Interfacing Glycosylated Carbon-Nanotube-Network Devices with Living Cells to Detect Dynamic Secretion of Biomolecules

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Biosensors

Interfacing Glycosylated Carbon-Nanotube-Network Devices with Living Cells to Detect Dynamic Secretion of Biomolecules**

Herry Gunadi Sudibya, Jimei Ma, Xiaochen Dong, Simon Ng, Lain-Jong Li, Xue-Wei Liu, and Peng Chen**

Carbon nanotubes (CNTs) have stimulated much interest in their biological applications ever since their discovery because of their remarkable electrical, structural, and physiochemical properties. For instance, they have been employed as nanoelectronic biosensors with extraordinary sensitivity for the rapid detection of biomolecules,[1–3] as nanovectors to deliver macromolecules into cells,[4] and as nanofibrous scaffolds for tissue engineering.[5,6] Despite all these promising potentials, the non-ideal (or even cytotoxic) interface between CNTs and the living cells, however, largely limits the nanotube applications in biology.[7,8] Herein, we present a strategy to functionalize carbon nanotubes with bioactive monosaccharides and demonstrate that glycosylated CNT network devices are able to biocompatibly interface with living cells and detect the dynamic secretion of biomolecules from them.

To achieve biocompatible interactions between CNTs and living cells, hydrophobic nanotubes have been coated with positively charged polyelectrolytes, such as poly(ethylene)imine (PEI)[9] and poly-L-lysine (PLL),[10] to promote adhesion of the negatively charged cell membrane. Charged groups can also be imparted to nanotube surface by chemical functionalization.[11] Non-physiological, strong electrostatic interactions between the highly charged surface and the cell membrane, nevertheless, may lead to cell death or cytotoxic effects.[12] Moreover, the introduced surface charges and chemical modifications often alter the electrical properties of nanotubes, which is undesirable when nanotubes are used as electrical sensing elements. Alternatively, CNTs have been coated with adhesion proteins found in the extracellular matrix.[13] But, the thick gel-like layer formed by the adhesion proteins precludes the intimate contact between the nanotubes and the cells. And the charge groups on these macromolecules may also adversely influence the nanotube properties. Therefore, new strategies are needed to modify CNTs, thereby ensuring biocompatible interactions between CNTs and living systems and the preservation of the functionalities of both. Carbohydrates, a major component of the cell membrane, have recently been used to decorate CNTs to establish natural contact with living cells.[14,15] Carbohydrates, in the forms of glycans, proteoglycans, glycoproteins, or glycolipids, populate the surfaces of both eukaryotic and bacterial cells and are critically involved in a wide spectrum of biological processes, such as cell–cell communication and interaction, cell growth, immune responses, and signal transduction.[16,17] Particularly, carbohydrates are important in mediating cell adhesion process through carbohydrate–carbohydrate or carbohydrate–receptor (lectin) interactions.[17,18] Herein we report that fabricated thin-film networks of single-walled carbon nanotubes (SWNTs) were glycosylated with monosaccharides abundantly found in living systems, specifically, *N*-acetyl-D-glucosamine (GlcNAc), D-glucose (Glc), or D-mannose (Man). These sugar moieties are anchored onto nanotubes by either a pyrene or a lipid tail. The synthetic routes of (16-allyloxy) hexadecyl-2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (*N*-acetyl-D-glucosamine with lipid tail or GlcNAc–lipid for short) and 4-(1-pyrenyl)butyl 2-acetamido-2-deoxy-β-D-glucopyranoside (*N*-acetyl-D-glucosamine with pyrene tail, or GlcNAc–pyrene) are depicted in Scheme 1 (details in Supporting Information).

