Covalent Attachment and Hybridization of DNA Oligonucleotides on Patterned Single-Walled Carbon Nanotube Films

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DNA oligonucleotides were covalently immobilized to prepatterned single-walled carbon nanotube (SWNT) multilayer films by amidation. SWNT multilayer films were constructed via consecutive condensation reactions creating stacks of functionalized SWNT layers linked together by 4,4'-oxydianiline. Aminated- or carboxylated-DNA oligonucleotides were covalently immobilized to the respective carboxylated or aminated SWNT multilayer films through amide bond formation using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. UV−vis−NIR spectroscopic analysis indicated that the SWNT film surface density increased uniformly according to the number of reaction cycles. Scanning electron microscopy and contact angle measurements of the SWNT multilayer film revealed a uniform coverage over the substrate surface. The covalent attachment of DNA oligonucleotides to the SWNT multilayer films and their subsequent hybridization with complementary oligonucleotides were verified using X-ray photoelectron spectroscopy and fluorescence-based measurements. This is the first report demonstrating that DNA oligonucleotides can be covalently attached to immobilized SWNT multilayer films. The anchored DNA oligonucleotides were shown to exhibit excellent specificity, realizing their potential in future biosensor applications.

Introduction

Recent advances from the convergence of nanotechnology and biotechnology are accelerating the development of fusion technology incorporating the highly selective binding capabilities of DNA in nanomaterials.1−6 Single-walled carbon nanotubes (SWNTs) are promising materials for highly sensitive DNA detection, because they have unique electric properties,7,8 which are suitable for use as electrochemical biosensors or chemical sensors.9,10 Most studies reported to date have concentrated on the applications using individual nanotubes,11 although ensembles of SWNTs are very important for practical electrochemical biosensors or chemical sensors.9,10 Most studies reported to date have concentrated on the applications using individual nanotubes,11 although ensembles of SWNTs are very important for practical applications in biosensors. For example, individual SWNTs are unsuitable as biomaterial matrices for mass production because of their limited length, fixation, and location on the solid substrate. Consequently, for advanced biosensor applications having immobilized biomaterials at desired locations on SWNTs, it is crucial to develop a SWNT film that is covalently anchored to a patterned solid substrate. In an earlier article,12 we reported the preparation of a high-density SWNT multilayer film comprising a micropatterned conducting array, fabricated via functionalization of the open ends and sidewall defect sites of the SWNTs.13,14 Stacked SWNT multilayer films comprising shortened SWNTs were constructed on aminated glass substrates via successive condensation reactions, using the 4,4′-oxydianiline (ODA) linker molecule and an appropriate condensation reagent, O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HATU). As-formed SWNT multilayer films are versatile substrates because they allow unrestricted patterning on the surface and the introduction of various functional groups, for use in the conjugation of biological materials. The interaction between a DNA oligonucleotide probe attached to a SWNT multilayer film and its complement represents an important molecular recognition process for use in detection and diagnostic processes. Here, we report a novel method for covalently linking DNA to SWNT films immobilized on solid surfaces. Motivated by the biological applications to solid substrates consisting of nanoparticle adsorbed DNA, we turned our attention to investigate (1) the formation of SWNT multilayer films, (2) their subsequent functionalization with DNA oligonucleotides, and (3) the successive hybridization reactions creating stacks of functionalized SWNT layers linked together by 4,4'-oxydianiline. The SWNT film surface density increased uniformly according to the number of reaction cycles.
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Figure 1. Overall scheme for the fabrication of patterned SWNT multilayer films and the selective immobilization of DNA: (a) surface treatment with t-BOC protecting group, (b) deposition of a layer of a PAG, (c) UV exposure (405 nm, 25 mW) using a photomask for patterning, (d) development process, (e) selective immobilization of SWNTs onto the aminated regions of the substrate, (f) chemical attachment of additional SWNT layers using an ODA linker molecule and a condensation agent (HATU), (g) covalent immobilization of oligonucleotide probes onto the patterned SWNT multilayer regions (functional groups (NH2 or COOH) terminated on the oligonucleotide), and (h) hybridization of the FITC-labeled complementary oligonucleotide.

