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Trinity DNA Detection Platform by Ultrasmooth and Functionalized PEDOT Biointerfaces

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ABSTRACT An oligonucleotide-grafted poly(3,4-ethylenedioxythiophene) (PEDOT) thin film is developed for three DNA biosensor detection methods, including fluorescence, quartz crystal microbalance, and electrochemical methods. By electrocopolymerization of hydroxyl-functionalized EDOT and carboxylic-functionalized EDOT in microemulsion solutions, ultrasmooth films with a controlled surface density of carboxylic groups are created. The probe oligonucleotides are immobilized on PEDOT thin films by using a *N*-hydroxysuccinimide and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride coupling method. By monitoring the DNA hybridization efficiency on thin films with different oligonucleotide densities, the optimized density for DNA hybridization is obtained. The feasibility and limitation of using this platform for electrochemical detection are also discussed.

KEYWORDS: conducting polymer • biosensor • electropolymerization • thin film

INTRODUCTION

The completion of a human genome project provides new dimensions for clinical diagnostics and drug development based on the understanding of DNA. As a result, there is a growing need for DNA-biosensing platforms ranging from genotyping to molecular diagnostics (1). Northern blotting (2), ribonuclease protection (3), and reverse transcription−polymerase chain reaction (4) are the most commonly used methods in gene expression analysis. However, each of these methods suffers from insufficient sensitivity to false signaling from amplified contaminations. Therefore, several new methods have been developed, such as fluorescence DNA biochips (5), a surface plasma resonance technique (6), gold nanoparticle-based DNA sensing (7), a quartz crystal microbalance (QCM) (8) and cantilevers (9), to detect mass changed during hybridization. Among these new technologies, there is a growing interest in developing electrochemical DNA biosensors (1d, 10) for the following reasons: (1) their cost-effectiveness coupled with modern semiconductor fabrication processes; (2) high sensitivity upon signal amplification through electrocatalysis; (3) rapid and direct detection regardless of light-absorbing chemicals; (4) ease of manufacturing of portable, robust, low-cost, and easy-to-handle detection instrumentation suitable for field tests and home-care usage. The key component for an electrochemical biosensor is the efficient construction of conductive biointerfaces. A self-assembled monolayer (SAM) based on thiol−Au interaction is the most popular method for conductive biointerface construction because of its simplicity, chemical availability, and ability to produce thin and uniform films (11). However, SAM suffered the following disadvantages, such as a limited selection of grafted electrode surfaces, extended time for immobilization, and potential-dependent instability of thiol−Au bonding (12). These drawbacks limit the development and large-scale manufacture of SAM-based electrochemical biosensors.

Previous studies have shown the feasibility of using conductive poly(3,4-ethylenedioxythiophene) (PEDOT)-coated electrodes to record the electrical activity of the central nervous system (13) because of their superior biocompatibility and conductivity. Furthermore, PEDOT-coated electrodes have also been successfully used for various biosensor applications, such as DNA and glucose detection (14). Recently, we have reported a strategy to make thin films based on PEDOT and its derivatives for biointerface applications (15). These films have several advantages, which make them a good fit for electrochemical DNA biosensor use. First, they are ultrasmooth with a roughness (R_{rms}) of <5 nm. It has been observed that the substrate morphology influenced the immobilization of capture probes (CPs) and hybridization of a target (16). Our PEDOT films with homogenous surface morphology provide an unhindered environment for both immobilization and hybridization. Second, the density of the functional groups for bioconjugation on films can be easily controlled by electrochemical copolymerization. Because the probe is immobilized via chemical reaction with these functional groups, control of the functional group density is identical with control of the probe density. For DNA sensors, the probe density is the key factor in determining the DNA hybridization efficiency (17). On the basis of the length and bases of DNA probes, hybridization is favored both thermodynamically and kinetically at a certain range of probe density. Third, films are highly conductive with thicknesses of less than 100 nm. Therefore, the electrical signal gener-

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Table 1. Oligonucleotide Sequences Used in This Work

function	5′ → 3′ sequence
capture probe (18 bp)	TTTGAGTCTGTTGCTTGG-AAAAAA-$(CH_2)_6$-NH_2
complementary target	CCAAGCAACAGACTCAAA[a]
one-base mismatch target	CCAAGCAACACACTCAAA[a]
noncomplementary target	CAAGCACTTGCTGACCAAGCAAC[a]

[a] Targets were 3′-labeled with Cy3 or biotin or nonlabeled for different detection schemes.

ated from events occurring on film surfaces can transmit through thin films to substrates instantly. Besides, these films can be deposited only on specified areas of the electrode surfaces within a short period of time. This advantage is important to device fabrication and benefits the manufacture of large-scale devices.