Ultra-thin networks comprising of small single-walled nanotube (SWNT) bundles (10–30 nm) were prepared through phase-separation facilitated self-assembly and drop-cast onto glass a coverslip as previously described.[19] An atomic force microscopy (AFM) image of such a network is presented in Figure 1 a. The SWNT-nets were functionalized by incubation with 5 mM glyco-conjugates equipped with either a lipid or pyrene tail for 24 h, followed by rinsing with deionized water to remove unbound molecules. The hydrophobic lipid tail anchors onto nanotubes by hydrophobic interactions, while the aromatic pyrene tail firmly attaches to the nanotube sidewall by π–π stacking interactions (Figure 1 b). The effectiveness of the glycosylation was visualized using fluorescein isothiocyanate (FITC) conjugated sugar binding proteins (lectins), specifically, FITC-conjugated Helix Pomatia Agglutinin (HPA) that selectively binds to GlcNAc and FITC-conjugated Concanavalin A (conA) that selectively binds to glucose and mannose. The functionalized SWNT-net was incubated with 5 mM FITC-conjugated lectin for 24 h, followed by rinsing to remove unattached protein and subsequent fluorescence imaging. As revealed by fluo-

[*] J. Ma,[+] S. Ng, Dr. X. Liu
School of Physical and Mathematical Sciences, Nanyang Technological University
21 Nanyang Drive, Singapore 637371 (Singapore)
E-mail: xuewei@ntu.edu.sg
H. G. Sudibya,[+] Dr. X. Dong, Dr. P. Chen
School of Chemical and Biomedical Engineering, Nanyang Technological University
70 Nanyang Drive, Singapore 637457 (Singapore)
E-mail: chenpeng@ntu.edu.sg
Dr. L. J. Li
School of Materials Science and Engineering, Nanyang Technological University (Singapore)
[+] These authors contributed equally.

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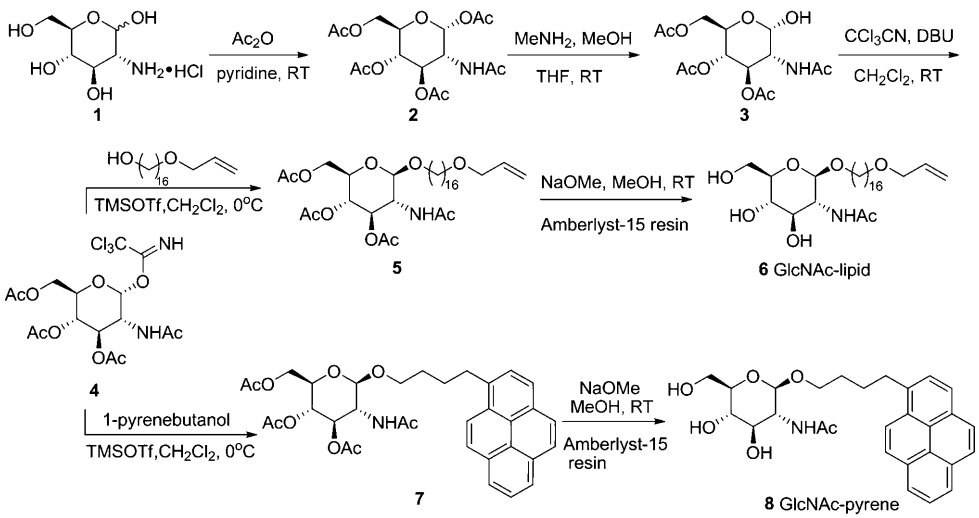

Scheme 1. Synthetic routes to GlcNAc–lipid and GlcNAc–pyrene. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene; TMSOTf = trimethylsilyl trifluoromethanesulfonate.

properties (Figure 1d). In comparison to single SWNT-based devices which are usually employed for biosensing with higher sensitivity, SWNT-net offers several advantages. They can be more readily fabricated and have lower 1/f (low frequency) noise that commonly plagues single-nanotube devices. The SWNT-net is especially advantageous to probe cellular activities, because it could serve as a cell-growth substrate and interface with cells through a large contact surface thus sensing area.

