Applications: Biosensors

10.1 Introduction ....................................................................... 237

Biomolecules such as nucleic acids and proteins carry important information of biological processes. The ability to measure extremely small amounts of specific biomarkers at molecular levels is highly desirable in biomedical research and healthcare. However, it is very challenging to find practical solutions to meet these needs. Current technologies rely on well-equipped central laboratories for molecular diagnosis, which is expensive and time consuming, often causing delay in medical treatments. There is a strong need for smaller, faster, cheaper, and simpler biosensors for molecular analysis [1]. The recent advancement in carbon nanotube (CNT) nanotechnologies has shown great potential in providing viable solutions. CNTs with well-defined nanoscale dimension and unique molecular structure can be used as bridges linking biomolecules to macro/micro- solid-state devices so that the information of bioevents can be transduced into measurable signals. Exciting new biosensing concepts and devices with extremely high sensitivities have been demonstrated using CNTs [2].

As the size of the materials reach the nanometer regime, approaching the size of biomolecules, they directly interact with individual biomolecules, in contrast to conventional macro- and micro-devices, which deal with assembly of relatively large amount of samples. Nanomaterials exhibit novel electronic, optical, and mechanical properties inherent with the nanoscale dimension. Such properties are more sensitive to the environment and target molecules in the samples. Although a big portion of nanomaterials are isotropic nanoparticles or thin films, high-aspect ratio one-dimensional nanomaterials such as CNTs and various inorganic nanowires (NWs) are more attractive as building blocks for device fabrication. The potential of CNTs and NWs as sensing elements and tools for biomolecular analysis as well as sensors for gases and small molecules have been recently recognized [3]. Promising results in improving sensitivity, lowering detection limit, reducing sample amount, and increasing detection speed have been reported using such nanosensors [4–6]. CNTs integrated with biological functionalities are expected to have great potential in future biomedical applications. This chapter summarizes the recent progress in the development of biological sensors using CNTs and highlights the potential future directions.

As described in previous chapters, CNTs are unique one-dimensional quantum wires with extremely high surface-to-volume ratio. As a result, their electronic properties are very sensitive to molecular adsorption. Particularly, in a semiconducting single-walled CNT (SWNT), all carbon atoms are exposed
at the surface so that a small partial charge induced by chemisorption of gas molecules is enough to deplete the local charge carrier and cause dramatic conductance change [4,7–10]. Because biomolecules typically carry many ions, they are expected to affect CNT sensing elements and transducers more dramatically than simple gases and small molecules [11,12]. Sensing devices have been fabricated for various applications using single CNTs [13–15], single semiconducting SWNT field-effect-transistors (FETs) [4,11,12,16], vertically aligned nanoelectrode arrays [6,17,18], and random networks or arrays [9,19–21]. Many studies have also reported complex nanostructure based on the hybrid bio-/nano-systems. These materials are heterogeneous assembly of biological molecules with solid-state nanomaterial, many of which use CNTs as templates for biomolecule assembly [22,23] or as conducting wires connecting biomolecules [24]. Such hybrid approaches have demonstrated the potential to combine the biorecognition functionalities with desired solid-state electronic properties in self-assembly of heterogeneous nano- and biosystems for biological sensing.

10.2 Fabrication of Carbon Nanotube Biosensors

10.2.1 CNT Growth

Even though much progress on CNT growth has been made in the past decade, it is still challenging to produce CNTs with desired properties for specific applications. Particularly, new methods are desired that can be directly integrated into device fabrication. High-temperature techniques such as electrical arc-discharge and laser ablation produce CNTs with the highest quality in terms of the graphitic structure. These techniques yield nanotubes along with other carbonaceous materials and metal catalysts. Individual CNTs were picked out of this mixture and placed on solid surfaces to fabricate devices such as FETs [25–27]. However, these techniques are slow and expensive, not applicable for mass-production of devices. Chemical vapor deposition (CVD) has provided a solution for the direct growth of CNTs in device fabrication at much lower temperature (about 850 to 1000°C). Wafer-scale SWNT FET arrays have been fabricated using CVD [28,29]. Incorporation of an electrical field was found to provide additional control of CNT growth along desired horizontal directions [30].

Plasma-enhanced CVD (PECVD), with the advantage of compatibility with semiconductor processing, has also attracted extensive attention in CNT growth for device fabrication [31–34]. A single CNT or an array of CNTs can be grown at sites defined by lithographic techniques down to tens of nanometers [35,36]. The alignment can be precisely controlled by an electrical field normal to the substrate surface. However, CNTs grown by PECVD are defective multiwalled nanotubes (MWNTs), or more appropriately referred to as multiwalled nanofibers (MWNFs) [37,38], (see Chapter 4) with the graphitic layers not perfectly parallel to the tube axis. Many bamboo-like closed shells are formed along the tube axis instead of presenting well-defined hollow channels running from one end all the way to the other end [32–34,36–38]. Such nanostructures, although not ideal, were found to be sufficient for many sensor applications [6,17,18]. With continued effort in the development of growth techniques, it is expected that CNTs with desired properties, quality, and quantity could be obtained for various applications.

