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A simple assay to amplify the electrochemical signal by the aptamer based biosensor modified with CdS hollow nanospheres

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ABSTRACT

A simple method to amplify the electrochemical signal by an aptamer with 22 bases modified with CdS hollow nanospheres (CdSHNs) was described. Using the thrombin as a model, the interaction between the aptamer and CdSHNs was characterized by cyclic voltammetry, electrochemical impedance spectroscopy and circular dichroism spectroscopy. CdSHNs promoted the electron transfer between the gold electrode and $K_3[Fe(CN)_6]$ and facilitated the conformation conversion of the aptamer from hairpin to G-quadruplex after the aptamer interacted with thrombin. Under optimal conditions, the modified electrode could be used for the determination of thrombin from 0 to 33 $\mu g\,mL^{-1}$ and the sensitivity was 1.34 $\mu A\,mL\,\mu g^{-1}\,cm^{-2}$, while the linear range of the modified electrode without the immobilization of CdSHNs was from 2.75 to 27.5 $\mu g\,mL^{-1}$ and the sensitivity was 0.062 $\mu A\,mL\,\mu g^{-1}\,cm^{-2}$. This constructed biosensor also had a good stability, specificity, reproducibility and accuracy which could provide a promising platform for fabrication of aptamer based biosensors.

1. Introduction

Aptamers are nucleic acid ligands which have been designed through an in vitro selection process. This process is called SELEX which is shortened from Systematic Evolution of Ligands by Exponential Enrichment (Tuerk and Gold, 1990; Ellington and Szostak, 1990). They show a large number of specific binding affinities with many substances such as proteins (thrombin and ATP) (Liu et al., 2009), carbohydrates, amino acids, drugs, lipids, and other small molecules (Bang et al., 2005; Xu et al., 2006; Centi et al., 2007). The aptamers have many advantages compared with antibodies, such as easier artificial synthesis, good stability and reproducibility, low cost and wider applicability (Zheng et al., 2007; Willner and Zayats, 2007; Lee et al., 2008). So far, various kinds of aptamer based protein biosensors have been developed, such as fluorescence detection assays (Pavlov et al., 2005; Wang and Liu, 2009; Wang et al., 2009), surface plasmon resonance (SPR) (Tang et al., 2007; Ostatná et al., 2008), quartz crystal microbalance (QCM) (Liss et al., 2002; Hianik et al., 2005, 2007), chromatographic assays (Zhao et al., 2008) and chemiluminescence (Li et al., 2010). However, they are difficult to be operated and they are also time consuming, expensive and even nonportable. Recently, the electrochemical methods have been receiving more attention than other assays mentioned above due to their rapid response, miniaturization of the instruments, low cost, high selectivity and sensitivity.

Thrombin is a very important serine protease in the blood coagulation cascade which facilitates the blood clotting by converting fibrinogen into fibrin. It is regarded as a tumor marker in the diagnosis of pulmonary metastasis (Lee et al., 2008; Liu et al., 2009) and the concentration of thrombin in blood is associated with coagulation abnormalities. A low concentration of thrombin in blood can cause oxidative stress, hypoglycemia, hypoxia, and growth supplement deprivation, while high concentration of thrombin can cause degeneration and cell death in both astrocyte and hippocampal neuron (Wang and Liu, 2009). It is of great importance to develop biosensors to monitor thrombin in blood serum with high sensitivity, selectivity, and simplicity for both clinical practice and diagnostic applications. The thrombin has two exosites: fibrinogen-recognition exosite and heparin-binding exosite, which can interact with aptamer.

