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A Field-Effect-Transistor Sensor Based on Polypyrrole Nanotubes Coupled with Heparin for Thrombin Detection

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Carboxylated polypyrrole (CPPy) nanotubes were fabricated by the chemical copolymerization of pyrrole and pyrrole-3-carboxylic acid (P3CA) using cylindrical micelle templates. Importantly, the chemical functionality of CPPy nanotubes was determined by the input amount of P3CA and their redox property was also retained without degradation. A field-effect-transistor (FET) sensor platform was constructed using liquid-ion gating. CPPy nanotubes were anchored on an amine-functionalized Au interdigitated microelectrode substrate to achieve high quality contact at the polymer/metal interface. Subsequently, heparin was covalently conjugated with the CPPy nanotubes via a diamino linker. The FET sensor based on heparin-conjugated CPPy nanotubes showed specific and fast response upon exposure to thrombin protein.

Keywords: biosensors; field-effect transistors; heparin; polypyrrole; thrombin

1. INTRODUCTION

Heparin is a sulfated polysaccharide that predominately consists of repeating units of D-glucuronate-2-sulfate and *N*-sulfo-D-glucosamine-6-sulfate [1,2]. Heparin binds specifically antithrombin III and can interact with thrombin, a coagulant protein, due to its high surface charge [3,4]. The antithrombin III inactivates thrombin and some of the activated coagulation factors. Owing to these properties, heparin has been widely utilized in medicine as an anticoagulant to prevent blood clotting in post-surgical patients. Another interesting

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application of heparin is the determination or separation of thrombin based on heparin-thrombin affinity. For this application, heparin is commonly immobilized on a support matrix. A number of methods have been developed for the immobilization of heparin, including adsorption [1,2,5], entrapment [6,7], and covalent attachment [8–10]. The noncovalent approaches such as adsorption and entrapment have attractive aspects including simplicity of reaction steps and little deterioration of physical properties of supports. However, the binding force by physical adsorption is susceptible to changes in pH, ionic strength, and temperature. In addition, the entrapment approach considerably suffers from limited biological activities of heparin by high diffusion barrier. On the other hand, covalent attachment offers significant advantages such as excellent biological activity and chemical/thermal stability.

Polypyrrole has been extensively studied as an immobilization matrix for versatile biomaterials due to its attractive properties such as facile synthesis, high conductivity, excellent environmental stability, and biocompatibility [11]. Polypyrrole-heparin systems have been also developed for protein separation [1,8], biomimic polymer coating [9], and improvement in blood compatibility [7,10]. Typically, Wallace and co-workers developed a new approach for the purification of thrombin based on specific interaction with heparin, which was incorporated as a dopant into polypyrrole during growth [1], and Shi *et al*. utilized heparin as an effective structure-directing agent for the preparation of polypyrrole nanowires [6]. Herein, we report a field-effect-transistor (FET) sensor based on heparin-conjugated polypyrrole (H-CPPy) nanotubes to detect the presence of thrombin. One of the most important challenges in biosensor design is achieving a stable immobilization of biomolecules on a substrate to preserve their specific binding ability and to attain reliable/reproducible responses [11]. We readily immobilized heparin entities on carboxylated polypyrrole (CPPy) nanotubes via covalent linkages and investigated the sensing behavior of H-CPPy nanotubes to detect thrombin in a FET configuration.

2. EXPERIMENTAL

Pyrrole (Aldrich, 98%) and pyrrole-3-carboxyl acid (P3CA) (Acros Organics, 95%) were used as received. Sodium bis(2-ethylhexyl) sulfosuccinate (AOT) (Aldrich, 98%) and ferric chloride (Aldrich, 97%) were employed as a surfactant and oxidizing agent, respectively. Hexane (Aldrich, 99%) was used as an apolar solvent. CPPy nanotubes were readily fabricated by copolymerizing pyrrole and P3CA in an AOT

reverse emulsion system. First, 7.9 mmol of AOT was dissolved in 20 mL of hexane, and 0.5 mL of 7 M aq. $FeCl_3$ solution was added into the AOT/hexane solution. Subsequently, the mixture of pyrrole (6 mmol) and P3CA (0.2 mmol) was added in the AOT/hexane solution, and then the chemical oxidation polymerization proceeded for 2 h at 15 °C. The resulting product was thoroughly washed with ethanol and allowed to dry in a vacuum oven at room temperature.