Bare nanotube substrates, however, are usually not suitable for cell growth. When PC12 cells were cultured on bare SWNT-nets, they abnormally aggregated into sparsely dispersed clumps (Figure 2a, left) and their growth was apparently impaired. For comparison, we examined growth of PC12 cells on SWNT-nets

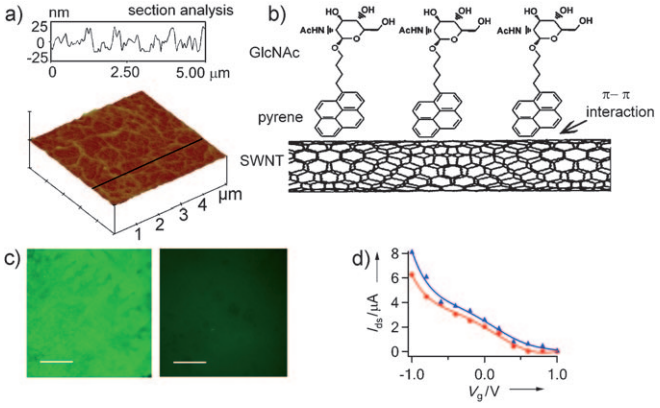

Figure 1. a) AFM image of a SWNT-net with a representative cross-section profile along the black line. b) Illustration of GlcNAc–pyrene functionalization of SWNTs. c) Fluorescence staining of GlcNAc-functionalized SWNT-net (left) and bare SWNT-net (right) by HPA-FITC. Scale bars = 500 µm. d) Electrical characterization of SWNT-net before (circles) and after (triangles) glycosylation of GlcNAc. Biasing at 400 mV, the source–drain current (I_{ds}) was measured while varying the gate voltage (V_g) applied to the bath solution by an Ag/AgCl electrode.

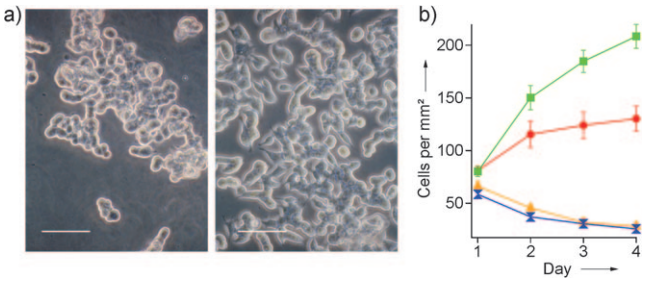

Figure 2. a) Phase-contrast images of PC12 cells cultured on a bare SWNT-net (left) and GlcNAc–SWNTs (right). Scale bars = 100 µm. b) Proliferation curves of PC12 cells grown on bare SWNT-net (red circles), or SWNT-net modified with GlcNAc (green squares), glucose (yellow triangles), or mannose (blue diamonds). Each data point is the average from 40 different areas (3 mm^2) in three different substrates. The error bars indicate the standard deviations.

rescence labeling, GlcNAc–lipid formed inhomogeneous patches on the SWNT-net (not shown) whereas GlcNAc–pyrene uniformly covered the SWNT-net (Figure 1c, left), indicating that pyrene is a better linker. The bare SWNT-net, in contrast, only showed background fluorescence (Figure 1c, right), because FITC–HPA was not able to bind nanotube substrate without the GlcNAc bait.

The conductance of the SWNT-net can be sensitivity modulated by the gate voltage applied to the solution that bathes the SWNTs. In a typical example shown in Figure 1d (circles), nanotube current dropped from 6.3 µA to 0.1 µA as the gate voltage varied from −1 to 1 V, manifesting *p*-type field-effect characteristics. It demonstrates that our SWNT-net device is highly responsive to the electrochemical perturbation in the solution, therefore desirable for electrical biosensing. Glycosylation does not compromise the nanotube noncovalently functionalized with glucosamine, glucose (Glc) or mannose (Man), which are common sugar residues of membrane glyco-conjugates. In particular, glucosamine is a major constitute of many proteoglycans which are important cell adhesion molecules found on both cell membrane and extracellular matrix.[20] PC12 cells have abundant proteoglycans, especially heparin sulfate that consists of a core protein with glycosaminoglycan branches polymerized from *N*-acetylglucosamine and glucuronic acid.[21] As anticipated, GlcNAc–pyrene coated SWNT-nets, in contrast to bare SWNT-nets, supported normal confluent growth of PC12 cells (Figure 2a, right), suggesting the facilitating role of glucosamine on the adhesion of PC12 cells. Noncovalent interactions between similar glycans can provide a strong adhesion force (ca. 250 pN) comparable to that of protein–

