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3,4,9,10-Perylenetetracarboxylic Acid/Hemin Nanocomposites Act as Redox Probes and Electrocatalysts for Constructing a Pseudobienzyme-Channeling Amplified Electrochemical Aptasensor

Yali Yuan, Ruo Yuan,* Yaqin Chai, Ying Zhuo, Xianxue Gan, and Lijuan Bai[a]

Abstract: A simple wet-chemical strategy for the synthesis of 3,4,9,10-perylenetetracarboxylic acid (PTCA)/hemin nanocomposites through π–π interactions is demonstrated. Significantly, the hemin successfully conciliates PTCA redox activity with a pair of well-defined redox peaks and intrinsic peroxidase-like activity, which provides potential application of the PTCA self-derived redox activity as redox probes. Additionally, PTCA/hemin nanocomposites exhibit a good membrane-forming property, which not only avoids the conventional fussy process for redox probe immobilization, but also reduces the participation of the membrane materials that act as a barrier of electron transfer. On the basis of these unique properties, a pseudobienzyme-channeling amplified electrochemical aptasensor is developed that is coupled with glucose oxidase (GOx) for thrombin detection by using PTCA/hemin nanocomposites as redox probes and electrocatalysts. With the addition of glucose to the electrolytic cell, the GOx on the aptasensor surface bioelectrocatalyzed the reduction of glucose to produce H_2O_2, which in turn was electrocatalyzed by the PTCA/hemin nanocomposites. Cascade schemes, in which an enzyme is catalytically linked to another enzyme, can produce signal amplification and therefore increase the biosensor sensitivity. As a result, a linear relationship for thrombin from 0.005 to 20 nM and a detection limit of 0.001 nM were obtained.

Keywords: aptamers · biosensors · electrochemistry · nanostructures · redox chemistry

Introduction

Nowadays, the application of nanomaterials for the design and fabrication of simple, sensitive, selective electrochemical biosensors has received much attention in biotechnology, biochemistry, and medicine. 3,4,9,10-Perylenetetracarboxylic acid (PTCA), an archetypal organic perylene dye with desirable organic electronic and optical properties, has recently emerged as one of the most fascinating materials.[1–5] Owing to its large specific surface area, good membrane-forming property, flat π system, and low manufacturing cost, the use of PTCA as the starting material to produce nanocomposites has turned out to be an efficient, simple, and economic process for constructing highly sensitive electrochemical sensors.[6–11] Commonly, among these studies, PTCA just served as a semiconducting template with the additional introduction of complicated redox probes by an immobilization process. The application of PTCA self-derived redox activity in redox probes has been rarely explored. In fact, the perylene dye has redox activity owing to its electronic properties; however, there were miscellaneous redox peaks in electrochemical characterization (e.g., cyclic voltammetry), which led to potential signal interference in target quantitative determination.[12,13] How to conciliate the PTCA redox activity with an appropriate material to obtain a pair of well-defined redox peaks that serve as a redox probe in electrochemical sensors is quite challenging.

Hemin (metalloporphyrin) is the active center of heme proteins, such as peroxidase, cytochromes, hemoglobin, and myoglobin.[14] It possesses a large delocalized π system that can be absorbed at substantial materials through π–π interaction. More importantly, it also has a peroxidase-like activity similar to that of peroxidase, which can catalyze H_2O_2 to oxidize the peroxidase substrate.[15] Herein, for the first time, we successfully conciliate the PTCA redox activity with hemin to obtain a pair of well-defined redox peaks. The novel PTCA/hemin nanocomposite can act as a redox probe and outstanding electrocatalyst at the same time with analogous properties to those of Prussian blue (artificial peroxidase), thus providing excellent opportunities for applications in the fields of artificial enzyme mimetics, electrochemical biosensors, and electrocatalysis. Additionally, the novel PTCA/hemin nanocomposite might inherit the property of PTCA that forms membranes directly on the electrode surface, which not only avoids conventional fussy redox probe immobilization methods such as entrapment,[16] covalent linking,[17] or self-assembly,[18,19] but also reduces the

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participation of the membrane materials that acted as a barrier to electron transfer.