Nanoparticles have been used to detect different proteins such as lysozyme, IgG and thrombin in recent years (Deng et al., 2009; Yang et al., 2009). Generally, the immobilization of the nanoparticles on the electrode increases the surface area of the electrode and promotes the absorption capability of the electrode. Sometimes the nanoparticles are used as electroactive labels in which the most common method is to detect the amplified detection signal by stripping voltammetry indirectly (Ho et al., 2009; Yu et al., 2009). For example, Numnuam et al. (2008) detected the thrombin with the solid contact Cd^{2+}-selective polymer membrane electrode

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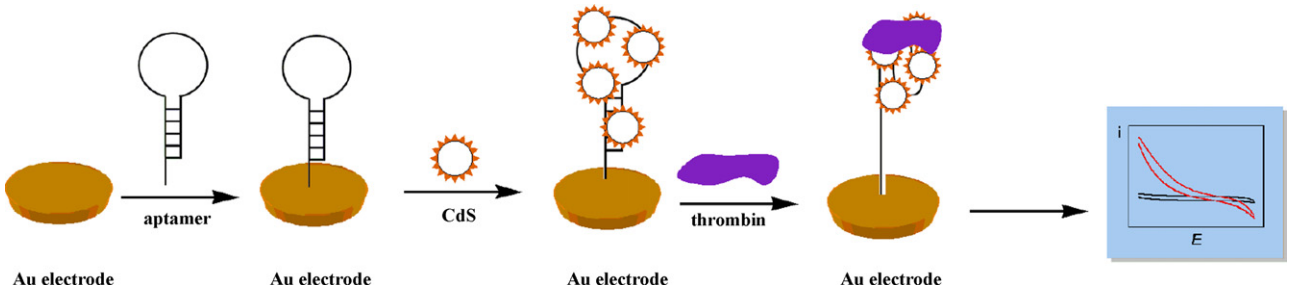

Scheme 1. Schematic representation of the modification process of the electrode and the detection of thrombin.

by dissolving CdS which was labeled on the secondary aptamer with hydrogen peroxide. Yang et al. (2009) used a sandwich type to detect the human thrombin using the CdSe quantum dots (QDs) as an electrochemical label, which was detected by the square wave stripping voltammetric analysis after dissolving CdSe with nitric acid. However, CdS or CdSe labeled aptamers were prepared by the interaction of biotin and streptavidin or avidin for a long time and they also needed to be dissolved to detect Cd^{2+} in the solution upon the detection of the thrombin. In this work, without preparing CdS labeled aptamer to detect Cd^{2+}, CdSHNs were directly dripped onto the aptamer modified gold electrode and the electrocatalytic responses were measured directly. At both the ends of the selected aptamer, the bases are CCAA and GGTT, respectively, which are complementary and can be matched easily. The middle bases are uncomplementary and they cannot match with each other, indicating that the aptamer was a hairpin form. The short stem structure also can be used as a transducing element of the signal generated by the loop aptamer of high binding affinity to molecules. The results demonstrated that the constructed biosensor had better responses compared with those without the immobilization of CdSHNs. The CdSHNs promoted the electron transfer between the gold electrode and $K_3[Fe(CN)_6]$ and facilitated the conformation conversion of the aptamer after addition of thrombin. To the best of our knowledge, there have been no reports similar to our assay which may have a great potential in clinical applications.

2. Experimental

2.1. Reagents and materials

The aptamer was purchased from Bioneer (Shanghai, China), the 5′-terminus of the aptamer was modified by the aliphatic amine and it contained 22 bases with its sequence as follows: 5′-NH_2-$(CH_2)_6$-TTC CAA CGG TTG GTG TGG TTG G-3′.

Thrombin (500 U), $C_8H_{17}N_3HCl$ (EDC), 11-mercaptoundecanoic acid (MUA), N-hydroxysuccinimide (NHS), chitosan, bovine serum albumin (BSA), lysozyme and IgG were purchased from Aldrich and were used without further purification. Serum samples were provided by Jiangsu Institute of Cancer Prevention and Cure and they were obtained by centrifugation of blood for about 5 min with the rotation rate of 3000–4000 rpm. Chitosan (0.5%, w/v) solution was prepared by dissolving 50 mg chitosan in 2 M acetic acid and then diluting to 10 mL with water. Other chemicals were all of analytical reagent grade.

