector. Scanning electron microscopy (SEM) images were obtained on a Hitachi S4700 field-
emission SEM system. Atomic force microscopy (AFM) analysis was conducted on a Molecular Imaging PicoPlus system equipped with a multipurpose scanner. The height profile analyses were assisted by using the SPIP software distributed by Image Metrology.

The spectrophotometric method with the use of anthrone reagent was used to determine sugar contents. As described in chapter 2.2, a solution of sugar-functionalized SWNTs (50 µL) was mixed with deionized water (50 µL), HCl (37%, 1 mL), formic acid (0.1 mL) and anthrone reagent (8 mL, 0.2 mg/mL in 80% H$_2$SO$_4$). The solution mixture was kept in a boiling water bath for 12 min, and then rapidly cooled in an ice bath for 30 min. The UV/vis absorption of the mixture was measured and corrected. Separately, the standard sugar (β-D-galactose or α-D-mannose) solutions of known concentration (1.0 mg/mL) were prepared. Various aliquots of the standard solutions (0, 25, 50, 75, and 100 µL) were tested in terms of the same procedure above to establish standard curves, from which the sugar content in the functionalized nanotube sample was obtained.

2.4.2.3 Synthesis of Sugar Dendrons

The sugar monomers (Gal- and Man-) were prepared using procedures already available in the literature (see chapter 2.2). For the sugar dendrons, the compound 1 (Scheme 2.4.2) was synthesized from t-butyl bromoacetate (through iodide substitution) and 3, 5-dihydroxybenzyl alcohol also according to procedures already reported in the literature.
1. At first, a mixture of sodium iodide (10 g, 75 mmol) and tert-butyl bromoacetate (9.75 g, 50 mmol) in acetone (50 mL) was refluxed for 12 hrs. The reaction mixture was allowed to cool and solvent was evaporated to dryness. The residue was then partitioned between water and chloroform. The organic layer was collected and dried with anhydrous MgSO₄, solvent was evaporated to dryness to afford tert-butyl iodoacetate as a brownish liquid (12 g, quantitative): \(^1\)H NMR (300 MHz, CDCl₃) δ 3.62 (s, 2H), 1.48 (s, 9H); \(^{13}\)C NMR (75.4 MHz, CDCl₃) δ 167.8, 82.3, 27.6, -2.6 ppm.

At r.t., 3, 5-dihydroxybenzyl alcohol (2.8 g, 20 mmol) was dissolved in dry acetone (100 mL). To this solution were added, K₂CO₃ (6.6 g, 48 mmol), 18-crown-6 (1.057 g, 4 mmol), and tert-butyl iodoacetate (10.65 g, 44 mmol). The reaction mixture was refluxed for 36 hrs. After been cooled to r.t., solvent was evaporated and the residue was partitioned between water and ethyl acetate. The water portion was extracted with ethyl acetate for 3 times. The combined organic layer was dried with anhydrous MgSO₄ and concentrated. The crude product was purified by chromatography with hexane/ethyl acetate (2:1.5) as the eluent to yield a colorless oil (7.1g, 96%): \(^1\)H NMR (500 MHz, CDCl₃) δ 6.56 (d, J = 2 Hz, 2H), 6.43 (t, J = 2.25 Hz, 1H), 4.64 (s, 2H), 4.51 (s, 4H), 1.51 (s, 18H); \(^{13}\)C NMR (125.7 MHz, CDCl₃) δ 167.8, 159.3, 143.6, 105.9, 101.2, 82.4, 65.8, 65.2, 28.1 ppm.

For the last step, at r.t., triphenyl phosphine (2.911 g, 11.1 mmol) and product of step 2 were stirred in dry THF (20 mL). After 7 mins, N-bromosuccinimide (1.976 g, 11.1 mmol) was added and stirred for another 5 mins. Water was then added to quench the reaction. Crude product was extracted by chloroform for three times, organic fraction
Scheme 2.4.2 Synthesis of serial sugar dendrons.
was dried (MgSO₄), condensed, and purified by silica gel chromatography with eluent of hexane/ethyl acetate (4:1). After dried in vacuum oven, 1 obtained as a whitish solid (3.018 g, 95%). H NMR (500 MHz, CDCl₃): δ 6.57 (d, J = 2.5 Hz, 2H), 6.44 (t, J = 2.25 Hz, 1H), 4.51 (s, 4H), 4.41 (s, 2H), 1.51 (s, 18H); ¹³C NMR (125.7 MHz, CDCl₃): δ 167.63, 159.17, 139.84, 108.38, 102.15, 82.52, 65.80, 33.16, 28.06 ppm. MALDI-TOF MS (M+Na⁺): (theoretically 453.09).

2. A solution of 1 (1.51 g, 3.5 mmol) in dry CH₂Cl₂ (10 mL) was prepared, and at 0 °C trifluoroacetic acid (4 mL) was added. The mixture was warmed back to room temperature and stirred for 12 h. The precipitate was collected via filtration and then dried in a vacuum oven to yield the hydrolyzed product 1’ as a white solid (1.1 g, quantitative): H NMR (500 MHz, MeOH-d₄) δ 6.66 (d, J = 2 Hz, 2H), 6.51 (t, J = 2.25 Hz, 1H), 4.67 (s, 4H), 4.51 (s, 2H); ¹³C NMR (125.7 MHz, MeOH-d₄) δ 171.0, 159.2, 140.5, 108.1, 101.6, 64.6, 32.3 ppm.

At 0 °C, a solution of N, N’-dicyclohexyl-carbodimide (990.3 mg, 4.8 mmol) in dry THF (20 mL) was added dropwise over 30 min to another THF solution (30 mL) of 1’ (638.7 mg, 2 mmol) and N-hydroxy-succinimide (552.4 mg, 4.8 mmol). The mixture was warmed back to room temperature and stirred in the dark for 12 h. The precipitated urea was removed via filtration, and the remaining solution was slowly dropped into a CH₂Cl₂ solution (20 mL) of freshly prepared Gal’-NH₂ (2.0 g, 4.8 mmol). After stirring for 12 h and then solvent removal, the reaction mixture was separated on a silica gel column (first hexane-ethyl acetate at 10/90 v/v, and then methanol-ethyl acetate at 5/95 v/v) to yield 2(Gal’) as a white solid (2.13 g, 99%): H NMR (500 MHz, CDCl₃) δ 6.96
(t, $J = 5.75$ Hz, 2H, NH), 6.71 (d, $J = 2$ Hz, 2H), 6.64 (t, $J = 2$ Hz, 1H), 5.41 (dd, $J = 2$ Hz, 2H), 5.23 (dd, $J = 8$ Hz, 2H), 5.03 (dd, $J = 3.5$ Hz, 2H), 4.52 (s, 4H), 4.51 (d, $J = 3$ Hz, 2H), 4.45 (s, 2H), 4.19-4.14 (m, 4H), 4.0-3.92 (m, 4H), 3.74-3.67 (m, 4H), 3.55-3.48 (m, 2H), 2.18 (s, 6H), 2.07 (s, 6H), 2.04 (s, 6H), 2.02 (s, 6H); $^{13}$C NMR (125.7 MHz, CDCl$_3$) $\delta$ 170.4, 170.2, 170.1, 169.5, 167.7, 158.6, 140.8, 109.1, 102.4, 101.3, 70.9, 70.7, 68.8, 68.5, 67.5, 67.0, 61.3, 38.8, 32.7, 20.7, 20.7(2C), 20.6 ppm. MALDI-TOF MS (M+Na$^+$): 1089.37 (theoretically 1087.24).

The same procedure was applied to the synthesis of 2(Man$'$) (2.13 g, 99%): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.08 (t, $J = 5.25$ Hz, 2H, NH), 7.01 (d, $J = 1.85$ Hz, 2H), 6.59 (t, $J = 2.25$ Hz, 1H), 5.37 (dd, $J = 3.2$ Hz, 2H), 5.31-5.27 (m, 4H), 4.87 (d, $J = 1.4$ Hz, 2H), 4.55 (s, 4H), 4.46 (s, 2H), 4.28-4.25 (dd, $J = 5.5$ Hz, 2H), 4.15-4.12 (m, 2H), 4.00-3.97 (m, 2H), 3.88-3.86 (m, 2H), 3.71-3.64 (m, 2H), 3.63-3.57 (m, 4H), 2.18 (s, 6H), 2.12 (s, 6H), 2.05 (s, 6H), 2.00 (s, 6H); $^{13}$C NMR (125.7 MHz, CDCl$_3$) $\delta$ 170.6, 170.1, 170.0, 169.7, 167.8, 158.4, 140.7, 109.0, 102.1, 97.6, 69.4, 69.0, 68.9, 67.3, 66.8, 66.0, 62.5, 38.4, 32.7, 20.9, 20.7 (2C), 20.6 ppm. MALDI-TOF MS (M+Na$^+$): 1088.54 (theoretically 1087.24).

Gal$_2$N$_3$ and Man$_2$N$_3$. The azide substitution and deprotection reactions of 2 were similar to those reported previously,$^{20}$ yielding quantitatively Gal$_2$N$_3$ [$^1$H NMR (500 MHz, MeOH-$d_4$) $\delta$ 6.71-6.68 (m, 3H), 4.56 (s, 4H), 4.35 (s, 2H), 4.25 (d, $J = 12.5$ Hz, 2H), 3.01-3.94 (m, 2H), 3.85-3.83 (dd, 2H), 3.77-3.67 (m, 6H), 3.62-3.43 (m, 10H); $^{13}$C NMR (125.7 MHz, MeOH-$d_4$) $\delta$ 169.6, 159.2, 138.6, 107.7, 103.9, 101.7, 75.4, 73.5, 71.1, 68.9, 68.1, 67.0, 61.1, 53.9, 39.0 ppm. MALDI-TOF MS (M+Na$^+$): 714.83
(theoretically 714.24]) and Man$_2$-N$_3$ [\(^1\)H NMR (500 MHz, MeOH-$d_4$) \(\delta\) 6.66-6.65 (m, 3H), 4.74 (d, \(J = 1.4\) Hz, 2H), 4.54 (s, 4H), 4.33 (s, 2H), 3.81-3.74 (m, 6H), 3.69-3.64 (m, 4H), 3.61-3.51 (m, 8H), 3.48-3.43 (m, 2H); \(^{13}\)C NMR (125.7 MHz, MeOH-$d_4$) \(\delta\) 169.7, 159.2, 138.7, 107.7, 101.6, 101.3, 73.5, 71.2, 70.7, 67.3, 67.0, 65.7, 61.6, 54.0, 38.6 ppm. MALDI-TOF MS (M$^+$): 690.77 (theoretically 691.25)]. These compounds were relatively stable, and they were freshly reduced to Gal$_2$-NH$_2$ and Man$_2$-NH$_2$ before the nanotube functionalization reaction.

3. There were two steps (Scheme 2.4.2), with the first being the conversion of the bromide in 1 to iodide (1$''$). 1 (3.018 g, 7 mmol) and sodium iodide (10 g, 67 mmol) were refluxed in acetone (50 mL) for 12 h. The reaction mixture was allowed to cool, and then the solvent was evaporated completely. The residue was partitioned between water and chloroform. The organic layer was collected and dried with anhydrous MgSO$_4$, followed by the removal of solvent to yield 1$''$ (3.56 g, quantitative): \(^1\)H NMR (500 MHz, CDCl$_3$) \(\delta\) 6.53 (d, \(J = 2.3\) Hz, 2H), 6.37 (t, \(J = 2.05\) Hz, 1H), 4.48 (s, 4H), 4.35 (s, 2H), 1.50 (s, 18H); \(^{13}\)C NMR (125.7 MHz, CDCl$_3$) \(\delta\) 167.7, 159.2, 141.4, 108.2, 101.7, 82.6, 65.8, 28.2, 5.1 ppm.

In the second step, a solution of 3,5-dihydroxybenzyl alcohol (490 mg, 3.5 mmol), K$_2$CO$_3$ (2 g, 14 mmol), and 18-crown-6 (172 mg, 0.7 mmol) in acetone (30 mL) was prepared, and to the solution was added 1$'''$ (3.56 g, 7 mmol). After refluxing for 36 h and then solvent removal, the reaction mixture was partitioned between water and ethyl acetate. The aqueous potion was repeatedly extracted with ethyl acetate. The combined ethyl acetate solution was dried with anhydrous MgSO$_4$, followed by silica gel column
(hexane-ethyl acetate at 1:1.5) separation to obtain 3 (2.55 g, 87% yield): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 6.56 (d, $J$ = 2.3 Hz, 4H), 6.55 (d, $J$ = 1.85 Hz, 2H), 6.45 (t, $J$ = 2.25 Hz, 1H), 6.40 (t, $J$ = 2.25 Hz, 2H), 4.93 (s, 4H), 4.60 (s, 2H), 4.47 (s, 8H), 1.46 (s, 36H); $^{13}$C NMR (125.7 MHz, CDCl$_3$) $\delta$ 167.9, 159.95, 159.2, 143.9, 139.6, 106.5, 105.7, 101.4, 101.2, 82.5, 69.7, 65.8, 65.0, 28.1 ppm. MALDI-TOF MS (M+Na$^+$): 863.29 (theoretically 863.38).

4. The first step was to convert the benzyl alcohol in 3 to benzyl bromide (3'). 3 (3.075 g, 3.675 mmol) was added to a THF (20 mL) solution of triphenyl phosphine (1.44 g, 5.5 mmol). After stirring for 7 min, N-bromosuccinimide (979 mg, 5.5 mmol) was added, and the mixture was stirred for 5 min more. Water was added to quench the reaction. The crude product was extracted with chloroform three times. The organic fraction was dried with anhydrous MgSO$_4$, condensed, and purified by silica gel chromatography with hexane-ethyl acetate (3/1 v/v) as eluent. 3' was obtained as a white solid (2.76 g, 83% yield): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 6.59 (d, $J$ = 2.5 Hz, 2H), 6.58 (d, $J$ = 2 Hz, 4H), 6.47 (t, $J$ = 2.25 Hz, 1H), 6.44 (t, $J$ = 2.25 Hz, 2H), 4.93 (s, 4H), 4.49 (s, 8H), 4.39 (s, 2H), 1.48 (s, 36H); $^{13}$C NMR (125.7 MHz, CDCl$_3$) $\delta$ 167.7, 159.9, 159.3, 139.8, 139.3, 108.3, 106.5, 102.2, 101.5, 82.4, 69.8, 65.8, 33.4, 28.0 ppm. MALDI-TOF MS (M+Na$^+$): 927.31 (theoretically 925.30).

A solution of 3' (2.4 g, 2.6 mmol) in CH$_2$Cl$_2$ (20 mL) was prepared, and at 0 °C trifluoroacetic acid (4 mL) was added. The mixture was warmed back to room temperature and stirred for 12 h. The precipitate was collected by vacuum filtration, and then dried in a vacuum oven to yield 4 as a white solid (1.78 g, quantitative): $^1$H NMR
(500 MHz, MeOH-$d_4$) $\delta$ 6.68 (d, $J = 2$ Hz, 2H), 6.67 (t, $J = 2.5$ Hz, 4H), 6.56 (t, $J = 2$ Hz, 1H), 6.51 (t, $J = 2.25$ Hz, 2H), 5.02 (s, 4H), 4.66 (s, 8H), 4.49 (s, 2H); $^{13}$C NMR (125.7 MHz, MeOH-$d_4$) $\delta$ 171.1, 160.0, 159.3, 140.4, 139.9, 108.3, 106.2, 101.8, 101.0, 69.4, 64.7, 32.6 ppm. MALDI-TOF MS (M+Na$^+$): 703.19 (theoretically 701.05).

5(Gal’) and 5(Man’). Two THF solutions (10 mL each) were prepared, with one for 4 (340 mg, 0.5 mmol) and N-hydroxy-succinimide (345 mg, 3 mmol) and the other for N, N’-dicyclohexyl-carbodiimide (619 mg, 3 mmol). At 0 ºC, the second solution was added to the first dropwise over 30 min. The reaction mixture was gradually warmed to room temperature and stirred in dark for 12 h. The insoluble urea was removed by filtration, and the filtrate was slowly dropped into a CH$_2$Cl$_2$ solution (20 mL) of freshly prepared Gal’-NH$_2$ (1.0 g, 2.4 mmol). After stirring for 12 h and then solvent removal, the reaction mixture was separated on silica gel column (first hexane-ethyl acetate at 1:9, and then methanol-ethyl acetate at 1:9) to afford 5(Gal’) as white solids (728 mg, 67% yield): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 6.94 (t, $J = 5.95$ Hz, 4H, NH), 6.66 (d, $J = 1.85$ Hz, 4H), 6.59 (d, 2H), 6.58 (t, 2H), 6.48 (t, 1H), 5.31 (d, $J = 3.2$ Hz, 4H), 5.13 (dd, $J = 7.8$ Hz, 4H), 4.98-4.92 (m, 8H), 4.44 (s, 8H), 4.42 (d, $J = 8.25$ Hz, 4H), 4.36(s, 2H), 4.10-4.02 (m, 8H), 3.90-3.82 (m, 8H), 3.68-3.56 (m, 8H), 3.46-3.40 (m, 4H), 2.07 (s, 12H), 1.97 (s, 12H), 1.94 (s, 12H), 1.92 (s, 12H); $^{13}$C NMR (125.7 MHz, CDCl$_3$) $\delta$ 170.4, 170.3, 170.2, 169.5, 167.9, 158.8, 158.7, 140.1, 140.0, 108.3, 107.0, 102.2, 102.1, 101.3, 70.8, 70.8, 69.6, 68.8, 68.6, 67.5, 67.0, 61.3, 38.8, 33.4, 20.8, 20.7 (2C), 20.6 ppm. MALDI-TOF MS (M+Na$^+$): 2195.56 (theoretically 2193.60).

The same procedure was applied to the synthesis of 5(Man’) (1.37 g, 63% yield):
1H NMR (500 MHz, CDCl₃) δ 7.06 (t, J = 5.95 Hz, 4H, NH), 6.68 (d, J = 1.85 Hz, 4H), 6.60 (d, J = 1.85 Hz, 2H), 6.56 (t, 2H), 6.50 (t, 1H), 5.32-5.29 (m, 4H), 5.24-5.18 (m, 8H), 4.97 (s, 4H), 4.81 (s, 4H), 4.50 (s, 8H), 4.38 (s, 2H), 4.23-4.19 (dd, J = 5.75 Hz, 4H), 4.09-4.03 (m, 4H), 3.95-3.92 (m, 4H), 3.82-3.78 (m, 4H), 3.66-3.49 (m, 12H), 2.11 (s, 12H), 2.06 (s, 12H), 1.96 (s, 12H), 1.94 (s, 12H); 13C NMR (125.7 MHz, CDCl₃): δ 170.7, 170.2 (2C), 169.8, 168.0, 159.9, 158.6, 140.1, 140.0, 108.3, 107.1, 102.2, 102.0, 97.7, 69.6, 69.4, 69.0, 68.9, 67.3, 66.9, 66.1, 62.6, 38.5, 33.5, 20.9, 20.8, 20.8, 20.7 ppm. MALDI-TOF MS (M+Na⁺): 2194.28 (theoretically 2193.60).

Gal₄-N₃ and Man₄-N₃. The first step was to convert the bromide in 5 to azide (Gal’₄-N₃ and Man’₄-N₃). For Gal’₄-N₃, a solution of 5(Gal’) (728 mg, 0.34 mmol) in acetone (20 mL) was prepared, and to the solution was added sodium azide (0.22 g, 3.4 mmol) and 18-crown-6 (54 mg, 0.2 mmol). The mixture was refluxed for 12 h, and then cooled to room temperature. The solvent was evaporated, and the residue was partitioned between water and ethyl acetate. The aqueous layer was extracted with ethyl acetate twice. All of the ethyl acetate fractions were combined and dried with anhydrous MgSO₄. The solvent was evaporated, and the residue was dried in a vacuum oven to yield Gal’₄-N₃ as a white solid (715 mg, quantitative): 1H NMR (500 MHz, CDCl₃) δ 6.98 (t, J = 5.5 Hz, 4H, NH), 6.65 (s, 4H), 6.59 (s, 2H), 6.52 (s, 1H), 6.50 (s, 2H), 5.32 (d, J = 3.25 Hz, 4H), 5.13 (dd, J = 7.55 Hz, 4H), 4.96 (s, 4H), 4.93 (d, J = 3.65 Hz, 4H), 4.44 (s, 8H), 4.42 (d, 4H), 4.22 (s, 2H), 4.07 (d, J = 6.85 Hz, 8H), 3.90-3.83 (m, 8H), 3.68-3.56 (m, 8H), 3.45-3.40 (m, 4H), 2.07 (s, 12H), 1.96 (s, 12H), 1.94 (s, 12H), 1.91 (s, 12H); 13C NMR (125.7 MHz, CDCl₃) δ 170.5, 170.3, 170.2, 169.6, 168.0, 160.0, 158.7, 140.0, 138.0,
107.3, 107.0, 102.1, 101.9, 101.3, 70.8, 70.8, 69.6, 68.8, 68.5, 67.4, 67.0, 61.3, 54.7, 38.8, 20.8, 20.7 (2C), 20.6 ppm. MALDI-TOF MS (M+Na\(^+\)): 2157.43 (theoretically 2156.69).

