Carbon Nanotubes for the Label-Free Detection of Biomarkers

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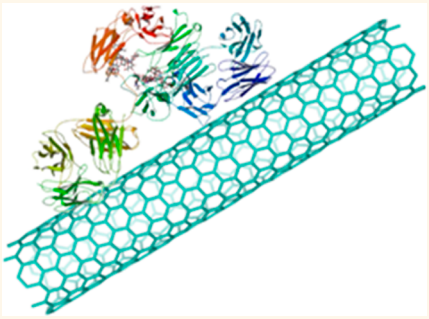
ABSTRACT Carbon nanotubes (CNTs) have been of high interest because of their potential to complement or to replace current biomedical sensor and assay techniques. By taking advantage of their unique electrical and optical properties, CNTs can be integrated into highly sensitive sensors and probes. We highlight recent advances toward applying CNTs to the biomedical field, focusing on a report by Reuel *et al.* in this issue of *ACS Nano*, wherein the inherent near-infrared (NIR) fluorescence of functionalized arrays of single-walled carbon nanotubes (SWNTs) is utilized for detection of several important biological markers.



Next-generation medical diagnostics demand novel biosensors and biological assays that can fulfill the requirements of high-throughput, cost-effective, and ultrasensitive alternatives to conventional methods. As a consequence of the discovery of nanomaterials and the feasibility of nano- and microfabrication, tremendous efforts have been made to develop platforms for highly efficient screening of biomarkers. These efforts have been focused on two approaches: substituting/complementing existing biomedical assays and engineering new tools that can give additional insight into biological and biophysical phenomena. In this Perspective, we will show that the inherent electronic, structural, and optical properties of carbon nanotubes offer the potential to create advanced biophysical tools that can compete with existing biomedical techniques and review recent research toward this goal.

Electronic Transducers. The most prominent biosensing approach based on carbon nanotubes (CNTs) is integration of this nanomaterial into chemiresistors and field-effect transistors (FETs). These devices can be composed of either a single, semiconducting SWNT or a network of SWNTs connecting the metallic source and drain electrodes. While the former has potential applications in single-molecule detection and monitoring subtle biological interactions, the process is time-consuming and requires demanding fabrication steps such as growing the CNTs on chip using chemical vapor deposition and subsequently employing lithography.[1] The latter, however, offers a cost-effective, simple production methodology. In Figure 1a, a common architecture for liquid-gated SWNT FET biosensors is depicted. This particular device is gated by a Ag/AgCl reference electrode directly immersed in the liquid dielectric. The biosensing merit of SWNTs lies in their one-dimensional shape combined with the fact that charge transport through a device can only take place at the nanotube surface. Therefore, even slight alterations in the environment surrounding the CNT surface will lead to electrical signals. Such alterations may result from charged moieties on the surfaces of proteins, or by a number of other mechanisms.[2] As a consequence, functionalization of nanotubes is a fundamental prerequisite for sensing applications. Ten years ago, research groups introduced SWNT FETs as highly specific and sensitive instruments to detect the presence of proteins.[3,4] Starting from this point, functionalized SWNT FETs have been established as robust biosensors for medical diagnostics and understanding the physical concepts behind different sensing mechanisms of these devices. While we highlight some recent examples, we refer to a review for more detailed discussions of SWNT FET-based biosensing.[5]