10.2.2 Device Integration

An essential task for device fabrication is to integrate nanoscale CNTs into functional devices that can pass information to the macroscopic world. Lithography-based micro- and nanofabrication techniques provide methods to fill this gap. Figure 10.1 summarizes four types of most commonly used biological sensing devices based on CNTs. Depending on the sensing applications, different device architectures and fabrication routes are required to successfully achieve the desired functions. Common to all, CNTs are the critical components of the sensing devices, which are integrated either directly or indirectly during the fabrication route. A variety of methods, ranging from advanced micro- or nanolithographic techniques to manual placing, has been used to date. Generally, CNTs are either the sensing elements whose
properties are changed upon specific biological events, or the transducers reduce that transfer the signal to the measuring units. The sensing device could use either a single CNT or an ensemble of CNTs.

The most straightforward biological sensing employs a single CNT to probe the biochemical environment in a single living cell or interrogate a single biomolecule (as shown in Figure 10.1A). The CNT probe can be attached to the pulled tip of an electrode [39] for electrical, electrochemical, and electrophysiology measurements. Normally, for such applications, the sidewalls of the CNT probe have to be shielded or insulated to reduce the background so that the small signal from the very end of the probe can be detected. The CNT probes provide the best spatial resolution as well as ultrahigh sensitivity and faster measuring speed than do conventional probes. The small size, high aspect ratio, and mechanical robustness of CNTs are also employed as the physical probe for high-resolution scanning-probe microscopy (SPM) as discussed in Chapter 6. This is a powerful technique that illustrates the structure of single molecules such as DNA and proteins with the resolution down to a few nanometers.

A single semiconducting CNT can be used to construct an FET as shown in Figure 10.1B using lithographic techniques. Such a device consists of a semiconducting CNT connected to two contact electrodes (source and drain) on an oxide-covered Si substrate, which could serve as the gate electrode [4,25–27]. Conventional FETs fabricated with semiconductors such as Si have been configured as sensors by modifying the gate oxide (without the gate electrode) with molecular receptors or ion-selective membranes for the analytes of interest [40]. The binding or adsorption of charged species could produce an electric field, which depletes or accumulates carriers within the semiconducting material similar to that by the gate potential. As a result, the conductance between the source and the drain electrodes is dramatically changed. Chemical sensors based on such a mechanism are referred to as chemical field-effect-transistors (ChemFET), which have been widely used in many applications [40].

The drawback of FET is the high cost of fabrication. Recent studies have demonstrated that random networks of SWNTs between two microelectrodes can behave as a thin film transistor and may be used as sensors [21]. Similar random CNT networks [34,41–43] and vertical CNT arrays [19,20] on metal electrodes can also be used as biosensors as shown in Figure 10.1C. The large surface-to-volume ratio and good electrical conductance along the tube axis make CNTs attractive for enzyme-based EC sensors. The highly porous CNT networks and arrays serve both as large immobilization matrices and as mediators to improve the electron transfer between the active enzyme site and the EC transducer. Improved electrochemical behavior of NADH [43], neurotransmitters [9], and enzymes [42] has been reported.

The fourth type of CNT device is also an EC sensor based on an array of vertically aligned CNTs embedded in SiO$_2$ matrices as schematically shown in Figure 10.1D. The EC signal is characteristic of the reduction/oxidation (redox) reaction of the analytes instead of nonspecific charges sensed by FETs, resulting in high specificity comparable with fluorescence-based optical techniques that are commonly used in today’s biology research. In addition, a high degree of miniaturization and multiplex detection can be realized for molecular diagnosis using an individually addressed microelectrode array integrated with microelectronics and microfluidics systems [1,44–46], which has advantages over optical techniques,
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particularly for field applications requiring quick and simple measurements. However, the sensitivity of EC techniques using traditional macro- and microelectrodes is orders of magnitude lower than laser-based fluorescence techniques, limiting their applications. Nanoscale-sensing elements such as CNT nanoelectrode arrays have been actively pursued to seek solutions for improving sensitivity of EC techniques.

The performance of electrodes with respect to temporal and spatial resolution is known to scale inversely with the electrode radius [47–49]. It is of interest in biosensing to reduce the radius of electrodes to 10 to 100 nm, approaching the size of biomolecules. It has been demonstrated that an MWNT array, with an average diameter of ~30 to 100 nm, can be integrated into a nanoelectrode array for ultrasensitive chemical and DNA detection [6,17,18]. The nanoelectrode array is fabricated with a bottom-up scheme resulting in a precisely positioned and well-aligned MWNT array embedded in a planarized SiO2 matrix [36,50]. MWNT arrays are first grown on metal films deposited on a Si surface using PECVD and then subjected to tetraethyloxysilicate (TEOS) CVD for gap-filling with SiO2. The excess SiO2 can be subsequently removed allowing exposure of the MWNT tips via a mechanical polishing step. The open ends of MWNTs exposed at the dielectric surface act as nanoelectrodes. Figure 10.2 shows scanning electron microscopy (SEM) images of a CNT array grown on 3 × 3 multiplex microelectrodes. Each microelectrode is about 200 × 200 μm2 and individually addressable. The location of CNTs can be controlled by the catalyst spots defined by UV or e-beam lithography. If the catalyst spot is less than 100 nm, a single CNT can be grown from each spot as shown in Figure 10.2D. Otherwise, a bundle of CNTs are formed as shown in Figure 10.2C. The dielectric encapsulation and polishing procedures ensure that only the very end of CNTs is exposed whereas the sidewall is insulated as shown in Figure 10.2E and F. Such nano-electrode array has shown characteristic electrochemical behavior for redox species both in bulk solution and immobilized at the CNT ends. Dramatic improvements in sensitivity and time constant were reported in References 6, 17, and 18.