The same procedure was applied to the synthesis of Man’4-N3 (1.02 g, quantitative): \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 7.01 (t, \(J = 5.5\) Hz, 4H, NH), 6.64 (d, \(J = 2\) Hz, 4H), 6.53 (t, \(J = 2\) Hz, 2H), 6.51 (t, \(J = 2\) Hz, 1H), 6.49 (d, \(J = 2\) Hz, 2H), 5.29-5.26 (m, 4H), 5.22-5.17 (m, 8H), 4.95 (s, 4H), 4.78 (d, \(J = 1\) Hz, 4H), 4.46 (s, 8H), 4.20-4.17 (m, 6H), 4.03 (dd, \(J = 2.75\) Hz, 4H), 3.93-3.89 (m, 4H), 3.79-3.74 (m, 4H), 3.63-3.57 (m, 4H), 3.57-3.46 (m, 8H), 2.08 (s, 12H), 2.02 (s, 12H), 1.93 (s, 12H), 1.91 (s, 12H); \(^{13}\)C NMR (125.7 MHz, CDCl\(_3\)) \(\delta\) 170.7, 170.2 (2C), 169.8, 168.0, 160.1, 158.5, 140.0, 138.0, 107.4, 107.0, 102.0, 101.9, 97.7, 69.6, 69.4, 69.0, 68.9, 67.3, 66.9, 66.1, 62.5, 54.8, 38.5, 21.0, 20.8, 20.7, 20.6 ppm. MALDI-TOF MS (M\(^+\)): 2134.08 (theoretically 2133.69).

For Gal4-N3, a solution of Gal’4-N3 (715 mg, 0.334 mmol) in CH\(_3\)ONa/CH\(_3\)OH (0.05 M, 30 mL) was prepared. After the solution was stirred for 12 h, Amberlite IR-120(plux) ion-exchange resin was added to adjust the pH to 7.0. The resin was removed via filtration, and the solution was dried with anhydrous MgSO\(_4\), followed by solvent evaporation to yield Gal4-N3 as a white solid (490 mg, quantitative): \(^1\)H NMR (500 MHz, D\(_2\)O) \(\delta\) 6.47 (s, 4H), 6.43 (s, 2H), 6.36 (s, 2H), 6.26 (s, 1H), 4.63 (s, 4H), 4.32 (d, \(J = 7.5\) Hz, 4H), 4.26 (s, 8H), 4.13 (s, 2H), 3.97-3.94 (m, 4H), 3.88 (d, 4H), 3.77-3.70 (m, 12H), 3.62-3.57 (m, 8H), 3.54-3.50 (m, 8H), 3.47-3.41 (m, 4H); \(^{13}\)C NMR (125.7 MHz, D\(_2\)O) \(\delta\) 170.5, 159.2, 158.3, 139.5, 138.4, 107.2, 106.9, 103.2, 101.4 (2C), 75.2, 72.8, 70.8, 69.2, 68.6, 68.3, 66.4, 61.0, 54.0, 39.1 ppm. MALDI-TOF MS (M+Na\(^+\)): 1484.50 (theoretically 1484.52). The same procedure was applied to the quantitative conversion of Man’4-N3 to
Man₄-N₃: ¹H NMR (500 MHz, D₂O) δ 6.41 (s, 4H), 6.37 (s, 2H), 6.34 (s, 2H), 6.23 (s, 1H), 4.75 (s, 4H), 4.61 (s, 4H), 4.24 (s, 8H), 4.05 (s, 2H), 3.81 (m, 4H), 3.76 (d, 4H), 3.70-3.65 (m, 12H), 3.62-3.57 (m, 4H), 3.54-3.50 (m, 8H), 3.47-3.42 (m, 4H), 3.36-3.32 (m, 4H); ¹³C NMR (125.7 MHz, D₂O) δ 169.4, 159.2, 158.3, 139.6, 138.4, 107.4, 106.8, 101.6, 101.3, 99.7, 72.9, 70.6, 70.1, 69.2, 66.7, 66.5, 65.7, 60.9, 54.0, 38.6 ppm. MALDI-TOF MS (M⁺): 1461.38 (theoretically 1461.53).

2.4.2.4 Nanotube Functionalization

Before the functionalization reaction, Gal₄-N₃ and Man₄-N₃ were reduced to Gal₄-NH₂ and Man₄-NH₂ by the classical palladium-catalyzed hydrogenation. At 0 ºC, to a solution of Gal₄-N₃ or Man₄-N₃ (800 mg, 0.55 mmol) in methanol-H₂O (10 mL) was added Pd/C (10 wt% palladium on activated carbon, 70 mg). The reaction mixture was gradually warmed to room temperature and stirred with the purging of hydrogen gas for 4 h. The Pd/C was removed by filtration, and the filtrate was evaporated to obtain Gal₄-NH₂ or Man₄-NH₂.

In the functionalization of SWNTs with Gal₄-NH₂, a purified nanotube sample (20 mg) was mixed with 1-ethyl-3-(3-dimethylaminoprop-yl) carbodiimide (EDAC, 112 mg, 60 mmol) in aqueous KH₂PO₄ buffer (45 mL, pH = 7.4). Upon sonication for 2 h, the freshly prepared Gal₄-NH₂ (0.55 mmol) was added. After sonication for another 36 h, the reaction mixture was loaded into a membrane tubing (cutoff molecular weight ~ 12,000) for dialysis against deionized water for 3 days. The suspension from the dialysis was centrifuged at 3,000g for 30 min to obtain a dark but optically transparent solution of
Gal-SWNT: $^1$H NMR (500 MHz, D$_2$O) $\delta$ 6.72 (broad), 6.61 (s, broad), 6.55 (broad), 6.50-6.40 (broad), 4.50 (s, broad), 4.35 (d, broad), 3.98 (s, broad), 3.93 (s, broad), 3.76 (s, broad), 3.64 (broad), 3.54 (broad), 3.50 (broad); $^{13}$C NMR (125.7 MHz, D$_2$O) $\delta$ 171.0, 158.5, 106.6, 103.2, 75.1, 72.7, 70.8, 69.6, 68.6, 68.4, 66.6, 60.9, 39.1 ppm. Similarly for Man-SWNT: $^1$H NMR (500 MHz, D$_2$O) $\delta$ 6.5-6.2 (broad), 4.35-4.10 (broad), 3.91 (s, broad), 3.85 (d, broad), 3.77 (d, broad), 3.67 (t, broad), 3.55-3.40 (broad), 3.43 (broad); $^{13}$C NMR (125.7 MHz, D$_2$O): $\delta$ 170.4, 159.2, 158.3, 139.5, 107.0, 101.2, 99.7, 72.9, 70.6, 70.1, 69.2, 66.7, 66.4, 65.7, 60.9, 38.6 ppm.

Gal$_2$N$_3$ and Man$_2$N$_3$ were similarly reduced and used to functionalize SWNTs resulting Gal$_2$-SWNT: $^1$H NMR (500 MHz, D$_2$O) $\delta$ 6.82 (s), 6.70 (s), 6.6-6.4 (broad), 4.71 (s), 4.40 (d, broad), 4.03 (m, broad), 3.95 (s, broad), 3.90-3.70 (broad), 3.70-3.60 (broad), 3.60-3.50 (broad); $^{13}$C NMR (125.7 MHz, D$_2$O) $\delta$ 103.1, 75.2, 72.7, 70.8, 68.6, 68.4, 66.9, 61.0, 39.1 ppm. Man$_2$-SWNT: $^1$H NMR (500 MHz, D$_2$O) $\delta$ 6.82 (s), 6.70 (s), 6.64 (s), 6.58 (s), 6.47 (s), 4.61 (s, broad), 4.53 (s, broad), 4.38 (d, broad), 3.86 (d, broad), 3.80-3.70 (m, broad), 3.70-3.60 (m, broad), 3.60-3.50 (m, broad); $^{13}$C NMR (125.7 MHz, D$_2$O): $\delta$ 171.1, 158.5, 99.6, 72.8, 70.5, 70.0, 66.7 (2C), 65.8, 60.9, 38.6 ppm.

2.4.2.5 Assay for E. coli

The green fluorescent protein (GFP)-expressing E. coli O157:H7/pWM1007 was transformed through electroporation. The frozen E. coli samples were recovered and cultured on tryptic soy agar (TSA) plates supplemented with kanamycin (50 µg/mL). After overnight growth at 37 °C, the bacteria were harvested.
and washed with phosphate buffer solution (PBS) via the centrifuging-suspending cycle three times. Finally the \textit{E. coli} cells were suspended in PBS to an optical density of 0.6 at 600 nm (~10^8 cells/mL).

In cell adhesion and precipitation experiment, a suspension (25 \(\mu\)L) of \textit{E. coli} O157:H7/pWM1007 cells was two-fold serially diluted and incubated with a solution (10 \(\mu\)L) of either Gal-SWNT (0.14 mg/mL) or Gal2-SWNT (0.13 mg/mL) for 3 h at room temperature with gentle inversions of the tubes at 15 min intervals. The precipitates at the tube bottom were collected and wet-mounted onto a glass slide for microscopy analyses.

For the colony-forming units (CFU) reduction assay, the same procedure described above was used, and at the end of incubation the mixture was centrifuged with a low force (300g) for 30 seconds. The supernatant was collected and ten-fold serially diluted. At each dilution 100 \(\mu\)L was plated out (in triplicate) onto TSA agar plates by using spread plate technique. The plates were incubated overnight at 37 °C. CFU was counted, and the percentage of CFU reduction was calculated and compared to that of the control without the sugar-functionalized SWNTs.

2.4.2.6 Assay for \textit{B. subtilis} Spores

\textit{B. subtilis} spores (strain ATCC33234) were supplied by American Type Culture Collection (Manassas, VA), and the spore suspension was prepared by following established procedures. In a typical binding experiment, an aqueous suspension of \textit{B. subtilis} spores (40 \(\mu\)L, 2.6 \(\times\) 10^8 CFU/mL) was mixed with a solution of mannose-functionalized SWNTs (40 \(\mu\)L, 0.2 mg/mL mannose equivalent concentration) or with
distilled water (40 µL) as control, followed by the addition of aqueous CaCl₂ (20 µL, 100 mM). The mixtures (sample and control) were rotated for 12 h. A small aliquot (10 µL) of the sample (or control) was dropped onto a glass slide (covered with cover glass slide) for optical microscopy analyses.

2.4.3 Results and Discussion

The sugar dendrons Gal₂-NH₂, Man₂-NH₂, Gal₄-NH₂, and Man₄-NH₂ were prepared by using tri-substituted benzenes (3,5-dihydroxybenzyl alcohol and 1) as building blocks in classical etherification and carbodiimide-activated amidation reactions (Scheme 2.4.2), with generally high product yields. The synthetic strategy was such that the dendritic framework was constructed first before being coupled with the amine-tethered monosaccharide. A significant advantage of the strategy is that sugar molecules are involved only in the end steps, thus difficulties associated with the sugar de-protection and stereo hindrance are avoided.

The same carbodiimide-activated amidation was used in the functionalization of SWNTs with the dendrons, with the tethered amino groups coupling with the surface defects-derived carboxylic acids on the nanotubes. The functionalization reaction is heterogeneous in nature, so that typically an excess amount of the functionalization agent is used. For the functionalization with 2’-aminoethyl-α-D-mannopyranoside (Man-), the molar ratio of Man- to the nanotube-bound carboxylic acids (estimated at 5% mole fraction of the nanotube carbons based on titration results) was varied. The results in Table 2.4.1 show that the amount of SWNTs solubilized in the functionalization reaches
Table 2.4.1. Results on Man-, Man$_2$-, and Man$_4$- in functionalization/solubilization of SWNTs.

<table>
<thead>
<tr>
<th>Molar ratio of sugar-NH$_2$ to nanotube-COOH</th>
<th>Starting SWNTs Solubilized (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Man-</td>
</tr>
<tr>
<td>1:1</td>
<td>7.5</td>
</tr>
<tr>
<td>2:1</td>
<td>13</td>
</tr>
<tr>
<td>4:1</td>
<td>20</td>
</tr>
<tr>
<td>5:1</td>
<td>24</td>
</tr>
<tr>
<td>6:1</td>
<td>23</td>
</tr>
<tr>
<td>20:1</td>
<td>24</td>
</tr>
<tr>
<td>100:1</td>
<td>22</td>
</tr>
</tbody>
</table>
a plateau at the Man-to-acid molar ratio of 5 or so. Thus, at the molar ratio of 100, the amount of solubilized nanotubes should represent the limit for the functionalization agent under the otherwise defined experimental parameters and conditions. As shown in Table 2.4.1, the percentage of the starting purified SWNTs solubilized in the functionalization reaction increases from about 25% for Man- to 35% for Man2- and to 50% for Man4-. The higher-order sugar dendrons are obviously more effective in the functionalization and solubilization of SWNTs.

The sugar dendron-functionalized carbon nanotube samples, Gal2-SWNT, Man2-SWNT, Gal4-SWNT, and Man4-SWNT, are all readily soluble in water, allowing their being investigated with solution-phase techniques. The first was the characterization of the samples by NMR, both in solution and in the gel phase. For example, the 1H NMR spectra of Gal2-SWNT and Gal2-N3 in solutions are compared in Figure 2.4.1a. The signals of the nanotube sample are obviously significantly broader than those of the corresponding free dendron, which may be attributed to the high molecular weight and low mobility of carbon nanotubes. For the gel-phase NMR measurement of the same Gal2-SWNT sample in a high resolution-magic angle spinning (HR-MAS) probe specifically for the gel-phase, the 1H spectrum is better resolved, with the signal patterns in the sugar region comparable with those of the free dendron (Figure 2.4.1a). The aromatic proton signals for Gal2-SWNT are slightly shifted downfield, 6.82 ppm and 6.70 ppm for the nanotube-bound dendron from 6.72 ppm and 6.66 ppm for the free dendron, respectively, which might be due to the formation of amide linkages affecting the aromatic ring in the close vicinity. The same effects could be responsible for the absence
of the benzyl proton signal in the spectrum of Gal$_2$-SWNT because the proton is right next to the nanotube. This proton has a chemical shift of 4.36 ppm in the spectrum of Gal$_2$-N$_3$. It is known in the literature that the effect of large aromatic ring currents in carbon nanotubes on the local magnetic environment of protons in close proximity causes their resonance to either shift into the region where it is difficult to be identified or to become too broad to be detected.$^{25-27}$

Similar $^1$H NMR results, including effects on the signal broadening and shifting, were found for other dendron-functionalized SWNTs. Shown in Figure 2.4.2a is a similar comparison between the $^1$H NMR spectra of Gal$_4$-SWNT and Gal$_4$-N$_3$ in solutions, and the comparison between Man$_4$-SWNT and Man$_4$-N$_3$ is largely the same.

$^{13}$C NMR spectra of the dendron-functionalized SWNTs, especially Gal$_4$-SWNT and Man$_4$-SWNT, were readily obtained in solution, unlike those of Gal-SWNT and Man-SWNT whose relatively poor solubility (thus low solution concentrations) made their $^{13}$C NMR measurement rather difficult.$^{17}$ For the comparisons between Gal$_2$-SWNT and Gal$_2$-N$_3$ (Figure 2.4.1b) and between Gal$_4$-SWNT and Gal$_4$-N$_3$ (Figure 2.4.2b), there are signal broadening effects from free dendrons to those attached to SWNTs, though relatively less so from Gal$_4$-N$_3$ to Gal$_4$-SWNT as one would expect. There are Gal carbon resonances in both Gal$_2$-SWNT (Figure 2.4.1b) and Gal$_4$-SWNT (Figure 2.4.2b) spectra, and the latter also exhibits some of the tether carbon signals due to the higher solution concentration and perhaps also to these carbons being farther away from the nanotubes (less affected by their large ring currents$^{26-27}$).

The optical absorption spectra of the functionalized SWNTs were measured both
Figure 2.4.1 Comparisons for (a) the $^1$H NMR spectra of Gal$_2$-SWNT (top; solution phase; middle: gel phase) and Gal$_2$-N$_3$ (bottom); and (b) the $^{13}$C NMR spectra of Gal$_2$-SWNT (top, with inset) and Gal$_2$-N$_3$ (bottom). (Peak with star mark is from solvent MeOH)
Figure 2.4.2 Comparisons for (a) the $^1$H NMR spectra of Gal$_4$-SWNT (top) and Gal$_4$-N$_3$ (bottom); and (b) the $^{13}$C NMR spectra of Gal$_4$-SWNT (top, with inset) and Gal$_4$-N$_3$ (bottom). (Peaks with star mark are from solvent MeOH)
Figure 2.4.3 Optical absorption spectra of (a)Gal$_2$-SWNT; (b) Gal$_4$-SWNT; (c) Man$_2$-SWNT; and (d) Man$_4$-SWNT in D$_2$O solution (solid line) and in the solid-state on glass substrate (dashed line).
Figure 2.4.4 Raman spectra (633 nm excitation) of the functionalized SWNTs and the starting purified nanotube sample are compared.
in solution (only down to 1,500 nm because of the overwhelming interference from D$_2$O beyond that) and in the solid state (samples deposited on the surface of glass slides). The S$_{11}$ (~1,880 nm) and S$_{22}$ (~1,050 nm) bands due to van Hove singularity transitions in semiconducting SWNTs$^{28}$ and the weak M$_{11}$ transition (~740 nm) in metallic SWNTs are all present in the spectra of dendron-functionalized nanotube samples (Figure 2.4.3), suggesting the electronic properties of the nanotubes are largely preserved in the functionalization targeting defects-derived carboxylic acid moieties on the nanotube surface, as also observed in many other such functionalization schemes.$^{17,25-26}$ The solution spectra of Gal$_4$-SWNT and Man$_4$-SWNT are somewhat better resolved than their solid-state counterparts (Figure 2.4.3). For Gal$_2$-SWNT and Man$_2$-SWNT, the generally weaker absorption bands in the solution spectra are probably a simple result of their lower solubility (thus lower solution concentrations).

The sugar dendron-functionalized SWNTs were characterized by Raman with 633 nm excitation. Interestingly, unlike in other well-functionalized nanotube samples,$^{26,29}$ no substantial luminescence interference was observed. Thus, resonance Raman spectra could be measured for all of the dendron-SWNT samples without their being thermally or chemically defunctionalized first. As compared in Figure 2.4.4, the Raman spectra of the different dendron-SWNT samples are largely similar among themselves and also similar to that of the starting purified nanotube sample, exhibiting the typical radial breathing mode peaks around 140 and 160 cm$^{-1}$, D-band around 1,300 cm$^{-1}$, tangential G-band around 1,580 cm$^{-1}$, and D*-band around 2,600 cm$^{-1}$.

Scanning electron microscopy (SEM) technique was used to look at the
morphology of sugar dendron-functionalized nanotube samples. Shown in Figure 2.4.5 are SEM images for the Gal$_2$-SWNT sample, for which the specimen was prepared by drop-casting an ultra-thin film from the sample solution. Abundant nanotubes are observed, with their being randomly oriented at the edge of the specimen (Figure 2.4.5a) but more ordered in the fractured portion of the same specimen (Figure 2.4.5b). For Gal$_4$-SWNT, a very dilute aqueous solution was used to prepare the SEM specimen (depositing a few drops and then evaporating the water). The image shows generally dispersed nanotubes (Figure 2.4.6).

The thermal defunctionalization behavior of the dendron-SWNT samples is very different from those of many other functionalized carbon nanotubes.$^{30}$ The functional groups (sugar dendrons) could not be removed from the nanotubes in thermogravimetric analysis (TGA) scans (under inert atmosphere to 800°C), and instead they were carbonized under the thermal defunctionalization conditions. Therefore, the usually effective TGA estimate of nanotube contents in the functionalized samples was not applicable, and the compositions in dendron-SWNT samples were determined in terms of sugar analyses.

The sugar contents in the dendron-SWNT samples were quantified by spectrophotometry with the anthrone reagent.$^{21}$ Results thus obtained for the sample compositions are shown in Table 2.4.2. There are no meaningful differences in sample compositions between Gal- and Man-, Gal$_2$- and Man$_2$-, or Gal$_4$- and Man$_4$- as functional moieties, despite the fact that the Gal-based functionalization agents are consistently less effective (a lower percentage of starting purified SWNTs solubilized) than their Man-
Figure 2.4.5 SEM images of a Gal$_2$-SWNT specimen (ultra-thin film): (a) at the edge of the specimen; and (b) in the fractured portion of the specimen.
Figure 2.4.6 SEM image of the Gal₄-SWNT specimen (on carbon-coated copper grid) prepared from a very dilute sample solution.
Table 2.4.2. Compositions in the Functionalized Nanotube Samples and Related Parameters.