2.2. Preparation of the CdSHNs and the modified electrode

The CdSHNs were prepared according to the literature (Dai et al., 2007). In brief, a solution of polyglycol was prepared by dissolving 5.0 g polyglycol (Mw 20,000) into 25 mL deionized water. Then 0.154 g $Cd(NO_3)_2{\cdot}4H_2O$ and 0.120 g $Na_2S{\cdot}9H_2O$ were dissolved in 10 mL and 12 mL solutions of polyglycol to make solutions A and B, respectively. Then 2 mL solution B was injected slowly into 2 mL solution A under sonication at 30–40 °C. After the injection was completed, the solution was further sonicated for 10 min. Finally, the produced yellow precipitate was centrifuged from the mixture. The deposit was collected, washed with deionized water for several times and dried under vacuum. Yellow CdSHNs were obtained.

The modification process of the electrode is shown in Scheme 1. The gold electrode (2 mm in diameter) was manually polished with 1, 0.3 and 0.05 μm Al_2O_3 successively, washed with deionized water, ultrasonically cleaned with ethanol and distilled water, and finally blown dry with nitrogen. The clean gold electrode was dipped into the MUA solution for about 18 h, then it was rinsed with freshly made EtOH:H_2O (v:v, 3:1) solution and the deionized water and dried with nitrogen. Next, the gold electrode was put into the phosphate buffer solution which contained EDC and NHS for about 1 h, then washed with deionized water and ethanol, respectively, and blown dry with nitrogen again. Finally, the gold electrode was dipped into the Tris–HCl buffer solution containing the aptamer for 2 h and then was rinsed with Tris–HCl butter solution and dried with nitrogen. Thus, the aptamer modified gold electrode was obtained. 10 mg CdSHNs were mixed with 0.5 mL water to obtain 20 mg mL^{-1} CdSHNs suspension. 2.5 μL of the CdSHNs suspension was dripped onto the aptamer modified gold electrode, followed by adding a chitosan membrane (after it was dried naturally) to block the CdSHNs leaking from the electrode. The control fluorescence method was followed by the literature (Jin et al., 2010). First, the fluorescence spectra of crystal violet were recorded on a fluorometer (F-7000, Hitachi) with excitation at 590 nm and an emission range from 615 to 750 nm. Then, a certain volume of 2.5 mM aptamer was added into the 5 mM crystal violet solution. The fluorescence of this mixture was recorded after incubating for 30 min. For the study of thrombin interaction with crystal violet, the solutions of thrombin varied from 0.1 to 10 μg mL^{-1} were added into the above mixture for fluorescence detection.

2.3. Measurements

Cyclic voltammetry (CV) was performed on CHI 660B electrochemical workstation (CH Instruments, USA) using the modified electrode as a working electrode, a platinum wire as an auxiliary electrode and Ag/AgCl as a reference electrode at room temperature (25 ± 2 °C). Electrochemical impedance spectroscopic (EIS) measurements were carried out on a PGSTAT30/FRA2 system (Autolab, Netherlands) in 0.1 M KCl solution containing 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. Circular dichroic (CD) measurements were made on JASCO Model J-810 dichrograph (Japan Spectroscopic Co. Ltd., Tokyo, Japan) at room temperature (25 ± 2 °C) in a 1 cm quartz cuvette. The morphology and particle size of the samples were characterized by JEM-200CX transmission electron microscopy (TEM) working at 200 kV.