10.2.3 Biofunctionalization

A common feature for biological sensing is the requirement of immobilization of biomolecules with specific functionalities on the sensing device. These biomolecules serve as probes to either specifically bind particular species in the testing sample or catalyze the reaction of a specific analyte. Such an event produces a change in chemical or physical properties that can be converted into a measurable signal by the transducer. The specific recognition of the target molecules is the essential feature for biological sensing. The common probe and target (analyte) recognition mechanisms include (a) antibody/antigen interactions, (b) nucleic acid hybridizations, (c) enzymatic reactions, and (d) cellular interactions. Depending on the device and its sensing mechanism, different functionalization methods have to be adapted. Current functionalization methods can be divided into two categories: (a) covalent binding to the open ends of CNTs and (b) covalent and noncovalent binding to the sidewall of CNTs.

CNTs, from a structural point of view, are very similar to a roll of graphitic sheets. The sidewalls have very inert chemical properties similar to graphite basal planes. The open ends of CNTs, on the other hand, are similar to graphite edge planes, which are much more reactive due to the dangling sp2 bonds [51,52]. For measuring chemical force of single molecules with CNT SPM tips [14] or using CNT nanoelectrodes for biosensing, the open end of the CNTs needs to be functionalized. Wong et al. [53] demonstrated that the open end of a SWNT is rich with –COOH group, which could be used for selective covalent bonding of primary amine molecules through amide bonds facilitated by the coupling reagents N-hydroxysuccinimide (NHS) sometimes aided with dicyclohexylcarbodiimide (DCC) [14,53]. Williams et al. [24] used similar methods to functionalize the open end of a SWNT with a peptide nucleic acid (PNA) with the sequence NH2-Glu-GTGCTCATGGTG-CONH2, where Glu is a glutamate amino-acid residue and the central block represents nucleic-acid bases. The primary amine terminated DNA oligo-probes can also be covalently functionalized to the open ends of a MWNT array embedded in SiO2 matrix by similar carbodiimide chemistry using water-soluble coupling reagents 1-ethyl-3(3-dimethyl amino-propyl carbodiimide hydrochloride (EDC) and N-hydroxysulfo-succinimide (sulfo-NHS) [54].
FIGURE 10.2  SEM images of (A) a 3 x 3 electrode array; (B) array of MWNT bundles on one of the microelectrode pads; (C) and (D) array of MWNTs at UV-lithography and e-beam patterned Ni catalyst spots, respectively; (E) and (F) the surface of polished MWNT nanoelectrode array grown on 2 µm and 100 nm spots, respectively [6]. (A) to (D) are 45° perspective views and (E) to (F) are top views. The scale bars are 200, 50, 2, 5, 2, and 2 µm, respectively.
For sensors using the FET configuration, functionalization of the sidewall of CNTs is required [11,12,55,56]. Because semiconducting SWNTs are used as the conducting channels whose electronic properties are monitored upon the binding of charged target molecules at the surface, the graphitic \( sp^2 \) sidewall structure has to be preserved to maintain its inherent properties. Such a sidewall structure is strongly hydrophobic and chemically inert, which raises problems in biocompatibility and biofunctionalization for specific recognition. It has been reported that proteins such as streptavidin and HupR can adsorb strongly onto the MWNT surface presumably via hydrophobic interactions between the aromatic CNT surface and the hydrophobic domains of the proteins [22]. A designed amphiphilic \( \alpha \)-helical peptide has been found to spontaneously assemble onto the SWNT surface in aqueous solution with the hydrophobic face of the helix noncovalently interacting with the CNT surface and the hydrophilic amino acid side chains extending outwards from the exterior surface [23]. Chen et al. [55] reported a noncovalent sidewall functionalization scheme whereby a variety of proteins were immobilized on SWNTs functionalized by \( \pi \)-stacking of the conjugate pyrenyl group of 1-pyrenebutanoic acid succinimidyl ester. The succinimidyl ester group reacts with amine groups on lysine residues of proteins to form covalent amide linkages. However, all such noncovalent interaction-based immobilization methods lack specificity, particularly the direct nonspecific binding of proteins to CNTs needs to be suppressed for biological sensing. Extensive washing using conventional protocols was not sufficient to remove such nonspecifically bound proteins. For this purpose, a surfactant (Triton-X 100) was coadsorbed on the CNT surface with poly(ethylene glycol) (PEG) [56]. Such a coating was found to be effective in resisting nonspecific adsorption of streptavidin. Amine-terminated PEG can be used so that the biotin moiety can be added to the PEG chains through covalently linking with an amine-reactive biotin reagent, biotinamidocaproic acid 3-sulfon-N-hydroxysuccinimide ester [56]. The functionalized CNTs have demonstrated specific recognition to streptavidin. A similar method using mixed polyethylene imine (PEI) and PEG coating was reported for functionalizing biotin to the sidewall of the SWNT in FET devices [11]. These methods can be extended to the recognition of other biomolecules based on specific interactions of antibody-antigen and complimentary DNA strands.