Experimental Section

Materials. As-prepared SWNTs were purified, shortened, and polished in accordance with literature methods. Aminopropylsilane-modified glass slides (Corning GAPS-coated slides, 25 × 75 mm) were obtained from Corning Inc. N,N-Dimethylformamide (DMF, Aldrich, 99.9%) was dried by refluxing it with triphenyl chlorosilane (5 g/L) at 130 °C for 20 h and then distilling it at about 5 mmHg pressure. Dichloromethane (Aldrich, 99.8%) was dried by refluxing it with 1-Ethyl-3-(3-dimethylaminopropyl)carbodimide (DMF, Aldrich, 99.9%) was dried by refluxing it with N,N-diisopropylethylamine (DIEA, 99%), and other organic solvents were all purchased from Aldrich and used as received. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2-(N-morpholino)ethanesulfonic acid (MES), SSC buffer (standard saline citrate), and sodium dodecyl sulfate (SDS) were purchased from Sigma and used as received. The 3′-aminated (or carboxylated) single-stranded DNA (ssDNA-NH2 or ssDNA-COOH) is a 20-base oligonucleotide having the following sequence: 5′-CAC AGC TTT TAG GTG GCA CA-FITC-3′. The noncomplementary single-stranded DNA (NC-ssDNA) is a 20-base oligonucleotide with the following sequence: 5′-TGT GCC ACC TAC AAG CTG TG-FITC-3′. Deionized water (18 MΩ·cm−1) was obtained from a Millipore system.

Preparation of Patterned SWNT Multilayer Films. The fabrication process for the formation of DNA-functionalized SWNT multilayer films, suitable for further hybridization experiments with complementary DNA, is illustrated in the scheme shown in Figure 1. To selectively immobilize DNA oligonucleotides on SWNT multilayer films, we formed a desired pattern on amine-modified glass, in which a photomask was used to create patterns in the SWNT multilayer films with microscale resolution. Briefly, patterned substrates were fabricated as previously described. Aminated-slide glass was treated with 6-(BOC-amino) caproic acid, and the entire BOC-protected surface was coated with an appropriate photocacid generator (PAG). The desired pattern was then formed by exposing the surface to UV light (405 nm, 25 mW) through a photolithographic mask, where the corresponding PAG selectively removed the BOC protecting group to leave free surface amine functionalities. Line widths of 1.25 and 2.5 μm and chessboard-like patterns (80-μm-width feature size), as well as raised and recessed regions, generated using relief and intaglio photomasks, respectively, were used to demonstrate the patterning capability of the SWNT multilayer films.

As-prepared nanotubes were shortened by oxidation in a mixture of concentrated sulfuric acid (98%) and nitric acid (70%) in a volume ratio of 3:1, under ultrasonication. This process has the added effect of creating carboxyl groups at the open ends and sidewall defect sites of the SWNTs. Immobilization of carboxylated SWNTs on prepatterned, aminated substrates was carried out by amide coupling, in the presence of the condensation reagent HATU. The first SWNT layer was prepared by immersing a prepatterned aminated-glass substrate into a DMF solution of carboxylated SWNTs, containing HATU and DIEA. The resulting SWNT-patterned substrate was then rinsed with copious amounts of water to remove any unreacted reactants. The resulting SWNT multilayer film was then immersed in a DMF solution of aminated DNA oligonucleotides, and the DNA oligonucleotides were covalently linked to the respective carboxylated nucleotides. Aminated or carboxylated DNA oligonucleotides were covalently linked to the respective carboxylated or aminated SWNT multilayer films, through appropriate coupling chemistries. We show that the resultant DNA-functionalized SWNT multilayer films exhibit excellent specificity and chemical stability under the conditions of DNA hybridization. This work provides an avenue for the development of devices in which the exquisite binding specificity of biomolecular recognition is directly coupled to electrodevices using SWNTs. Furthermore, this system is expected to find numerous applications in biological, medical, pharmaceutical, and forensic fields.


of anhydrous DMF and CH$_2$Cl$_2$, respectively. After drying in a stream of high-purity argon, the substrate was immersed in a DMF solution of DIEA (5 mL, 1.2 mM) and stirred for 30 min. Additional DMF solutions of fresh HATU (5 mL, 1.0 mM) and ODA (20 mL, 10 mM) were then added to the first solution, and the reaction mixture was stirred for a further 2 h. In this reaction cycle, ODA is coupled to the patterned aminated surface through one of its amino groups. The remaining amine groups on the immobilized ODA are then allowed to react with the carboxyl functionalities of further SWNTs to form a second SWNT layer. Repeated reaction cycles led to the formation of SWNT multilayer films. All reaction steps were performed at room temperature. The patterned SWNT multilayer films were fabricated at the desired locations using the fabrication procedure outlined above.