In this article, we demonstrate a versatile platform for DNA detection by using oligonucleotide-grafted PEDOT thin films. The oligonucleotide-grafted PEDOT thin films were prepared by electrocopolymerization of two functionalized EDOT monomers followed by CP immobilization by a bioconjugation reaction. This platform can be used in various DNA detection protocols, including fluorescence, QCM, and electrochemical methods. By comparing the signal readout from substrates of different densities of surface functional groups, we can calculate the optimized density of CPs for DNA sensing. An electrochemical detection protocol was applied on our PEDOT system. The resulting detection showed a nice linear dynamic range from 10 to 1000 nM. The main issue to limit the detection for our platform is also discussed.

EXPERIMENTAL SECTION

Materials and Reagents. Ethylenedioxythiophene (EDOT; Sigma-Aldrich), lithium perchlorate ($LiClO_4$; Fluka), sodium dodecyl sulfate (SDS; Alfa Aesar), and D-(+)-glucose (Sigma) were used as received. Hydroxymethyl-functionalized EDOT (EDOT-OH) was synthesized according to the literature procedure (18). Carboxylic acid-functionalized EDOT (C_2-EDOT-COOH) was synthesized in the same manner as that described previously (19). A phosphate-buffered saline (PBS) consisting of 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer was used as the supporting electrolyte solution. The redox polymer used in this study was a poly(vinylimidazole)−poly(acrylamide) copolymer partially imidazole-complexed with Os(4,4′-dimethyl-2,2′-bipyridine)$_2Cl^{+/2+}$ (PVA-Os), which was synthesized as described previously (20). Glucose oxidase-avidin D (GOD-A; Vector Laboratories) was diluted in PBS by 100 times in volume to form 50 μg/mL. The probe and target oligonucleotide sequences in Table 1 were custom-prepared by 1st Base, Inc. Indium−tin oxide (ITO)-coated glass (Delta-Technologies, Ltd.) was cleaned by immersion in detergent solutions, acetone, dichloromethane, and then finally isopropyl alcohol with ultrasonic agitation for a period of 30 min prior to use. Au and Pt disk working electrodes (CHI Instruments) were polished by Polishing Kits (PK-4, Bioanalytical Systems, Inc.) with 0.05-μm alumina (Gamma Micropolish, Buehler) before use.

Electropolymerization and Film Syntheses. PEDOT films from different EDOT monomers were electropolymerized on Au, Pt, and ITO electrodes from 10 mM EDOT aqueous solutions containing 0.1 M $LiClO_4$ as the supporting electrolyte in the presence of 1 mM HCl and 0.05 M SDS by applying cyclic potentials (−0.6 to +1.1 V vs Ag/AgCl at a scan rate of 100 mV/s) or potentiostatic methods.

Bioconjugation and Immobilization. Carboxylic acid groups on polymer films were activated by *N*-hydroxysulfosuccinimide (sulfo-NHS; Pierce) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC; Pierce) coupling. After 15−20 min of incubation at room temperature, extra sulfo-NHS and EDC were removed by deionized (DI) water. Amine-modified CP oligonucleotides were immobilized onto electrodes by immersing the electrodes in PBS containing 1 μM CP. After adsorption, the electrodes were rinsed with PBS and water and then blown dry with nitrogen to remove nonspecifically adsorbed materials.

Hybridization and Detection. The hybridization of target oligonucleotides was performed in a moisture-saturated chamber maintained at room temperature. CP-immobilized electrodes were immersed in PBS with 1 μM target oligonucleotides overnight. Cy3- and biotin-labeled target oligonucleotides were used for fluorescence detection and electrochemical detection, respectively. After rinsing thoroughly with PBS and DI water, electrodes of biotin-labeled target oligonucleotides were immersed in GOD-A solutions. GOD was attached to the target oligonucleotides via biotin−avidin interaction. The glucose electrooxidation current was measured amperometrically in PBS containing 40 mM glucose with a potential of 0.4 V (vs Ag/AgCl) in a faradaic cage. Details of this electrochemical response were described before (11a). Fluorescence measurement was performed with a fluorescence microscope (BX-51, Olympus) with a CCD camera (DP70, Olympus). Images were then analyzed with Image-Pro 3D Suite software (MediaCybernetics) to calculate the fluorescence intensity.