Heparin sodium salt (from porcine intestinal mucosa, 180 USP units mg^{-1}) was purchased from Sigma. In addition, 4,-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was synthesized and used as an efficient condensing agent [11–13]. To covalently attach heparin on CPPy nanotubes, 0.1 wt% CPPy nanotube ethanol solution (20 μL) was mixed with 10 wt% aq. DMT-MM solution (20 μL) and 10 wt% aq. ethylenediamine solution (20 μL) for 12 h at 1800 rpm. After washing with distilled water, the amine-functionalized nanotubes were mixed with 10 wt% aq. DMT-MM solution (20 μL) and 30 wt% aq. heparin solution (20 μL) for 2 h at 1800 rpm and then washed with distilled water.

A microarray consisting of a pair of Au interdigitated microelectrodes with 25 fingers was patterned on a glass substrate through a photolithographic process. In order to construct a FET sensor platform, the microelectrode substrate was treated with 5 wt% aq. (3-aminopropyl)trimethoxysilane (APS) solution for 6 h and then expose to the mixture of 0.1 wt% aq. CPPy nanotube solution (10 μL) and 10 wt% DMT-MM ethanol solution (10 μL) during 12 h. At the first stage, the substrate surface was modified with primary amine groups through a condensation reaction between the surface hydroxyl group and the hydrolyzed APS. The second stage involves the covalent immobilization of CPPy nanotubes via the condensation reaction between the amine groups of APS and the carboxyl groups of CPPy nanotubes. The resulting CPPy nanotubes-immobilized substrate was rinsed with distilled water. Subsequently, according to the above-mentioned procedure, heparin was covalently conjugated with the nanotubes immobilized on the microelectrode substrate.

Human α-thrombin was purchased from Sigma, and a phosphate buffered saline (PBS) solution (pH 7.4) was used for liquid-ion gating. All electrical measurements were carried out using a Kiethley 2400 SourceMeter and a Wonatech WBCS 3000 potentiostat.

3. RESULTS AND DISCUSSION

The copolymerization of pyrrole and P3CA makes it possible to introduce carboxyl side-chain groups into the polymer backbone without

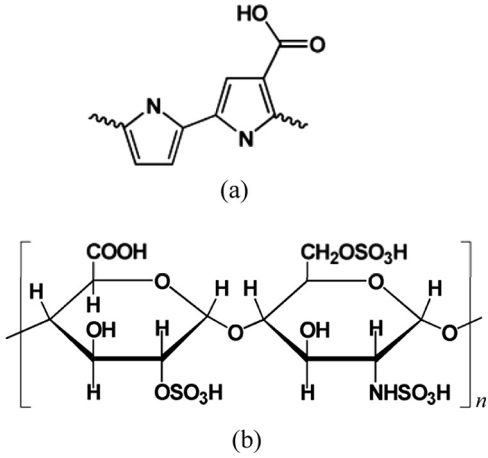
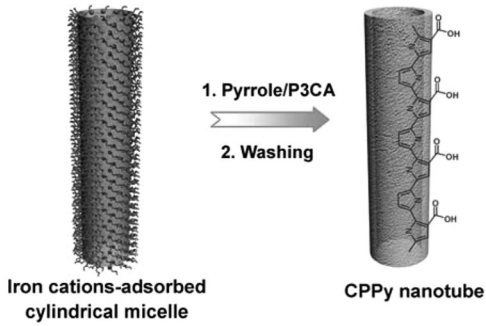


FIGURE 1 Chemical structures of (a) CPPy and (b) heparin.