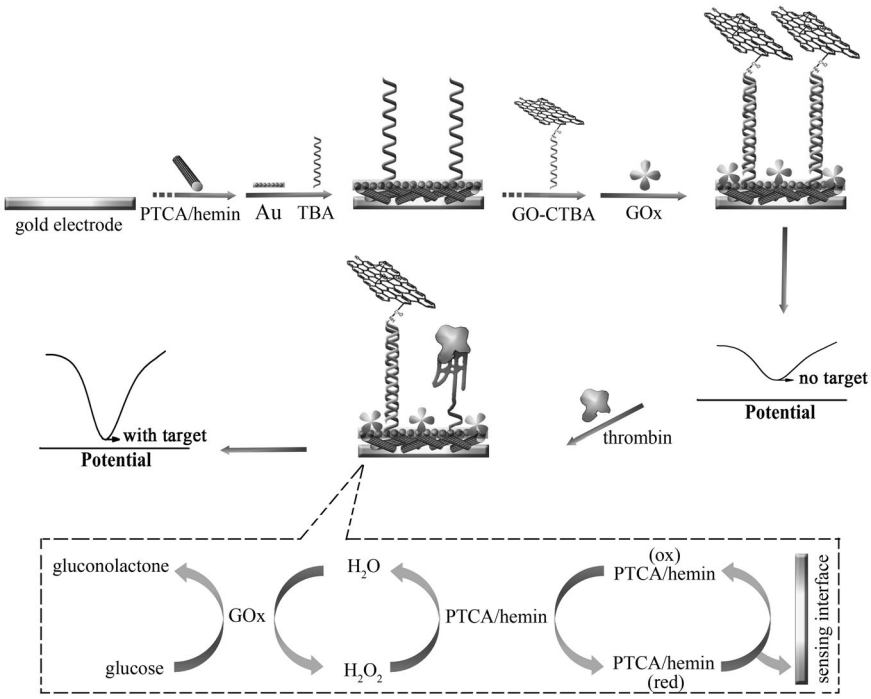
On the basis of this novel nanocomposite, we have developed a pseudobienzyme-channeling amplified electrochemical aptasensor for thrombin detection that couples glucose oxidase (GOx) and PTCA/hemin nanocomposites. Here, GOx also serves as a blocking reagent to block possible remaining active sites and avoid nonspecific adsorption, instead of requiring the use of a conventional reagent such as bovine serum albumin (BSA),[20] hexanethiol,[21] or 6-mercapto-1-hexanol.[22] With the addition of glucose to the electrolytic cell, the GOx on the aptasensor surface bioelectrocatalyzed reduction of glucose to produce H_2O_2, which in turn was electrocatalyzed by the PTCA/hemin nanocomposites. Cascade schemes, in which an enzyme is catalytically linked to another enzyme, can produce signal amplification and therefore increase the biosensor sensitivity.[23] Furthermore, in comparison to the employment of H_2O_2 as the enzyme substrate, the presence of glucose might improve the stability and prolong the lifetime of the aptasensor, due to its inert electrochemical property. A schematic diagram of the stepwise self-assembly procedure of the aptasensor and electrocatalysis detection principle is shown in Scheme 1.

Results and Discussion

Characterization of the PTCA/hemin nanocomposites: The morphology of the as-synthesized samples was studied by scanning electron microscopy (SEM) and high-resolution transmission electron microscopy (HRTEM), with images shown in Figure 1. The PTCA/hemin nanocomposites show irregular quadrate-shaped molecular islands with large specific surface area (Figure 1A). Figure 1B exhibits a magnified HRTEM image of one of the quadrate-shaped molecular islands. Additionally, a further magnified image for PTCA/hemin nanocomposites is displayed in Figure 1C.