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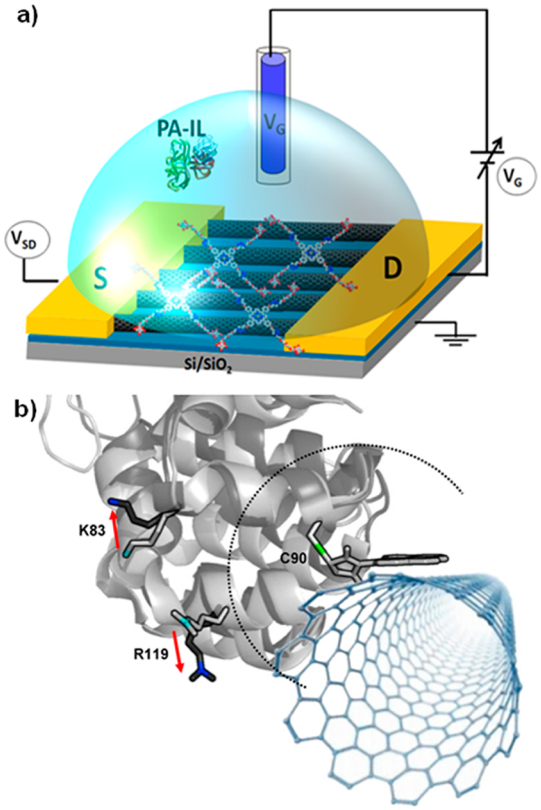

Figure 1. (a) Schematic illustration of single-walled carbon nanotube (SWNT) field-effect transistor device functionalized with glycosylated porphyrins for selective detection of bacterial lectins (PA-IL). Aligned SWNTs connect source and drain electrodes, and a Ag/AgCl reference electrode directly immersed in the buffer solution gates the transistor. Reproduced from ref 6. Copyright 2011 American Chemical Society. (b) Depiction of a single lysozyme attached to a SWNT *via* a maleimide-pyrene linker. The catalytic activity of the enzyme can be probed due to the motion of the charges at residues 83 and 119. Reproduced from ref 8. Copyright 2013 American Chemical Society.

In this Perspective, we will show that the inherent electronic, structural, and optical properties of carbon nanotubes offer the potential to create advanced biophysical tools that can compete with existing biomedical techniques and review recent research toward this goal.

Recently, the Star research group reported a sensor specific to certain bacterial lectins.[6] An AC dielectrophoresis (DEP) approach, which could also be applied on the wafer scale, was used to fabricate the CNT devices. The devices demonstrated excellent behavior in terms of sensitivity, selectivity, and robustness. Specific detection of bacterial lectins was enabled by porphyrin-based glycoconjugates, synthesized using azide–alkyne "click" chemistry, immobilized on the CNTs. A detection limit of about 2 nM lectin concentration was observed, and interactions between lectins and glycoconjugates could be probed by extracting the dissociation constant (K_D). This method can compete with standard techniques such as isothermal titration calorimetry (ITC), electrochemical surface plasmon resonance (E-SPR), and electrochemical impedance spectroscopy (EIS).

Not only do nanotube devices have the potential to be integrated into standard biomedical assays, but they may also enable gaining additional scientific insight into biological processes. For example, understanding enzymatic activities is of fundamental importance as they are involved in numerous physiological tasks and thus are connected to many genetic diseases. Enzyme catalysis is accompanied by conformational changes of specific domains of the protein. Such motions, which occur at different rates, are commonly observed by fluorescent techniques such as Förster Resonance Energy Transfer (FRET).[7] One of the major drawbacks of FRET, however, is bleaching and quenching of the fluorophore labels attached to certain parts of the enzyme. The fluorophore itself also must be conjugated to the target enzyme, which can cause unwanted effects such as steric hindrance and conformational changes.

The Collins research group overcame the aforementioned limitations of FRET by using a label-free electronic method, namely, a FET employing a single semiconducting SWNT bridging source and drain electrodes.[1] A noncovalent functionalization scheme using a pyrene–maleimide linker was utilized to immobilize a single lysozyme on the surface of the carbon nanotube (Figure 1b). Consequently, the dynamics of this lysozyme were monitored for several minutes, which had not previously been achieved by any other method. Notably, the Collins group observed that by introducing peptidoglycan (a polysaccharide found in bacterial cell walls), 100 glycosidic bonds were progressively hydrolyzed by the lysozyme at 15 Hz, followed by nonproductive hinge bending at a rate of 330 Hz. Considering that most of the surface charges of this molecule are screened by the electrical double layer (EDL), these rates are solely probed due to the 1 to 2 Å motion of

two positively charged residues taking place at a distance of about 1−1.2 nm from the nanotube surface. Furthermore, they were able to probe the catalytic activity of lysozymes at different pH conditions leading to a set of independent parameters characterizing that system, such as the mean time the enzyme resides in each of its physical states and the separation energy between those states.