<table>
<thead>
<tr>
<th></th>
<th>Sugar Content $^a$</th>
<th>SWNT Content $^a$</th>
<th>Average No. of Nanotube Carbon per Sugar Unit</th>
<th>Aqueous Solubility (SWNT-equivalent, mg/mL)</th>
<th>Starting SWNTs Solubilized (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal-SWNT</td>
<td>45%</td>
<td>43%</td>
<td>14</td>
<td>0.83</td>
<td>11%</td>
</tr>
<tr>
<td>Man-SWNT</td>
<td>47%</td>
<td>42%</td>
<td>13</td>
<td>0.84</td>
<td>22%</td>
</tr>
<tr>
<td>Gal$_2$-SWNT</td>
<td>44%</td>
<td>19%</td>
<td>6</td>
<td>1.7</td>
<td>20%</td>
</tr>
<tr>
<td>Man$_2$-SWNT</td>
<td>45%</td>
<td>18%</td>
<td>6</td>
<td>1.6</td>
<td>35%</td>
</tr>
<tr>
<td>Gal$_4$-SWNT</td>
<td>37%</td>
<td>23%</td>
<td>9</td>
<td>3.1</td>
<td>27%</td>
</tr>
<tr>
<td>Man$_4$-SWNT</td>
<td>35%</td>
<td>29%</td>
<td>11</td>
<td>4.3</td>
<td>50%</td>
</tr>
</tbody>
</table>

$^a$ The remaining (100% - sugar content - SWNT content) is the content of linkers/tethers.
based counterparts in the nanotube solubilization (Table 2.4.2). The poorer solubilization performance of the Gal-based agents is probably due at least in part to the significantly lower (by a factor of 3 to 4) aqueous solubility of galactose than that of mannose.\textsuperscript{31}

The known sample compositions also allow the calculation for the average number of nanotube carbons per sugar unit in the samples (Table 2.4.2). From Gal- (or Man-) to Gal$_2$- (or Man$_2$-), the number decreases significantly, namely that more sugars are displayed on the same amount of SWNTs. For Gal$_4$-SWNT and Man$_4$-SWNT, even though there is no advantage in the number of sugars displayed (because of the relatively larger amount of tethering moieties for the display), the improved aqueous solubility and the specific configuration of β-D-galactoses or α-D-mannoses displayed in quartet may offer properties that are not available to other configurations.

It has been reported that Gal-SWNT binds to \textit{E. coli} O157:H7 to result in significant cell agglutination.\textsuperscript{17} The same assay was used to evaluate the binding of Gal$_2$-SWNT with the same \textit{E. coli} strain. As shown in Figure 2.4.7, there was obviously significant aggregation of the cells in the presence of Gal$_2$-SWNT. The amount of aggregates (precipitates at the bottom of the centrifuge tube) was larger for a higher starting \textit{E. coli} concentration of $10^8$ cells/mL than that of $5 \times 10^7$ cells/mL (Figure 2.4.7). Between Gal$_2$-SWNT and Gal-SWNT under similar experimental conditions, the amount of recovered aggregates was clearly larger in the former than in the latter (Figure 2.4.7). This was reflected more quantitatively in results from the CFU reduction assay. As compared in Figure 2.4.8, Gal$_2$-SWNT was obviously more effective in the agglutination of \textit{E. coli} O157:H7 cells to result in a more significant CFU reduction. Mechanistically,
Figure 2.4.7 Fluorescence microscopy images (10 μm for all scale bars) on the agglutination of *E. coli* O157:H7 (GFP-expressing) cells by (a) Gal2-SWNT, (b) Gal-SWNT, and (c) the control (cells only). (d) A visual comparison on the amount of precipitates (in centrifuge tubes) associated with the agglutination of *E. coli* O157:H7 cells (top: $5 \times 10^7$ cells/mL, and bottom: $10^8$ cells/mL) by Gal2-SWNT (left) and Gal-SWNT (right). (Courtesy of Dr. P. J. Luo)
Figure 2.4.8 Results from the CFU reduction assay for *E. coli* O157:H7 in the presence of Gal2-SWNT, Gal-SWNT, and PBS only as control. (Courtesy of Dr. P. J. Luo)
Figure 2.4.9 Optical microscopy images (75 μm for all scale bars) on the aggregation of *B. subtilis* spores (a) Man-SWNT, (b) Man₂-SWNT, and (c) Man₄-SWNT in the presence of calcium cation. (Courtesy of Dr. H. Wang)
the binding responsible for the cell agglutination is attributed to specific ligand-receptor interactions of the nanotube-displayed β-D-galactoses with *E. coli* surface galactose-binding-protein. The more favorable binding by Gal₂-SWNT seems to suggest that the paired galactoses could be more effective in the specific interactions. However, the multivalent binding of carbohydrates with cell surface receptors is a complicated phenomenon. A more definitive conclusion requires more systematic investigations with these and other assays.

The complexity with desired quantitative evaluations of the sugar-functionalized SWNTs in binding assays is also reflected in the results on *B. subtilis* spores. *B. subtilis* is a commonly used nonvirulent simulant for *Bacillus anthracis* (anthrax), with surface expressed with various carbohydrates. As reported previously for *B. anthracis* (Sterne) spores, Man-SWNT also binds to *B. subtilis* spores for their significant aggregation in the presence of calcium cation (Figure 2.4.9). Similarly, Man₂-SWNT and Man₄-SWNT are both capable of binding and aggregating *B. subtilis* spores in the same assay, as also illustrated in Figure 2.4.9. However, a more quantitative comparison of the different α-D-mannose-functionalized SWNTs samples was hindered by other issues beyond simply the different displays of mannoses in Man₂-SWNT and Man₄-SWNT. For example, it was found that for Man₂-SWNT samples of somewhat different Man/nanotube ratios (from slight variations in reaction conditions in the synthesis) their bindings with the spores (and the associated degrees of aggregation) were obviously different. Such effects were more pronounced in the binding assay of Man₄-SWNT with *B. subtilis* spores in the presence of calcium cation. Especially for Man₄-SWNT samples of slightly higher
Man/nanotube ratios, the binding assay resulted in well-dispersed aggregates of an irregular rod-like shape (20-50 microns in length and 5-10 microns in diameter according to optical microscopy analyses). We speculate that some of these complications might be due to the divalent cation-mediated interactions between the paired α-D-mannoses in Man$_2$-SWNT and Man$_4$-SWNT, which could be competing with the binding with cell surface receptors. Further investigations are required for a better understanding of the results.

2.4.4 Summary

In summary, a series of dendritic β-D-galactopyranosides and α-D-mannopyranosides with a terminal amino group were synthesized and used for the functionalization of SWNTs targeting the defect-derived carboxylic acid moieties on the nanotube surface. The functionalized nanotube samples were characterized by using established NMR, optical spectroscopy, and microscopy techniques. The results suggest that the higher-order sugar dendrons are more effective in the solubilization of SWNTs, with the corresponding functionalized nanotube samples of improved aqueous solubility characteristics, and that the nanotube is indeed a unique pseudo-one-dimensional scaffold for displaying multiple copies of the sugar molecules in pairs or quartets. These multivalent carbohydrate configurations may potentially offer interesting chemical and biochemical properties and functions, despite some of the complications that are yet to be understood (as reflected in the results of the binding assay with $B$. $subtilis$ spores).
Reference


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31. According to the MSDS data sheets (www.fishersci.com).


CHAPTER III

OTHER INTERESTING OR UNIQUE PROPERTIES OF SUGAR-FUNCTIONALIZED CARBON NANOTUBES

3.1 Carbohydrate-Carbohydrate Interactions Unique To Monosaccharide Ligands Tethered Single-Walled Carbon Nanotubes

3.1.1 Introduction

Cell recognition and adhesion are the initial step in a set of basic cellular processes such as cell migration and differentiation.\textsuperscript{1} Three known interactions, protein-protein interaction, protein-carbohydrate interaction, and carbohydrate-carbohydrate interaction, are the forces manipulating cell recognition and adhesion.\textsuperscript{2} Studying these interactions thus is fundamentally important to an understanding of basic cellular behaviors. Among tremendous studies in this field, most are limited to the self recognition of proteins, only a few focused on carbohydrates involving interactions, especially interactions among carbohydrates. This is because carbohydrate-carbohydrate interaction is usually very weak and co-exists with two other interactions.\textsuperscript{3} This problem was partially solved in early 1990’s, by mimicking natural multivalent ligands, the interaction of two carbohydrate clusters could be orders of magnitude stronger than that of the two single carbohydrates.\textsuperscript{4} Since then, many multivalent carbohydrate models have been developed to study carbohydrate interactions, such as three-dimensional (3D) glycol gold nanoparticles employed to study self adhesion process of trisaccharide Lewis\textsuperscript{x} and disaccharide lactose,\textsuperscript{5} glycol dendrimers,\textsuperscript{6} glycol polymers,\textsuperscript{7} and two-dimensional (2D) self assembled carbohydrate cluster on gold surface\textsuperscript{8} for the investigation of protein-
carbohydrate interaction.

Recently, our group reported a new pseudo-one-dimensional multivalent model by using single walled carbon nanotube (SWNT) as scaffold to display monosaccharides (such as β-D-galactose) as multivalent ligands. The galactose-functionalized SWNT (Gal-SWNT) material exhibits special ability of aggregating *E. Coli* O157:H7 cells through the likely ligand-receptor interaction between the tethered galactose and galactose-binding protein on the cell surface (see chapter 2.2). Another recent study of the same material showed a unique cation (Ca$^{2+}$)-mediated binding ability to *Bacillus anthracis* (Sterne) spores. This study inspired us that there could be Ca$^{2+}$ ion-mediated self-recognition in the nanotube-based multivalent monosaccharide system. After systematic studies, we reported here that there are indeed monosaccharide-monosaccharide interactions that are unique to the nanotube-bound sugars.

### 3.1.2 Experimental Section

**Materials.** CaCl$_2$ and ethylenediaminetetraacetic acid (EDTA) were purchased from Fisher. The series of sugar-functionalized SWNT samples: Man-SWNT, Gal-SWNT, Man$_2$-SWNT, Gal$_2$-SWNT, Man$_4$-SWNT, and Gal$_4$-SWNT, were prepared via typical carbodiimide-activated amidation between the sugar-tethered amino group and the nanotube-bound carboxylic acid group. The as-produced sugar-functionalized nanotube samples formed blackish but optically transparent aqueous solutions that were stable for months’ storage. The microscopy studies showed that the nanotubes were substantially debundled to individual tubes or thin bundles with their electrical properties being largely
unchanged. More details on the preparation and characterization have been provided in chapter 2.4.

**Atomic Force Microscopy (AFM).** Analysis of the samples was conducted on a Molecular Imaging PicoPlus system equipped with a multipurpose scanner. The height profile analyses were assisted by using the SPIP software distributed by Image Metrology. Sample specimen was prepared by spaying onto a heated mica substrate.

**Optical Microscopy.** Sample specimen was prepared by dropping a small aliquot (10 µl) of the sample onto a glass slide, then covered with cover glass and imaged with the differential interference contrast (DIC) model on a Leica DMIRE2 microscopy coupled to a Leica TCS SP2 laser scanning device and equipped with Leica HC PL APO 10*/*0.30 and 20*/*0.70 objectives.

**Scanning Electron Microscopy (SEM).** SEM images were obtained on a Hitachi S4700 field-emission SEM system. Sample specimen was prepared by first depositing the sample onto the filter paper (Whatman, nuclear pore polycarbon filter paper, 200 nm diameter) by filtration. Upon washing with ethanol solution and critical point drying, the final specimen was mounted on an aluminum stub with double-sided carbon tape and coated with platinum.

3.1.3 Results and Discussion

It is well-known that carbohydrate-carbohydrate interaction is closely related to divalent cation, especially Ca$^{2+}$ ion which has the ideal ionic radii (0.99 Å) to form stable carbohydrate-metal complex. In the literature, Ca$^{2+}$ ion has been effectively used
as the interacting medium to study self-adhesion among the trisaccharides\textsuperscript{3,5} and the disaccharides.\textsuperscript{12} In this study, CaCl\textsubscript{2} (20 mM, 200 µL) was first added into a 2mL Man-SWNT sample (mannose concentration around 0.4mg/mL, Figure 3.1.1a). The tiny black particles (aggregates) were formed immediately (observed easily by naked eyes), the small particles kept aggregating and started precipitation within 10 mins (Figure 3.1.1b-d), and after 1 hr, all the aggregates dropped down to the bottom of the vial and the upper solution changed to colorless (Figure 3.1.1e). The optical absorption spectrum of the upper solution became totally blank, meaning that it hardly contained any Man-SWNT sample. The same aggregation was observed in the experiment with Gal-SWNT sample (one tenth diluted from as-produced Gal-SWNT sample), and the results are showed in Figure 3.1.2.

To study the morphology of the initially formed aggregates at microscopic level, optical microscopy images of the freshly generated (within half min) Man-SWNT or Gal-SWNT aggregates were captured. As shown in Figure 3.1.3, the shape of the aggregates is either round or irregular. According to a statistical measurement of 1,202 Man-SWNT particles, the particle size varies from few micrometers to 100 µm, but most are within the range of 10 to 20 µm (Figure 3.1.3d). Considering that the size of an individual Man-SWNT is around several nanometers wide and a few micrometers long, those large aggregates suggest an ultra-fast self-aggregation among Man-SWNTs or Gal-SWNT upon adding Ca\textsuperscript{2+}.

The scanning electron microscopy (SEM) was also conducted to have a closer look at the morphology of the Man-SWNT/Gal-SWNT aggregates formed at very early
stage. As shown in Figure 3.1.4, the Man-SWNT/Gal-SWNT was randomly aggregated to form particles with different size varying from hundreds of nanometers to a few micrometers. A high-resolution image of Gal-SWNT particles showed nanotubes were closely aggregated together, and each particle contained more than 10 individual or thin-bundled nanotubes (Figure 3.1.4b).

To further examine the reason behind the aggregation, an EDTA-Na solution (40 mM, 200 μL) was added into the Man-SWNT precipitates. The Man-SWNT precipitates re-dispersed into the solution very fast and soon a blackish transparent Man-SWNT solution was recovered (Figure 3.1.1f-g). The re-dispersed Man-SWNT solution preserved same absorption curve as that of starting Man-SWNT sample (Figure 3.1.5b), and two typical absorption bands of the SWNTs at ~1036 nm (S_{22}) and ~718 nm (M_{11}) were preserved (Figure 3.1.6), which means that the aggregation/re-dispersion cycle does not affect the electronic properties of the Man-SWNT samples, and the Ca^{2+}-induced Man-SWNT aggregation is reversible by adding EDTA-Na. The similar EDTA-enabled re-dispersion of nanotubes was also observed in Gal-SWNT aggregation.

The reversibility of the aggregation is also supported by atomic force microscopy studies. Showed in Figure 3.1.7 is the AFM comparison of the original and re-dispersed Man-SWNT/Gal-SWNT samples. Most re-dispersed nanotubes stayed individually or in thin bundles as the original nanotubes, and no entangled nanotubes like those in Figure 3.1.3-b were found. Comparing to the starting Man-SWNT/Gal-SWNT samples (Figure 3.1.7a,b), the surface of the re-dispersed nanotubes is not very smooth, probably due to the deposition of the EDTA-Ca salts during the preparation of the AFM specimen. The
Ca^{2+}/EDTA mediated aggregation/redispersion cycle of Man-SWNT/Gal-SWNT samples can be repeated for many times (Figure 3.1.1h-n and 3.1.2e-g). In addition to Ca^{2+} cation, other divalent cations, such as Mg^{2+} and Ba^{2+}, are also capable to aggregate Man-SWNT/Gal-SWNT (Figure 3.1.8).

Before drawing the final conclusion on the force causing the aggregation of Man-SWNT/Gal-SWNT samples, a set of control experiments were conducted. CaCl\(_2\) (100 mM) was added into four controls including the mannose-functionalized polymeric nanoparticles (Man-NP, the mannose content about 5 wt%), the galactose-functionalized polymeric nanoparticles (Gal-NP, the galactose content about 4.7 wt%), the BSA-functionalized SWNTs (BSA-SWNT), and the poly(ethylene glycol)-functionalized SWNTs (PEG-SWNT), none of them generated aggregates (Figure 3.1.9a-d). Another control experiment was carried out by adding NaCl (50 mM) into the Man-SWNT/Gal-SWNT solution, and no aggregation was observed either (Figure 3.1.9e-f).

Combining all these results, we now have sufficient evidence to conclude that the aggregation is caused by the divalent cation-mediated monosaccharide-monosaccharide interaction. First, Ca\(^{2+}\) ion only induces the aggregation of mannose- or galactose-functionalized SWNTs, not the BSA- or PEG-functionalized SWNTs at all, indicating that monosaccharide is an essential element for aggregation. Second, removing Ca\(^{2+}\) ion by adding EDTA reverses the aggregation, characteristic of the kind of carbohydrate-carbohydrate interactions. Third, the aggregation is only triggered by divalent cation (Ca\(^{2+}\), Mg\(^{2+}\) or Ba\(^{2+}\)) instead of cations of other valence (such as Na\(^{+}\) ion), also characteristic of the kind of carbohydrate-carbohydrate interactions.\(^{3,5,11-12}\) Therefore, the
Figure 3.1.1 The repeatable solution-aggregation/precipitation-redispersion cycle of the mannose-functionalized SWNT sample. An as-prepared Man-SWNT solution (a); after adding CaCl₂ solution and waited for 5 (b), 10 (c), 30 (d), 60 (e) minutes to observe the aggregates and precipitates; precipitation re-dispersed into the solution by adding EDTA-Na (f and g). A repeat of the same precipitation (h-k) and re-dispersion (l-n) cycle.
Figure 3.1.2 In a solution of 100 µL of original Gal-SWNT sample mixed with 900 µL of water (a), 100 µl of 20mM CaCl$_2$ solution was added and waited for 5 min (b), 60 min (c), then 50 µL of 40 mM EDTA-Na solution was added (d), another 100 µL 20 mM CaCl$_2$ was added again and waited for 5 min (e), 30 min (f), and finally, the mixture was added 200 µL of 40 mM EDTA-Na solution (g).
Figure 3.1.3 Optical micrographs showing the Ca$^{2+}$-induced aggregation of (a,c) Man-SWNT, (b) Gal-SWNT, and (d) a size distribution analysis of freshly formed Man-SWNT aggregates upon adding CaCl$_2$. 
Figure 3.1.4 SEM images showing the Ca$^{2+}$-mediated aggregates of (a): Man-SWNT at a low magnification and (b): Gal-SWNT at a high magnification.
Figure 3.1.5 Optical absorption spectra (in H₂O) of (a): the supernatant of the post centrifuged sample shown in Figure 3.1.1e; (b): the aqueous Man-SWNT solution before adding Ca²⁺; and (c): the recovered Man-SWNT solution from the sample showed in Figure 3.1.1g.
Figure 3.1.6 Vis/NIR absorption spectra of the Man-SWNT solution in D$_2$O (solid line) and the same solution but after experiencing one aggregation/re-dispersion cycle induced by adding Ca$^{2+}$ and then EDTA (dashed line).
Figure 3.1.7 AFM phase images of (a): Man-SWNT and (b): Gal-SWNT; and the amplitude images of (c): Man-SWNT with Ca\(^{2+}\) then EDTA-Na and (d): Gal-SWNT with Ca\(^{2+}\) then EDTA-Na.
Figure 3.1.8 Photos of (a): a Man-SWNT solution; (b): the Man-SWNT aggregation upon adding Mg\textsuperscript{2+} cation; and (c): the re-dispersed Man-SWNT solution after adding EDTA-Na solution; and similarly (d): a Gal-SWNT solution; (e): the Gal-SWNT aggregation upon adding Mg\textsuperscript{2+} cation; and (f): the re-dispersed Gal-SWNT solution after adding EDTA-Na solution.
(Note: The results were similar with the use of divalent cation Ba\textsuperscript{2+} instead of Mg\textsuperscript{2+}.)}
Figure 3.1.9 Controls of (a): Gal-NP + Ca$^{2+}$ solution, (b): Man-NP + Ca$^{2+}$ solution; (c): BSA-SWNT + Ca$^{2+}$ solution; (d): PEG-SWNT + Ca$^{2+}$ solution; (e): Man-SWNT + Na$^+$ solution; and (f): Gal-SWNT + Na$^+$ solution.
Figure 3.1.10 The mixtures of Ca$^{2+}$ (100 mM) with sugar dendron-functionalized SWNTs (from left to right): Man$_2$-SWNT, Man$_4$-SWNT, Gal$_2$-SWNT, and Gal$_4$-SWNT.
specific binding effect between the divalent cation and the nanotube-tethered monosaccharides is the only reasonable explanation on the self-aggregation of the monosaccharide-functionalized SWNT samples (Scheme 3.1.1a).

An interesting fact here is that although Man-SWNT/Gal-SWNT aggregates strongly in the presence of Ca$^{2+}$, Man-NP/Gal-NP hardly aggregates at all. In the literature on those spherical gold glyconanoparticle models, Penadés’s group reported self-recognition of the trisaccharides Lewis$^x$ on 2nm gold nanoparticles,$^5$ and Russell et al. observed interaction among disaccharide lactoses-coated 16nm gold nanoparticles,$^{12b}$ but no similar interaction between monosaccharides was ever reported. It seems that the observed monosaccharide-monosaccharide interaction in our work is unique to the SWNT-displayed monosaccharides.

Ca$^{2+}$ involves in sugar-sugar interaction through forming coordinate bonds among sugars.$^{2c,12}$ Most naturally enriched monosaccharides, such as mannose, galactose and glucose, are short of $\alpha\text{-e-}\alpha$ sequence of hydroxyl group (which is the optimal geometry for the complexing site with Ca$^{2+}$) in their most stable state.$^{12}$ Therefore, in general, free monosaccharides rarely coordinate with Ca$^{2+}$ in aqueous solution. However, the studies of the C-type (Ca$^{2+}$ dependent) mannose-binding protein (MBP)$^{13}$ and galactose-binding lectin$^{14}$ all suggest that monosaccharides, under certain condition, could bind with Ca$^{2+}$ in solution.