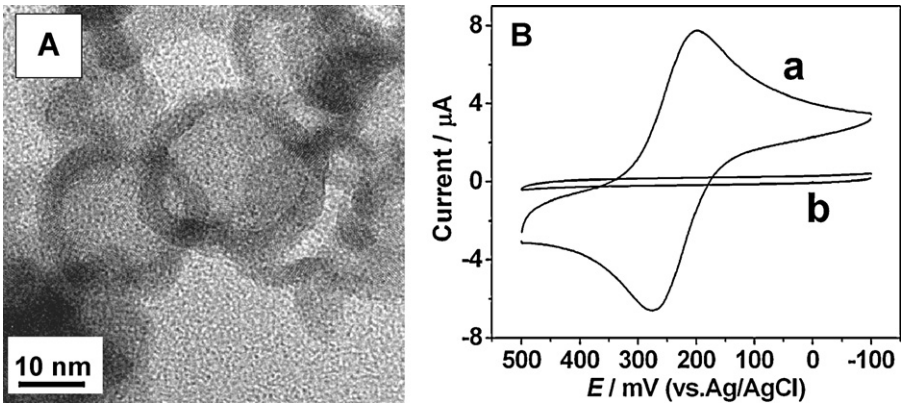

Fig. 1. (A) The TEM image of the CdSHNs; (B) CVs of the bare (a) and the aptamer modified (b) gold electrodes in 1 mM $K_3[Fe(CN)_6]$ solution containing 0.1 M KCl. Scan rate: 100 mV s^{-1}.

3. Results and discussion

3.1. The TEM of the CdSHNs

The TEM image of the CdSHNs is shown in Fig. 1A. From the image, we can see the pale colour regions in the central parts in contrast to dark edges, implying a hollow spherical structure. Moreover, the unchanged contrast difference between the center and edge in the TEM images of one sphere is obtained when the sample grid is rotated by different degrees which proves their hollow structures. The average diameter of the hollow spheres is about 15 nm mainly ranging from 12 to 18 nm.

3.2. The immobilization of aptamer on the gold electrode

To demonstrate that the aptamer was immobilized on the gold electrode, CV signals of the bare and the aptamer modified gold electrodes were performed, respectively. An obvious quasi-reversible redox peak on the bare gold electrode in 1 mM $K_3[Fe(CN)_6]$ solution containing 0.1 M KCl was obtained (curve a in Fig. 1B). When the gold electrode was immobilized with aptamer, the redox peaks disappeared (curve b in Fig. 1B). Because the backbone phosphate group of the aptamer and the $Fe(CN)_6^{3-}$ both had negative charges, the electrostatic repulsive interactions would block the electron transfer (Bang et al., 2005). This experiment demonstrated that the aptamer was immobilized onto the gold electrode.

3.3. Function of the CdSHNs in the detection of thrombin

Different from the previous reports (Numnuam et al., 2008), in this work, CdSHNs were dripped on the aptamer modified gold electrode directly instead of dissolving the CdSHNs to detect Cd^{2+} by the square wave stripping voltammetric analysis. To evaluate the function of CdSHNs, using the thrombin as a model, we made a comparison of the aptamer modified gold electrode with and without the immobilization of CdSHNs. Fig. 2A shows the CVs of aptamer modified gold electrode to the thrombin with and without the immobilization of CdSHNs, respectively. It can be seen that the electrocatalytic response to the thrombin of aptamer modified gold electrode with the immobilization of CdSHNs is much larger than that of the aptamer modified gold electrode without the immobilization of CdSHNs at the potential of 490 mV, which indicates that CdSHNs promote the electron transfer between the gold electrode and $K_3[Fe(CN)_6]$. Fig. 2B shows the EIS of aptamer modified gold electrode to the thrombin with and without the immobilization of CdSHNs, respectively. At aptamer modified GCE, the charge transfer resistance (R_{et}), as determined from the width of the semicircle is 1625 Ω, and the presence of CdSHNs decreases the charge transfer impedance from 1625 Ω to 850 Ω. The double-layer capacitance (C_{dl}) of aptamer is calculated to be 0.128 μF cm^{-2} while in the presence of CdSHNs, the capacitance increases to 0.178 μF cm^{-2}. ($C_{dl} = 1/\omega^* R_{et}$, ω^* corresponded to frequency of the top of the semicircle). These results indicate that the presence of CdSHNs decreased the interfacial resistance and increased the capacitance of the double layers. The increased capacitance and decreased resistance are due to the increase in electroactive area of the electrode (e.g. increasing the surface roughness) (Pauliukaite et al., 2010). A high capacitance corresponds with a rough electrode surface (He et al., 2006). Inset in Fig. 2B represents the equivalent circuit. All currents pass through the solution resistance, therefore the other resistor (R_Ω) is as a series element to represent this effect. From Fig. 2B, we can see R_Ω is almost zero. So R_Ω is not shown in