While great strides have been made using CNTs as electronic biosensors, electronic detection of protein−protein interactions and direct examination of interior cell components remain demanding.

In another recent publication, the Collins group used engineered S90C lysozyme variants containing differently charged residues, such as negatively charged glutamates and neutral alanines, to ascertain the net charge of the dominant (*i.e.*, closest to the CNT surface) sites of the lysozyme. This was accomplished by analyzing the effective change in gate voltage from the dynamic signals during catalytic activity of the lysozyme variants.[8] In other words, depending on the net charge of a residue that is moving relatively close to the CNT surface, either a positive or a negative shift in the effective gate voltage is observed, which leads to quantitative conclusions about the electronic landscape on the surface of the enzyme. In general, their findings represent an innovative way to monitor enzyme activity and dynamics. Because of the simplicity of the underlying physical mechanisms, this process can be applied to a vast number of proteins and enzymes without restricting their catalytic activity.

While significant advances have been made toward the applications of CNTs to the field, medical diagnostics require high-throughput methods for realistic applications. For instance, Heath and co-workers have successfully exploited the Zweifach-Fung effect to separate blood cells from whole blood and direct small volumes of protein-rich plasma to an array of sensors.[9] Future techniques involving SWNT electronic devices may rely on microarray chips integrated with microfluidics to enable the specific detection of bacteria, proteins, or other biomarkers in crude samples including soil, water, or animal/human specimens. While requiring only minimal sample volume, these SWNT-based point-of-care (POC) diagnostic systems will demonstrate rapid, specific detection.

Intracellular Probes. In addition to the detection of small biomolecules such as proteins and enzymes, one-dimensional semiconductors have emerged as attractive probes for living cells. Two approaches for accomplishing this in living cells have become apparent: intracellular interrogation and extracellular recording of cell activities.[10] Examining intracellular activities with one-terminal devices entails a balancing act between the small probe size and impedance limitations that can cause low temporal resolution and small signal-to-noise ratios.

The Lieber group addressed this issue by fabricating silicon nanowire (SiNW) three-terminal devices that are not limited by impedance; therefore, this platform can be downscaled to probe living cells in a nondestructive manner.[10] The SiNW probes and FET devices were constructed utilizing multistep processes, yielding a SiO_2 nanotube-tipped SiNW FET device capable of puncturing a cell and taking electrical measurements. Because of the small size of the tapered tip (∼55 nm outer diameter) and phospholipid functionalization, cells readily internalized the nanotube tip without significant interruption of cellular processes. By monitoring conductance, the intracellular action potential of embryonic chicken cardiomyocyte cells was observed. Later, the group also developed a variety of kinked nanowire structure (KNW) devices consisting of different morphologies toward the goal of creating highly compact and multiplexed three-dimensional probes for biomedical applications.[11]

In this context, the applicability of multiwalled carbon nanotubes (MWNTs) for intracellular probing was demonstrated by Singhal *et al.*[12] Multiwalled carbon nanotubes with diameters ranging from 50−200 nm and lengths of 50−60 μm were mounted onto a glass pipet tip in order to penetrate cell membranes in a minimally invasive approach. Additionally, attoliter fluid handling, intracellular surface-enhanced Raman spectroscopy (SERS), and the potential for submicrometer tip manipulation were demonstrated with the same device. Similarly, high aspect ratio CNT probes (1 mm length and 5−10 μm diameter) were developed to address the need for *in vivo* neural recordings;[13] however, devices based on SWNTs for intracellular recordings have not yet been sufficiently explored.

Living cell studies in combination with carbon nanotubes are also found in platform technologies such as SWNT FETs and optical array sensors. Huang *et al.* showed that ATP (negatively charged) release from living astrocytes can be monitored online upon the addition of glutamate.[14] Another promising report demonstrated that membrane proteins can be separately probed when embedded in a supported lipid membrane.[15] In this case, the activity of the protein ion pump Na^+/K^+-ATPase was studied. In short, SWNT FET devices are useful for tracking changes in external cell media and isolated proteins, but intracellular studies in live specimens remain challenging.