The nanotube-displayed multivalent sugars, when compared with the particle-displayed ones, apparently have at least two unique characters: one is their one-dimensionally displayed morphology due to SWNT; and the other is their non-uniform
distribution on the nanotube due to the nature of the nanotube defect-site functionalization. Therefore, it is possible that the nanotube-displayed monosaccharides have a special local morphology that tends to bind to Ca\(^{2+}\) even in solution. On the other hand, due to the intrinsically hydrophobic nature and the size of the nanotubes and the limited number of the carbohydrates that are attached to the nanotubes, the monosaccharide-functionalized SWNTs might have a lower solubility than glycol gold nanoparticles, which makes them more sensitive to weak interactions among monosaccharides.

Another interesting observation is, against our expectation, that the monosaccharide (galactose and mannose)-based dendrons in their functionalized nanotube samples, including Man\(_2\)-SWNT, Gal\(_2\)-SWNT, Man\(_4\)-SWNT, and Gal\(_4\)-SWNT, have very weak aggregation response toward Ca\(^{2+}\). There was no immediate aggregation observed even at a very high Ca\(^{2+}\) concentration (100 mM), though there were some aggregates formed after a few days, but with most of the functionalized nanotubes still well dispersed in solution (Figure 3.1.10). Comparing the \(\alpha\)-D-mannose- or \(\beta\)-D-galactose- functionalized nanotubes to their dendron-functionalized ones, a major difference is that the latter have much higher sugar density and better aqueous solubility, thus may be less sensitive to monosaccharide-monosaccharide interactions. Another possible explanation is that since sugars are more densely packed in the sugar dendron functionalized SWNTs, they may tend to interacted with each other near by instead of with the one on the other nanotubes, thus cause much less nanotube aggregations (Scheme 3.1.1b).
Scheme 3.1.1 A schematic illustration for Ca$^{2+}$ mediated carbohydrate-carbohydrate interaction in (a) mono-sugar functionalized SWNTs and (b) sugar dendron functionalized SWNTs.
As we implied before, the research on carbohydrate-carbohydrate interactions is still at an early stage, and most current multivalent models are only suitable for a qualitative demonstration of those weak interactions. The efficiency of the demonstration is largely affected by the nature of the models, such as the size, morphology, solubility, and so on. Nerveless, our α-D-mannose- and β-D-galactose-functionalized nanotube systems obviously demonstrate the kind of unique interactions among monosaccharides.

3.1.4 Summary

In summary, we have firstly demonstrated the self recognition of the monosaccharides in solution. The interaction is unique to the monosaccharide ligands displayed on SWNTs. Although the detail mechanism of the interaction is still unknown, the special conformation of the nanotube bonded sugars and the unique nature of the nanotube might play the critical roles for this monosaccharide-monosaccharide interaction. And the SWNT carried multivalent carbohydrate ligands might be used as a versatile model to further study similar carbohydrate involved weak interactions.
References


3.2 Selective Interactions of Sugar-Functionalized Single-Walled Carbon Nanotubes with Bacillus Spores

3.2.1 Introduction

Potential applications of nanomaterials in biological field, such as biosensors and drug delivery, have attracted significant interest in recent years due in part to the dimensional proximity of these materials to biomacromolecules (e.g. proteins and DNAs) and some small organisms (e.g. viruses and bacteria).\textsuperscript{1,2} In these applications, the nanomaterials are usually used as scaffolds for the display of multiple copies of bioactive functional groups,\textsuperscript{2-5} making interactions of the bio-nano conjugates more significant than simply the free bioactive molecules with target biological species. For example, α-D-mannose-tethered polymeric nanoparticles were shown to bind to \textit{E. coli} cells (with mannose receptors) to induce massive cell agglutination, but no similar binding and agglutination were found with the use of free α-D-mannose molecules.\textsuperscript{2b}

Carbon nanotubes, especially single-walled ones (SWNTs), are typical “one-dimensional” nanomaterials because of their small diameters and large aspect ratios. The unique structure of the carbon nanotubes makes it a favored platform than other nanoscale carriers in the above mentioned interactions with biological species.\textsuperscript{4,5} For example, we previously reported that SWNTs displaying multiple monosaccharides, either α-D-mannose (Man-SWNT) or β-D-galactose (Gal-SWNT), could bind to \textit{Bacillus anthracis} spores in the presence of Ca\textsuperscript{2+}, resulting in significant spore aggregation and substantial reduction in colony forming units (CFU).\textsuperscript{5} These were attributed to the Ca\textsuperscript{2+}-mediated multivalent interactions between SWNT-displayed carbohydrates and spore
surface carbohydrates. The aggregation phenomenon was observed only with the use of nanotube scaffolds, and absent if polymeric nanoparticles or no nano-substrate was used instead.\(^5\)

The ongoing exploration of *Bacillus* spores and their surface structure related to the binding with various species has been of significant interest due to the importance in the countermeasure of bioterrorism threats with *B. anthracis*.\(^6\) Although only a partial understanding of the outermost layers of the *Bacillus* spores is available, a number of studies have been carried out to investigate the interactions of these spores with molecules such as peptides,\(^7\) lectins,\(^8\) and carbohydrates.\(^9\) Following our previous investigation on *B. anthracis* spores, here we reported a similar Ca\(^{2+}\) mediated binding of monosaccharide-coated SWNTs (Man-SWNT and Gal-SWNT) to *Bacillus subtilis* (a nonvirulent simulant for *B. anthracis*) spores. Beyond that, it was found that Man-SWNT can bind to and aggregate *B. subtilis* spores in the absence of Ca\(^{2+}\). The binding was specific to \(\alpha\)-D-mannose-functionalized SWNTs and *B. subtilis* spores, suggesting carbohydrate-protein interactions as a possible mechanism for the direct binding (without divalent cation mediation) of Man-SWNT to *B. subtilis* spores.

It is gratefully acknowledged that all spore-related experiments reported and discussed here were performed by Dr. Haifang Wang, also assisted and participated by Dr. Pengju (George) Luo and others, in our research group.

3.2.2 Experimental Section

**Materials.** The monosaccharide-functionalized single-walled carbon nanotubes
(Man-SWNT and Gal-SWNT) were prepared by using the same procedures detailed in chapter 2.2. The α-D-mannose- and β-D-galactose-coated polymeric nanoparticles were synthesized according to the literature.\textsuperscript{2b-c}

**Bacillus Spore Preparation.** *B. subtilis* ATCC33234 (American Type Culture Collection, Manassas, VA) spores were prepared by using a modified procedure from the literature.\textsuperscript{10} Briefly, *B. subtilis* cells were grown at 37 °C in solid Difco sporulation medium (DSM) until sporulation was essentially complete. Vegetative cells and spores were collected from medium plates, washed extensively with cold (4 °C) sterile distilled water, and pelleted by centrifugation. The pellet was re-suspended in a solution of Tris-Mg buffer (pH 8.0) that contained lysozyme at a concentration of 1 mg/mL. Subsequently, the suspension was shaken slowly for 1 h at 37 °C. Then \( N \)-Laurylsarcosine was added to a concentration of 2%, and the solution was incubated for 30 min. The spores were then pelleted and washed 4 times with sterile cold distilled water. Finally the spores were re-suspended in cold sterile distilled water and stored at 4 °C until further use. The purification of spores was further checked through spore staining and phase contrast microscopy.

**Binding Assays.** \( \text{Ca}^{2+} \)-mediated binding assays of Man-SWNT or Gal-SWNT and *B. subtilis* spores were carried out by following previous procedures.\textsuperscript{5} The binding assays without \( \text{Ca}^{2+} \) were conducted as follows. An aqueous suspension of *B. subtilis* spores (20 \( \mu \text{L}, 10^5 \text{ CFU/\muL} \)) was mixed with a test solution (20 \( \mu \text{L} \)) or with distilled water (20 \( \mu \text{L} \), as control). The mixtures (sample and control) were rotated for 16 h before being examined visually and under microscopes.
**Optical Microscopy.** A small aliquot of 10 µL sample from above binding assay was dropped onto a glass slide and covered with a cover slip. The specimen was imaged in the differential interference contrast (DIC) mode on a Leica DMIRE2 microscopy equipped with a Leica TCS SP2 laser scanning device and Leica HC PL APO 10*/0.30 and 20*/0.70 objectives.

**Scanning Electron Microscopy (SEM).** The sample mixture from above binding assay was centrifuged at 6800g for 5 min. The pellet was washed with distilled water in centrifuging-suspending cycles, and the final pellet was suspended for the fixing and post-fixing treatment according to established procedures. The final specimen upon critical point drying was mounted on an aluminum stub with double-sided carbon tape and coated with platinum. SEM images were obtained on a Hitachi S4700 field-emission SEM system.

**Colony Forming Units (CFU) Reduction Assays.** Similar to the CFU reduction assay used previously for the Ca²⁺-mediated binding of *B. anthracies*, a suspension of *B. subtilis* with the spore count of 2.6 × 10⁸ (in 40 µL) was mixed with a test sample (40 µL) or with distilled water as control. After 30 min, an aqueous CaCl₂ solution (20 µL, 100 mM) was added with gentle mixing, followed by rotation at room temperature for about 12 h. The mixtures (samples and control) were centrifuged at a low speed (25 g) for 1 min. A portion of each supernatant (40 µL) was used for the assay. Upon serial dilution, the diluted samples (0.1 mL each in triplicate) were spread evenly on solid TSA medium plates for incubation at 37 °C for about 12 h. The plates were counted, and the percentage CFU reduction was calculated in reference to the control.
For the binding without Ca\(^{2+}\), a suspension of *B. subtilis* with the spore count of 2 \(\times 10^8\) (in 40 \(\mu\)L) was mixed with a solution of test sample (40 \(\mu\)L) or with distilled water (as control). After being rotated at room temperature for about 16 h, the mixtures (sample and control) were treated in the same procedures as described above for the Ca\(^{2+}\)-mediated binding.

### 3.2.3 Results and Discussion

Due to high pathogenicity of *B. anthracis* spore, the genetically closely related *B. subtilis* spore has usually been used as a simulant. Albeit only a little has been known about the spore surface, current knowledge suggests that the spore surface is mainly constructed by glycoproteins and carbohydrates.\(^{12-13}\) For example, various carbohydrates including rhamnose, 3-O-methyl rhamnose, and galactosamine, were identified on *B. anthracis* spore surface,\(^{12}\) whereas quinovose and other unique sugars were isolated on the coat of *B. subtilis* spore.\(^{13}\)

We recently reported Ca\(^{2+}\)-mediated carbohydrate-carbohydrate interactions between monosaccharide-functionalized SWNTs (Man-SWNT and Gal-SWNT) and *B. anthracis* spores, resulting in significant spore aggregation.\(^{5}\) Since *B. subtilis* spore surface also expresses various carbohydrates, this kind of interaction should exist in *B. subtilis* system as well. To explore this possibility, binding assays of Man-SWNT and Gal-SWNT with *B. subtilis* spores were conducted in the presence of Ca\(^{2+}\). Both Man-SWNT and Gal-SWNT were found binding and aggregating *B. subtilis* spores in the presence of Ca\(^{2+}\). As shown in Figure 3.2.1a, the spores were aggregated to form larger
clumps to the size of 20-100 microns. Considering the normal size of a single spore at ~1 micron in diameter and ~2 microns in length, these spore clumps definitely contained hundreds to thousands of aggregated spores. The SEM images gave a closer view of the spore aggregation (Figure 3.2.1b-c). The spores were clearly embedded in a network constructed by the nanotubes. The spore aggregation could be re-dispersed by the addition of the EDTA sodium salt solution (100 µL, 20 mM), suggesting the existence of the expected carbohydrate-carbohydrate interactions between the nanotube-displayed monosaccharides and sugars on the B. subtilis spore surface.

Under the experimental condition, the CFU reductions for B. subtilis spores aggregated owing to the Ca\(^{2+}\)-mediated binding with Man-SWNT (0.2 mg/mL mannose equivalent) and Gal-SWNT (0.15 mg/mL galactose equivalent) were found to be 80% and 67%, respectively (Figure 3.2.2). The free mannose and mannan (a polymannose) as well as mannose- or galactose-coated polymeric nanoparticles (data not shown) did not show any spore reduction compared to the control, indicating that the binding was unique to carbon nanotube-displayed multiple sugars.

To further evaluate the binding potential of Man-SWNT to B. subtilis spores, the CFU reductions in the presence of various divalent cations and at different Ca\(^{2+}\) concentrations were conducted (Figure 3.2.3). The results showed that the increase in Ca\(^{2+}\) concentration would help to achieve higher CFU reduction. At the Ca\(^{2+}\) concentration of 25 mM, the CFU reduction of the spores was 70%, and improved to 87% when the Ca\(^{2+}\) concentration was raised to 250 mM.

The Ca\(^{2+}\) mediates carbohydrate-carbohydrate interactions by forming coordinate
Figure 3.2.1 Optical micrograph (a) and SEM image (b) of the aggregation of *B. subtilis* spores induced by Ca$^{2+}$-mediated binding with Man-SWNT; (c) A high-resolution SEM image showing the Ca$^{2+}$-mediated interactions of Gal-SWNT with *B. subtilis* spores. (Courtesy of Dr. H. Wang and Dr. P. J. Luo)
Figure 3.2.2 The CFU reduction (% of control) for *B. subtilis* spores aggregated owing to the Ca$^{2+}$-mediated binding with (a) 5 mg/mL Mannose; (b) 5 mg/mL Mannan; (c) Man-SWNT, 0.2 mg/mL mannose equivalent; and (d) Gal-SWNT, 0.15 mg/mL galactose equivalent. The Ca$^{2+}$ concentration is 100 mM. (Courtesy of Dr. H. Wang and Dr. P. J. Luo)
Figure 3.2.3 The CFU reduction (% of control) for *B. subtilis* spores aggregated owing to the binding with Man-SWNT in the presence of various divalent cations (Mg$^{2+}$, Ca$^{2+}$, and Ba$^{2+}$) and at different concentration of Ca$^{2+}$ (25 mM to 250 mM). (Courtesy of Dr. H. Wang and Dr. P. J. Luo)
bonds among sugars. Other divalent cations, such as Mg$^{2+}$ and Ba$^{2+}$, have been shown in the literature for the similar coordinating effect on carbohydrates.$^{14}$ In our study here, we found that Mg$^{2+}$ and Ba$^{2+}$ could indeed mediate the binding of Man-SWNT to *B. subtilis* spores. As suggested by CFU reduction results, at a divalent cation concentration of 100 mM, the binding efficiency in terms of CFU reduction is in the order of Mg$^{2+} < $ Ca$^{2+} < $ Ba$^{2+}$ (Figure 3.2.3). Apparently a larger diameter cation is favored in mediating the binding of Man-SWNT to *B. subtilis* spores, which is consistent with the literature report.$^{14d}$

The carbohydrate-carbohydrate interactions rely on divalent cations, which explains our previous finding of the Ca$^{2+}$-dependency in binding of Man-SWNT or Gal-SWNT to *B. anthracis* spores.$^5$ A similar Ca$^{2+}$-dependence was found in the *B. subtilis* spore aggregation study with Gal-SWNT. In the absence of divalent cation, Gal-SWNT can not induce any detectable spore aggregation (Figure 3.2.4d). However, the Man-SWNT was found to aggregate *B. subtilis* spores even in the absence of the divalent cation. As shown in Figure 3.2.4a, the *B. subtilis* spore aggregates could easily be identified in optical micrograph. A closer examination with SEM imaging showed that *B. subtilis* spores in the aggregates are “wrapped” by the functionalized SWNTs (Figure 3.2.4b-c). Although these spore aggregates were relatively small in size and loosely packed as compared to what were observed in Ca$^{2+}$-mediated aggregations, it was obviously caused by specific binding instead of nonspecific adsorption.

The CFU reduction due to *B. subtilis* spore aggregation in direct binding with sugar-coated nanomaterials showed that Man-SWNT had a 53% reduction while Gal-
Figure 3.2.4 Optical micrograph (a) and SEM images (b, c) of the aggregation of *B. subtilis* spores induced by direct binding with Man-SWNT; and a SEM image (d) showing no binding between Gal-SWNT and *B. subtilis* spores. (Courtesy of Dr. H. Wang and Dr. P. J. Luo)
Figure 3.2.5 The CFU reduction (% of control) for *B. subtilis* spores aggregation due to the direct binding with (a) Man-SWNT, 0.2 mg/mL mannose equivalent; (b) Mannose functionalized polymeric nanoparticles, 0.25 mg/mL mannose equivalent; (c) Gal-SWNT, 0.15 mg/mL galactose equivalent; and (d) Galactose functionalized polymeric nanoparticles, 0.52 mg/mL galactose equivalent. (Courtesy of Dr. H. Wang and Dr. P. J. Luo)
SWNT and α-D-mannose/β-D-galactose-functionalized polymeric nanoparticles did not cause any reduction (Figure 3.2.5). Therefore, the binding of Man-SWNT to B. subtilis spores is unique to the α-D-mannose-functionalized SWNTs and also specific to B. subtilis spores (no direct binding to B. anthracis spores observed).

Although the detailed binding mechanism of Man-SWNT to B. subtilis spores is still under investigation, it is unlikely due to the carbohydrate-carbohydrate interactions because it is Ca\(^{2+}\)-independent. Since the B. subtilis spore coat is composed primarily of proteins, the observed highly specific binding could most likely be attributed to carbohydrate-protein interactions between the nanotube-bound α-D-mannose moieties with the B. subtilis spore surface protein receptors. Although such carbohydrate-protein interactions are relatively weak, they do exist and are highly specific, with the addition of the divalent cation Ca\(^{2+}\) to bring in the additional binding due to carbohydrate-carbohydrate interactions, both kinds of interactions contributing to the binding and aggregating of B. subtilis spores with Man-SWNT. This may partially explain the results that Man-SWNT has a higher binding efficiency (as suggested by the CFU reduction) than Gal-SWNT in the Ca\(^{2+}\)-mediated aggregation of B. subtilis spores.

### 3.2.4 Summary

Carbon nanotubes represent a unique displaying scaffold for multivalent monosaccharide ligands. The monosaccharide-functionalized SWNTs were found to effectively bind and aggregate B. subtilis spores in the presence of a divalent cation. The observation was consistent with our previous report on B. anthracis spores. A new
finding reported here is that the $\alpha$-D-mannose-functionalized SWNTs were capable of binding to *B. subtilis* spores without the divalent cation. This direct binding is unique to the nanotube-bound $\alpha$-D-mannose and specific to *B. subtilis* but not *B. anthracis* spores. The selectivity may be exploited in the study of spores in the anthrax family for improved understanding of their surface structures and functions.
References


APPENDICES
List of the Abbreviations

Atomic force microscope ................................................................. AFM

*Bacillus anthracis* ................................................................. *B. anthracis*

*Bacillus subtilis* ................................................................. *B. subtilis*

Bovine Serum Albumin ................................................................. BSA

Colony-forming units ................................................................. CFU

*N, N’-dicyclohexyl-carbodiimide* ........................................... DCC

*N,N’-dimethyl-4-aminopyridin* ........................................... DMAP

*N, N’-dimethyl Formamide* ................................................... DMF

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide .................. EDAC

Ethylendiaminetetraacetic acid ................................................ EDTA

*Escherichia coli* ................................................................. *E. coli*

β-D-Galactose ................................................................. Gal-

Matrix-assisted laser desorption/ionization .......................... MALDI

α-D-Galactose ................................................................. Man-

Multi walled carbon nanotubes ................................................ MWNTs

*N-hydroxysuccinimide* ....................................................... NHS

Nuclear magnetic resonance ...................................................... NMR

Phosphate Buffered Saline ....................................................... PBS

Scanning electron microscope ................................................... SEM

Single walled carbon nanotubes ................................................ SWNTs
Transmission electron microscope................................................................. TEM
Thermo Gravimetric Analysis.............................................................................. TGA
Tetrahydrofuran ................................................................................................. THF
Time-of-flight mass spectrometry...................................................................... TOF-MS
Coauthored Publications Not Included in the Dissertation


S.; Sun, Y.-P., “Immuno-Carbon Nanotubes and Recognition of Pathogens”


Unique Aggregation of Anthrax (Bacillus anthracis) Spores by Sugar-Coated Single-Walled Carbon Nanotubes

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There has been significant interest in the surface structure and characteristics of anthrax (Bacillus anthracis) spores as related to their binding by molecular species. The investigation of such binding is obviously important to the development of countermeasure technologies for the detection and decontamination of anthrax spores. Various ligands based on natural and synthetic bioactive oligomers and polymers have been explored. For example, Tumborough and co-workers reported that a family of short peptides with a specific sequence could bind selectively to B. anthracis spores. Separately, Cole et al. showed that some lectins were able to agglutinate B. anthracis spores, for which the binding was attributed to the presence of lectin-specific α-galactose or N-acetylglucosamine on the spore surface.