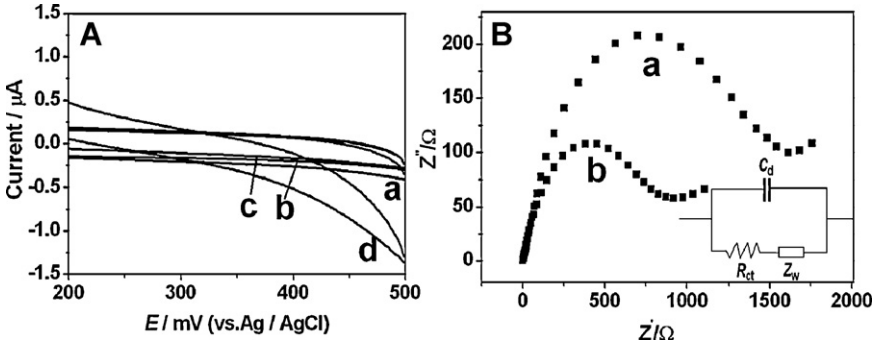

Fig. 2. (A) CVs of aptamer (a and b) and aptamer/CdSHNs (c and d) modified gold electrodes without (a and c) and with the addition of 11 μg mL^{-1} thrombin (b and d) in 1 mM $K_3[Fe(CN)_6]$ containing 0.1 M KCl. Scan rate: 100 mV s^{-1}. (B) EIS recorded at aptamer (a) and aptamer/CdSHNs (b) modified gold electrodes with the addition of 11 μg mL^{-1} thrombin in 0.1 M KCl solution containing 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. Frequency range: 0.01–100,000 Hz. Amplitude: 5 mV. Inset: Equivalent circuit applied to fit impedance measurements. C_{dl}, double-layer capacitance; Z_w, *Warburg* impedance; R_{ct}, electron transfer resistance.

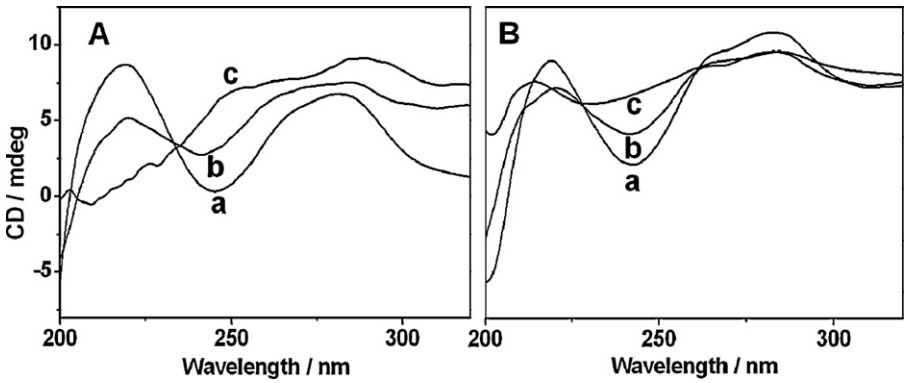

Fig. 3. CD spectra (A) of the aptamer/CdSHNs with 0 (a), 20 μg mL^{-1} (b) and 50 μg mL^{-1} (c) thrombin and CD spectra (B) of the aptamer with 0 (a), 20 μg mL^{-1} (b) and 70 μg mL^{-1} (c) thrombin in a 10 mM Tris–HCl buffer. Scan speed: 100 nm/min, response time: 2 s, scan range: 200–320 nm.

the equivalent circuit. Z_W is the *Warburg* impedance, which represents a kind of resistance to mass transfer. It cannot be calculated precisely because it changes with the frequency.