protein interaction.[22] Therefore, it is plausible that a nanotube displaying many glucosamine residues can strongly interact with glycosaminoglycans on the PC12 cell membrane. In addition, glucosamine-specific lectins on the cell membrane can bind with glucosamine on the nanotube and promote cell adhesion as well.[23] In comparison to GlcNAc–pyrene, Glc–pyrene and Man–pyrene appear to seriously inhibit cell adhesion and thus cell growth. Proliferation curves of cells cultured on bare SWNT-net or SWNT-net functionalized with different sugar moieties are shown in Figure 2b. These experiments were conducted in parallel and PC12 cells of the same initial density were introduced onto the respective substrates. As seen, PC12 cells grown on GlcNAc-functionalized SWNT-nets can proliferate well, as shown by a 160.0% increase in cell density after three days. On the other hand, proliferation of PC12 cells was largely inhibited on bare SWNT-nets. In this case, the cell density only increased by 60.9% after three days. On both glucose and mannose coated nanotubes, PC12 cells cannot adhere well and their density decreased by 57.5% and 56.1%, respectively.

As demonstrated, appropriate glycosylation enables biocompatible interfacing between SWNT-net and living cells without sacrificing SWNT functionalities. Hence, glycosylated SWNT-net provides a novel platform to probe dynamic cellular activities, for example, to continuously monitor biomolecular release from living cells. Carbon nanotube devices have already been utilized as nanoelectronic biosensors to detect various biomolecules.[1–3] But these realizations are essentially limited to static in vitro measurements.

PC12 cells are a popular cell model to study regulated secretion (or exocytosis), a fundamental and dynamic biological process underlying hormone secretion from endocrine cells and neurotransmitter secretion from neurons. As illustrated in Figure 3a, membrane depolarization (increase in intracellular potential) resulting from a physiological stimulation opens voltage-gated Ca^{2+} ion channels. The influx of Ca^{2+} ions through these channels, in turn, triggers the actions of several sets of proteins which drive immobilization and final fusion of large dense core secretory vesicles. Upon vesicle fusion with the plasma membrane, catecholamine molecules (dopamine, norepinephrine, and epinephrine) inside the vesicle are discharged into the extracellular space. Once catecholamines are released into the narrow interface gap between the cell and the SWNT-net, they quickly diffuse onto the nanotubes and intimately interact with them by π–π stacking between the aromatic ring of the catecholamine molecule and the nanotube sidewall. As the current flows solely at the surface of the nanotube, the conductance of nanotube is highly sensitive to the electrochemical perturbations at the surface induced by the interacting molecules. So, it is possible that triggered catecholamine release from PC12 cells can be reported by changes in nanotube current.

Indeed, when high potassium (K^+) solution, which triggers exocytosis by membrane depolarization, was introduced to PC12 cells cultured on top of a glycosylated SWNT-net biased at 400 mV, a train of current spikes was detected (Figure 3b). When another secretagogue, calcymycin (1 mM) which inserts into cell membrane and forms Ca^{2+} selective ion channels, was