There have been other studies suggesting that various carbohydrate derivatives are expressed on the surface of B. anthracis spores, including rhizomuc, 3-O-methyl glucosamine, and galactosamine. The surface carbohydrate residue may be exploited for the binding and agglutination of B. anthracis spores through interactions with synthetic multivalent carbohydrate ligands. As reported recently, single-walled carbon nanotubes (SWNTs) serves as an excellent pseudo one-dimensional scaffold for the ligand display, with the SWNTs related to more stable carbohydrate species exhibiting strong cell adhesion to result in efficient agglutination of pathogenic Escherichia coli. In the work reported here, we found that functionalized SWNTs displaying a large number of mono- or disaccharide (derivatives including mannose or galactose, Scheme 1) molecules bind to B. anthracis spores with the mediation of a divalent cation and that the binding is unique to the nanotube-displayed carbohydrates. The resulting significant agglutination of B. anthracis spores and the associated substantial reduction in colony forming units (CFU) may potentially find valuable applications in the detection and decontamination efforts.

Figure 1: Optical micrographs showing the aggregation of 20 µL of B. anthracis spores incubated with Man-SWNT (a and b) and Gal-SWNT (c). Scale bar = 100 µm (a) and 20 µm (b, c).

The sugar-functionalized nanotube samples Man-SWNT and Gal-SWNT were synthesized by coupling 2-aminomethyl-α-D-mannopyranoside and 2-aminomethyl-β-D-galactopyranoside, respectively, to purified SWNTs via surface conjugation of the nanotube surface-bound carboxylic acid moieties, as reported previously. The Man-SWNT and Gal-SWNT samples are readily soluble in water or aqueous buffer, fully compatible with biological species and assays.

B. anthracis spores (Strain 3-957), a nonvirulent strain, but still requires careful handling in a biosafety level 2 laboratory, was supplied by Colorado Serum Company (Denver, Colorado), and the spore suspension was prepared by following established procedures. It was found that when a sugar-functionalized nanotube solution was added to spore suspension to form an apparently homogeneous mixture, the further addition of divalent cations CaCl₂ resulted in substantial aggregation of the spores (Figure 1). In a typical experimental procedure, an aqueous suspension of B. anthracis spores (20 µL, 10⁷ CFU/µL) was mixed with a Man-SWNT solution (20 µL, nanomole equivalent concentration 0.1 mg mL⁻¹) or with distilled water (20 µL) as control. A few minutes later, an aqueous CaCl₂ solution (10 µL, 10 mM) was added with gentle mixing. There was immediate formation of aggregates visible to naked eyes in the mixture with Man-SWNT. Nevertheless, the mixture (spiral and control) were retained for 4 h. There were obvious large aggregates in the sample mixture, while no changes were found in the control (even after retention for more than 12 h). A small aliquot (10 µL) of the sample was dropped onto a glass slide (covered with cover glass slide) for optical microscopy analyses. As shown in Figure 1, the specimen contained predominantly large aggregates of at least 10 spores in size (compared with a typical spore size on the order of 1 micron in diameter and 3 microns in length).

The aggregates in the spore mixture were analyzed at a higher resolution by using scanning electron microscopy (SEM). For the preparation of the SEM specimen, the sample mixture was centrifuged at 8000 g for 5 min. The pellet was washed with distilled water in centrifuging--suspending cycles, and the final pellet was
The observed aggregation and CFE reduction of \textit{B. aneurits} spores is attributed to divalent cation-modulated multidimensional carbohydrate-carbohydrate interactions.\textsuperscript{15,18} Specifically, the carbohydrates tethered to SWNTs with those expressed on the spore surface. The role of Ca\textsuperscript{2+} mediation was confirmed by the experiment in which the aggregation of \textit{B. aneurits} spores was reversed with the addition of EDTA, a strong chelating agent of Ca\textsuperscript{2+}. While \textit{B. aneurits} spores were aggregated substantially by Man-SWNT with Ca\textsuperscript{2+} under the specific experimental conditions described above, the further addition of an excess solution of EDTA reduced their 150 (20 mM) with gentle shaking resulted in an immediate and complete reaggregation of the spores and mannosides (no aggregates at all). The observation is consistent with what is known in the literature on similar carbohydrate-carbohydrate interactions.\textsuperscript{14,15}

In summary, the carbon nanotubes represent a unique displaying scaffold for multidimensional monosaccharide ligands that bind effectively with \textit{B. aneurits} spores in the presence of a divalent cation. The binding results in substantial aggregation of the spores and corresponding CFE reduction. While an optimization of the binding and the elucidation of related mechanistic details remain to be investigated, the aggregation of spores may be exploited for anti-biofouling applications. For example, aggregated \textit{B. aneurits} spores are considerably less potent for aerosol inhalation,\textsuperscript{19} which is considered as the most lethal transmission pathway for fatal infection.

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References

Galactosylated Polymeric Nanoparticles: Synthesis and Adhesion Interactions with Escherichia coli

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Polymeric nanoparticles of a polystyrene core and polyethylene glycol corona were prepared via dispersion copolymerization of styrene with a specifically synthesized macromonomer. The particle surface tethered covalently dextrinized galactose moieties. These galactosylated polymeric nanoparticles were characterized by using dynamic light scattering, electron microscopy, NMR, and other techniques. Bioactivity of the nanoparticles were evaluated and demonstrated by their significant adhesion interactions with several Escherichia coli strains.

Keywords: Polymeric Nanoparticles, Escherichia coli, Galactose, Adhesion Interactions, Cell Agglutination.

1. INTRODUCTION

Microspheres or beads have been widely used in biological and medical assays. However, their relatively large sizes are disadvantageous in some applications, such as in the capturing of bacterial cells in immunosorptions.1 Recently, nanoscale particles have been developed and studied as novel or improved carriers for bioactive functionalities.2,3 For example, gold nanoparticles of a few nanometers in diameter were coated with mannose for binding with bacterial type I pili of Escherichia coli.2 Similarly, lactose-conjugated gold nanoparticles were found to exhibit selective aggregation when exposed to Recinella communis agglutinin, accompanied by significant color changes for optical detection.4 On the other hand, polymeric nanoparticles can be prepared conveniently in sizes on the order of 100 nm,5,6 which are between those of microspheres and gold nanoparticles. The particles can be tethered covalently with the desired bioactive groups or biological species, providing a versatile and robust platform in the design of specific nanoscale sensors or delivery vehicles for various purposes. In the reported work we synthesized and characterized nanoparticles of a polystyrene core and a large number of oligomeric polyethylene glycol (PEG) surface ethers. As an evaluation of their ability to carry bioactive functional groups, these polymeric nanoparticles were used to covalently display multiple copies of galactose for adhesion to several E. coli strains in physiological solutions.

There has been considerable interest in the literature concerning the interactions between carbohydrates and bacterial surface receptors (adhesins).7,8,9 Such interactions are generally weak and in nature they are enhanced by the presentation of multiple carbohydrate ligands to individual receptors.9,10 In an effort toward mimicking bacterial adhesion-specific interactions for various purposes, such as pathogen detection and treatment of bacterial infections, a variety of synthetic multivalent inhibitors have been explored. Among widely employed carriers for multivalent carbohydrate ligands are linear and branched polymers,11

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Galactosylated Polymeric Nanoparticles: Synthesis and Adsorption Interactions with Escherichia coli
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2. EXPERIMENTAL DETAILS

2.1. Materials and Measurements

Materials. 1,3-D-Galactosopentaacetate, p-chloromethylstyrene (98%), 2-chloroethanol, 4-dimethylaminopyridine (DMAP, 99%), hydrochloric acid, N-hydroxysuccinimide (93%), MES hydrate (99%), pyridine (99%), sodium hydride (60%, dispersed in mineral oil), sodium bicarbonate (powder), palladium on activated carbon (10% Pd), and succinimide hydrate (99%) were purchased from Acros; azobis(isobutyronitrile) (AIBN, 98%) was from Aldrich; 1,10-diacyclohexylcarbodiimide, triphenylphosphine (99%), sodium azide (99%), and p-toluenesulfonyl chloride (98%) were from Alfa Aesar; styrene was from Baker; polyethylene glycol (PEG, Mₚ ~ 2000) was from Fluka; tetrahydroammonium iodide (98%) was from Lancaster; and ethanol, chloroform, methylene chloride, THF, DMF, and diethyl ether were from Fisher. THF and methylene chloride were distilled over sodium and calcium hydride, respectively, and DMF was dried with calcium hydride and distilled under reduced pressure before use. Deuterium oxide (99.9%) and deuterated chloroform for NMR measurements were supplied by Cambridge Isotope Laboratories. Cellulose membrane tubing for dialysis was obtained from Sigma.

Measurements. NMR measurements were performed on a JEOL Eclipse 500 NMR spectrometer. Dynamic light scattering characterization was carried out on a Coulter N4 Plus particle sizer. Transmission electron microscopy (TEM) analyses were conducted on an Hitachi HD-2000 TEM/STEM system equipped with a CCD camera for digital imaging.

2.2. Nanoparticle Synthesis

The polymeric nanoparticles were prepared via copolymerization of the macromonomer with styrene. The macromonomer was synthesized in terms of procedures shown in Scheme 1.

α-Tosylate-St-PEG Macromonomer (1). NaI (0.6 g, 15 mmol) was added to a THF solution (100 mL) of PEG (20 g, 10 mmol). The slurry was kept at 40 °C for 4 h under constant stirring. p-Chloromethylstyrene (1.83 g, 12 mmol) was added to the slurry at 34 °C. The reaction mixture was stirred at 30 °C for 24 h and then neutralized to pH 7.0 with diluted HCl. Upon the removal of insoluble salts via filtration, the filtrate was concentrated and precipitated into cold ether. The precipitated was collected and dried in a vacuum oven at room temperature to obtain 1 (20.1 g, 95% yield). 1H NMR (400 MHz, CDCl₃): δ 7.38 (d, J = 7.3 Hz, 2 H), 7.30 (d, J = 6.9 Hz, 2 H), 6.72 (m, 1 H), 5.74 (dd, J = 17.8, 0.9 Hz, 1 H), 5.24 (dd, J = 10.3, 0.9 Hz, 1 H), 4.65 (s, 2 H), 3.44-4.0 (m, 180 H) ppm. 13C NMR (125 MHz, CDCl₃): δ 137.95, 136.99, 136.60, 128.01, 112.82, 73.00, 72.60, 70.41, 69.45, 61.78 ppm.

α-Tosylate-St-PEG Macromonomer (1). Pyridine (5.2 g, 65 mmol) was added to a dry solution (150 mL) of macromonomer 1 (10 g, 4.3 mmol) in CH₂Cl₂. After the mixture was cooled to 0 °C, p-toluenesulfonyl chloride (12.65 g, 65 mmol) was added. The resulting mixture was stirred at room temperature for 12 h, and then the solvent methylene chloride was removed on a rotary


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evaporator. The crude product was dissolved in chloroform (20 ml) and washed with HCl (2 M), aqueous NaHCO₃ (5%), and then water. The organic layer was separated, dried over anhydrous MgSO₄, and filtered. The filtrate was precipitated into cold ether. The precipitates were collected and dried in a vacuum oven at room temperature overnight to obtain 2 (9.5 g, 92% yield).¹ H NMR (500 MHz, CDCl₃): δ 7.78 (d, J = 8.2 Hz, 2 H), 7.25-7.44 (m, 6 H), 6.69 (m, 1 H), 5.72 (dd, J = 17.8, 0.9 Hz, 1 H), 5.22 (dd, J = 11.0, 0.9 Hz, 1 H), 4.55 (s, 2 H), 4.15 (t, J = 4.8 Hz, 2 H), 3.40-3.80 (m, 180 H), 2.44 (s, 3 H) ppm.¹³C NMR (125 MHz, CDCl₃): δ 144.83, 137.05, 136.96, 136.59, 133.04, 129.89, 128.03, 127.59, 126.25, 115.81, 72.97, 70.88, 70.78, 70.69, 70.61, 69.44, 69.30, 68.71, 21.71 ppm.

α-Azide-St-PEG Macromonomer (3). NaN₃ (2.76 g, 42 mmol) and tetraethylammonium iodide (0.2 g, 0.54 mmol) were added to a solution of macromonomer 2 (8 g, 4.2 mmol) in DMF (60 ml). The mixture was stirred at 40 °C for 4 h and precipitated into cold ether. The precipitates were collected and redissolved in chloroform. The resulting solution was dried over anhydrous MgSO₄ and filtered. The filtrate was concentrated and reprecipitated into cold ether. The precipitates were collected via filtration and then dried in a vacuum oven at room temperature to obtain 3 (8.3 g, 95% yield).¹ H NMR (500 MHz, CDCl₃): δ 7.38 (d, J = 8.2 Hz, 2 H), 7.30 (d, J = 7.3 Hz, 2 H), 6.70 (m, 1 H), 5.73 (dd, J = 16.6, 0.9 Hz, 1 H), 5.23 (dd, J = 11.6, 0.9 Hz, 1 H), 4.54 (s, 2 H), 3.80-3.54 (m, 180 H), 3.38 (t, J = 5.0 Hz, 2 H) ppm.¹³C NMR (125 MHz, CDCl₃): δ 137.95, 137.00, 136.90, 136.20, 136.00, 135.82, 113.82, 73.00, 70.70, 70.63, 69.45, 50.76 ppm.

α-Amine-St-PEG Macromonomer (4). Macromonomer 3 (3 g, 1.38 mmol), PPh₃ (4.4 g, 16.5 mmol), and H₂O (37.3 g, 2.1 mmol) were dissolved in THF (25 ml), and the mixture was stirred at room temperature for 36 h. The reaction mixture was concentrated and precipitated into cold ether. The precipitates were collected and dried under vacuum at room temperature to obtain 4 (2.6 g, 94% yield).¹ H NMR (500 MHz, CDCl₃): δ 7.39 (d, J = 8.2 Hz, 2 H), 7.30 (d, J = 8.2 Hz, 2 H), 6.71 (m, 1 H), 5.74 (dd, J = 18.3, 0.9 Hz, 1 H), 5.23 (dd, J = 11.4, 0.9 Hz, 1 H), 4.56 (s, 2 H), 3.8-3.4 (m, 180 H), 2.88 (t, J = 5.2 Hz, 2 H) ppm.¹³C NMR (125 MHz, CDCl₃): δ 137.94, 136.97, 136.59, 127.59, 126.25, 113.81, 73.10, 72.98, 70.70, 70.63, 69.43, 41.84 ppm.

α-Carboxyl Acid-St-PEG Macromonomer (5). Macromonomer 4 (1.96 g, 9.2 mmol), succinic anhydride (0.18 g, 18.4 mmol), and DMAP (10 mg, 0.08 mmol) were dissolved in methylene chloride (20 ml), and the solution was stirred at room temperature for 36 h. The reaction mixture was washed with water (5 ml x 3). The organic layer was separated, dried over anhydrous MgSO₄, and filtered. The filtrate was concentrated and precipitated into cold ether. The precipitates were collected and dried under vacuum at room temperature to obtain 5 (1.63 g, 80% yield).¹ H NMR (500 MHz, CDCl₃): δ 7.40 (d, J = 8.2 Hz, 2 H), 7.34 (d, J = 7.8 Hz, 2 H), 6.27 (m, 1 H), 5.76 (dd, J = 18.4, 0.9 Hz, 1 H), 5.24 (dd, J = 10.1, 0.9 Hz, 1 H), 4.58 (s, 2 H), 3.50-3.40 (m, 180 H), 2.67 (t, J = 6.6 Hz, 2 H), 2.56 (t, J = 6.6 Hz, 2 H) ppm.¹³C NMR (125 MHz, CDCl₃): δ 174.26, 172.67, 137.92, 136.97, 136.58, 128.00, 126.26, 113.83, 73.00, 72.00-70.3 (m), 70.25, 69.65, 69.42, 59.51, 51.03, 50.27 ppm.

Aminoethyl-st-Galactopyranoside (6). The compound was prepared according to the procedure already in the literature.¹ H NMR (500 MHz, D₂O): δ 4.33 (d, J = 7.5 Hz, 1 H), 3.84-3.89 (m, 2 H), 3.74-3.56 (m, 5 H), 3.50-3.40 (m, 1 H), 2.80-2.75 (m, 2 H) ppm.¹³C NMR (125 MHz, D₂O): δ 103.83, 75.39, 75.34, 71.29, 70.81, 68.96, 61.20, 60.97 ppm.

Galactosylated Macromonomer (7). Galactosylated macromonomer was prepared via the classical carbodiimide-activated coupling of 5 and 6 (Scheme I). In the reaction, to a cold solution of 5 (0.3 g, 0.226 mmol) and N-hydroxysuccinimide (28.6 mg, 0.249 mmol) in methylene chloride (10 ml) was added N,N-dicyclohexylcarbodiimide (56 mg, 0.271 mmol), and the mixture was reacted at 0 °C for 1 h. The reaction mixture was filtered, concentrated on a rotary evaporator, and precipitated into cold diethyl ether. The precipitation process was repeated twice to obtain 5a. To the solution of 5a in DMAP (10 ml) was added 6 (463 mg, 2.08 mmol), and the mixture was reacted at room temperature for 24 h. Then, the reaction mixture in a cellulose membrane tubing (cut-off molecular weight ~500) was dialyzed against fresh deionized water for 48 h to obtain 7 (70% yield).¹ H NMR (500 MHz, CDCl₃): δ 7.37 (d, J = 8.2 Hz, 2 H), 7.29 (d, J = 8.2 Hz, 2 H), 6.69 (m, 1 H), 5.73 (dd, J = 17.8, 0.9 Hz, 1 H), 5.22 (dd, J = 11.4, 0.9 Hz, 1 H), 4.54 (s, 2 H), 5.26 (d, J = 6.9 Hz, 4.00-3.20 (m), 2.56 (m, 4 H) ppm.¹³C NMR (125 MHz, CDCl₃): δ 174.05, 174.91, 137.94, 137.01, 136.61, 128.04, 126.29, 113.85, 103.67, 74.73, 73.77, 73.02, 71.55, 70.62, 70.18, 69.53, 69.42, 68.85, 62.21, 59.71, 51.96 ppm.

Galactosylated Nanoparticles. The dispersion co-polymerization shown in Scheme II was used to prepare the nanoparticles with a polystyrene core and surface PEG ethers,¹ which are terminated with galactose moieties. In a typical copolymerization reaction, the macromonomer 7 (0.62 g, 0.26 mmol), styrene (0.55 g, 5.3 mmol), and the polymerization initiator AIBN (9 mg, 0.055 mmol) were mixed in an ethanol-water mixture (4/1 v/v, 5 ml) in a round flask. Upon degassing via freeze-thaw cycles, the
flask was sealed, and the mixture was stirred at 60 °C for 24 h to form the nanoparticles. The suspended nanoparticles were transferred to a cellulose membrane tubing (cut-off molecular weight ~12,000) for dialysis against freshly deionized water for 3 days.

2.3. Experiments with E. coli

The E. coli D157: H7 strain EDL931 was obtained from Centers for Disease Control and Prevention. The E. coli ORN178 was kindly provided by Prof. Chao-Cheng Lin of National Taiwan Normal University. The frozen E. coli samples were recovered and cultured in tryptic soy broth. After overnight incubation at 37 °C, the bacterial culture was harvested and resuspended in sterile phosphate buffered saline (PBS, pH 7.4).

In a typical experiment for cell adhesion, the galactosylated polymeric nanoparticles were suspended in PBS. An aliquot of the suspension was mixed with the E. coli suspension in PBS for 15 min. The mixture was centrifuged at 8000 g for 5 min, which resulted in the precipitation of the bacterial cells with any adherent nanoparticles, while the unbound nanoparticles remained in solution. The supernatant containing free nanoparticles was discarded, and the pellet was washed twice with PBS in suspending-centrifuging cycles. The binding of the galactosylated polymeric nanoparticles with E. coli cells was probed by using TEM. The sample for the preparation of the TEM specimen was fixed in cacodylate buffered glutaraldehyde (3.5%, pH ~7.2) at 4 °C for 12 h. A droplet of the sample was deposited onto a carbon-coated copper grid, stained with uranyl acetate, and dried in air for 30 min.

3. RESULTS AND DISCUSSION

3.1. Nanoparticle Characterization

The galactosylated polymeric nanoparticles were in a stable aqueous suspension following the dialysis as the final step of the preparation process. The suspension appeared milky at the original high concentration but became optically more transparent upon dilution.

Dynamic light scattering (DLS) was used to characterize the nanoparticles in aqueous suspension. Shown in Figure 1 is the DLS result on the particles. The average diameter of the galactosylated polymeric nanoparticles is around 200 nm, with a relatively narrow size distribution standard deviation of 16 nm. Since the DLS measurement was in aqueous suspension, the particle size thus determined also includes the PEG corona over the polystyrene core (Scheme II). On the other hand, the dried nanoparticles on a carbon-coated copper grid were analyzed by SEM. The specimen was prepared by depositing a small drop of the diluted particle suspension onto the grid, followed by evaporation to remove water. A typical SEM image of the galactosylated polymeric nanoparticles is shown in Figure 2. By counting the particles on the surface layer in SEM images, the average particle size and size distribution standard deviation are 178 and 14 nm, respectively. It is reasonable that the average particle size estimated from SEM is somewhat smaller than that from DLS measurement, because the PEG corona is likely shrunk under the conditions for SEM imaging.
The galactosylated polymeric nanoparticles were characterized by several NMR methods. In these nanoparticles, the particle core is made essentially of linear polystyrene polymers, and the tethered galactoses are pendant functional groups on the polymers. Thus, the nanoparticles can be dissolved in a solvent good for both the polystyrene backbones and the pendant galactose moieties, resulting in a homogeneous solution. Chloroform is such a solvent, so that NMR measurement was carried out in deuterated chloroform solution. As compared with that of the macromonomer before polymerization in Figure 3, the proton NMR spectrum of the dissolved nanoparticles is considerably broader (due to much lower mobility of the polymer chain), with characteristic polysaccharide peaks in the 7.2–6.1 and 2.3–1.2 ppm regions. The PEG signal remains at ~3.65 ppm. However, the relative concentration of the tethered galactoses is too low to be detected in the NMR measurement of the nanoparticles in the homogeneous chloroform solution. According to $^1$H NMR signal integrations for the PEG and styrene peaks, the macromonomer mole fraction in the copolymer is on the order of 5%.