QCM Measurement. A QCM experiment was conducted by a Q-Sense E4 (Q-Sense) system. The details of this technique are described elsewhere (21). PEDOT was electropolymerized directly on Au-coated crystals with 14 mm diameter. Measurement was conducted at a fundamental frequency of 4.95 MHz. The concentrations of CPs and target oligonucleotide solutions are 100 nM. Solutions were conducted by using a microprocessor-controlled dispensing pump (IPC-4, Ismatec) at a flow rate of 50 μL/min.

Surface Characterization. The surface density of carboxylic acid groups (−COOH) on polymer films was determined by toluidine blue (TBO) staining (22). Polymer films were immersed in a 0.5 mM TBO solution at pH 10. After removal of noncomplexed dye with water, the dye on the polymer films was desorbed in a 50% acetic acid solution, and the dye concentration was determined by measuring the absorbance at 633 nm (Agilent 8453). The density was then obtained from a calibration curve of the absorbance intensity versus the dye concentration.

RESULTS AND DISCUSSION

Formation of Oligonucleotide-Grafted PEDOT Thin Films. We have previously reported the formation of thin, ultrasmooth, noncytotoxic, and functionalized PEDOT films by acid-catalyzed microemulsion electropolymerization (15). Electropolymerization of carboxylic acid-functionalized EDOT (C_2-EDOT-COOH) and EDOT-OH in a 0.1 M $LiClO_4$ aqueous solution containing 10 mM respective monomers, 1 mM HCl, and 0.05 M SDS yielded an ultrasmooth polymer film. In this work, the potentiostatic method was used to retain the consistency of the amount of polymer deposited on the electrode surface. The films were deposited at a constant potential (1.0 V) (23), and the electropolymerization was cut off when the charge exceeded 0.15 mC. To apply these films as biointerfaces, we first identified the accessibility of surface −COOH functional groups by a TBO

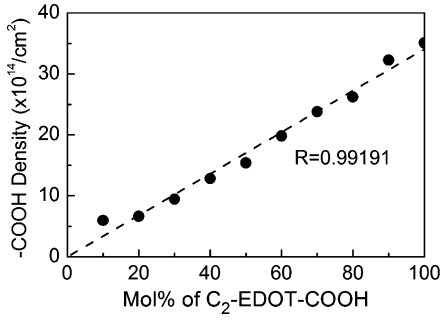


FIGURE 1. Calculated surface density of −COOH groups with increased mole percent of C_2-EDOT-COOH in the monomer mixture.

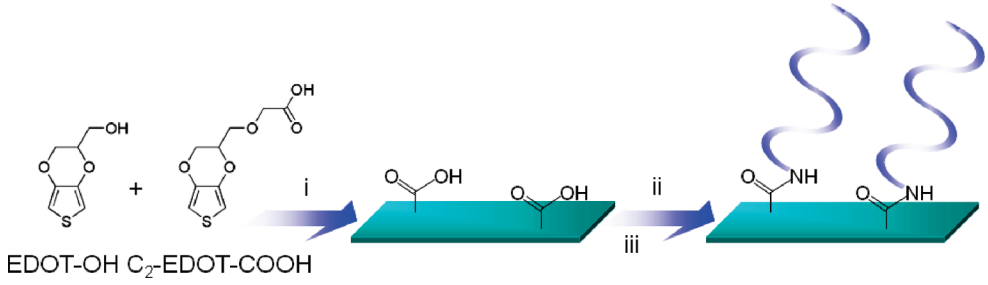
assay (22). TBO dye was a cationic molecule, which would bind to surface −COOH groups when these groups were deprotonated as COO^- at high pH. After washing, the surface-bound TBO dye was redissolved in a 50% acetic acid solution, and the presence of TBO absorption at 633 nm would signify the presence of −COOH on the PEDOT film surface. Assuming that TBO was bound to a surface −COOH group in a 1:1 molar ratio, the density of the surface functional carboxylic acid group was quantified as $3.5 \times 10^{15}/cm^2$ based on the absorbance of redissolved TBO. Theoretical calculations (24) and previous reports suggested that the efficiency of DNA hybridization to a surface-bound probe was maximized when the probe density was $10^{12}-10^{13}/cm^2$ (25). This value was 2−3 orders of magnitude lower than what we obtained with the poly(C_2-EDOT-COOH) film. To reduce the −COOH surface density, copolymers of C_2-EDOT-COOH and EDOT were synthesized from a mixture of monomers. From the mechanistic view of random electropolymerization, the −COOH density should be controlled by the percentage of C_2-EDOT-COOH in the monomer mixture. A linear relationship ($R = 0.99179$) between the −COOH surface density and the molar percentage of C_2-EDOT-COOH in the mixture confirmed our hypothesis, as shown Figure 1. Unfortunately, we found the TBO showing slightly nonspecific adsorption on pure PEDOT-OH thin films. The nonspecific adsorption obviously influenced the linearity when the content of C_2-EDOT-COOH was lower, as shown in Figure 1. This issue prevents us from using this method to quantify the −COOH surface density for even lower content C_2-EDOT-COOH mixtures.