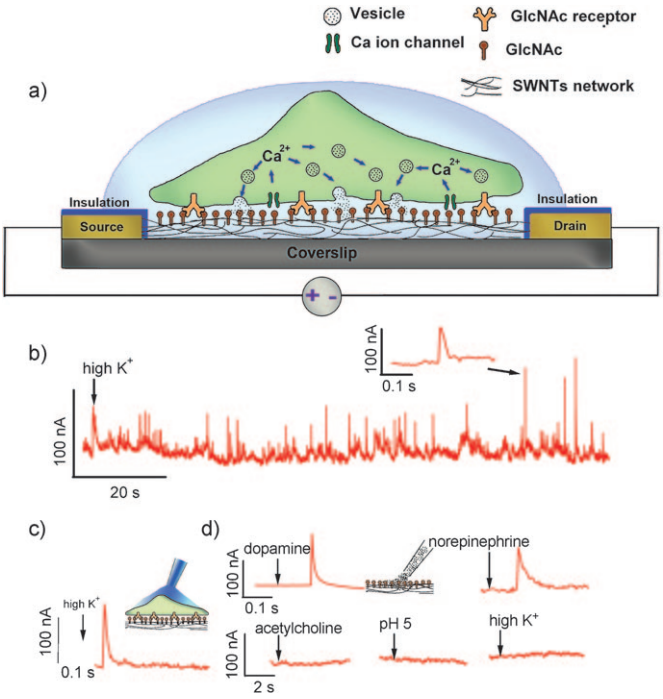

Figure 3. a) Triggered exocytosis and SWNT-net detection. b) Nanotube responses to exocytosis of PC12 cells triggered by high K^+ stimulation. The SWNT-net was biased at $V_{ds} = 0.4$ V. c) Stimulation of single PC12 cell through micropippete perfusion of high K^+ solution. d) Transient perfusion of 1 mM dopamine or norepinephrine on glycosylated SWNTs-net results in current spikes, while perfusion of acetylcholine, acidic solution (pH 5.0), and high K^+ did not cause appreciable responses. The arrows in (c) and (d), roughly indicate where the stimulations were applied.

applied, similar current spikes were elicited (Supporting Information). We hypothesize that each spike corresponds to Ca^{2+} dependent exocytosis of catecholamines from single PC12 cells. To test this hypothesis, we used an application pipette (tip size of ca. 1 μm) positioned approximately 1 μm away from the target cell to locally deliver high K^+ solution or calcymycin, which exclusively stimulates this one cell. A single current spike, with similar amplitude and time scale as observed in Figure 3b, was generated by such local stimulation (Figure 3c and Supporting Information), suggesting that each recorded spike in Figure 3b is due to single-cell activity. Furthermore, when a drop of bath solution containing 1 mM dopamine or norepinephrine was delivered onto a cell-free and 400 mV-biased glycosylated SWNT-net by a micropipette, a current spike was also induced (Figure 3d), indicating that local discharge of catecholamines can cause a transient increase in the nanotube current like those observed in the experiment depicted in Figure 3b. High K^+ solution and calcymycin did not produce any signals when added to the glycosylated SWNTs-net in the absence of cells (Figure 3d and Supporting Information). It is arguable that the observed spike may be due to the acidic vesicular fluids (ca. pH 5.5). This possibility was ruled out by the observation that nanotubes were not responsive to the micropipette application of bath solution with low pH (5.0) (Figure 3d). Upon Ca^{2+} triggering, PC12 cells also release acetylcholine from small

synaptic-like vesicles. But as shown in Figure 3d, acetylcholine was not able to modulate nanotube current. Moreover, it is not likely that the recorded transient signals originate from constitutively secreted molecules (e.g., metabolites) from the cells because, in comparison to triggered rapid exocytosis, the constitutive secretion is a slow and constant process. The spiky nanotube responses were only observed after application of the secretagogues which lead to Ca^{2+} dependent exocytosis.

Exocytosis of biomolecules are conventionally analyzed by biochemical assays which, however, only offer averaged ensemble measurements from a population of cells with low temporal resolution and sensitivity. Although the state-of-the-art electrophysiological methods, such as membrane capacitance measurement and carbon microfiber amperometry, provide high sensitivity and temporal resolution, they require highly skilled operators, specialized equipment and are very low throughput techniques. In addition, membrane capacitance measurement is invasive and carbon fiber microelectrodes (ca. 5 μm in diameter) can only electrochemically detect the release of electroactive molecules from approximately 5% of the total area of the cell surface. Comparing to these methods, our nanotube approach provides a novel alternative to dynamically detect exocytosis from individual cells.

In summary, we demonstrate that noncovalent functionalization with bioactive sugar moieties confers biocompatibility to carbon nanotubes without compromising their sensing capabilities. Glycosylated SWNT-net can directly interface with living cells by supporting their adhesion and growth, and detect dynamic biomolecular release from these cells. Compared to current methods to detect exocytosis, our nanotube approach provides real-time and non-invasive measurements from living cells with high sensitivity, high temporal resolution, high throughput, and ease of detection. The study represents another example of how nanotechnology and nanomaterials can bring new opportunities to biology.[24,25]

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