Gel-phase NMR was employed to analyze the nanoparticle-bound galactose moieties. For the measurement, the nanoparticles were suspended in deuterated water. Under such a condition, the polystyrene core was in the solid state, thus contributing no NMR signals. Only the hydrophilic tethers and the galactoses could be detected. The gel-phase proton NMR spectrum of the galactosylated polymeric nanoparticles is also compared in Figure 3. It exhibits no signals from the polystyrene core, as predicted. The PEG signal at 3.65 ppm is strong and broad, covering the expected peaks from the tethered galactoses. However, the overlapping between the PEG and galactose signals is less significant in the gel-phase $^1$C NMR spectrum. The peaks due to the tethers at 174 ppm (carboxyl), 39 ppm (CH$_2$-NH$_2$), and 32 ppm (COCH$_2$CH$_2$CO) and the galactose carbons at 103 and 52 ppm could be clearly identified. Other galactose carbon signals are still mixed up with those from the PEG moieties. Nevertheless, the NMR results are consistent with the nanoparticle structure of a polystyrene core and flexible surface tethers terminated with galactoses.

### 3.2. Quantification of Nanoparticle-Bound Galactoses

The amount of tethered galactoses in the nanoparticles was estimated by using the classical anthranilic acid method, which is widely employed in the determination of sugar content in an analyte based on the oxidation of sugar moieties. In this test, the nanoparticle-bound galactoses were oxidized in an aqueous H$_2$SO$_4$–HCl acidic solution of anthran at 100 °C. The absorbance at 625.5 nm was used for the quantitation in reference to a standard curve. A technical difficulty in the measurement was due to the scattering effect of the nanoparticle suspension. To compensate the scattering effect, a suspension of the polymeric nanoparticles containing no galactoses, but with comparable total particle counts, was used as the blank. The galactose content in the nanoparticle sample was estimated to be 6.5% (w/v%). On the other hand, the macromonomer mole fraction in the copolymer estimated above in terms of solution-phase $^1$H NMR signal integrations puts the galactose content on the order of 4.5% (w/v%). By using the average particle diameter from the SEM analysis for the spherical polystyrene core, with the assumption that the core has the same density as bulk polystyrene (1.047 g/cm$^3$), these galactose content values suggest that there are on average 0.8–1.3 million tethered galactoses on each particle.

### 3.3. Cell Adhesion

The bioactivities of the galactosylated polymeric nanoparticles were evaluated in terms of their adhesion to several E. coli strains. For the pathogenic E. coli O157:H7, as an example, the bacterial cells in PBS were mixed
with the suspended nanoparticles, followed by centrifuging to separate the cells from free nanoparticles in the supernatant. The pellet was washed repeatedly with PBS through a suspending–centrifuging routine. The interactions of the galactosylated polymeric nanoparticles with E. coli O157:H7 cells were visualized in TEM analyses. Shown in Figure 4 is a typical TEM image of the sample. The galactosylated polymeric nanoparticles are apparently bioactive, binding with multiple E. coli cells to result in significant cell agglutination. This is consistent with the report that there are periplasmic galactose-binding proteins on the E. coli cell that couple with galactose ligands.21

The E. coli ORN178 strain is known to contain receptors toward D-mannoses.22 Interestingly, however, the galactosylated polymeric nanoparticles can also bind with E. coli ORN178. The TEM image in Figure 5 shows that an E. coli cell is surrounded by a large number of the nanoparticles. This could not be a result of simple adsorption because no binding was observed with the use of bare polymeric nanoparticles without the galactose moieties.22

Multivalent carbohydrate ligands carried on linear and branched polymers or dendrimers are more potent than their monovalent counterparts in cell adhesion.23–25 The results reported here show that polymeric nanoparticles are also very effective in the display of multivalent ligands for interactions with specific cell adhesions. The nanoparticles are easily imaged in microscopy analyses, allowing the visualization of the interactions. In addition, the polymeric nanoparticles displaying galactoses and other bioactive ligands may also be developed as potent inhibitors or effectors for specific cellular responses.

4. SUMMARY

Polymeric nanoparticles of a polystyrene core and oligomeric PEG-based tethers can be prepared via dispersion copolymerization of styrene with specifically synthesized macromonomer. The tethers can be used to carry covalently derivatized galactose moieties, and the particle surface functionalities can be characterized by using NMR and other techniques. The galactosylated polymeric nanoparticles are bioactive, exhibiting significant adhesion interactions with several E. coli strains. The reported work demonstrates that polymeric nanoparticles may serve as a versatile vehicle for the delivery of drug or biological groups in potential biomedical applications.

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Superior optical limiting performance of simple metalloporphyrin derivatives

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Abstract

Several simple metalloporphyrins based on 5,10,15,20-tetraakis(2,4,6-trimethoxyphenyl)-21H,23H-porphine were found to be excellent optical limiters toward nanosecond laser pulses at 532 nm. In particular, the optical limiting performance of the porphyrin derivative with lead as the central metal matches that of currently the best benchmark optical limiter chloroindium tetra(2-butyldiphenyl)phthalocyanine. The optical limiting results of the molecules were correlated with their ground- and excited-state parameters in terms of the five-level reverse saturable absorption model.

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1. Introduction

Organic and inorganic nonlinear optical materials suitable for passive-mode optical limiting and potential optical switching applications have attracted much attention [1–6]. An ideal optical limiter exhibits linear transmittance at low incident light fluences, but becomes opaque at high incident light fluences. Among the most promising optical limiting materials are those showing strong nonlinear absorptions, which are commonly referred to as reverse saturable absorbers. The primary mechanism for the nonlinear absorptive optical limiting is a large ratio of excited-state to ground-state absorption cross-sections [1,3,6]. Thus, typical reverse saturable absorbers are molecules with weak ground state absorptions at the concerned wavelengths, such as metallophthalocyanines [3,6–9], fullerences [4,5,10–12], and mixed metal complexes [13–16] for optical limiting in the green (532 nm).

Porphyins and metalloporphyrins have been widely studied for their optical limiting properties [1,4,17–22]. For example, Sevian et al. investigated the limiting of picosecond laser pulses at 532 nm by nonplanar porphyrin derivatives [19]. Zhong et al. reported the limiting of nanosecond laser pulses by an indium porphyrin-based complex in solution [16]. There have also been many reports on various derivatization schemes in the search for porphyrin derivatives of superior optical limiting performance, including meta-substituted porphyrins with different center metals and the porphyrins with peripheral substitution on the ring. Generally speaking, however, porphyrins and their metallo and other derivatives have not been considered as being among the best optical limiters [4]. While some metalloporphyrins have strong optical limiting responses, very few of them can reach the performance level of many metallophthalocyanines. Here we report a surprising finding that some simple metalloporphyrin derivatives can be used as excellent optical limiters toward nanosecond laser pulses at 532 nm. In fact, the performance of the compound with lead as the center metal matches that of currently the best benchmark optical limiter.

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2. Experimental

2.1 Materials

4-Hydroxybenzaldehyde (99%), methyl 4-formylbenzoate (99%), 1-bromohexadecane (99%) and thionyl chloride (99.5±%) were purchased from Acros, zinc acetate, 5,10,15,20-tetrakis(p-hydroxyphenyl)-21H,23H-porphine were purchased from Aldrich, indium(III) chloride anhydrous from Alfa, and lead acetate tribromide from J.T. Baker. Spectrophotometry or HPLC grade solvents were used in the optical measurements. THF was first dried over molecular sieves, distilled, and then distilled over sodium before use. Other solvents for chemical synthesis were purified via simple distillation. Deuterated NMR solvents were supplied by Cambridge Isotope Laboratories.

2.2 Measurements

$^1$H and $^{13}$C NMR spectra were obtained on a JEOL Eclipse +500 NMR spectrometer. MALDI-TOF MS measurements were carried out on a Bruker OmniFlex instrument. UV/vis absorption spectra were recorded on a Shimadzu UV2550 spectrophotometer. Fluorescence spectra were measured on a Spex Fluorolog-2 photon-counting emission spectrometer equipped with a 450-W xenon source, a Spex 340S dual-grating and dual-exit emission monochromator, and two detectors. The room-temperature detector consisted of a Hamamatsu R928P PMT operated at ~950 V, and the thermoelectrically cooled detector consisted of a NIR-sensitive Hamamatsu R5108 PMT operated at ~1500 V. All emission spectra were corrected for nonlinear instrumental response by use of predetermined correction factors.

The experimental setup for optical limiting measurements consisted of a Continuum Powerlite 7010 Q-switched Nd:YAG laser operated in the single shot mode. The infrared fundamental was frequency doubled to generate the second harmonic at 532 nm, with the maximum energy of 320 mJ/pulse and a pulse width (FWHM) of ~5 ns. The laser output was collimated and varied from 7 to 300 mJ/pulse by using a waveplate-polarizer combination. The laser beam diameter was determined in individual measurements for the calculation of energy density (generally ~7 mm, corresponding to energy densities in the range of 0.02-0.8 J/cm²). For higher energy densities, the laser beam diameter was reduced to ~3.5 mm using a galvanic style telescope consisting of a plano-concave lens and a plano-convex lens. The detector was a Scientech Vector AC2501 calorimeter controlled by a Scientech Vector S310 meter. All measurements were performed with a 2-mm glass cuvette, and surface losses were corrected by using the corresponding neat solvents as references.

2.3. 5,10,15,20-Tetrakis(p-hexadeoxyxyloxyphenyl)-21H,23H-porphine (H$_2$-THPP) [23]

5,10,15,20-Tetra(p-hydroxyphenyl)porphyrin (150 mg, 0.221 mmol) and bromohexadecane (0.674 g, 2.21 mmol) were dissolved in DMF in the presence of K$_2$CO$_3$ and 18-crown-6 (catalytic quantity). After the mixture was refluxed for 24 h, the solvent DMF was removed on a rotary evaporator. The solid sample was washed with deionized water and then acetone, followed by silica gel column chromatography separation with hexanes-chloroform (4:1) as eluent. H$_2$-THPP was obtained as a purple solid (90% yield). $^1$H NMR (CDCl$_3$, 500 MHz) δ 8.86 (s, 8H), 8.10 (d, J = 8.25 Hz, 8H), 7.24 (d, J = 8.25 Hz, 8H), 4.22 (t, J = 6.4 Hz, 8H), 2.02-1.88 (m, 8H), 1.67-1.55 (m, 8H), 1.51-1.46 (m, 8H), 1.27 (s, broad, 8H), 0.88 (t, J = 7.35 Hz, 12H), 2.75 (s, 2H). $^{13}$C NMR (CDCl$_3$, 125 MHz) δ 159.05, 135.70, 134.54, 119.92, 112.77, 68.41, 32.06, 29.85, 29.81, 29.68, 29.64, 29.51, 26.37, 22.83, 14.29. MALDI-TOF MS: 1574.

2.4. Metalloporphyrins

For the preparation of Pb-THPP [24], H$_2$-THPP (100 mg, 0.064 mmol) and excess lead acetate were dissolved in pyridine, and the solution was diluted for 24 h. Upon cooling to ambient temperature, the reaction mixture was filtered to keep the green-colored precipitates. The solid sample was washed with deionized water and then acetone to obtain Pb-THPP in quantitative yield. $^1$H NMR (CDCl$_3$, 500 MHz) δ 7.89 (s, 8H), 8.6-7.5 (br, 16H), 4.25 (t, J = 6.4 Hz, 8H), 2.00-1.96 (m, 8H), 1.67-1.58 (m, 8H), 1.50-1.42 (m, 8H), 1.27 (s, br, 96H), 0.88 (t, J = 7.35 Hz, 12H). $^{13}$C NMR (CDCl$_3$, 125 MHz) δ 158.87, 149.64, 135.37, 131.96, 122.00, 112.53, 68.42, 32.03, 29.82, 29.78, 29.66, 29.47, 26.37, 22.79, 14.22. MALDI-TOF MS: 1782. Zn-THPP and InCl$_3$-THPP were prepared in a similar procedure, except for the use of acetic acid as the solvent [24]. Zn-THPP: $^1$H NMR (CDCl$_3$, 500 MHz) δ 8.97 (s, 8H), 8.10 (d, J = 8.25 Hz, 8H), 7.26 (d, J = 8.25 Hz, 8H), 4.24 (t, J = 6.4 Hz, 8H), 2.06-1.95 (m, 8H), 1.66-1.58 (m, 8H), 1.48-1.27 (s, broad, 96H), 0.88 (t, J = 7.35 Hz, 12H). $^{13}$C NMR (CDCl$_3$, 125 MHz) δ 158.88, 150.58, 135.49, 135.16, 131.95, 120.88, 112.64, 68.42, 32.03, 29.84, 29.79, 29.66, 29.48, 26.36, 22.80, 14.23. MALDI-TOF MS: 1640. InCl$_3$-THPP: $^1$H NMR (CDCl$_3$, 500 MHz) δ 9.09 (s, 8H), 8.26 (d, J = 8.25 Hz, 4H), 8.0 (d, J = 8.25 Hz, 4H), 7.32 (d, J = 8.25 Hz, 4H), 7.26 (d, J = 8.25 Hz, 4H), 4.26 (t, J = 6.4 Hz, 8H), 2.02-1.96 (m, 8H), 1.66-1.58 (m, 8H), 1.50-1.42 (m, 8H), 1.27 (s, br, 96H), 0.88 (t, J = 7.3 Hz, 12H). $^{13}$C NMR (CDCl$_3$, 125 MHz) δ 159.29, 149.84, 136.20, 135.44, 134.09, 132.78, 121.59, 113.06, 112.80, 68.48,
2.5. Other compounds

Chloroaurium tetra(t-butyl)-phthalocyanine (InClPc) [7,25] and zinc 2,3,7,8,12,13,17,18-octabromo-5,10,15, 20-tetraphenylporphyrin (ZnOBP) [20,26] were synthesized and purified by using experimental procedures similar to those already reported in the literature.

3. Results and discussion

Ground-state absorption spectra of H$_2$-THPP and Pb-THPP in room temperature THF solutions are shown in Fig. 1. The spectrum of H$_2$-THPP has four absorption bands in the visible, typical of a simple free-base porphyrin [27]. However, the spectrum of Pb-THPP is noticeably different, with the Q-bands red-shifted to result in weak absorption at 532 nm. The molar absorptivity of Pb-THPP is $\sim 2000 \text{ M}^{-1}\text{cm}^{-1}$ at 532 nm, much lower than that of H$_2$-THPP (more than 7000 M$^{-1}$ W$^{-1}$).

The free-base porphyrin H$_2$-THPP is not a strong optical limiter toward 5 ns laser pulses at 532 nm, while Pb-THPP exhibits excellent limiting response. As compared in Fig. 2 for H$_2$-THPP and Pb-THPP in room-temperature THF solutions of the same 70% linear transmittance, the saturated output fluence $J_{\text{out}}$ of Pb-THPP at the limiting plateau is 0.02 J/cm$^2$, considerably lower than that of H$_2$-THPP. In fact, the optical limiting response of Pb-THPP in the THF solution compares favorably with that of the benchmark material fullerene $C_{60}$ in toluene under the same experimental conditions (Fig. 2).

![Fig. 1. Absorption spectra of H$_2$-THPP (---) and Pb-THPP (-----) in room-temperature toluene solutions.](image)

![Fig. 2. Optical limiting results of H$_2$-THPP (○) and Pb-THPP (△) in room-temperature THF solutions of 70% linear transmittance at 532 nm. The results of $C_{60}$ in room-temperature toluene (□) are also shown for comparison.](image)
Chlorinoidium (tetra-2-buty)-phthalocyanine (InClPc) is often considered as the best dye for limiting nanosecond laser pulses at 532 nm \[7\]. For example, Perry and coworkers reported that InClPc could be used to achieve an attenuation factor of up to 340 \[7\]. Thus, InClPc was used as a reference for Pb-THPP in optical limiting performance. For the comparison, solutions of InClPc in toluene with 85% and 70% linear transmittances were prepared. Optical limiting responses of the solutions were measured in a cuvette of 2 mm optical path length at room temperature. The results thus obtained with the collimated beam configuration agree well with those reported by Perry and coworkers (Fig. 3 inset) \[7b\], which serves as a calibration of experimental setups and procedures in different laboratories. The other comparison in Fig. 3 shows that Pb-THPP and InClPc are essentially equal in optical limiting performance for nanosecond laser pulses at 532 nm under the same experimental conditions.

It has been rare for porphyrins to exhibit strong optical limiting response in the visible wavelength region. A noticeable exception is the zinc octabromotetraphenylporphyrin (ZnOBP) \[20\]. Su et al. reported that ZnOBP is an excellent optical limiter for nanosecond laser pulses at 532 nm, with the performance comparable with those of the benchmark optical limiters \[20\]. For a comparison between ZnOBP and Pb-THPP, a solution of ZnOBP in methylene chloride with 70% linear transmittance was prepared. The optical limiting results of the solution from measurements using the collimated beam configuration are in good agreement with those reported by Su et al. (Fig. 4 inset) \[20\]. However, as compared in Fig. 4, Pb-THPP in THF has better optical limiting performance than ZnOBP in methylene chloride for nanosecond laser pulses at 532 nm under the same experimental conditions.

The center metals in InClPc and ZnOBP are obviously In and Zn, respectively. The same metals were incorporated into THPP in the search for a metallo-THPP with even stronger optical limiting response. The optical limiting responses of InCl-THPP and Zn-THPP were measured both in room-temperature THF. As compared in Fig. 5, however, neither of the two compounds has as strong limiting response as Pb-THPP. The saturated $I_{50}$ values are 0.057 for InCl-THPP and 0.223 for Zn-THPP.

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1 It is noted that atom-substitution of the indium phthalocyanine may result in improved optical limiting performance \[8\].

Fig. 3. Optical limiting results of Pb-THPP in room-temperature THF (C) are compared with those of InClPc in room-temperature DMF (V) of the same 70% linear transmittance at 532 nm. Shown in the inset is a comparison for calibration between results of InClPc obtained in this work (V) and those reported by Perry et al. (V) \[7b\] at the same linear transmittance of 85% at 532 nm.

Fig. 4. Optical limiting results of Pb-THPP in room-temperature THF (C) are compared with those of ZnOBP in methylene chloride (Δ) of the same 70% linear transmittance at 532 nm. Shown in the inset is a comparison between the results obtained in this work (Δ) and by Su et al. (O) \[20\] at the same linear transmittance of 70% at 532 nm.

Fig. 5. A comparison of optical limiting results between Pb-THPP (C), Zn-THPP (V), and InCl-THPP (Δ) in THF solutions of the same 70% linear transmittance at 532 nm.
and 0.056 for Zn-THPP, higher than the value of 0.02J/cm² for Pb-THPP under the same experimental conditions. Nevertheless, both InCl-THPP and Zn-THPP are still excellent optical limiters toward nanosecond laser pulses at 532nm.

The optical limiting results of ZnOBP were explained in terms of the five-level reverse saturable absorption (RSA) model (Fig. 6) [20]. The same model may be applied to the results of metallo-THPP compounds reported here. For RSA in the metalloporphyrins, the optical limiting response is due primarily to the stronger excited singlet-state absorption than the ground-state absorption because the excited singlet-state is too short-lived to have any significant contributions to the limiting of the nanosecond laser pulses. Thus, the different limiting responses between the metallo-THPP compounds may be correlated with their ground- and excited-state parameters. The porphyrin compound with a lower ground-state absorptivity at the laser wavelength and a higher intersystem crossing efficiency is likely to have a better optical limiting performance. Fig. 7 shows a correlation of the saturated output fluence at the limiting plateau with the molar absorptivity at 532 nm for the different THPPs. The dependence is consistent with the expectation based on the RSA model.

The intersystem crossing in porphyrins competes with the fluorescence. The metallo-THPPs are less fluorescent than the free-base THPP due to more efficient intersystem crossing associated with the introduction of the center metal [20,27]. Shown in Fig. 8 are fluorescence spectra of the different THPP compounds in room-temperature solutions. The quantum yields of fluorescence ($\Phi_F$) and intersystem crossing ($\Phi_{ISC}$) have the following relationship:

$$\Phi_{ISC} = 1 - \Phi_F - \Phi_{NR},$$

where $\Phi_{NR}$ is a sum of quantum yields for other excited singlet-state nonradiative processes. If $\Phi_{NR}$ is assumed to be similar among the THPP compounds, the different $\Phi_F$ values represent changes in $\Phi_{ISC}$. As shown in Fig. 7, the correlation of the saturated output fluence at the limiting plateau with $\Phi_F$ for the different THPPs is similar to that for the molar absorptivity in the same figure. The results suggest that in the context of the five-level RSA model the better optical limiting performance of Pb-THPP is likely due to a combination of lower ground-state absorptivity at laser wavelength 532nm and higher intersystem crossing yield. The latter should be expected because of the known heavy metal effect on the intersystem crossing process.