To make oligonucleotide-grafted PEDOT thin films, the surface −COOH groups were activated by sulfo-NHS and EDC (26) and then coupled with 3′-amino-functionalized oligonucleotides, as shown in Scheme 1. This whole assembly process combining polymerization, activation, and immobilization required less than a few hours. Compared to the assembling process for SAM devices (27), which usually takes overnight time, our procedures were more suitable for the efficient manufacturing and array applications. Here we adapted a primer design targeting viral neuraminidase gene N1 for avian flu detection as the CP on polymer surfaces, and the sequences of CP are listed in Table 1.

Fluorescent Detection of a CP-Grafted PEDOT Biointerface. Fluorescent detection remained the most popular platform for DNA detection because of its simplicity and availability. Several reports applied a strategy similar to that for the construction of a fluorescent DNA detection platform based on polypyrrole (28). However, the films electropolymerized on the surface of the electrodes were not uniform under the conditions applied. The rough surface could have affected the hybridization efficiency and the detection limit if a long DNA sequence from sample tissues or blood was targeted. In contrast, the uniform PEDOT film that we developed would increase the accessibility of each CP, allowing efficient target hybridization and lowering the detection limit. On the basis of our previous discussion, a copolymer electropolymerized from a EDOT monomer mixture consisting of 0.1−1% C_2-EDOT-COOH provided an optimized surface −COOH density for detection. Therefore, we examined the fluorescent response with PEDOTs of these compositions to confirm the hypothesis. Initially, unfunctionalized EDOT was employed to modulate the monomer composition, but there was undesired attachment of polymer films to the substrate surface. Therefore, the more hydrophilic EDOT-OH was subsequently used in place of EDOT for copolymerization. The resulting poly(EDOT-OH)-*co*-poly(C_2-EDOT-COOH) system greatly enhanced the interfacial adhesion between the polymer film and the substrates such as Au, Pt, or ITO.

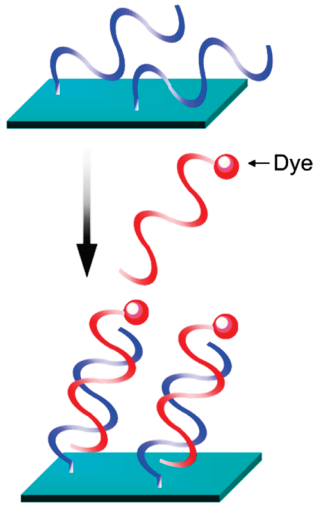
A general method applied for fluorescent DNA detection involved dye-labeled targets or probes. Our system can be applied toward this type of detection, as shown in Scheme 2. After hybridization with Cy3-labeled targets on our CP-grafted biointerface, much greater fluorescence intensity was obtained with complementary targets, compared to noncomplementary and one-base mismatch targets as shown

Scheme 1. Preparation of an Oligonucleotide-Grafted PEDOT Nanobiointerface with Microemulsion Electropolymerization[a]



[a] Steps: (i) electropolymerization; (ii) sulfo-NHS/EDC coupling; (iii) oligonucleotide conjugation.

Scheme 2. Schematic Presentation of Fluorescence Detection on PEDOT Nanobiointerfaces[a]



[a] Fluorescence detection of DNA on poly(EDOT-OH)-*co*-poly(C_2-EDOT-COOH) films was done by hybridization with dye-labeled target DNA.