The combination of PTCA with hemin was characterized by Fourier transform infrared (FTIR) spectroscopy. The FTIR spectra of hemin, PTCA, and PTCA/hemin nanocomposites are shown in Figure 1D. The hemin shows typical absorption peaks at 937.3 and 1701.0 cm^{-1} (Figure 1D, curve a),[24] which correspond to porphyrin cyclic organic structures and the C=O stretch of the carboxylic acid groups, respectively. In the FTIR spectrum of PTCA (Figure 1D, curve b), the peak at 1593 cm^{-1} is assigned to the aromatic $\tilde{\nu}$(C=C) stretching vibration in PTCA. The very broad stretching bands $\tilde{\nu}$(C=O) at 1772.4 cm^{-1} are assigned to the carboxylic acid groups of PTCA.[10,25] Some characteristic peaks of hemin and PTCA are observed in the spectrum of the PTCA/hemin nanocomposites (e.g., 1593.0, 1631.6, and 1772.4 cm^{-1}; Figure 1D, curve c). However, the absorption peaks of PTCA/hemin nanocomposites shifted from 937.3 and 1701.0 cm^{-1} to 1020.2 and 1755.0 cm^{-1}, respectively, with the evaporation of the absorption peak at 1701.0 cm^{-1} and decrease in the band at 1772.4 cm^{-1}, which indicates the successful combination of PTCA with hemin through the π–π interaction and hydrogen bonding between PTCA and the porphyrin cyclic organic structures of hemin.[26]

X-ray photoelectron spectroscopy (XPS) analysis provides effective information on the chemical composition of the as-prepared PTCA/hemin nanocomposites. To verify the modification process, besides PTCA/hemin nanocomposites we employed PTCA without the addition of hemin as a blank sample. As shown in Figure 1E, both the C and O peaks appeared in the XPS survey spectrum for PTCA (curve a) and PTCA/hemin nanocomposites (curve b), whereas the Fe and N peaks appeared in the XPS survey spectrum only for PTCA/hemin nanocomposites, thereby suggesting the possible combination of PTCA with hemin.



Scheme 1. Schematic representation of the stepwise self-assembly procedure and electrocatalysis detection principle of the proposed electrochemical aptasensor. TBA = thrombin aptamer, GO = graphene oxide, CTBA = complementary thrombin aptamer, GOx = glucose oxidase.

Electrochemical redox behavior and peroxidase-like activity of PTCA/hemin nanocompo-

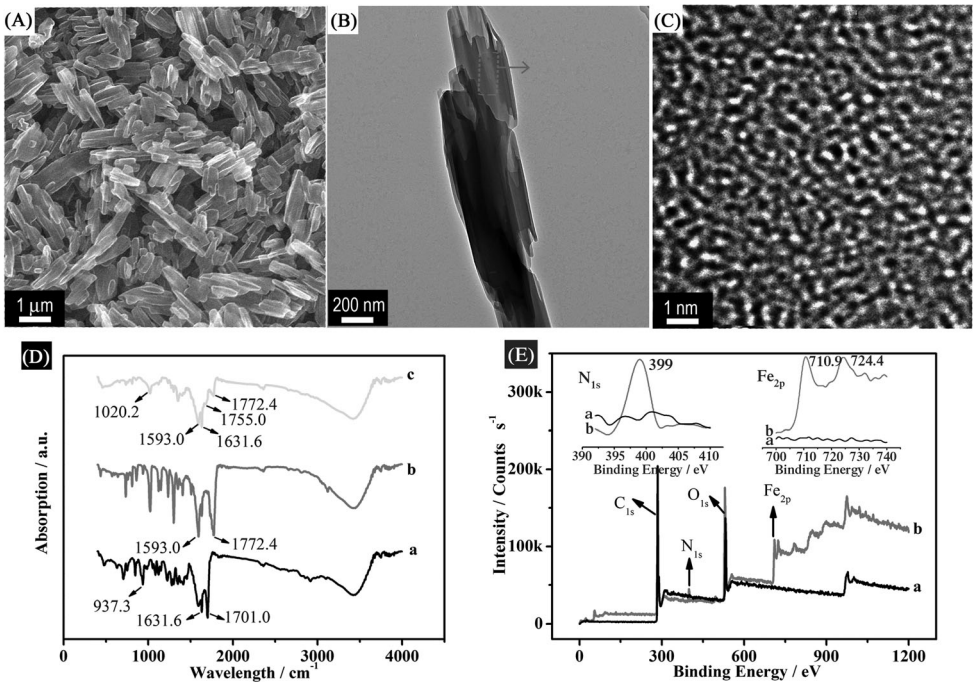


Figure 1. A) SEM image and B,C) HRTEM images of PTCA/hemin nanocomposites. D) FTIR spectra of hemin (curve a), PTCA (curve b), and PTCA/hemin nanocomposites (curve c). E) XPS of PTCA (curve a) and PTCA/hemin nanocomposites (curve b).