In summary, the reported results show that simple metalloporphyrin derivatives can be used as state-of-the-art optical limiters for nanosecond laser pulses in the green, competitive to metallophthalocyanines. The observed optical limiting by these molecules is likely nonlinear absorption in origin, consistent with the five-level reverse saturable absorption model. These metal-
lophyrin derivatives with the long alkyl chains are readily soluble in a variety of common organic solvents, making it easier to be incorporated into polymer or other matrices in the limited device fabrication. The long alkyl chains and the associated solubility should prevent the formation of any intermolecular complexes, as found in other porphyrin and phthalocyanine systems. The possible contribution of such effect to observed superior optical limiting performance in these metallophyrin derivatives is under investigation.

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References

Selective Interactions of Porphyrins with Semiconducting Single-Walled Carbon Nanotubes

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Single-walled carbon nanotube (SWNT) samples produced via various techniques are generally mixtures of metallic and semiconducting SWNTs, which correspond to different electrical conductivities and other related properties. These properties are important to a variety of technological applications of SWNTs, including, for example, the electrical conductivity and charge dissipation in polymer/carbon nanocomposites and thin films.2,3 The enrichment of metallic over semiconducting SWNTs or vice versa in a nanotube sample toward a complete separation of the two different kinds of SWNTs has attracted much recent attention.2,4 In this communication, we report on the selectivity of a derivatized porphyrin toward semiconducting SWNTs as presumably nonconductive interactions, resulting in significantly enriched semiconducting SWNTs in the solubilized sample and predominantly metallic SWNTs in the residual solid sample according to Raman, near-IR absorption, and bulk conductivity characteristics.

The SWNT sample was produced in Professor A. M. Rao’s laboratory (Physics Department, Clemson University) by using the arc-discharge method. It was purified via oxidative acid treatment according to an established procedure.5,6 10,15,20,25-Tetrakis(3-hexadecyloxyphenyl)-21H,25H-porphine (THPP) was synthesized by coupling p-hexadecylbenzaldehyde with pyrrole in glacial acetic acid, followed by column chromatography separation and structural characterizations.

In a typical noncovalent solubilization experiment, a purified SWNT sample (100 mg) was added to a solution of THPP in chloroform (10 mg/mL, 20 mL), and the mixture was vacuumsaturated (Fisher Scientific PG3, 70 W, 42 kPa) for 48 h. After the removal of chloroform on a rotary evaporator, the solid mixture was extracted repeatedly with hexanes, coupled with vigorous centrifuging (~3100g) for 20 min, to remove free THPP. Then, THPP (10 mg/mL) was added to the solid sample to dissolve the THPP-attached SWNTs. Upon vigorous centrifuging for 10 min, the solid residue was collected and then used to go through the same noncovalent solubilization procedure again (twice), the third repeat was tried, but no further solubilization was observed. The functionalized SWNT sample largely free from the porphyrin was obtained as the final solid residue (99 mg, denoted as “free-SWNT” sample). The solubilized THPP-SWNT samples from the two repeated experiments were combined, followed by a complete evaporation of the solvent THF. The removal of THPP from the combined soluble sample was accomplished via washing the sample with acetic acid, coupled with vigorous centrifuging, resulting in the recovery of insoluble SWNTs (denoted as “recovered-SWNT” sample). The results from scanning electron microscopy analyses show that the two samples are largely similar. To ensure a rigorous comparison between the free-SWNT and recovered-SWNT samples in subsequent spectroscopy and conductivity characterizations, both solid-state samples were thermally treated under the same experimental condition of 800 °C for 1 h in a nitrogen atmosphere.

The recovered-SWNT sample is enriched in semiconducting SWNTs, while the free-SWNT sample contains predominantly metallic SWNTs. Their Raman spectra obtained with 785 nm (1.58 eV) excitation exhibit different features (Figure 1a).7,8 According to the widely used work of Dresselhaus and co-workers,9,10 while semiconducting and metallic SWNTs may have different tangential mode G bands, the difference is not expected to be obvious for the arc-produced SWNTs used in this work with the 785 nm excitation. The recent results and analyses by Papakonstantopoulou and co-workers on laser ablation-produced SWNTs reaffirm such a conclusion (Figure 4 in ref 5). As shown in Figure 1a, however, the G-band of the free-SWNT sample is broader and more asymmetric than that of the recovered-SWNT sample, indicative of substantial enrichment in metallic SWNTs (Raman—Wigner—Fano line shape).11,12 The Raman features in the radial breathing mode region are also consistent with an enrichment of metallic SWNTs in the free-SWNT sample.5

The significant separation of semiconducting nanotubes (in recovered-SWNT sample) from metallic nanotubes (in free-SWNT sample) is made more evident by a quantitative comparison of the near-IR absorption spectra. The spectra shown in Figure 1b were obtained by measuring (Thermo-Nicolet Nexus 670) the thermally treated solid-state samples of the same quantity under the same experimental conditions. No surfactant or other dispersing agents were used in the preparation of the specimen for measurements to avoid even the remote possibility of any doping effects. For the semiconducting recovered SWNT sample, the near-IR absorption spectrum contains significant bands at ~530 nm (1.95 μm, 0.67 eV) and ~970 nm (~1.25 μm), corresponding to transitions associated with the first (S₁₅) and second (S₂₂) pairs, respectively, of van Hove singularities in the electronic density of states for semiconducting SWNTs.13 On the other hand, the absorption of the metallic free-SWNT sample is negligible over the near-IR region.12 The results suggest that most of the semiconducting SWNTs in the starting sample ended up in the
Figure 1. (a) Raman spectral features (785 nm excitation) and (b) near-IR absorption spectra of the semiconducting recovered-SWNT (--) and metallic SWNT (---) samples after the same thermal treatment.

recovered-SWNT sample, namely that the porphyrin interactions were selective toward semiconducting SWNTs.

The bulk electrical conductivities of the free-SWNT and recovered-SWNT samples (thermally treated under the same conditions, as described above) are very different. In a comparative conductivity evaluation, the two samples were pressed separately into pellets of approximately 9 mm × 3 mm × 1 mm in dimension. Results from the classical four-probe conductivity measurements under identical conditions show that the bulk conductivities of the two samples differ by more than two orders of magnitude. 1.1 S cm⁻¹ for the metallic free-SWNT vs 0.007 S cm⁻¹ for the semiconducting recovered-SWNT.

To examine the effects of the nanorube on the THPP species on the surface, the soluble THPP–SWNT sample in solution was characterized by 1H NMR (data not shown). The NMR signals of THPP are significantly broadened upon the attachment to the SWNTs, which is likely due largely to the significantly reduced mobility of the THPP species. Consistent with the NMR results, it seems that the porphyrin ring is probably bound to the SWNT surface, leading to localized charge transfer around in solution. Such a picture is supported by the high-resolution TEM results, which show soft materials on the surfaces of individual nanorubes (Figure 2).

Figure 2. High-resolution TEM images (scale bar = 5 nm) of the soluble THPP–SWNT sample.

The interactions of THPP with SWNT are apparently specific to the porphyrin free base, hindered upon the location of a metal atom. For example, the Zn–THPP was used in the place of THPP under the same experimental conditions, but no interaction leading to the solubilization of SWNTs was observed. The negative results with the use of metallo-porphyrins also suggest that the selectivity toward semiconducting SWNTs reported above is associated with THPP, not fragments of decomposed THPP, because porphyrins with and without the center-chelated metal show smaller decomposition properties. 15

In summary, the derivatized porphyrin THPP selectively interacts with and solubilizes semiconducting SWNTs, resulting in the "extraction" of most of the semiconducting SWNTs from the starting purified nanorube sample. Mechanistically, we suspect that the semiconducting and metallic SWNTs have significantly different surface properties. A speculation is that a semiconducting SWNT is more like a conjugated macrocycle than the nanorube surface properties conceptually similar to those of a nucleic acid pair, thus amenable to interactions with the free-base porphyrin molecules. Since the porphyrin is readily prepared and largely recoverable from the extraction experiment, it may become an effective and convenient method for the separation of semiconducting SWNTs from metallic SWNTs.

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Supporting Information Available: Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

Original Paper

Preparation, Characterization, and Evaluation of Immuno Carbon Nanotubes

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Abstract. Single-walled and multiple-walled carbon nanotubes were functionalized with bovine serum albumin protein to attain aqueous solubility and biocompatibility. These aqueous soluble carbon nanotube-protein conjugates were further conjugated with pathogen-specific antibody to form immuno carbon nanotubes. The experimental details on the conjugation and results from the characterization of the conjugates are presented. The potential of these immuno carbon nanotubes in the detection of pathogenic E. coli O157:H7 was evaluated, and the evaluation results are discussed.

Key words: Carbon nanotubes; conjugation; E. coli O157:H7; immunoasay; pathogen detection.

There has been much recent attention on potential biological applications of both single-walled (SWNT) and multiple-walled (MWNT) carbon nanotubes for their unique combination of properties, such as one-dimensional structure, high surface area, chemical inertness, excellent mechanical properties and rich electronic properties [1, 2]. For the targeted applications, there have been rationally designed chemical modifications to carbon nanotubes with biologically active species [3], including carbohydrates, amino acids, peptides, proteins, and nucleic acids. Practical uses of these bio-functionalized carbon nanotube samples in vitro or even in vivo have been explored, with examples such as drug and gene delivery [4–6] and specific recognition of bacteria [7, 8].

Aqueous soluble functionalized carbon nanotubes upon being coupled with antibodies may serve as excellent bioanalytical reagents in immunoassays. Their unique properties include the flexible and essentially linear structure with high aspect ratio and large surface area, and the availability of biofunctionalities in arrays. There might also be significant difference between SWNTs and MWNTs in bioanalyses, such that SWNTs are more flexible and thus relatively easier in the binding with the cell surface at multiple sites, while MWNTs are better dispersed in the functionalization and contain less impurity but more encapsulated ferromagnetic species for potentially magnetic separation purposes. We have previously reported some results on the conjugation of carbon nanotubes with proteins and antibodies for potential bioanalytical applications [7, 9, 10]. Here we provide a more detailed account for the preparation, characterization, and biological evaluation in the followup experiments toward the practical applications of the
immuno carbon nanotubes in immunoassays in general and the rapid and sensitive pathogen detection in particular.

Experimental

Materials

Bovine serum albumin (BSA) protein (Fraction V, 98%), Micro Lowry Total Protein Kit (Gibco & Earl modification), and Bicinchoninic Acid (BCA) Kit were purchased from Sigma-Aldrich, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, 98%) and sodium azide (99%) from Alfa Aesar, the cellulose ester dialysis tubing from Spectrum Laboratories, affinity purified goat anti-E. coli O157 antibody from Ketajgaard & Perry Laboratories and the green fluorescence protein (plasmid vector pGFP-P69(P), pGFP-P) from Clontech.

The SWNT and MWNT samples were either obtained commercially (Carbon Solutions, Inc. and Nanostructure & Nanoporous Materials, Inc., respectively) or produced in the laboratory of Prof. A. M. Rao (Department of Physics and Astronomy, Clemson University) by using the arc-discharge (Ni/Y as catalyst) and CVD (xylene as precursor and ferrocene as catalyst) methods, respectively. Similar purification procedures were used for SWNTs and MWNTs. Typically, a nanotube sample (100 mg) was refluxed in diluted HNO₃ solution (2.6 M, 50 mL) for 5–24 h. The mixture was cooled to room temperature and then centrifuged at 3,000 g. The supernatant was discarded, and the remaining solid sample was washed repeatedly with deionized water until neutral and then dried in a vacuum oven to obtain the purified nanotube sample.

Measurements

Optical absorption spectra were recorded on a Shimadzu UV2101 or UV2000 spectrophotometer and a Thermo-Nicolet Nexus 670 FT-IR spectrometer. Raman spectra were obtained on a Renishaw Raman spectrometer equipped with a 514 nm diode laser source for 780 nm excitation or a John Yvon T64000 Raman spectrometer equipped with a 355 nm He-Ne laser source for 628 nm excitation. Thermogravimetric analysis (TGA) was performed on a Metter- Toledo TGA/SDTA851e system. Scanning (SEM) and transmission (TEM) electron microscopy analyses were carried out on Hitachi S4700 FE-SEM and Hitachi HD-2000 Scanning-TEM systems, respectively. Atomic force microscopy (AFM) measurements were conducted in the acoustic AC mode on a Molecular Imaging Pro-Plus system.

Carbon Nanotube-BSA Conjugate

In a typical experiment to prepare the conjugate of SWNT with BSA (SWNT-BSA, Scheme 1), a purified SWNT sample (50 mg) was dispersed in a KH₂PO₄ buffer solution (20 mL, pH=7.4) with EDAC (250 mg, 1.2 mmol), and the mixture was sonicated for 2 h. Then, BSA (500 mg) was added, and the mixture was stirred at room temperature for 24 h. The suspension from the reaction was centrifuged at 3,000 g to collect the supernatant for dialysis in a cellulose ester tubing (cutoff molecular weight 300,000) against deionized water for 5 days, in which any excess BSA and other byproducts and reagents were removed. The resulting aqueous solution was evaporated to yield SWNT-BSA as a dark-colored solid sample.

The preparation of MWNT-BSA was only slightly different. In a typical procedure, a purified MWNT sample (50 mg) was dispersed in a KH₂PO₄ buffer solution (20 mL, pH=7.4) with EDAC (250 mg, 1.2 mmol). The mixture was sonicated for 2 h, followed by centrifuging at 3,000 g to disc the supernatant. The remaining solid sample containing EDAC-treated MWNTs was re-suspended in the same buffer solution (20 mL) and to which was added BSA (500 mg). The mixture was stirred at room temperature for 24 h, and the subsequent work-up procedure was the same as that for SWNT-BSA described above. The MWNT-BSA was also obtained as a dark-colored solid sample.

Immuno Carbon Nanotubes

In a typical conjugation experiment, a SWNT-BSA sample was suspended in phosphate buffer (PBS, 0.1 M, pH=7.4). The suspension was mixed with a separately prepared PBS solution of goat anti-E. coli O157, and the mixture was subjected to slow rotation at 40 rpm for 20–24 h at room temperature, followed by centrifuging at 14,000 g (Eppendorf Centrifuge 5415R) to remove any free antibody molecules. The sediment was washed several times by being suspended in PBS and then centrifugation to discard the supernatant. The final suspension contained the conjugate of SWNT-BSA with the E. coli O157 antibody (immuno-SWNTs).

The same procedure was applied to the preparation of immuno-MWNTs.

Bacterial Cell Culture and Bio-Evaluation of Immuno-Nanotubes

The E. coli O157:H7 strain C972 was kindly provided by Prof. M. P. Doyle (University of Georgia). The cells were labeled with GIP by using a common procedure [11]. The GIP-labeled E. coli cells were grown in Tryptic soy broth supplemented with ampicillin (100 μg/mL–1) in a shaker incubator at 37°C for 22 h. The as-cultured bacteria cells were washed several times with a sterile NaCl solution (0.85%), and then suspended and diluted in the same NaCl solution to an optical density of 0.7 at 630 nm (corresponding to bacteria concentration of ~10⁹ CFU mL⁻¹). Serial dilutions were then performed to obtain different concentrations of pure E. coli cultures. In the enumeration of bacterial culture via colony formation, an aliquot (100 μL) of the culture was spread on a Tryptic soy agar (TSA) plate (supplemented with ampicillin) and incubated at 37°C overnight. The enumeration was performed in duplicate.
In a typical experiment for the bio-evaluation, solutions of fresh-cultured E. coli cells and immuno carbon nanotubes were mixed. The mixture was subjected to slow rotation at 40 rpm for 1h at room temperature, followed by centrifugation to remove unbound cells. The captured E. coli cells were re-suspended in PBS buffer for either colony enumeration or the fixation required for SEM imaging. The procedures for fixation and the preparation of SEM specimen have been reported elsewhere [8].

Results and Discussion

Carbon Nanotube-BSA Conjugates

Both SWNTs and MWNTs were functionalized with BSA via carbodiimide-activated amidation of the defect-derived carboxylic acid groups (generated during acid-treatment in the purification) on the carbon nanotube surface with the available pendant amino moieties (especially lysine residues) on BSA (Scheme 1) [9]. The SWNT-BSA and MWNT-BSA conjugates thus obtained were readily soluble in water, with solubilities over 5 mg mL\(^{-1}\) and 1 mg mL\(^{-1}\), respectively, to result in dark-colored but still transparent and homogeneous solutions. The dark solution color must be due to well-dispersed carbon nanotubes because the protein is colorless. This was confirmed in the absorption spectral measurements. The spectrum of aqueous SWNT-BSA solution exhibits features at around 1,010 nm and 700 nm (Fig. 1), corresponding to the electronic transitions of the semiconducting (second van Hove singularity pair S\(_{22}\)) and metallic SWNTs, respectively [12, 13]. These absorption features suggest that the nanotube electronic structures are largely undisturbed in the conjugation with BSA, consistent with the expectation that the functionalization reaction targets primarily the surface defect sites of SWNTs [9, 10, 14]. Also as expected, the absorption spectrum of the MWNT-BSA conjugate is featureless, with the continuously decreasing absorption toward longer wavelengths, similar to those found in other functionalized MWNTs [14].

The Raman measurements of the SWNT-BSA and MWNT-BSA conjugates were subject to significant luminescence interference, as commonly observed in the same measurements of most other functionalized carbon nanotube samples [9, 14, 15]. In fact, the presence of strong luminescence has been considered as an indication that the carbon nanotubes are well-dispersed in these functionalized samples, because the luminescence is associated only with the well-dispersed nanotubes (inter-tube quenching minimized) in which the defects are well-passivated as a result of efficient functionalization [15]. However, since the luminescence is dependent on the nanotube dispersion and functionalization, it is diminished upon the removal of the functional groups either thermally or chemically [9, 14, 15]. In this work, the nanotube-bound BSA species in the conjugates could be decomposed and evaporated selectively in the thermogravimetric analysis (TGA) under inert atmosphere (a process often called thermal defunctionalization). The defunctionalized samples exhibited typical Raman features of, for example, G-band at 1,596 cm\(^{-1}\), D-band at 1,330 cm\(^{-1}\) for MWNTs.

The TGA analysis in which the protein species could largely be removed from the nanotube surface (at 400 °C or less) was also used to estimate the nanotube contents in the conjugate samples [14, 16]. In this study, the SWNT-BSA and MWNT-BSA samples typically contained around 10–30% w/w wt of the nanotubes according to the TGA results. On the other hand, the aqueous solubility of the conjugate samples and the optical transparency of the resulting solutions also allowed the determination of protein contents by using classical analytical methods for proteins. The modified Lowry assay based on coloring the solution for quantitative spectrophotometry was employed [17]. In the analysis, a standard curve of optical density vs protein concentration was generated by using BSA solutions of known concentrations. However, because of the background of carbon nanotubes at the detection wavelength, the observed optical density values of the conjugate solutions that were treated with the Lowry reagents must be subtracted by those of the
same solutions without the treatment as blanks (Fig. 2). According to the modified Lowry assay, the total protein contents in SWNT-BSA and MWNT-BSA conjugates were 60–70% wt/wt, generally consistent with the estimates from TGA results.

The conjugate samples were also characterized by using microscopy techniques. As shown in Fig. 3, the AFM images suggest that in both SWNT-BSA and MWNT-BSA, there is a direct association of the proteins with the nanotubes [9], thus consistent with the expected conjugation. According to the TEM image (dark field, Fig. 4), the nanotubes in MWNT-BSA are well-dispersed. There are soft materials, likely BSA proteins, attached to the nanotube surface. In addition, the TEM images show encapsulated metal species (appearing bright) in the nanotube hollow interior, which are readily attributed to residues from the metal catalysts (iron-related compounds) used in the CVD production of MWNTs. These encapsulated species apparently survived the purification and chemical functionalization procedures. Similar catalyst residues were found in the functionalized SWNTs [18, 19], though the TEM imaging of well-dispersed SWNTs to examine the structural relationship between the nanotubes and the residual metal species was proven to be more difficult. Nevertheless, since these residues embedded in the functionalized nanotube samples are ferromagnetic, they may be explored in applications such as magnetic separations.

**Immuno Carbon Nanotubes**

As reported earlier [7], the nanotube-BSA conjugates could be further conjugated via simple adsorption with pathogen specific antibodies such as goat anti-*E. coli* O157 to yield immuno carbon nanotubes. In this work, an optimization in the amount of antibody adsorption was investigated in terms of the BCA (Bicinchoninic Acid) protein assay [20]. The assay

![Graph showing absorption spectra](image)

![AFM images of SWNT-BSA](image)

![TEM images of MWNT-BSA](image)
is sensitive in the low protein concentration regime in the determination of the maximum protein content when different amounts of the antibody were used. For example, in the preparation of immuno-MWNTs, different volumes (50–80 μL) of the antibody solution (10 μg/mL−1) were used in the mixing with a constant volume (800 μL) of the MWNT-BSA solution (10 μg/mL−1). Then, free antibody species were removed via centrifugation, and the resulting MWNT-BSA-antibody conjugate was subject to the BCA assay. In the assay, a higher observed optical density corresponds to the adsorption of more antibody species in the conjugation. The results shown in Fig. 5 suggest that the amount of antibody attachment reached the maximum when 70 μL of the antibody solution was used, or about 8.8 μL antibody solution per 100 μL of the MWNT-BSA solution (concentration: 10 μg/mL−1) [21]. In a similar set of experiments for immuno-SWNTs, the optimal ratio was found to be 80 μL of antibody solution per 100 μL of the SWNT-BSA solution (concentration: 8 μg/mL−1).