Besides the application in FET-sensing devices, the biofunctionalized CNTs also show much better solubility so that further chemistry or biochemistry can be applied. In addition, the biorecognition can be employed to regulate the assembly of supramolecular structures, which may lead to new sensing devices. Because the integrity of the CNT structure is not as critical in such applications as in FETs, covalent sidewall functionalizations are also actively pursued by directly attaching functional groups to the graphitic surface such as fluorination and hydrogenation or using carboxylic acid groups at the defect sites to form amide or ester linkages [57]. Such sidewall functionalizations may particularly be applicable to MWNT sensors in which CNTs serve only as the probe materials to transduce signals from the measured molecules, and their own electronic properties are insensitive to the environment. For example, a MWNT electrode functionalized with antibody or enzyme can be used as a single EC probe to study biochemistry in a single cell [39]. Sidewall functionalization of the MWNT array can also greatly increase the enzyme (glucose oxidase) loading in electrochemical glucose sensors [20]. Some other studies on enzyme-based sensors using CNT-casted glassy carbon electrodes have demonstrated that even spontaneous adsorption or polymer wrapping can improve the enzyme loading and improve the electron transfer [41–43]. From the biological side, peptides with selective affinity for carbon nanotubes were recently reported [58], which could lead to new methods for bio-/nanointegration.

### 10.3 Biosensing Applications

#### 10.3.1 Single-Cell and Single-Molecule Sensors

The most direct application of CNTs for biological sensing is to use them as single probes to gain great spatial resolution. With the small size, such probes can be inserted into a single cell for \textit{in situ} measurements with minimum disturbance and ultrahigh sensitivity. Vo-Dinh et al. [59] demonstrated an antibody-based nanobiosensor for the detection of benzo[a] pyrene tetrol (BPT), a biomarker for human
exposure to the known carcinogen benzo[a]pyrene (BaP) by simply pulling an optical fiber to nanometer size at the tip. The distal end was covalently coated with anti-BPT antibodies through silane linkers. The nanobiosensors were inserted into individual cells, incubated 5 minutes to allow antigen-antibody binding, and then removed for the detection. Such a nanobiosensor, based on fluorescence spectroscopy, shows a sensitivity down to $1.0 \times 10^{-10}$ M for BPT [59,60] and an absolute limit of detection for BPT of $\sim 300$ zeptomoles ($10^{-21}$ mole) [61]. A single MWNT nanoelectrode probe [39] can be adapted using similar techniques to study the electrophysiology phenomenon in a single cell or the reactivity of a single molecule. The electrical signal has the potential to reach single molecular sensitivity [49].

Single CNTs attached to an SPM tip have also attracted intensive interests, as discussed in Chapter 6, due to their small diameter, high aspect ratio, large Young’s modulus, and mechanical resilience [13]. They can be used as physical probes to obtain high-resolution image of macromolecules or cell surfaces. Li et al. [15] demonstrated the method for implementing and characterizing CNTs as SPM tips for *in situ* imaging of DNA molecules on mica surface within a buffer solution. A magnetically driven oscillating probe in an atomic force microscope (AFM) with a silicon nitride cantilever (spring constant of $k = 0.1 \, \text{N/m}$) was used at a frequency of $\sim 30 \, \text{kHz}$. A bundle of MWNTs was attached to the pyramidal tip with an acrylic adhesive. The diameter of the bundles ranged from tens to hundreds of nanometers. Typically, a single MWNT extended out and was used as a probe for AFM imaging. Lambda DNA molecules were spontaneously bound to the surface from 2 $\mu$g/ml solutions with the presence of $\sim 1 \, \text{mM MgCl}_2$ for enhancing the DNA/mica interaction. Figure 10.3 shows an AFM image of DNA molecules in a $2.3 \times 2.3 \, \mu\text{m}^2$ area. Single DNA molecules were clearly resolved. The resolution of DNA images is very uniform and consistent with the diameter of the CNT tip ($\sim 5 \, \text{nm}$ in diameter). SWNT tips could provide even higher resolution [14,53], and the double-helix structure of DNA may be resolved using SWNT tips. The tip can also be functionalized to provide additional chemical force information [14,53].

Woolley et al. [62] reported the direct haplotyping of kilobase-size DNA using SWNT AFM probes. The haplotype of a subject — the specific alleles associated with each chromosome homolog — is a critical element in single nucleotide polymorphisms (SNPs) mapping that leads to a greater comprehension of the genetic contribution to risk for common diseases such as cancer and heart disease. However, the current methods for determining haplotypes have significant limitations that have prevented their use in large-scale genetic screening. For example, molecular techniques for determining haplotypes, such
as allele-specific or single-molecule polymerase chain reaction (PCR) amplification, are hampered by the need to optimize stringent reaction conditions and the potential for significant error rates. Using AFM with high-resolution SWNT probes, multiple polymorphic sites can be directly visualized by hybridizing specifically labeled oligonucleotides with the template DNA fragments of ~100 to 10,000 bases. The positions of streptavidin and IRD800 labels at two sequences in M13mp18 were demonstrated. The SWNT tips, with tip radii less than 3 nm (~10 base resolution), made it possible for high-resolution multiplex detection to differentiate different labels such as streptavidin and the fluorophore IRD800 based on their size. This concept has been further applied for the determination of haplotypes on the UGT1A7 gene [62], which is studied for its role in cancer epidemiology. The direct haplotyping using SWNT AFM probes represents a significant advance over conventional approaches and could facilitate the use of SNPs for association and linkage studies of inherited diseases and genetic risk [63].