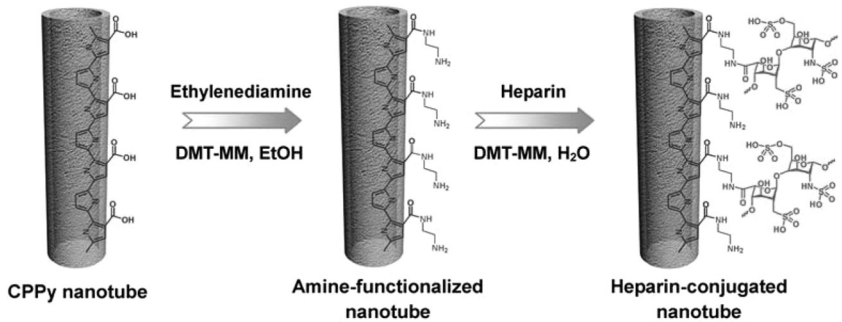
degradation in major physical properties (Fig. 1a). The chemical functionality of the copolymer is also tunable by adjusting the feeding amount of P3CA. Heparin has a carboxylic acid group in the repeating unit (Fig. 1b). Importantly, the carboxylic acid group can serve as a chemical reactive site for covalent functionalization [11–13].

Figure 2 depicts schematically the fabrication of CPPy nanotubes and the immobilization of heparin on the nanotubes. Above all, AOT cylindrical micelles were generated in an apolar solvent via a cooperative interaction between aqueous $FeCl_3$ solution and AOT [14–17]. Subsequently, CPPy nanotubes were fabricated through the chemical oxidation copolymerization of pyrrole and P3CA monomers by iron cations at the surface of AOT cylindrical micelles [18–21]. For the covalent attachment of heparin on CPPy nanotubes, at the first stage, the surface functional group of the nanotubes was modified with a diamino linker (ethylenediamine) via a condensation reaction using DMT-MM. The newly attached amino groups can act as the anchoring site for versatile molecules bearing carboxyl groups. Accordingly, the subsequent condensation reaction between the amino group of the nanotube surface and the carboxyl group of heparin allowed the covalent immobilization of heparin on the nanotubes.

Diffuse-reflectance infrared Fourier-transform (DRIFT) spectroscopy, a surface-sensitive technique, was carried out to confirm the immobilization of heparin on the nanotube surface (Fig. 3). On the spectrum of CPPy nanotubes, the peaks at 1554, 1473, and 1294/1195 cm^{-1} were attributed to pyrrole ring stretching, conjugated



(a) ***Fabrication of CPPy nanotubes***



(b) ***Immobilization of Heparin***

FIGURE 2 Schematic illustrations of (a) the fabrication of CPPy nanotubes and (b) the immobilization of heparin onto the nanotubes.

C−N stretching, and =C−H in-plane vibration, respectively. The peak of the carboxylic acid functional group was also observed at 1715 cm^{-1} due to the stretching vibration of C=O. After the immobilization of heparin, the DRIFT spectrum indicated a −OH or −NH− stretching peak around 3450 cm^{-1}, an amide stretching peak around 1620 cm^{-1}, and $-SO_3^-$ asymmetric and symmetric stretching peaks at 1235 and 1038 cm^{-1}, respectively. The $-SO_3^-$ peaks have been frequently used for the quantitative and qualitative analyses of heparin since the intensity of the peaks is not affected by the presence of water [22]. The characteristic peaks at 1235 and 1038 cm^{-1} were clearly observed as shown in Figure 3b, and this result confirmed that heparin was successfully immobilized on the nanotubes.