The display of abundant antibody species on the surface of immuno carbon nanotubes facilitated the specific cell recognition. As reported earlier for the binding of the immuno-SWNTs with pathogenic E. coli O157:H7 cells [7], the immuno-MWNTs could serve the same purpose (Fig. 6). The binding was specific (antibody–antigen), because there was no meaningful attachment to the bacterial cells by the MWNT-BSA conjugate species without the antibody. However, the immuno-MWNTs appeared somewhat less efficient than the immuno-SWNTs in the targeting of the pathogenic cells, presumably due to the relatively larger sizes and less structural flexibility of MWNTs.

More quantitatively, the binding of immuno-SWNTs with the E. coli cells was evaluated by using colony enumeration. A serial dilutions of relatively low bacterial concentrations were used. As shown in Table 1, the immuno-SWNTs could apparently detect and capture the E. coli cells effectively. For example, at the initial cell count of 15 CFU per 100 μL, more than half of the cells were recovered by the immuno-SWNTs. The bacterial recovery could be as high as 80–90% with the use of a larger amount of immuno-SWNTs and/or a higher initial bacterial count (Table 1).

The presence of residual metal catalysts-derived species in the SWNT-BSA conjugate sample was
Table I. Evaluation on Detection of E. coli O157:H7 by Immuno-SWNTs

<table>
<thead>
<tr>
<th>Immuno-SWNTs (8 μg/ml)</th>
<th>Initial cell count (CFU per 100 μL)</th>
<th>Detected (CFU per 100 μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 μL</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>87</td>
<td>60</td>
</tr>
<tr>
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<td>252</td>
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<tr>
<td>200 μL</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>132</td>
<td>106</td>
</tr>
</tbody>
</table>

explored for the purpose of immunomagnetic separation. In the experiment, a solution of SWNT-BSA was placed in a magnetic separator (Dynal Biotech Model MFC-L) to isolate the magnetically active portion of the sample. Only a small fraction of the original sample was isolated within a few hours of the magnetic separation. The isolated SWNT-BSA sample was used in the conjugation with goat anti-E. coli O157 to yield magnetically responsive immuno-SWNTs. However, the subsequent experiment of using the immuno-SWNTs to capture E. coli cells for magnetic separation was not successful, with generally unsatisfactory recovery efficiency (few or no CFU detected over a long separation time). These results, though still preliminary, suggest that the magnetic residues in the immuno-SWNTs are probably insufficient for the purpose of immunomagnetic separation in the capturing and detection of pathogenic E. coli. A possible reason is that in SWNT samples the carbon cavities containing the magnetic residues are generally independent of the nanotubes, so that the magnetic separation step applied to the SWNT-BSA sample likely isolates mostly species associated with carbon impurities [18]. Therefore, the immuno-MWNTs with magnetic elements encapsulated in the nanotube structures may be more suitable for the intended application.

Conclusions

Carbon nanotube-BSA conjugates were synthesized via the classical carbodiimide-activated amidation reaction. The conjugates were characterized extensively by using optical spectroscopy, TGA, protein assay, and various microscopy methods. The results are consistent with the expected functionalization scheme targeting defect sites on the nanotube surface. The conjugates were further conjugated via direct adsorption with goat anti-E. coli O157 antibody species to form immuno carbon nanotubes. These are unique bioanalytical reagents for the targeting and detection of pathogenic E. coli cells. The microscopy and colony enumeration results suggest that the immuno carbon nanotubes are capable of sensitively capturing and effectively recovering the bacteria. Further investigation to couple with the immunomagnetic separation technique for rapid and ultrasensitive pathogen detection is in progress.

Acknowledgement. We thank Profs. M. P. Doyle for providing the E. coli strains and Prof. A. M. Rao for supplying the nanotube samples. Financial support from NSF is gratefully acknowledged. J. B., R. J., and E. J. were participants of the Summer Undergraduate Research Program sponsored jointly by NSF and Clemson University.

References

[13] The water absorption overhydrodorized the Si bond of the semiconducting SWNTs at around 1,800 nm
[21] The observed decrease of adsorption of antibody at higher concentration is probably due to the leaching effect as a result of over-coating.

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Immuno-Carbon Nanotubes and Recognition of Pathogens
Tara Elkin, Xiuping Jiang, Shelby Taylor, Yi Lin, Lingrong Gu, Hua Yang, Jessica Brown, Susan Collins, and Ya-Ping Sun

Carbon nanotubes have been widely investigated for their unique combination of properties including their one-dimensional hollow structure, high strength with flexibility, large surface area, and chemical stability. There has also been growing interest in their potential biological applications, especially in biosensors and vaccine- and drug-delivery systems. Both single- and multi-walled carbon nanotubes (SWNT and MWNT, respectively) can be modified and conjugated with biological molecules and biological species including carbohydrates, amino acids and peptides, nucleic acids and analogues, and proteins. We have previously demonstrated that SWNTs are naturally protein-affinity in water and that covalent modification of the nanotubes with proteins gives stable conjugation and excellent aqueous solubility of the conjugates. Here we report the preparation of immuno-carbon nanotubes by conjugating pathogen-specific antibodies to the SWNT that are functionalized with bovine serum albumin (BSA) protein (Scheme 1) and, for the first time, the recognition of target pathogen cells by the immuno-carbon nanotubes via antibody-antigen interactions in physiological environment.

The nanotube-bound protein species can be selectively removed in the thermal gravimetric analysis (TGA) to allow an estimate of the nanotube content. According to the TGA result, 25±5% (w/w) of a typical SWNT-BSA conjugate sample is nanotube. The conjugate sample was also analyzed in a modified Lowry assay to determine the total protein content. The result of ~70% (w/w) total protein in the sample (with the rest being primarily SWNTs) is consistent with the nanotube content estimate based on TGA.

The SWNT-BSA conjugate was coated with the antibody goat anti-Esherichia coli O157 (Ab) by direct adsorption. In a typical procedure, a solution of Ab, 0.1 μg/mL, 90 μL in phosphate-buffered saline (PBS; 0.1 M, pH 7.4) was added to the SWNT-BSA solution (20 mg/mL, 100 μL, 0.1 M PBS). The mixture was subjected to slow rotation (DynaTech Biotech Shaker Model 947.01) at 40 rpm for 20–24 h at room temperature, and then separated in a centrifuge at 14,000 rpm (Eppendorf Centrifuge 5417R) to remove free Ab (in the discarded clear supernatant). The black pellet was resuspended in PBS buffer (100 μL) and another round of the centrifuging/suspending process followed. The final sample was again suspended in PBS buffer (100 μL) to yield a homogeneous dispersion of the SWNT-BSA-Ab conjugate (or "Immuno-SWNT").

A solution of freshly cultured E. coli O157:H7 cells (100 μL, 0.85% NaCl, ~10^8 CFU/mL) was added to the immuno-SWNT solution (100 μL, 0.1 M PBS). The mixture was subjected to slow rotation at 40 rpm for 1 h at room temperature, and then separated in a centrifuge at 14,000 rpm. The pellet was repeatedly

Figure 1. An AFM image of the SWNT-BSA sample on a mica surface (scale bar = 1 μm).

Scheme 1.

Supporting Information for this article is available on the WWW under http://www.chembiochem.org or from the author.

References:
[1] T. Elkin, Prof. X. Jiang, S. Taylor, Dr. Y. Lin, L. Gu, H. Yang, J. Brown, S. Collins, Prof. Y-P Sun
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Supporting Information for this article is available on the WWW under http://www.chembiochem.org or from the author.
washed with PBS buffer to remove unbounded immuno-SWNT species, and the final suspension in PBS was passed through a membrane filter (0.2 μm). The filtrate with the immuno-SWNT-bound E.coli cells was used as the specimen (after the application of a thin conductive coating) for scanning electron microscopy (SEM) analysis. The SEM images thus obtained provide visual evidence for the conclusion that there are indeed strong interactions between the immuno-SWNT and E.coli cells. In fact, the E.coli cells are covered by SWNTs, as shown in Figure 2.

In the confocal microscopy imaging, the green fluorescence spots (Figure 4A) identify the E.coli cells (GFP-labeled), and the red fluorescence spots (Figure 4B) identify the Ab3 species (rhodamine-labeled). Figure 4C shows a combination of the two images, in which the yellow fluorescence spots are due to the superposition of the green and red signals. Since Ab3 only binds to Ab2, but not to E.coli and there was no free Ab2, as a result of the thorough washing, the confocal microscopy results suggest strongly that the antibody-antigen interactions with the E.coli cells are specific to Ab2 in the immuno-SWNT.

The interactions of the immuno-SWNT with E.coli cells are specific. In a control experiment, the SWNT-BSA conjugate rather than the immuno-SWNT was used with the E.coli cells in the same experimental procedure. However, the SEM results suggest no meaningful binding of the nanotube conjugates with the E.coli cells (Figure 3). The cells in these images are in sharp contrast to those from the E.coli-immuno-SWNT sample (Figure 2). Evidently, the immunofunctionality induced by the coating of SWNT-BSA with Ab2 is critical for the binding with the E.coli cells. The results also suggest that SWNTs conjugated with immunofunctionalities are indeed biaxial and capable of specific antibody-antigen interactions with pathogenic cells.

The specific antibody-antigen interactions were verified in terms of the use of a secondary antibody, goat anti-rabbit IgG (H+L) (Ab4). The secondary antibody Ab4 is such that it binds only to the primary antibody (Ab2) but not to the E.coli.20 To enable confocal microscopy imaging, E.coli cells labeled with a green fluorescent protein (GFP) marker21 and secondary antibody Ab4 labeled with rhodamine (red fluorescence) were used. In the experiment, a solution of Ab3 (5 μg/mL, 15 μL, 0.1 w/v PBS) was added to a mixture of the immuno-SWNT and E.coli cells (15 μL, 0.01 w/v PBS), and the resulting sample was incubated at 37°C for 30 min. After centrifuging at 10000 g, the sample was washed repeatedly with PBS (0.01M) to remove unbound Ab3, and then resuspended in PBS. A small drop of the suspension was placed on a glass slide and sealed with a glass cover slip for confocal microscopy analysis.

Figure 2. SEM images of E.coli cells bound with immuno-SWNT species. There was the possibility occasionally to find two cells bound together by the nanotubes (b). This became even more when the bacteria concentration was lower.

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The immunoswnt-E.coli interactions are through the nanotube-bound Ab2 species and the cell surface antigen sites. In immuno-SWNT, the initial nanotube conjugation with BSA introduces the necessary aqueous solubility, making it easier to further modify the nanotubes in a physiologically compatible environment.20 However, the adsorption mode of the antibodies onto the SWNT-BSA is probably complex, including the issue of whether the nanotube-bound BSA species repel or facilitate the conjugation with the antibodies. Several possibilities for the adsorption mode will be evaluated in further investigations.23

It is known that the conformational structures of the antibody proteins might play a critical role in some antibody-antigen interactions.32 For the antibody Ab2, possible changes in its conformation upon being attached to the SWNT-BSA conjugate remain to be explored and understood. It does appear, however, that whatever the effect might be, it does not fundamentally change the immunological function of the SWNT-bound antibody species. The observation of clearly strong interactions between the immuno-SWNT and the target E.coli cell suggests that the conjugated antibodies are capable of specific recognition toward their antigen counterparts. Similarly, Dai and co-workers reported that some antigens that were immobilized onto SWNT devices by covalent linkages to surfac-
tant molecules still retained activity toward their antibody counterparts.

In summary, we have shown that the antibody goat anti-
E. coli O157 can be conjugated to the SWNT-BSA conjugate to
form the immuno-SWNT under physiologically compatible con-
ditions. The immuno-SWNT is capable of recognizing patho-
genic E. coli O157:H7 cells through specific antibody–antigen
interactions. Carbon nanotubes are species of unique proper-
ties, such as large aspect ratio and high surface area. These
nanomaterial properties are being explored for biosensors and
related bioapplications. The work reported here might prove
valuable to the potential development of rapid and ultrasensi-
tive pathogen-detection techniques based on the use of
carbon nanotubes.

Acknowledgements

We thank Prof. M. P. Doyle for providing the E. coli strain, Prof.
A. M. Rao for supplying the nanotube sample, and S. Fernando
and B. Zhou for experimental assistance. Financial support from
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by NSF and Clemson University.

Keywords: biological activity - immunoassays - nanostructures - nanotubes

Carbon Nanotubes for Immunomagnetic Separation of Escherichia Coli O157:H7

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Keywords: Carbon Nanotubes, Functionalization, Filling, Escherichia Coli O157:H7, Immunoassay, Immunomagnetic Separation.

Bovine serum albumin-functionalized multiple-walled carbon nanotubes with encapsulated ferromagnetic elements were conjugated with pathogen-specific antibody, and the conjugate was evaluated for immunomagnetic separation of Escherichia coli O157:H7 in pure and mixed (with Salmonella Typhimurium) cultures.

Carbon nanotubes, both single-walled (SWNT) and multiple-walled (MWNT), have been attracting much attention. A recent focus in the carbon nanotube research is on potential biological applications that take advantage of their unique properties, such as their one-dimensional nano-structure with large aspect ratio, high surface area, and unique electronic and optical features. Chemical modification or functionalization of carbon nanotubes is often used for their homogenous dispersion into aqueous environments for in vitro and in vivo evaluations. For example, water-soluble carbon nanotubes were used to deliver DNA into mammalian cells in a feasibility study of potentially using nanotubes in gene delivery systems, and SWNTs with covalently linked peptide functionalities were found to exhibit strong anti-peptide antibody responses in vivo. According to Gu, et al., a carbon nanotube could serve as a one-dimensional scaffold with covalently attached β-D-galactose molecules. These nanotube-bound galactose arrays were found to bind specifically with pathogenic Escherichia coli O157:H7 via ligand-receptor interactions, resulting in significant cell agglutination.

Similarly, carbon nanotubes may serve as scaffolds to express arrays of various proteins, especially antibodies, for immunoassays. Potential advantages of immuno-nanotubes may include the ability to carry a large number of immuno-functionalities per unit weight of the material while remaining stable in optically transparent solutions. The immuno-nanotube's one-dimensional structure and large aspect ratio may also allow effective targeting of cells via binding with multiple sites on the cell surface. In addition, we demonstrate here that immune-MWNTs with encapsulated ferromagnetic elements may be used for immunomagnetic separation (IMS) of pathogenic cells in pure and mixed cultures (Scheme 1), thus offering a potentially attractive alternative in the development of IMS materials and technology.

The MWNT sample produced by CVD (xylene precursor with ferrocene as catalyst) contained ferromagnetic catalyst residues (~5 wt%), both mixed with (or attached to) and encapsulated in the nanotubes. The purification to remove the attached iron species from the sample was performed properly under relatively mild conditions in order to prevent breaking the nanotubes and thus to preserve the encapsulation. A typical procedure was to reflux the as-prepared MWNT sample (100 mg) in aqueous HNO3 (14%, 50 mL) for 5 h.

The covalent functionalization of MWNTs with bovine serum albumin (BSA) protein has been reported. The resulting MWNT-BSA conjugates were soluble in aqueous buffer to form a colored but optically transparent solution. The solution in a test tube was placed in a magnetic separator (Dynal Biotech Model MPCM) for 30 min to isolate the magnetic fraction of the MWNT-BSA conjugate sample (yield ~70%). The conjugate sample was prepared typically containing 20–40 wt% MWNTs, as estimated in thermogravimetric analysis (TGA) and in modified Lowry assay for proteins. Figure 1 shows microscopy images for the MWNT-BSA conjugates. The TEM images suggest well-dispersed MWNTs of different lengths and diameters in the conjugate sample. In addition, imaging in the Z-contrast mode (making metals more visible) reveals

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clearly the encapsulation of metal elements in the nanotube structures (Fig. 1, top). High magnification TEM reveals this more clearly (Fig. 1, bottom-left). The atomic force microscopy (AFM) results are consistent with the conjugation of protein species with MWNTs, as reported previously.16,17

The magnetic conjugates (magnetic-MWNT-BSA) were dissolved in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) for the coating of goat anti-E. coli O157 antibody (Kirkegaard & Perry Laboratories). In a typical procedure, an antibody solution in PBS (10 μg/mL, 8.75 μL) was added to the magnetic-MWNT-BSA solution in PBS (10 μg/mL, 100 μL). After slow rotation (40 rpm) for 22 h at room temperature, the mixture was magnetically separated for 15 min to remove any free antibody (left in the clear supernatant), and the black-colored pellet was redissolved in PBS (100 μL). The separation process was repeated twice more to yield the final magnetic-MWNT-BSA-anti-E. coli O157 conjugate ("immunomagnetic-MWNT") in PBS. It should be noted that the amount of antibody used in the coating experiment was optimized on the basis of bicinchoninic acid (BCA) assay.16 The antibody content in the immunomagnetic-MWNT sample was on the order of a few percent. The mode of conjugation for the antibody is probably protein-protein interactions, though structural and mechanistic details remain to be explored in further investigations.16,17

The immunomagnetic-MWNT was used to capture E. coli O157:H7 in an aqueous solution for IMS. The E. coli cells were labeled with green fluorescent protein (GFP) with an ampicillin resistance selectable marker and then grown by following established procedures.19 The overnight cultures of E. coli O157:H7 were washed in repeats with sterile NaCl solution (0.85%) and then suspended and diluted in the same solution to obtain E. coli cultures of different concentrations. In a typical IMS experiment, a fresh E. coli culture (100 μL) was mixed with a PBS solution of immunomagnetic-MWNT (100 μL). Upon slow rotation (40 rpm) for 1 h at room temperature, the mixture in a test tube was placed in the magnetic separator for 5 min. The supernatant was carefully pipetted out, and the magnetic precipitate was resuspended in PBS for another round of the magnetic separation. After two repeats, the final magnetic fraction was used for microscopy analysis and quantitative colony enumeration.

For SEM imaging, the final fraction from IMS was resuspended in a glutaraldehyde solution (2.5% in 0.2 M sodium cacodylate HCl-buffer, 1 mL), fixed at room temperature for 30 min, and then passed through a membrane filter (0.2 μm, Whatman Nucleopore Polycarbonate). The sample on the filter was prepared into an SEM specimen by following established procedures.18 Typical SEM images of the specimen are shown in Figure 2. The captured E. coli cells are apparently surrounded by the nanotubes. This is consistent with the expected immunomagnetic-MWNT binding specifically with the pathogenic cells, followed by efficient magnetic separation.

The detection efficiency and sensitivity of immunomagnetic-MWNT toward E. coli O157:H7 in pure culture were evaluated by using the colony enumeration on the final fraction from IMS.17 For the evaluation, various initial bacterial counts and immunomagnetic-MWNT concentrations were used. In a typical experiment, an aliquot of
the bacterial culture (100 μL) was spread onto a Tryptic soy agar plate (TSA, supplemented with 100 μg/mL ampicillin), followed by incubation overnight at 37 °C. The enumeration, in duplicate, was based on the green colonies corresponding to the GFP-labeled E. coli strain. As shown in Table I, the immunomagnetic-MWNT was capable of capturing the pathogenic E. coli at a relatively low population (40 CFU/100 μL) in pure culture for the magnetic separation. The IMS results were little changed with use of different immunomagnetic-MWNT concentrations (from 10 to 50 μg/mL).

Table I. Detection of E. coli O157:H7 cells with immunomagnetic-MWNT.

<table>
<thead>
<tr>
<th>Bacterial concentration (CFU/100 μL)</th>
<th>Detected (CFU/100 μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>48</td>
<td>1</td>
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<td>170</td>
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</tr>
<tr>
<td>370</td>
<td>11</td>
</tr>
<tr>
<td>~800μL</td>
<td>35</td>
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</table>

*Mixed culture with S. Typhimurium (~3000 CFU/100 μL) as the background flora.

The IMS for the detection of E. coli cells in a mixed culture was also successful. In the presence of Salmonella Typhimurium DT104 cells (3000 CFU/100 μL) as the background flora, the immunomagnetic-MWNT was able to recover 10.6% of the initial E. coli cells (800 CFU/100 μL), as shown in Table I. There was no presence of any S. Typhimurium colony (white as opposed to the green E. coli colonies) in the enumeration, suggesting a high specificity in IMS with immunomagnetic-MWNT.

The results reported here suggest that immunomagnetic-MWNT may serve as an alternative IMS platform for rapid and highly sensitive detection of pathogens. Further investigations will include a systematic evaluation of the detection in more complex matrices and the simultaneous detection of multiple pathogens. In addition, preliminary studies have shown that similarly prepared immunomagnetic-SWNTs are not as effective as the immunomagnetic-MWNT used here, due primarily to decreased encapsulation of ferromagnetic elements in SWNTs and consequently poorer magnetic separation. Thus, an increase in the amount of magnetic elements in both SWNTs and MWNTs via post-production encapsulation will be pursued for more efficient and sensitive IMS of cells and other biological species.

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References and Notes


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