The electronic properties of biomolecules such as DNAs have been extensively studied in the past few years due to their potential for single molecular sensing. However, DNA molecules were found not to be very conductive even in the duplex form. There are also difficulties in assembling them into reliable devices. Williams et al. [24] reported a study to couple SWNTs covalently with PNA, an uncharged DNA analogue. The hybridization of DNA fragments to the PNA sequence was measured with an AFM under ambient conditions and indicated that the functionalization is specific to the open ends of SWNTs with rare attachment to the sidewall. PNA was chosen due to its chemical and biological stability. The uncharged PNA backbone gives rise to PNA–DNA duplexes that are more thermally stable than their DNA–DNA counterparts because there is no electrostatic repulsion. This method unites the unique properties of SWNTs with the specific molecular recognition features of DNA, which may provide new means to incorporate SWNTs into larger electronic devices by recognition-based assembly as well as electronic biological sensing.

10.3.2 FET-Based Biosensors

The extreme high sensitivity and potential for fabricating high-density sensor array make nanoscale FETs very attractive for biosensing, particularly because biomolecules such as DNA and proteins are heavily charged under normal conditions. SWNT FETs are expected to be more sensitive to the binding of such charged species than chemisorbed gas molecules. However, the wet chemical environment with the presence of various ions and presence of other biomolecules makes it much more complicated than gas sensors. Extensive efforts have been made to understand the fundamental issues of SWNT FET in wet environments.

First, the behavior of SWNT FETs has to be well understood in ambient environment. Several studies indicated that SWNT FETs fabricated on SiO$_2$/Si substrates with the structure similar to that in Figure 10.2B exhibit hysteresis in current versus gate voltage. This is not desired for electronics applications but is the nature of nanoscale chemical sensors relying on the interaction between SWNT and molecular species in the environment. The hysteresis was attributed to charge traps either in SiO$_2$ film or SWNTs [64–66]. However, Kim et al. [67] found that the major cause can be attributed to water molecules, especially the strong SiO$_2$ surface-bound species, which are difficult to remove even by pumping in vacuum. Passivating the SWNT FET with poly(methyl methacrylate) (PMMA) can nearly remove the hysteresis. The choice of passivation polymers and the humidity in the environment can strongly affect electronics properties of SWNT FETs. Bradley et al. [68] coated the FET device with a charged polymer and found that the hysteresis can be used as a humidity sensor. The mobile ions in the hydrated polymer film were suspected to be responsible for the hysteresis.

If water adsorption and mobile ions can significantly affect the performance of SWNT FETs, the measurement of the binding of charged biomolecules on SWNT FETs in liquid environments is even more challenging. Star et al. [11] explored the device response in a buffer and found that it is essentially obscured by the abundant ions in the environment. So far, not much success has been reported in using SWNT FETs for biosensing in practical biological environments. To get around this problem, the SWNT FET after incubation was washed and dried before characterization in an ambient environment [11].
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The device was indeed effective for specific biotin-streptavidin binding. A mixed PEI and PEG polymer coating has been used to avoid nonspecific binding. Biotin molecules were attached to the polymer layer for specific molecular recognition. The SWNT FET device coated with PEI/PEG polymer alone changes from p-type to n-type device characteristics probably due to the electron-donating properties of the NH2 groups of the polymer. The attachment of biotin molecules through covalent binding to the primary NH2 group reduced the overall electron-donating function of PEI and convert the device back to p-type characteristics. Finally, the specific binding of streptavidin with biotin was found to almost totally remove the gating effect and cause dramatic decrease in source-drain current at negative gate voltages. Nonspecific binding was observed in devices without the polymer coating, whereas no binding was found for polymer-coated but not biotinylated devices. Streptavidin, in which the biotin-binding sites were blocked by reaction with excess biotin, produced essentially no change in device characteristic of the biotinylated polymer-coated devices.

Despite the complexity, Rosenblatt et al. [16] successfully demonstrated a high-performance electrolyte-gated SWNT FET. The uncoated device was submerged in a drop of water solution containing 10 mM NaCl. A water gate voltage \( V_{wg} \) was applied to the droplet through a silver wire. Such electrolyte-gated SWNT FET showed p-type characteristics with high device mobilities and transconductances, very promising for biosensing applications. Interestingly, the electrolyte gating approaches the ultimate limit where the capacitance is governed by quantum effects and not electrostatics.