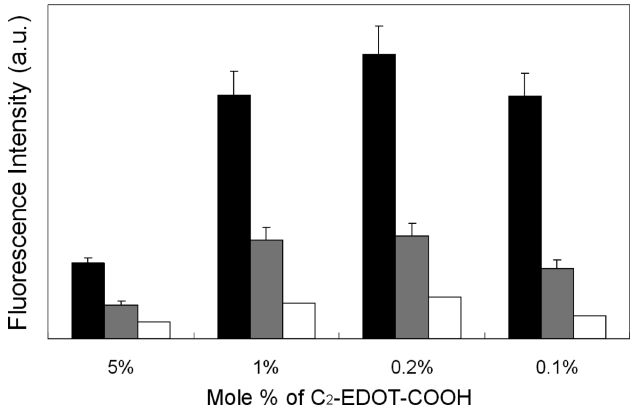


FIGURE 2. Fluorescence intensity in the presence of a 1 μM Cy3-labeled complementary target (black), a one-base mismatch target (gray), and a noncomplementary target (white) in the fluorescence labeling experiments (consisting of the mole percent of the C_2-EDOT-COOH monomer specified) on ITO substrates.

regardless of the surface density of CPs, as shown in Figure 2. The intensity from one-base mismatch targets was also higher than that from noncomplementary targets, which is mainly due to the hybridization kinetics of mismatch targets (29). The one-base mismatch targets showed a certain level of hybridization on our platform at room temperature. For examination of our hypothesis for optimized −COOH density, our data clearly supported the theoretical prediction. Copolymers with 5% C_2-EDOT-COOH displayed <60% fluorescence intensity after hybridization of complementary targets compared to copolymers with 1−0.1% C_2-EDOT-COOH. The copolymers with 0.2% C_2-EDOT-COOH delivered the highest fluorescence intensity, indicated as the optimized probe density for hybridization in our platform.

QCM with Energy Dissipation (QCM-D) of a CP-Grafted PEDOT Biointerface. In addition to fluorescence detection, our conductive biointerfaces were also suitable for QCM-D detection. The monitoring of CP immobilization and target hybridization processes on copolymer thin films with 0.2% and 5% C_2-EDOT-COOH by using a QCM-D is shown in Figure 3a. For CP immobilization process, compared to the film with 0.2% C_2-EDOT-COOH, the film with 5% C_2-EDOT-COOH showed a larger frequency decrease, which indicated that more CPs were immobilized. This is due to a higher density of −COOH groups for immobilization. However, for the hybridization process, the frequency decreased more for the film with 0.2% C_2-EDOT-COOH than for the film with 5% C_2-EDOT-COOH, which indicated that more targets hybridized on the film with 0.2% C_2-EDOT-COOH (less CPs) instead of 5% C_2-EDOT-COOH (more CPs). The result is consistent to what we observed by using fluorescence detection. A QCM-D system also monitors the energy dissipation change (ΔD), which is used to observe the viscoelasticity of film surfaces (30). During the immobilization process, although CPs were immobilized on film surfaces and increased the weight crystals, ΔD did not change a lot. This indicated that the surface viscoelasticity was not affected much after attachment of single-strand oligonucleotides. However, ΔD increased relatively more when targets hybridized on surfaces. This is mainly due to the formation of a double-helix structure after hybridization,

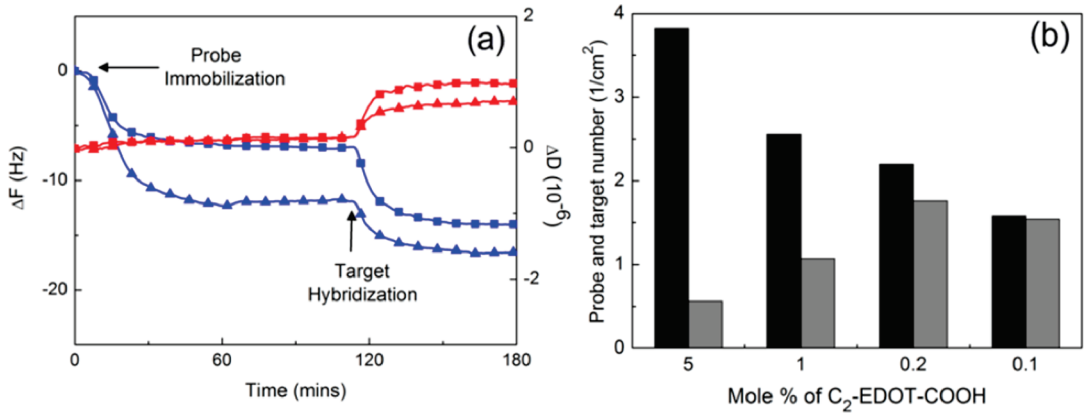
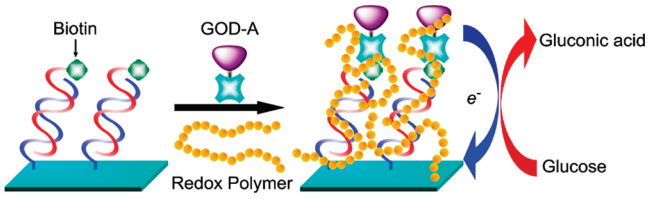