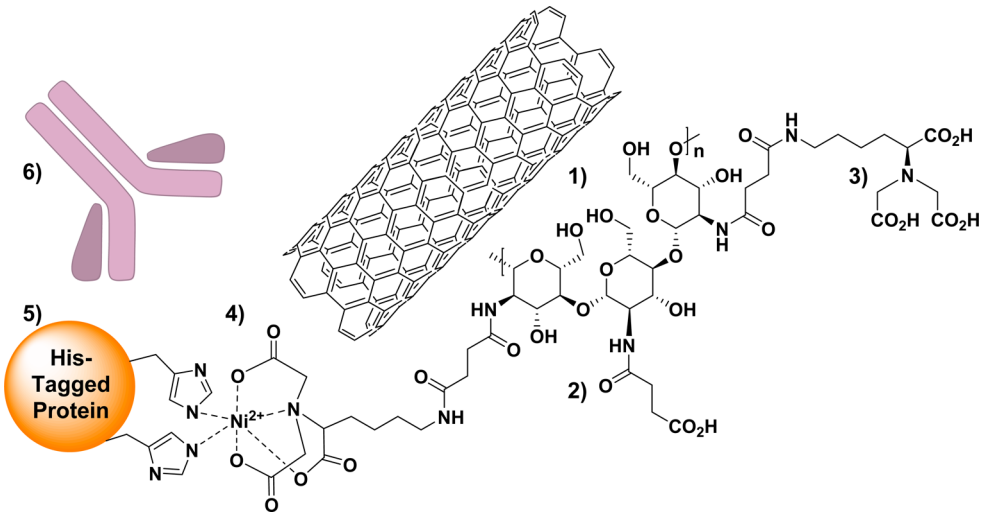

Figure 2. Functionalization scheme for creating a single-walled carbon nanotube-based sensor array. (1) Nanotubes are wrapped with chitosan. (2) Chitosan is reacted with succinic anhydride. (3) Nitrilotriacetic acid (NTA) groups are attached using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/*N*-hydroxysuccinimide (NHS) coupling to the resulting carboxylic acid groups. (4) Ni^{2+} is chelated to NTA group. (5) His-tagged protein is attached to Ni^{2+}. (6) Protein−protein interactions lead to Ni^{2+}-displacement relative to the carbon nanotube, causing a change in near-infrared fluorescence. Adapted from ref 18. Copyright 2011 American Chemical Society.

In this issue of *ACS Nano*, the Strano group emphasized the benefit of nanosensor arrays not only to demonstrate their obvious potential to be used as multiplexing sensors for a large number of analytes, but also to gain stochastic insight into affinity distributions of proteins.