Recently, Besteman et al. [12] realized that proteins carry pH-dependent charged groups that could electrostatically gate a semiconducting SWNT, creating the possibility of constructing a nanosize protein or pH FET sensors. Even more interesting, redox enzymes go through a catalytic reaction cycle where groups in the enzyme temporarily change their charge state resulting in conformational changes in the enzyme. They successfully demonstrated that the enzyme-coated SWNT FETs can be used as single-molecule biosensors. As shown in Figure 10.4A, the redox enzyme glucose oxidase (GOx) is immobilized on SWNT using a linking molecule, which on one side binds to the SWNT through van der Waals coupling with a pyrene group and on the other side covalently binds the enzyme through an amide bond as developed by Chen et al. [55]. The FET preserves the p-type characteristic but shows much lower conductance upon GOx immobilization, which is likely the result of the decrease in the capacitance of the tube due to GOx immobilization because GOx blocks the liquid from access to the SWNT surface.

The GOx-coated SWNT did show a strong pH dependence as well as high sensitivity to glucose. Figure 10.4B shows the real-time measurements where the conductance of a GOx-coated SWNT FET has been recorded as a function of time in milli-Q water. No significant change in conductance is observed when more milli-Q water is added as indicated by the first arrow. However, when 0.1 M glucose is added, the conductance increases by 10% as indicated by the second arrow. The inset (a) of Figure 10.4B shows the similar behavior repeated with another GOx-coated device in contrast to no change with a bare device shown in inset (b). Clearly, GOx activity is responsible for the measured increase in conductance upon the addition of glucose.

Both studies on liquid gated FETs use very low ionic strength with 10 mM NaCl [16] and 0.1 mM KCl [12], respectively. The salt concentrations are more than 10 times lower than physiology buffers. Experiments are needed to explore the measurements under more practical physiological buffers to confirm whether the high ionic concentration will obscure the response. This is critical for further biosensor applications.

10.3.3 Nanoelectrode Array–Based Electronic Chips

An embedded CNT array minimizes the background from the sidewalls whereas the well-defined graphitic chemistry at the exposed open ends allows the selective functionalization of –COOH groups with primary amine terminated oligonucleotide probes through amide bonds. The wide electropotential window of carbon makes it possible to directly measure the oxidation signal of guanine bases immobilized at the electrode surface. Such nanoelectrode array can be used as ultrasensitive DNA sensors based on an electrochemical platform [6,17,18]. As shown in Figure 10.1D and Figure 10.5A (see color insert following...
oligonucleotide probes of 18 bases with a sequence of [Cy3]5-CTIIATTTCICAIITCCT-3[AmC7-Q] are covalently attached to the open end of MWNTs exposed at the SiO\textsubscript{2} surface. This sequence is related to the wild-type allele (Arg1443stop) of BRCA1 gene [69]. The guanine bases in the probe molecules are replaced with nonelectroactive inosine bases, which have the same base-pairing properties as guanine bases. The oligonucleotide target molecule has a complimentary sequence [Cy5]5-AGGAC-CTGCGAAATCCAGGGGGGGGG-3 including a 10 mer polyG as the signal moieties. Hybridization was carried out at 40°C for about 1 hour in ~100 nM target solution in 3 × SSC buffer. Rigorous washing in three steps using 3 × SSC, 2 × SSC with 0.1% SDS, and 1 × SSC respectively at 40°C for 15 minutes after each probe functionalization and target hybridization process was applied in order to get rid of nonspecifically bound DNA molecules, which is critical for getting reliable electrochemical data.

Such solid-state nanoelectrode arrays have great advantages in stability and processing reliability over other electrochemical DNA sensors based on mixed self-assembled monolayers of small organic molecules. The density of nanoelectrodes can be controlled precisely using lithographic techniques, which in turn define the number of probe molecules. The detection limit can be optimized by lowering the nanoelectrode density. However, the electrochemical signal is defined by the number of electrons that can be transferred between the electrode and the analytes. Particularly, the guanine oxidation occurs at rather high potential (~1.05 V vs. saturated calomel electrode [SCE]) at which a high background is produced by carbon oxidation and water electrolysis. This problem can be solved by introducing Ru(bpy)	extsubscript{3}\textsuperscript{2+} mediators to amplify the signal based on an electrocatalytic mechanism [70]. Combining the...

![FIGURE 10.4](image)

(A) Schematic of an SWNT FET biosensor with GOx immobilized on the SWNT surface. (B) Real-time electronic response of the SWNT sensor to glucose, the substrate of GOx. The conductance is measured in 5 µl-milli-Q water. The source-drain and liquid-gate voltage is kept constant at 9.1 mV and ~500 mV, respectively. At the time indicated by the first arrow, 2 µl milli-Q water is added to the solution. After a while (at the second arrow), 2 µl 0.1 mM glucose in milli-Q water is added. Inset (a) shows experiment repeated with a second device and inset (b) is the same experiment on a device without GOx. (Reprinted from Besteman et al. Nano Lett., 3 (6), 727–730 (2003). With permission.)
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MWNT nanoelectrode array with Ru(bpy)$_3^{2+}$ mediated guanine oxidation (as schematically shown in Figure 10.5B), the hybridization of less than ~1000 oligonucleotide targets can be detected with a 20 × 20 µm$^2$ electrode, with orders of magnitude improvement in sensitivity compared with previous EC-based DNA detections [6,17].