An interdigitated microelectrode array was patterned on a glass substrate (finger dimensions: interfinger gap 2 μm, width 2 μm, length

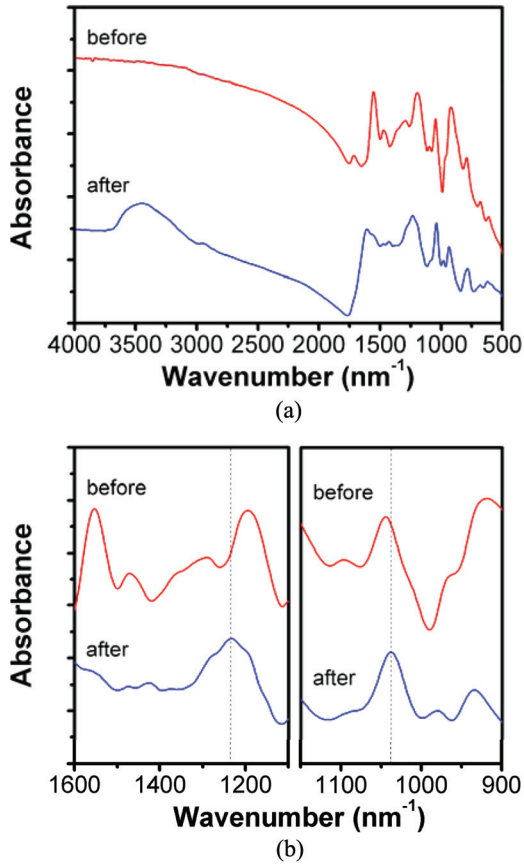


FIGURE 3 (a) DRIFT spectra of CPPy nanotubes before and after heparin attachment recorded in the range of 4000–500 cm^{-1}. (b) Close-up comparison of characteristic IR bands centered at 1235 and 1038 cm^{-1}, respectively. The spectra were obtained in transmittance mode.

1000 μm, thickness Au 20 nm/Ti 10 nm) (Fig. 4a). Conducting polymer nanomaterials are generally deposited on electrode substrates for application in electrochemical sensors. However, most conducting polymers intrinsically have poor adhesion to the substrates, and thus the electrical contact between the nanomaterials and the electrodes is one of the important factors affecting the response of conducting polymer sensors [23]. To resolve this obstacle, CPPy nanotubes were anchored onto a microelectrode substrate by an aminosilane. The substrate surface was first modified by treatment of APS and then the

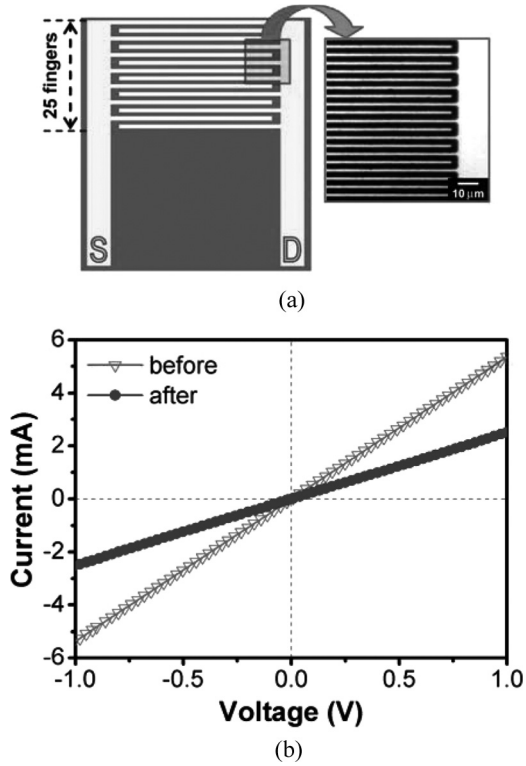
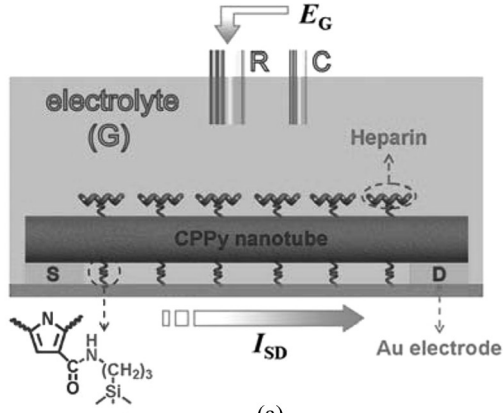


FIGURE 4 (a) Schematic illustration and optical micrograph of an interdigitated microelectrode array on glass substrate and (b) *I-V* curves of CPPy nanotubes deposited on the microelectrode before and after heparin attachment (scan rate: 10 mV s^{-1}).