sites: To demonstrate that the interaction between hemin and PTCA would produce a novel redox probe and electrocatalyst, PTCA and the PTCA/hemin nanocomposite were directly dropped on gold electrode surfaces and subjected to cyclic voltammetry analysis in 0.10 M sodium phosphate buffer (PBS) containing 10 mM KCl (pH 7.0). As shown in Figure 2 A, owing to the desirable organic electronics of PTCA, the PTCA showed miscellaneous redox peaks in the potential region from −0.7 to 0.1 V. However, interestingly, the PTCA/hemin nanocomposites showed a pair of well-defined redox peaks. This phenomenon could be ascribed to the π–π interaction and hydrogen bonding between PTCA and porphyrin cyclic organic structures of hemin, which increase the resonance energy and delocalize charge effectively, finally bringing about a remarkable synergistic action with a pair of well-defined redox peaks.[27] Figure 2 B displays the PTCA/hemin nanocomposite-modified Au electrode investigated in PBS with and without H_2O_2. With the addition of H_2O_2, the biosensor triggers a bioelectrocatalytic reaction, which dramatically changes the cyclic voltammogram with an obvious increase in reduction peak current and a concomitant small increase in the oxidation peak current, which suggests that the PTCA/hemin nanocomposites have superior peroxidase-like activity similar to that of peroxidase enzyme. These results demonstrate that the PTCA/hemin nanocomposites act as not only novel redox probes, but also outstanding electrocatalysts towards H_2O_2. Thus, we developed a pseudobienzyme-channeling amplified electrochemical aptasensor that was coupled with GOx for thrombin detection by using PTCA/hemin nanocomposites as redox probes and electrocatalysts. The characteristics of the stepwise immobilization for aptasensor fabrication are shown in Figure S1 (see the Supporting Information).

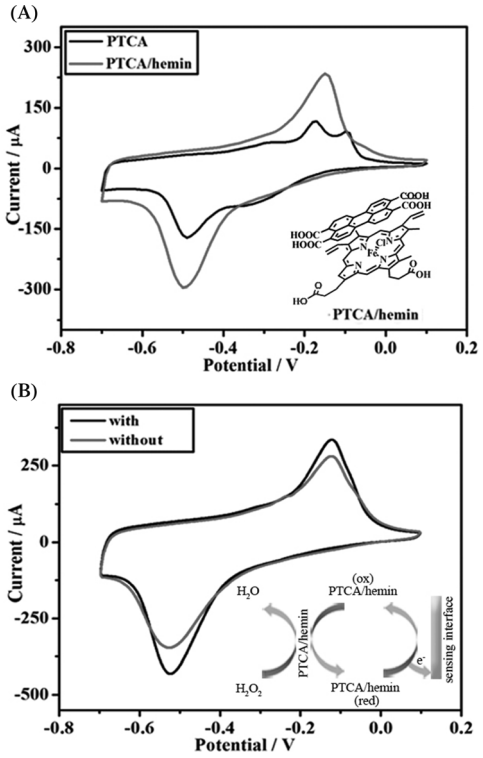


Figure 2. A) Cyclic voltammetry analysis of PTCA and PTCA/hemin nanocomposite-modified electrode in 0.10 M PBS. B) Cyclic voltammetry analysis of PTCA/hemin nanocomposite-modified electrode in 0.10 M PBS without and with H_2O_2.