FIGURE 3. QCM results of oligonucleotide immobilization and target hybridization. The frequency change (ΔF) is shown in blue and dissipation in red. (a) QCM monitoring of the DNA immobilization and hybridization processes on poly(EDOT-OH)-*co*-poly(C_2-EDOT-COOH) films. The mole percent of the C_2-EDOT-COOH monomer is 5% (▲) and 0.1% (■). (b) Probe density (black) and target density (gray) calculated from the Sauerbrey relationship (consisting of the mole percent of the C_2-EDOT-COOH monomer specified).

Scheme 3. Schematic Presentation of Amperometric Detection on PEDOT Nanobiointerfaces[a]



[a] Glucose oxidase–avidin D (GOD-A) and redox polymer were added in solution after hybridization with biotin-labeled DNA on poly(EDOT-OH)-*co*-poly(C_2-EDOT-COOH) films. Electrical signals were generated and transferred to substrates after adding a glucose solution.

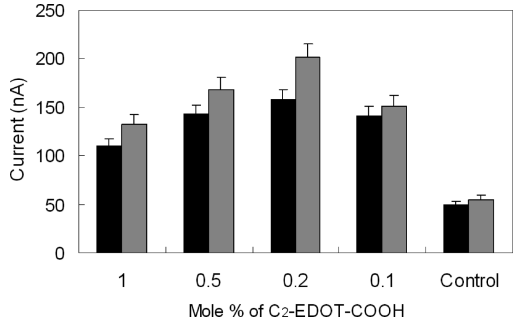


FIGURE 4. Summary of the amperometric response of glucose oxidation after DNA hybridization on poly(EDOT-OH)-*co*-poly(C_2-EDOT-COOH) films (consisting of the mole percent of the C_2-EDOT-COOH monomer specified) on Au (black) and Pt (gray) substrates. The control experiment was conducted on films containing 0.2 mol % of C_2-EDOT-COOH and hybridized with a noncomplementary target.

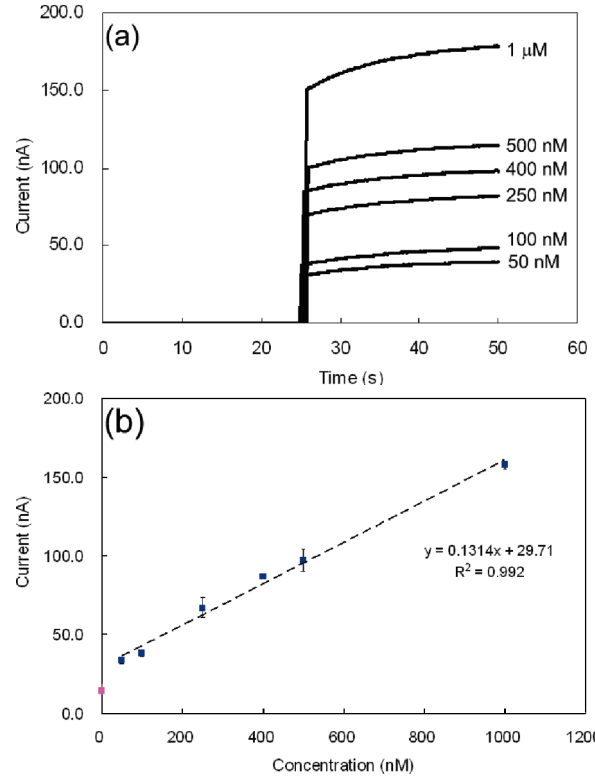


FIGURE 5. (a) In situ amperometric response of glucose oxidation on poly(EDOT-OH)-*co*-poly(C_2-EDOT-COOH) films (prepared from 0.2 mol % of C_2-EDOT-COOH solutions) after hybridization with target DNA in different concentration. (b) Dynamic range and detection limit for PEDOT-based electrochemical platform.

and the "standing-up" DNA increased the surface viscoelasticity and led to a fast decay of the damped oscillation.