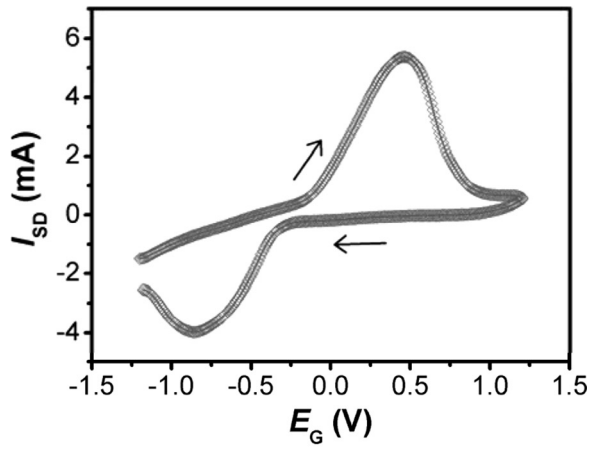
carboxyl group of the nanotubes was linked to the surface amino group of the substrate via a condensation reaction. Subsequently, heparin was covalently immobilized onto the nanotubes deposited on the microelectrode substrate according to the above-mentioned procedure. The *I-V* characteristics of CPPy nanotubes were investigated to evaluate the quality of the electrical contact on the microelectrode substrate (Fig. 4). Both before and after the immobilization of heparin, the CPPy nanotubes exhibited linear *I-V* characteristics, which indicate Ohmic behavior. When the electrical contact is poor, the *I-V* characteristic often reveals nonlinearity likely due to the formation of Schottky barriers at electrode contact [23]. As a result, it is evident that the covalent attachment of CPPy nanotubes on the substrate provides excellent electrical contact at the polymer/metal interface. The

conductivity values (dI/dV) of CPPy nanotubes on the microelectrodes were found to be around 10^{-3} s.

A FET sensor platform was constructed with the interdigitated microelectrode array using CPPy nanotubes as the conductive channel (Fig. 5a). The phosphate-buffered electrolyte (pH 7.4) was employed as a liquid-ion gate, and two interdigitated microelectrode bands were



(a)



(b)

FIGURE 5 (a) Schematic representation of an FET sensor platform based on H-CPPy nanotubs (only one nanotube is shown for clarity): the source (S), drain (D), liquid gate (G), and Ag/AgCl reference (R) and Pt counter (C) electrodes are labeled, and (b) I_{SD}-E_G curve of the FET sensor platform at $V_{SD} = 10$ mV (scan rate: ± 10 mV s^{-1}).

used as source and drain electrodes, respectively. The nanotubes were spanned over 25 source-drain electrodes, and the gate potential (E_G) was applied to the electrolyte through a reference electrode. The general feature of FETs is the modulation of source-drain current (I_{SD}) by the application of E_G. To examine the charge transport behavior of H-CPPy nanotubes in the FET configuration, the $I_{SD} - E_G$ curve was