Pseudobienzyme-channeling amplified aptasensor: Herein, GOx was employed for blocking nonspecific binding sites to construct the pseudobienzyme-channeling amplified electrochemical aptasensor. Figure 3 shows the electrochemical

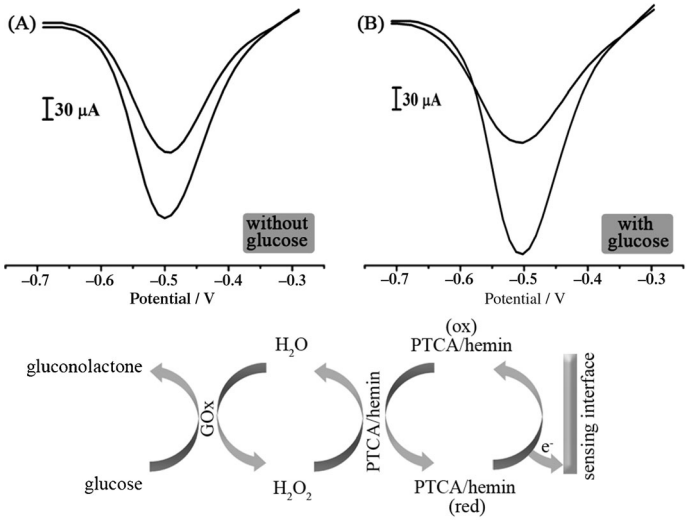


Figure 3. Signal changes of the cathodic peak (before and after 10 nM thrombin incubation) with (A) and without (B) 3.0 mM glucose in the electrolytic cell.

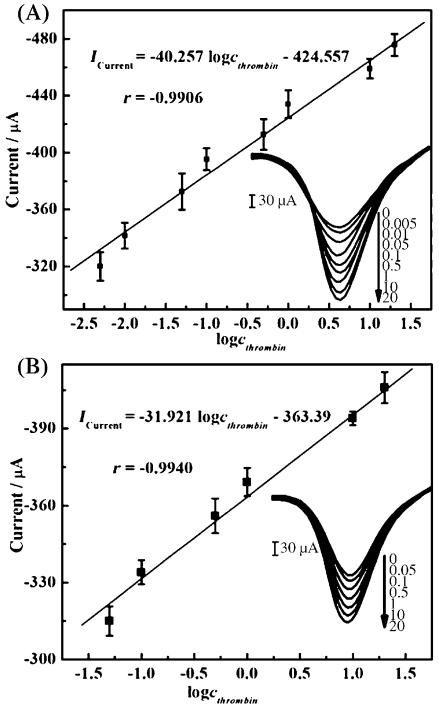


Figure 4. Calibration plots of the cathodic peak current response versus concentration of thrombin. The aptasensors were investigated in electrolytic cells with (A) and without (B) 3.0 mM glucose. The insets show the partial cyclic voltammograms of the cathodic peak at different concentrations.

signal changes (before and after incubation) with (Figure 3A) and without (Figure 3B) 3.0 mM glucose in the electrolytic cell. With glucose in the electrolytic cell, the GOx on the aptasensor surface bioelectrocatalyzed the reduction of glucose to produce H_2O_2, which in turn was electrocatalyzed by the PTCA/hemin nanocomposites. As a result, the electrochemical signal with glucose in the electrolytic cell changed much more, thereby indicating that the pseudobienzyme-channeling strategy could effectively improve the sensitivity of the proposed aptasensor as expected.

Analytical performance of protein detection: In protein quantitative analysis, the pseudobienzyme-channeling amplified electrochemical aptasensor was incubated with different concentrations of thrombin and then subjected to cyclic voltammetry analysis in an electrolytic cell containing 3.0 mM glucose. After 40 min of incubation, thrombin was captured on the aptasensor sensing interface, which led to the release of inert graphene oxide labeled complementary thrombin aptamer (GO-CTBA) from the self-assembled duplex on the electrode into solution, thus decreasing the steric hindrance of the aptasensor and facilitating pseudobienzyme-channeling amplification with an enhanced electrochemical signal. As a result, the reduction peak current increased with the increasing concentration of thrombin in the range from 0.005 to 20 nM and showed a linear relationship with the logarithm of thrombin concentration (Figure 4A). The linear equation was $I\,[\mu A] = -40.2578 \log c_{\text{thrombin}}\,[\text{nM}] - 424.457$ with a correlation coefficient of 0.9906, and an evaluated detection limit of 0.001 nM (defined as 3σ, in which σ is the standard deviation of the blank sample) could be obtained. To demonstrate that the pseudobienzyme-channeling strategy could effectively improve the sensitivity of the proposed aptasensor, a comparative study of the electrochemical responses was carried out by placing the aptasensor in an electrolytic cell without 3.0 mM glucose under analogous conditions. The aptasensor showed a much lower reduction peak current and inconspicuous signal changes with a linear range of thrombin from 0.05 to 20 nM (Figure 4B). Thus, the pseudobienzyme-channeling strategy effectively improved the sensitivity of the proposed aptasensor, as expected.