Figure 10.6A shows three consecutive AC voltammetry (ACV) scans in 5.0 mM Ru(bpy)$_3^{2+}$ in 0.20 M NaOAc buffer solutions after hybridizing the polyG tagged BRCA1 targets on a MWNT array electrode (with average tube-tube spacing of ~1.5 µm). The AC current is measured by applying a sinusoidal wave of 10 Hz frequency and 25 mV amplitude on a staircase potential ramp. Well-defined peaks are observed around 1.04 V with the first scan clearly higher than the almost superimposed subsequent scans. The background is almost a flat line at zero. The peak current at ~1.04 V consisting of two parts with:

$$I_p = I_{\text{mediator}} + I_{\text{amplifiedG}}$$

(10.1)

where $I_{\text{mediator}}$ is the oxidation current of bulk Ru(bpy)$_3^{2+}$ mediators coincidently superimposed with the mediator amplified oxidation current of guanine bases $I_{\text{amplifiedG}}$ at almost the same potential. Due to the electrocatalytical mechanism, the signal of guanine is much larger than that from guanine bases alone ($I_G$), i.e.,

$$I_{\text{amplifiedG}} = N \times I_G \gg I_G$$

(10.2)

where $N$ is the amplification factor (normally >5). However, guanine oxidation is irreversible and contributes only in the first scan, and the current in subsequent scans mainly corresponds to $I_{\text{mediator}}$. As a result, the net signal associated with guanine bases can be derived as

$$I_{p1} - I_{p2} = I_{\text{amplifiedG}} \propto [G].$$

(10.3)

As shown in Figure 10.6B, subtracting the second scan from the first one gives a well-defined positive peak (continuous line) whereas subtracting the third scan from the second gives a much smaller negative peak (dotted line). The difference between further scans is almost a flat line at zero. The high quality of the data indicates that there is still plenty of room to lower the detection limit of target DNAs.

In practical applications, the target DNA typically consists of over hundreds of bases, with a large quantity of guanine bases in each target molecule. The Ru(bpy)$_3^{2+}$ mediators can efficiently transport electrons between guanine bases and the CNT electrode. As a result, all inherent guanine bases in the DNA target that dangle in the hemispherical diffusion layer of the Ru(bpy)$_3^{2+}$ mediators can efficiently
serve as signal moieties, resulting in a large signal compared with the techniques using redox tags [45]. Thus the detection limit can be lowered to less than 1000 target molecules, approaching the limit of laser-based fluorescence techniques [17]. The use of inherent guanine bases as signal moieties makes it possible to skip the expensive and time-consuming labeling procedure. Other advantages of EC detection such as the ability to apply extra stringent control using local electrical field could be realized with this system. It is also applicable in enzymatic biosensors for pathogen detection by immobilizing proteins such as enzymes and antibodies at the electrodes.

10.3.4 Nanonetworks and Thin Films

Besides being used as building elements in well-defined devices, both SWNTs and MWNTs can be cast as a random network or thin film on conventional electrodes [41–43] or used as a three-dimensional porous film [9,19,20]. CNTs serve both as large immobilization matrices and as mediators to improve the electron transfer between the active enzyme site and the electrochemical transducer. CNT-modified glassy carbon electrodes exhibit a substantial (~490 mV) decrease in the overpotential for -Nicotinamide adenine dinucleotide (NADH) oxidation. Various enzymes such as GOx and flavin adenine dinucleotide (FAD) can spontaneously adsorb onto CNT surface and maintain their substrate-specific enzyme activity.

FIGURE 10.6 (A) Three consecutive AC voltammetry measurements of the low-density MWCNT array electrode functionalized with oligonucleotide probes with the sequence [Cy3]-5’-CTIIATTTCICAIITCCT-3’[AmC7-Q] and hybridized with oligonucleotide targets with the sequence [Cy5]-5’-AGGACCTGCAGATCCAGGGGGGGGGG-3’ [6]. The thick, thin, and dotted lines correspond to the first, second, and third scan, respectively. The measurements were carried out in 5 mM Ru(bpy)$_3^{2+}$ in 0.20 M NaOAC supporting electrolyte (at pH=4.8) with an AC sinusoidal wave of 10 Hz and 25 mV amplitude on top of a staircase DC ramp. (B) The difference between the first and the second scans (solid line) and between the second and the third scans (dotted line) [6]. The positive peak corresponds to the increase in Ru(bpy)$_3^{2+}$ oxidation signal due to the guanine bases on the surface. The negative peak serves as a control representing the behavior of a bare electrode.
over prolonged time [41]. Biosensors based on enzymes that catalyze important biological redox reactions (such as glucose oxidation) can be developed.

### 10.3.5 CNT Templated Bioassembly

Besides being used as building blocks in device fabrication, CNTs have also attracted extensive interest as nanoscale templates for self-assembly of CNT-biomolecular complex. Such bio-/nanomolecular assembly could incorporate the specific recognition properties of biomolecules with desired electronic properties of CNTs to develop novel biosensors and bioelectronic materials. SWNT-PNA hybrid molecular wires by covalently functionalizing PNA fragments to the open end of SWNTs was described in Section 10.3.1. Several studies have also been carried out to investigate the self-assembly processes driven by the hydrophobic forces both at the inner and outer surfaces of CNTs.