Another evidence to demonstrate the function of CdSHNs comes from the CD spectroscopy, which is a good way to investigate the structure of DNA or RNA, especially to detect the changing of the structure when they interacted with proteins. Bang reported that upon addition of thrombin, the conformation conversion of the aptamer from hairpin form to G-quadruplex form took place. The thrombin is bound to both loop regions and a part of the stem structure of the aptamer. When the aptamer on the gold electrode surface encounters the thrombin, the hairpin forming aptamer is expected to be broken by subsequent conformational changes in the structure of the aptamer (Bang et al., 2005). Our work is similar to this case. Fig. 3A shows the CD spectra of aptamer/CdSHNs with the different concentrations of thrombin. It shows two positive peaks near 218 and 282 nm and a negative peak near 244 nm without thrombin (curve a). Upon the addition of 20 μg mL^{-1} thrombin, three peaks all decrease and the negative peak shifts to about 241 nm (curve b). With the increase of concentrations of thrombin to 50 μg mL^{-1}, a dramatic change in CD spectra is observed (curve c). A positive band at about 251 nm starts to appear, indicating formation of a G-quadruplex (Jin et al., 2009). For comparison, CD spectra for aptamer without the immobilization of CdSHNs are also carried out. As shown in Fig. 3B, CD spectra shows two positive peaks near 219 and 283 nm and a negative peak near 242 nm without thrombin. Upon the addition of 20 μg mL^{-1} thrombin, three peaks also decrease, however, the change is smaller than those of the immobilization of CdSHNs. When the concentration of thrombin increases even to 70 μg mL^{-1}, the positive peaks and the negative peaks still decrease but no positive band is observed. Thus, the major function of CdSHNs is to favor the conformation conversion of aptamer, i.e. changing from the hairpin form to the G-quadruplex form.

According to Scheme 1, combining with the experimental results, the mechanism for the electrochemical biosensor was proposed as follows. The aptamer with the conformation of hairpin which was immobilized on the surface of the gold electrode blocked the electron transfer between the $K_3[Fe(CN)_6]$ and the gold electrode greatly. When the aptamer encountered the thrombin, it caused conformation conversion of aptamer from the hairpin to G-quadruplex. The aptamer unfolded and one of the ends of the aptamer was away from the surface of the gold electrode, leading to the electron transfer, however the signal was weak (curve b in Fig. 2A). When the CdSHNs were introduced to the aptamer modified gold electrode, the signal increased significantly (curve d in Fig. 2A), indicating CdSHNs improved the electron transfer between the $K_3[Fe(CN)_6]$ and the gold electrode.

Chitosan was used to prevent the aptamer and CdSHNs from breaking away from the gold electrode surface. As it is well known, chitosan is a biocompatible, biodegradable and high mechanical strength biopolymer for enzyme or biomacromolecule immobilization (Pauliukaite et al., 2010). In our work, aptamer was immobilized on the electrode, and then followed by dripping CdSHNs. Because the scale of thrombin was much smaller than that of CdSHNs, thrombin can penetrate chitosan membrane while aptamer and CdSHNs cannot.

3.4. Quantitative determination of thrombin

The electrocatalysis of aptamer/CdSHNs modified gold electrode to thrombin is shown as inset A in Fig. 4. Upon addition of thrombin to the solution, CV changed with an increase of oxidation current at 490 mV. Furthermore, the oxidation current increased with the increase of concentrations of thrombin. Fig. 4 shows the calibration curve of peak current to thrombin on the aptamer/CdSHNs modified gold electrode (curve a). The linear response range of concentrations of thrombin was from 0 to 33 μg mL^{-1} with a correlation coefficient of 0.9956 (n = 12), which was wider than 0–20 μg mL^{-1} thrombin by ECL method (Huang and Zhu, 2009). At the concentration of thrombin higher than 33 μg mL^{-1}, the calibration curve showed a platform. In comparison, the calibration curve of the aptamer modified gold electrode without the immobilization of CdSHNs was also carried out (curve b in Fig. 4). The