Specificity and analytical application of the proposed electrochemical aptasensor: The specificity of the proposed electrochemical aptasensor was evaluated by challenging it against other biomolecules, and the results are presented in Figure 5. With many nontarget molecules, such as BSA and bovine hemoglobin (BHB), no apparent change in the current was observed compared with that of the thrombin test. Moreover, the mix (containing thrombin, BSA, and BHB with similar concentrations to each component mentioned above) also showed negligible changes compared with that of the thrombin test. However, the presence of the target analytes resulted in a dramatic increase in the current, which suggests that the electrochemical aptasensor is specific to thrombin and possesses high specificity. To demonstrate the applicability of the proposed protein detection

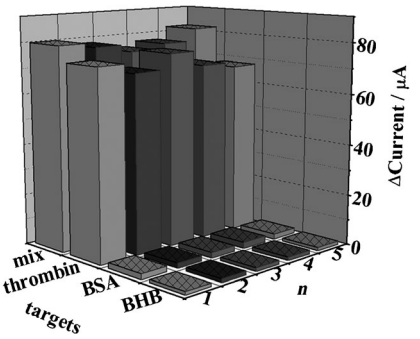


Figure 5. Specificity of the proposed electrochemical aptasensor (*n* denotes the number of parallel experiments).

technology to clinical diagnosis, the standard addition method was employed and a series of samples was prepared by adding thrombin of different concentrations to human blood serum (obtained from Ninth People's Hospital of Chongqing, China). Significantly, we found that the recovery and relative standard deviation with acceptable values ranged from 90.3 to 109% and 4.7 to 8.1%, respectively. This means that the electrochemical aptasensor is promising for analytical application in real biological samples.

Conclusion

PTCA/hemin nanocomposites have been successfully prepared by a simple wet-chemical strategy through π–π interactions. These new nanocomposites exhibit large specific surface area, good membrane-forming property, intrinsic peroxidase-like activity, and a pair of well-defined redox peaks. On the basis of the unique properties of the PTCA/hemin nanocomposites, we have fabricated a highly sensitive electrochemical aptasensor that uses a pseudobienzyme-channeling amplification strategy in cooperation with blocking reagent GOx. Compared with previous enzyme-based aptasenors, this strategy was much simpler. Moreover, owing to the unique properties of the PTCA/hemin nanocomposites, they will provide excellent opportunities for applications in fields such as artificial enzyme mimetics, electrochemical biosensors, and electrocatalysis.

Experimental Section

Materials and reagents: 3,4,9,10-Perylenetetracarboxylic dianhydride ($C_{24}H_8O_6$, PTCDA) was bought from Lian Gang Dyestuff Chemical Industry Co. Ltd. (Liaoning, China). Graphene oxide (GO) was obtained from Nanjing Xianfeng Nano Co. (Nanjing, China). Thrombin, hemin, glucose oxidase (GOx), bovine serum albumin (BSA), hexanethiol (96%), bovine hemoglobin (BHB), and gold chloride ($HAuCl_4$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *N*-Hydroxysuccinimide and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride were acquired from Shanghai Medpep Co. (Shanghai, China). The DNA sequences used in this study were:

thrombin aptamer (TBA): 5′-SH-$(CH_2)_6$-GGTTGGTGTGGTTGG-3′

complementary thrombin aptamer (CTBA): 5′-NH_2-$(CH_2)_6$-CCAACCACACCACCACC-3′

All oligonucleotides were synthesized by TaKaRa (Dalian, China). GO-labeled CTBA was prepared by covalent bonding between the carboxyl of GO and amido group of CTBA. All other chemicals were of reagent grade and used as received. Doubly distilled water was used throughout.