Attachment of DNA Oligonucleotides to SWNT Multilayer Films. A ssDNA-NH$_2$ solution (94.8 nmol) was prepared at a concentration of 100 μM (pH 6.5) in 25.0 mM MES buffer. The SWNT multilayer film was activated by the dropwise addition of a 25 mM MES buffer (pH 6.5) solution containing 100 μL of NHS (0.42 μmol) and 200 μL of EDC (6.34 μmol; NHS/EDC ratio of 1:15), which was then left to incubate in a humidity chamber at 25 °C for 30 min. Following this, 100 μL of a 94.8 nmol ssDNA-NH$_2$ solution was added to the activated film, and then the film was left to incubate for 10 h at room temperature. The substrate was washed with copious amounts of deionized water. The ssDNA-COOH oligonucleotide probes were immobilized on ODA-functionalized SWNT multilayer films using the same method.

Hybridization of DNA Oligonucleotides on SWNT Multilayer Films. Hybridization was carried out using C-ssDNA. NC-ssDNA was also investigated as a negative control. Lyophilized C-ssDNA and NC-ssDNA were diluted in 3× concentrated SSC and 0.3% SDS to give final concentrations of 100 μM. The C-ssDNA (or NC-ss DNA) was denatured at 95 °C and snap-cooled on ice. The collected C-ssDNA (or NC-ssDNA) solution (40 μL) was deposited on the surface of freshly prepared SWNT multilayer films using a micropipet. For hybridization, the specimen was placed under a coverslip (22 × 22 mm Hybrid-Slips) and incubated for 10 h at 55 °C in a hybridization chamber. The coverslip was removed by immersing the slide in 2× concentrated SSC solution, and the SWNT multilayer film was then washed five times with 0.1× concentrated SSC and 0.1% SDS solution at room temperature, immersed in 0.1× concentrated SSC solution for 5 min, and then dried.

Characterization. To quantify the amount of nanotubes linked per reaction cycle, the SWNT film surface density was measured using an ultraviolet–visible–near-infrared spectrophotometer (UV–vis–NIR; JASCO V-570) at a resolution of 1 nm$^{-1}$. Images of SWNT multilayer films were taken at various magnifications using a field emission scanning electron microscope (XL 300 FE G, Philips) equipped with a Schottky based field emission gun. X-ray photoelectron spectroscopy (XPS) spectral data was obtained using a V.G. Scientific ESCALAB MK II spectrometer equipped with a Mg K$_\alpha$ X-ray source (1253.6 eV photons) and a hemispherical energy analyzer. The X-ray source was operated at 12 kV and a filament current of 10 mA, and measurements were taken at a takeoff angle of 75° to the sample surface. The binding energy was referenced to the C(1s) peak for benzene and assigned a value of 284.6 eV. Data normalization and quantitative and peak-fitting analyses were carried out using XPSPeak Version 4.1 (programmed by R. Kwok of the Chinese University). The XPS P(2p) spectrum was peak-fitted using a Gaussian function. Images of the chessboard-like patterned SWNT multilayers were taken with an optical microscope (OM, Leica DMLB) at a magnification of 10 × 5. The OM micrographs were obtained using a Leica M6 MP 80 photomaton. Detection of the fluorescence signals was performed on a ScanArray 5000 unit (Packard Bioscience, BioChip Technologies LLC) and analyzed using the QuantArray 3.0 software package (GSI Lumonics, Billerica, U.S.A.).

Results and Discussion

High-density SWNT multilayer structures were constructed via successive condensation reactions, creating stacks of SWNT layers, covalently bound together through the use of an appropriate linker molecule and condensation agent. A SEM investigation of the SWNT multilayer film formed after six reaction cycles confirmed that the successive condensation reactions produced a uniform film over the entire aminated glass surface (Figure 2), with the exception of a few areas where scores of nanosized particles of debris were observed. Closer inspection of the surface revealed that the film was completely covered by randomly oriented SWNT ropes 400–600 nm in length (Figure 2, inset).

UV–vis–NIR spectroscopy was used to monitor the stacked SWNT films to determine whether the number of SWNTs linked per reaction cycle was uniform. Because the linker molecule (ODA) and condensation agent (HATU) do not absorb in the spectral region monitored, the measured absorbance is due only to the SWNTs. The surface density of the SWNT multilayer films was measured using an ultraviolet–visible spectrophotometer (UV–vis; Lumonics, Billerica, U.S.A.). The absorbance at 250 nm indicated that the SWNT multilayer film was not absorbing in the spectral region monitored.
determined by analyzing the UV absorbance at 1020 nm. The color of the SWNT multilayer films on amine-modified glass (Figure 3a) was found to darken as the number of reaction cycles was increased. This was corroborated by the absorbance measurements, which were found to increase linearly (Figure 3b). In contrast, the corresponding reaction between SWNTs and nonfunctionalized glass substrates revealed no change in color or absorbance (data not shown), implying that SWNT multilayer films can only be formed successfully though covalent bonding.