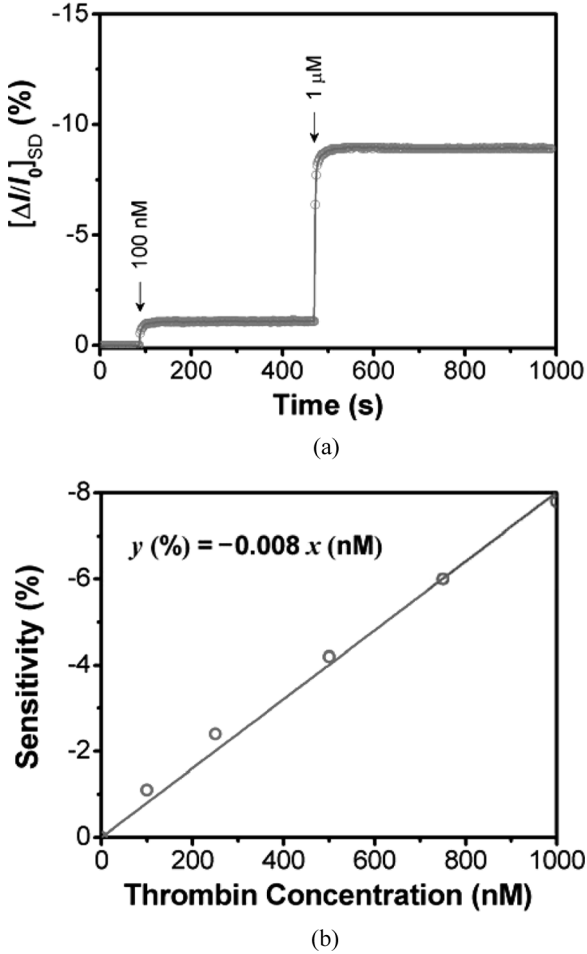


FIGURE 6 Response of the FET sensor to thrombin measured at $V_{SD} = 10$ mV: (a) real-time response (the I_{SD} change was normalized as $[(I - I_0)/I_0]_{SD} = [\Delta I/I_0]_{SD}$, where I and I_0 denote the real-time and initial current, respectively) and (b) calibration curve (the sensitivity was determined from the maximum $[\Delta I/I]_{SD}$ recorded after exposure to thrombin).

recorded at a constant source-drain voltage (V_{SD}). As shown in Fig. 5b, the dependence of I_{SD} on E_G was confirmed: namely, the redox state of H-CPPy nanotubes was dependent on the E_G applied [11,24]. The oxidation and reduction peaks at 0.46 and -0.86 V indicated that the pyrrole repeating units conjugated with heparin were accessible to oxidation/reduction events.

Figure 6 exhibits the response of the H-CPPy nanotube FET sensor upon exposure to thrombin. The I_{SD} was monitored in real-time at a constant V_{SD} (10 mV). The FET sensor displayed an abrupt decrease in I_{SD} when exposed to thrombin (Fig. 6a). The response time was less than 5 s. Heparin has a considerably high negative charge density due to its polar moieties such as sulfate and carboxyl groups. The negative charge of heparin is partially screened when the heparin-thrombin complex is formed. This has an influence on the I_{SD} flow in a manner similar to applying less negative E_G [25,26]. Consequently, the positive charge density is lowered at the surface region of CPPy nanotubes. Polypyrrole is a p-type semiconductor. Hence, the hopping rate of charge carriers decreases in CPPy nanotubes, giving rise to the decrease in I_{SD}. A control experiment was performed using CPPy nanotubes with no heparin attached. The FET sensor did not give any measurable changes in I_{SD} when exposed to thrombin. This result supports that the sensor response arises from specific interaction between heparin and thrombin. The change in I_{SD} was susceptible to the concentration of thrombin. The calibration curve is plotted in Figure 6b. The sensitivity became higher with increasing the concentration of thrombin and presented linear behavior in the concentration range of 10^2-10^3 nM.

4. CONCLUSIONS

A liquid-ion gated FET sensor for the recognition of thrombin protein was successfully fabricated with H-CPPy nanotubes. The carboxyl groups of CPPy nanotubes made it possible to immobilize the nanotubes on a sensor substrate and to bind heparin on the nanotubes without any sophisticated surface treatment. The FET sensor gave a rapid decrease in I_{SD} upon exposure to thrombin. The sensing mechanism involved the reduction of charge carrier density in CPPy nanotubes by screening the negative charge of heparin.

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