Optical Arrays. While great strides have been made using CNTs as electronic biosensors, electronic detection of protein−protein interactions and direct examination of interior cell components remain demanding. For example, EDL formation at the solid−electrolyte interface results in electrostatic screening of charged moieties outside of the Debye screening length (relative to the nanotube surface) from the semiconducting channel of CNTs, leading to a significant decrease in transistor response. The Strano research group circumvents these issues by exploiting the near-infrared (NIR) fluorescent optical properties of SWNTs. Employing a general, adaptable scheme, they developed CNT array-based sensors for a broad spectrum of applications. The key feature of this approach is the noncovalent functionalization of fluorescent CNTs with chitosan, a biocompatible polymer with pendant nitrilotriacetic acid (NTA) groups for chelating nickel. After Ni^{2+}-loading, NTA-Ni^{2+} complexes bind to histidine-tagged (His-tagged) proteins. This allows for a library of His-tagged proteins to be attached to the sidewalls of the nanotubes (Figure 2). Interestingly, the role of nickel ions is to act not only as a specific binding site for His-tagged proteins, but also as a proximity quencher and thus as the biosensor's transducer. Operating similarly to FRET, the relative distance between the CNT and Ni^{2+} is altered upon interaction of analyte molecules with the His-tagged target, leading to responses in the NIR signal that can be directly correlated to adsorption or desorption of the analyte proteins.[16] Moreover, this functionalization approach permits the subsequent elution of the His-tagged protein upon exposure to imidazole, which has a similar chemical structure to His-tag and thus acts as a competitive binder. The group proposed several applications for this type of microarray sensor. For instance, they were able to tailor their sensor to the needs of the fast-developing field of glycobiology. Herein, the strong pharmacological interest concerning glycan profiling and monitoring interactions between glycans and carbohydrate recognition domains is worth noting. Especially for drug production lines, sensors would be required that monitor different production steps online without prior labeling of the proteins or liberation of glycans from the proteins polypeptide side-chains.[17] In this context, His-tagged lectins were attached to the nanotubes, which enabled the detection of glycans as well as glycoproteins.[18] Binding kinetics of these systems were monitored, leading to the quantitative determination of binding constants and outperforming conventional surface plasmon resonance (SPR) sensors in required sample volume and assay time. In this issue of *ACS Nano*, the

Strano group emphasized the benefit of nanosensor arrays not only to demonstrate their obvious potential to be used as multiplexing sensors for a large number of analytes, but also to gain stochastic insight into affinity distributions of proteins. For this purpose, a His-tagged protein A was immobilized on all sensors of the array (more than 10 000) for specific interaction with three different variants of immunoglobulin G (IgG): commercial lyophilized IgG, murine IgG, and human IgG from CHO cell lines.[19] A stochastic analysis of the luminescent response yielded K_D distributions that correlated with values for IgG-protein A interactions obtained from the literature. Furthermore, utilizing a stochastic method of analysis also paves the way for monitoring weak affinities. For example, such an approach was demonstrated through a His-tagged, mannose-specific plant lectin employed to monitor hypermannosylation in cell culture supernatant. Additionally, this process was also applied to the field of biomanufacturing by studying localized production of IgG in cultures growing on the array, potentially significantly improving throughput of colony selection. Finally, this technique moves further toward exploiting nanotubes as optical transducers in living organisms, as NIR fluorescent SWNTs emit in a range that has low tissue absorbance.

Carbon nanotubes are on their way to being established as a versatile component in next-generation biomedical assays.

Outlook and Future Challenges. Herein, we have reported that carbon nanotubes are on their way to being established as a versatile component in next-generation biomedical assays. They can be utilized as high-end biophysical tools to reveal subtle dynamics of single biomolecules and can compete with common biological assays. For glycoprofiling, liquid chromatographic techniques (LC, for compound separation) are typically used in combination with mass spectrometry (MS, for detailed structural analyses). However, LC/MS requires relatively large sample volumes, is time-consuming, and glycan liberation from the protein is needed.[16] Other traditional techniques rely on labeling with fluorescently active molecules (ELISA, resazurin-reduction assay), complex and expensive equipment, and are severely limited by photobleaching. In contrast, as the Strano group demonstrates, SWNTs fluoresce in the NIR, are not susceptible to photobleaching, and samples can be analyzed in a matter of minutes. Carbon nanotube array-based platforms could outperform conventional methods in terms of label-free and rapid, online monitoring of biological interactions without suffering from a loss in simplicity and sensitivity.

While recent progress toward advancing biomedical techniques with CNTs has been significant, attention still must be paid to several areas. First, electronic sensors built on nanotube structures are highly susceptible to Debye screening, and therefore, the surrounding media and size of functionalization molecules are vitally important. Second, further work must be directed toward adapting these sensors for applications in complex media while retaining selectivity and sensitivity. In contrast, optical arrays have the ability to overcome many of the shortcomings of electronic measurements, and further development of these platforms will facilitate the development of many noninvasive, cost-effective POC techniques that current, standard techniques are unable to address fully.

Conflict of Interest: The authors declare no competing financial interest.

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