We also used QCM-D to test the immobilization and hybridization processes on films with 1% and 0.1% C_2-EDOT-COOH. By using the Sauerbrey relationship (31), we can calculate the mass of adhering probes and targets. Divided by their molecular weights, the densities then can be estimated, as summarized in Figure 3b. This shows that the optimized probe density for our platform is around $2 \times 10^{12}/cm^2$, which is achieved on the film with 0.2% C_2-EDOT-COOH.

Electrochemical Detection of a CP-Grafted PEDOT Biointerface. In addition to fluorescence detection, our conductive biointerfaces were also suitable for electrochemical detection. We adapted a SAM-based detection protocol previously developed in our institute for feasibility demonstration, as shown in Scheme 3 (32). After the biotin-labeled target was hybridized onto the CP-grafted biointerface, GOD-A and PVA-Os were introduced subsequently by dripping an aliquot on the surface of the PEDOT films or soaking the electrode in the solution. The amperometric signal was calculated from the difference in the current output between soaking the electrodes in a PBS buffer and soaking the electrodes in a PBS buffer containing 40 mM glucose. Experiments were first conducted on films with 0.2% C_2-EDOT-COOH coated on a Au substrate with a GOD-A concentration (5 μg/mL) in our detection protocols. The concentration of biotin-labeled targets was 1 μM. A much greater amperometric signal (151 nA) with complementary target hybridization was obtained, compared to the signal (50 nA) from the control experiment using noncomplementary targets, as shown in Figure 4. An even greater difference in the signal was attained between complementary (222 nA) and noncomplementary (55 nA) targets when a Pt substrate was employed. This was attributed to the better work function matching between Pt and PEDOT. The comparison between the signal outputs from different surface probe densities also agreed with the theoretical prediction and the results from fluorescent detection (25). PEDOT films containing 0.2% C_2-EDOT-COOH consistently yielded the largest signal output.

Figure 5a shows the amperometric response of glucose oxidation after hybridization with various concentrations of biotin-labeled targets. The electrodes are initially stabilized in PBS for 25 s and then immersed in PBS containing 40 mM glucose. Signal curves revealed prompt responses after the addition of glucose for all electrodes. The responses were summarized, as shown in Figure 5b. It clearly showed a linear dynamic range at target concentrations between 10 and 1000 nM. However, when we tested the amperometric response for detecting those samples with even lower target concentrations, we found inconsistent results with relatively large deviation. We then realized the main reason for this problem was a high background signal. As shown in Figure 5b, even without any target hybridization, an amperometric

response can still be detected (~15 nA). This is mainly due to a nonspecific binding of a glucose enzyme on PEDOT thin films.

CONCLUSIONS

Our studies demonstrate the feasibility of applying PEDOT biointerfaces for fluorescent, QCM, and electrochemical DNA detection. Compared to SAM or other conducting polymer-based methods for constructing DNA-grafted conducting surfaces, PEDOT biointerfaces are advantageous because they are uniform, compositionally tunable, and allow for efficient manufacturing. By varying the molar fraction of C_2-EDOT-COOH in the monomer mixture, we are able to synthesize PEDOT biointerfaces with different CP densities. Our initial results show that PEDOT films containing 0.1−1 % C_2-EDOT-COOH gave rise to the largest signal output. This finding agrees with the theoretical predictions. The current challenge for this platform is to solve the problem caused by a nonspecific binding of a glucose enzyme. The nonspecific binding of a glucose enzyme leads to a high background signal and limits the detection sensitivity. Further development of this system would lead to an excellent platform technology for disease diagnostics and biomarker identification. Other than the three detection platforms that we have demonstrated, we also believe PEDOT thin films can be used as substrates in surface plasmon resonance (SPR) spectroscopy. SPR spectroscopy is another powerful technique for bioconjugation study. SPR spectroscopy measurement requires Au substrates, which we have demonstrated a PEDOT thin film can be coated on. The application of PEDOT thin films for SPR spectroscopy needs further studies.

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