The phenomenon of molecules inserting into the confined space in the inner channel of CNTs, particularly in liquid environments, is of great fundamental interest. Even though it is a very challenging problem for experimental exploration due to the lack of proper characterization techniques, computer simulation studies did show interesting results. Recent molecular dynamics simulations demonstrated that SWNTs can be used as molecular channels for water transport [71]. Gao et al. [72] further demonstrated that a DNA molecule could be spontaneously inserted into a SWNT in a water solute environment. The van der Waals and hydrophobic forces were found to be important for the insertion process, with the former playing a more dominant role in the DNA-CNT interaction. It suggests that the encapsulated CNT-DNA molecular complex can be exploited for applications such as DNA modulated molecular electronics, molecular sensors, electronic DNA sequencing, and nanotechnology of gene delivery systems.

On the other hand, the external CNT surface has hydrophobic properties similar to graphite surface, which is known to be a model substrate to study molecular self-assembly. The decoration of a CNT surface with self-assembly of biological molecules has been demonstrated in various systems including lipids [73], oligonucleotides [74], proteins [22,55], and peptides [23], etc. Richard et al. [73] found that both sodium dodecyl sulfate (SDS) and synthetic lipids form supramolecular structures made of rolled-up half-cylinders on the nanotube surface. Depending on the symmetry and the diameter of the CNT, it can form rings, helices, or double helices. Permanent assemblies can be produced from the mixed micelles of SDS and different water-insoluble double-chain lipids after dialysis of the surfactant. Biomolecules such as histidine-tagged proteins can be immobilized on such lipid-decorated CNTs for the development of new biosensors and bioelectronic materials.

For sensor applications, biomolecules with specific recognition functions such as proteins can also be directly packed on the external surface of CNTs, but they have to remain functional. A good criterion for the conservation of the functional properties of the protein is its ability to form ordered arrays. Balavoine et al. [22] reported a study of streptavidin assembly on CNTs to form helical crystalization. Streptavidin is very useful in many biochemical assays, such as labeling and affinity chromatography due to its high affinity for (+) biotin (Ka ~ 10^{15}). The assembly was carried out in solutions by spontaneous adsorption. MWNTs were prepared by the arc-discharge method and stored as a suspension in methanol (~2 mg/ml). A 100-µl aliquot of MWNT suspension was dried under an ethane gas flow and resuspended in 20 ml of a 40% aqueous solution of methanol. This suspension was sonicated to disperse the MWNTs before the addition of 20 µl streptavidin solution (~10 µg/ml) in a buffer containing 10 mM Tris (pH = 8) and 50 mM NaCl, and allowed to stand at room temperature for 45 minutes. Such a sample was deposited on carbon film covered grid and was negatively stained with a 2% uranyl acetate solution for transmission electron microscopy imaging. In appropriate conditions, the MWNT surface was found almost completely covered with streptavidin, presumably due to the interaction with its hydrophobic domains. Even though most assemblies are disordered, some regular-spaced helical structures were observed at proper conditions. Another water-soluble protein, HupR, was also studied and showed ordered arrays on a wider range of MWNT diameters than streptavidin.

A 29-residue amphiphilic α-helical peptide was also specifically designed to coat and solubilize CNTs as well as control the assembly of the peptide-coated CNTs into macromolecular structures through
peptide-peptide interactions [23]. Figure 10.7 shows the cross-sectional view of the molecular structure and the perspective view of the helical backbones of the model illustrating the assembly of such molecules on a SWNT surface. Six such α-helices are sufficient to surround the circumference of an individual SWNT while maintaining typical interhelical interactions. The hydrophobic face of the helix with apolar amino acid side chains (Val and Phe) presumably interacts noncovalently with the aromatic surface of CNTs, and the hydrophilic face extends outward to promote self-assembly through charged peptide-peptide interactions. Electron microscopy and polarized Raman studies reveal that the peptide-coated CNTs assemble into fibers with CNTs aligned along the fiber axis. The size and morphology of the fibers can be controlled by manipulating solution conditions that affect peptide-peptide interactions.

Whereas the above-mentioned studies are based on non specific adsorptions, Wang et al. [58] have used phage display to identify peptides with selective affinity for CNTs. Binding specificity has been confirmed by demonstrating direct attachment of nanotubes to phage and free peptides immobilized on microspheres. Consensus binding sequences show a motif rich in histidine and tryptophan at specific locations. The analysis of peptide conformations shows that the binding sequence is flexible and folds into a structure matching the geometry of carbon nanotubes. The hydrophobic structure of the peptide chains suggests that they act as symmetric detergents. An IgG monoclonal antibody against the fullerene $C_{60}$ [75] was also studied to show binding to CNTs with some selectivity [76].

### 10.4 Summary and Future Directions

The potential of CNT-based nanodevices for ultrasensitive biological sensing has been recognized, and several demonstrations confirmed the exciting possibilities ahead. The reduction of the size of sensing and transducing elements to the size of biomolecules, i.e., 1 to 100 nm, makes it possible to detect down to single molecules. The development in this direction may revolutionize the filed of biotechnology. However, though the sensitivity improves, the reliability may pose a problem. Particularly at the level to detect a handful of molecules in the sample, the signal will fluctuate in a large range due to statistical reasons. Using a high-density array of such devices is desired to increase the statistics and improve the reliability. In the meantime, the dynamic range can also be increased. Extensive efforts have to be made in both device fabrication and assay development to solve these issues before the great potential for practical applications can be realized.
Applications: Biosensors

References