To further confirm the uniformity and surface density over large areas, we performed advancing water contact angle measurements (see in Figure 3b). The wettability of solid surfaces is an important surface property, which is governed by both chemical and surface structural factors. Because chemical composition is an intrinsic property of materials, wettability is usually enhanced by increases in surface roughness (three-dimensional microgeometry), especially by fractal structures. We have, therefore, paid particular attention to the geometrical structure of the solid surfaces and found that the SWNT surface density has remarkable effects on its wettability properties. The contact angle results show that the aminated glass surface is somewhat hydrophobic, with a water contact angle of about 64°. However, attachment of the first SWNT layer resulted in a decrease in the contact angle (ca. 50°). Although graphite surfaces are more hydrophobic than aminated surfaces, these results indicate that the SWNTs were attached so sparsely on the aminated surface that “island formation” occurred. For this reason, it is safe to say that the contact angle of a liquid on the substrate depends not only on the surface tension of the liquid and the substrate but also on the surface roughness of the substrate. The subsequent attachment of a second layer of SWNTs through the use of an ODA linker resulted in an increase in the contact angle (ca. 70°), while after the third reaction cycle, the contact angle reached a plateau, suggesting that the majority of the surface was covered by SWNTs after just three reaction cycles. The measured water contact angle of the uniformly covered SWNT surface was determined to be about 73°, which is quite similar to that of graphite surfaces (lit. value 86°). Although the surface appears to show a uniform and complete SWNT coverage, the difference between the experimental data and the literature value for graphite indicates that the SWNT multilayer film surface is not perfectly flat.

Photolithography was used to prepare various SWNT multilayer patterns on aminated glass substrates. Briefly, the aminated glass was first treated with 6-(BOC-amino) caproic acid to yield a BOC-protected surface. This was
then coated with a PAG layer and UV exposed through appropriate photomasks to generate specific patterns of amine functionalities, while leaving the unexposed BOC-protected amine groups untouched. Following this, the successive condensation reaction cycles led to the formation of patterned SWNT multilayer films on the prepatterned substrate. OM and SEM were employed to confirm the selective attachment of SWNT multilayers to the UV-patterned substrate. Regular arrays of selectively deposited SWNT multilayers, corresponding to the photomask pattern, were clearly observed, as confirmed by SEM (Figure 4; taken after the sixth reaction cycle). To demonstrate the unrestricted patterning capability of immobilized SWNT multilayer films, the films were also UV exposed to produce chessboard-like arrays (feature widths of 80 μm) and parallel lines (line widths of 1.25 and 2.5 μm). Moreover, relief and intaglio photomasks were used to demonstrate the patterning of raised and recessed regions, respectively, where the SEM images clearly show the boundaries between the SWNT regions (light gray) and the areas representing the clean substrate surface (dark gray; Figure 4). Consequently, the micropatterned SWNT multilayer films are uniform and correspond to predefined locations on the substrate. Further studies are in progress to investigate the behaviors and electric signals for biomaterials on the patterned SWNT multilayer film at the submicrometer scale.

The covalent attachment of aminated or carboxylated single-stranded DNA to an immobilized SWNT multilayer film by EDC-catalyzed amidation was found to proceed with excellent efficiency. Figure 5a shows the OM image (10 × 5) of the micropatterned SWNT multilayer film immobilized on aminated glass, where the dark squares represent the regions containing the SWNT multilayers. To show general hybridization specificity on the patterned regions, ssDNA-COOH was covalently attached to a micropatterned, amine functionalized SWNT multilayer film. The ssDNA-modified SWNT multilayer film was then exposed to a solution containing its complementary strand (C-ssDNA). After hybridization, the patterned substrate was washed with copious amounts of deionized water and the fluorescence was measured. The fluorescence image of the hybridized oligonucleotide regions shows remarkable similarity to that of the SWNT multilayer film pattern (Figure 5b), indicating that a high proportion of DNA assemblies are specifically bound to the nanotube sites, with excellent efficiency. Figure 5a shows the OM image (10 × 5) of the micropatterned SWNT multilayer film immobilized on aminated glass, where the dark squares represent the regions containing the SWNT multilayers. To show general hybridization specificity on the patterned regions, ssDNA-COOH was covalently attached to a micropatterned, amine functionalized SWNT multilayer film. The ssDNA-modified SWNT multilayer film was then exposed to a solution containing its complementary strand (C-ssDNA). After hybridization, the patterned substrate was washed with copious amounts of deionized water and the fluorescence was measured. The fluorescence image of the hybridized oligonucleotide regions shows remarkable similarity to that of the SWNT multilayer film pattern (Figure 5b), indicating that a high proportion of DNA assemblies are specifically bound to the nanotube sites, with excellent efficiency.