Apparatus: Atomic force microscopy (AFM) images were acquired by scanning probe microscopy (SPM; Vecco, USA). FTIR characterization was performed by using a Spectrum GX FTIR spectroscopy system (Perkin–Elmer, USA). Scanning electron micrographs were taken with a scanning electron microscope (S-4800, Hitachi). XPS measurements were conducted with a VG Scientific ESCALAB 250 spectrometer, with $Al_{K\alpha}$ X-rays (1486.6 eV) as the light source. All electrochemical measurements were performed on a CHI 660C electrochemical workstation (Shanghai Chenhua Instrument Co. Ltd., China) with a conventional three-electrode system composed of platinum wire as auxiliary electrode, a saturated calomel electrode as reference, and a modified gold electrode as working electrode. The scan rates were 100 $mV\,s^{-1}$.

Preparation of PTCA/hemin nanocomposites: PTCA/hemin nanocomposites were prepared with PTCA as a template by a simple π–π stacking approach. In a typical experiment, hemin (1 mg) was dispersed in an aqueous solution (1 mL) containing ammonia (20 μL). At the same time, PTCA was synthesized from hydrolyzed PTCDA by a modified Caruso method.[28] Then, the prepared PTCA (1 mg) was added to the hemin aqueous solution with magnetic stirring for 36 h at 25°C. After centrifugation at 12000 rpm for 15 min to remove surplus hemin, the sediment of the resulting PTCA/hemin nanocomposites was resuspended in doubly distilled water and stored at 4°C for further use.

Fabrication of the pseudobienzyme-channeling amplified electrochemical aptasensor: Prior to modification, a gold electrode (4 mm in diameter) was polished with 0.3 and 0.05 μm alumina slurries, rinsed thoroughly with doubly distilled water between each polishing step, washed successively with ethanol and doubly distilled water in an ultrasonic bath, and dried in air. A PTCA/hemin nanocomposite-modified Au electrode (PTCA/hemin/Au) was obtained by casting the prepared PTCA/hemin suspension (5 μL) on the surface of the well-polished Au electrode, which was dried in air. For thiol-terminated TBA immobilization, the PTCA/hemin/Au was immersed in an aqueous solution containing 1% $HAuCl_4$ for 30 s of electrodeposition. Afterwards, thiol-terminated TBA was immobilized on the PTCA/hemin/Au substrate by placing a droplet (20 μL) of 2.5 μM TBA solution (in 20 mM Tris–HCl buffer, 140 M NaCl, pH 7.4) on the surface for 16 h and producing a self-assembled monolayer of TBA. After modification, a droplet of graphene oxide labeled complementary thrombin aptamer (GO-CTBA) solution (20 μL; 2.5 μM cetyltrimethylammonium bromide in 20 mM Tris–HCl buffer), in which inert GO was employed to increase the steric hindrance effect of CTBA, was placed on the modified electrode for 2 h to obtain a GO-CTBA/TBA/nano-Au/PTCA/hemin/Au electrode by hybridization between TBA and CTBA. Finally, the resulting electrode was incubated for 6 h in GOx solution (1 $mg\,mL^{-1}$) to block nonspecific binding sites on the electrode surface. After every step, the electrode was extensively rinsed with washing buffer (0.1 M sodium phosphate buffer, PBS, pH 7.0) and dried under a stream of nitrogen prior to electrochemical characterization.

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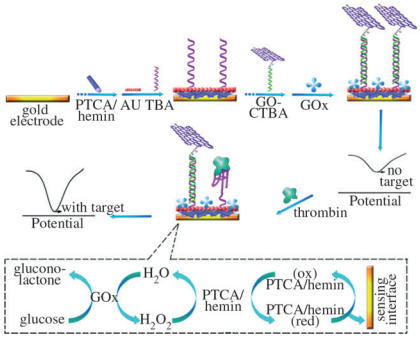

Aptasensors

Y. Yuan, R. Yuan, Y. Chai, Y. Zhuo, X. Gan, L. Bai* ..... ▮▮▮▮–▮▮▮▮

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