Figure 6. Scanned fluorescence images of hybridized oligonucleotides on SWNT multilayer films. (a) Hybridization of the FITC-labeled complementary oligonucleotide on the carboxylated-SWNT multilayer film, (b) hybridization of the non-complementary oligonucleotide, (c) hybridization of the FITC-labeled complementary oligonucleotide on the ODA-functionalized-SWNT multilayer film, and (d) hybridization of the noncomplementary oligonucleotide.

Figure 7. Peak-fitted XPS spectra of P(2p1/2) (133.9 eV) and P(2p3/2) (133.1 eV) corresponding to the phosphate groups present in the oligonucleotide probe immobilized on the SWNT multilayer film.

rather than the BOC-protected regions. Hybridization experiments were then performed to confirm that the DNA oligonucleotides are indeed incorporated into the patterned SWNT multilayer film. The hybridization studies of both ssDNA-NH₂ and ssDNA-COOH oligonucleotides, coupled to the carboxylated- and aminated-SWNT films, respectively, were tested using perfect complements (C-ssDNA) and controlled mismatches (NC-ssDNA). Figure 6 shows a composite of fluorescence results obtained from the hybridization experiments. Figure 6a,c shows scanned fluorescence images of the hybridized oligonucleotides on the two ssDNA-functionalized SWNT multilayer films. The fluorescence is clearly evident in both cases, and with near equal intensity. To test whether the binding of ssDNA is selective enough to distinguish a mismatch of sequences, equivalent experiments were carried out using the NC-ssDNA containing a mismatch sequence. Although the same binding is performed on both substrates, the fluorescence signal was barely visible for the mismatched DNA (NC-ssDNA), as shown in Figure 6b,d. Thus, we conclude that the selectivity of the ssDNA-functionalized SWNT multilayer film is sufficient to discriminate the mismatched DNA. This system may, therefore, serve as a novel method to improve sensitivity, reproducibility, stability, and low noise and serve to be an effective hybridization test for chip-based assays.

The phosphate group in the DNA nucleotide was analyzed using XPS to further confirm the presence of conjugated ssDNA probes on the SWNT multilayer film. Figure 7 shows the peak-fitted X-ray photoelectron spectra in the phosphorus region (2p₁/₂ and 2p₃/₂). The observed phosphorus signal can be deconvoluted to give two component peaks: the first at 133.9 eV, attributed to 2p₁/₂, and a second peak at 133.1 eV, attributed to 2p₃/₂. The presence of the 2p₁/₂ and 2p₃/₂ peaks provides further evidence indicating that the ssDNA oligonucleotide probes are indeed conjugated to the immobilized SWNT multilayer film.

Conclusions
The functionalization chemistry of the open ends and defect sites on the sidewalls of SWNTs has played a vital role in tailoring the material properties and engineering of nanotube devices. As a fine example of the application of SWNT to biotechnology, the covalent bonding of DNA oligonucleotides on the SWNT multilayer film yields interfacial layers showing high selectivity in subsequent hybridization processes, in which the SWNT multilayer film can be patterned to predefined locations on the substrate. UV–vis–NIR analysis of the surface density of the SWNT films revealed a linear increase as a function of the number of reaction cycles, while SEM images showed uniform coverage of the high-surface-density SWNT multilayer films on the prepatterned glass slide. XPS and fluorescence-based measurements confirmed that single-stranded DNA oligonucleotides can be conjugated to SWNT multilayer films, and their specificity was confirmed through hybridization experiments with their corresponding DNA complement. The resultant conjugated ssDNA-SWNT multilayer films were shown to exhibit excellent specificity and chemical stability under conditions of DNA hybridization. Consequently, DNA oligonucleotides covalently attached to SWNT conducting arrays on solid substrates represent a novel biosubstrate with excellent potential for both immobilization and hybridization applications and for use in bio-electronic devices.

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