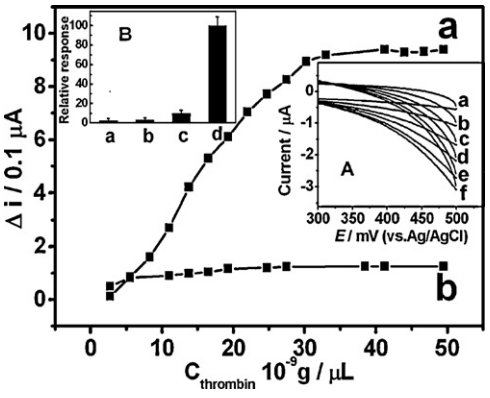

Fig. 4. Calibration curve of peak current for thrombin with the aptamer/CdSHNs (a) and aptamer (b) modified gold electrodes. Inset A: CVs of aptamer/CdSHNs modified gold electrode with the concentrations of thrombin of 0 (a), 2.75 (b), 5.5 (c), 8.25 (d), 11.0 (e) and 13.75 μg mL^{-1} (f) in 1 mM $K_3[Fe(CN)_6]$ containing 0.1 M KCl. Scan rate: 100 mV s^{-1}. Inset B: Detecting specificity in a solution containing 1 μM BSA (a), 1 μM lysozyme (b), 0.1 mM IgG (c), and 0.1 μM of thrombin (d). It is an average value of the response of three measurements for each sample.

Table 1
Determination of concentrations of thrombin in the human serum.

Sample no.	Measured by our biosensor		Measured by fluorescence	
	Concentrations($\mu g\,mL^{-1}$)	RSD (%)	Concentrations($\mu g\,mL^{-1}$)	RSD (%)
1	0.31	4.10	0.33	4.12
2	1.05	3.20	1.13	4.63
3	1.67	4.27	1.78	4.89
4	2.39	3.82	2.51	4.65

It is an average value of the response of three measurements for each sample.

aptamer modified gold electrode showed a smaller response to thrombin than aptamer/CdSHNs modified gold electrode at the same concentrations of thrombin. The linear range of concentrations of thrombin was from 2.75 to 27.5 $\mu g\,mL^{-1}$ and sensitivity was 0.062 $\mu A\,mL\,\mu g^{-1}\,cm^{-2}$. Thus it can be concluded that the CdSHNs had a great influence on the electrochemical signal.

3.5. Stability and reproducibility of the aptamer biosensors

The aptamer/CdSHNs modified gold electrode in our protocol had a good stability. The sensor could retain 96.0% of its initial activity to thrombin within a storage period of 10 days in Tris–HCl butter solution, while 87.3% of activity to thrombin was retained when stored in air.

The fabrication reproducibility of five electrodes, made independently, showed an acceptable reproducibility with the average relative standard deviation (RSD) of 6.40% for the currents determined at a concentration of thrombin of 20 $\mu g\,mL^{-1}$.

3.6. Interference

For testing the specific recognition to thrombin of the sensor designed, BSA, lysozyme and IgG which are usually used in control experiments, were adopted. The modified electrode was immersed in the solution of 1 μM BSA, 1 μM lysozyme and 0.1 mM IgG for 30 min without the presence of thrombin, respectively. Relative response from inset B in Fig. 4 was obtained by signals of the proteins dividing the signal of the thrombin then multiplying by 100. It was found that BSA and lysozyme had almost no interference with the determination of thrombin and IgG produced the relative response of about 10.8%, indicating that these species coexisting in the sample matrix did not affect the determination of thrombin and the sensor had a good specificity.

3.7. Application of the aptamer-based system in human serum

Four blood samples were analyzed using four independently prepared electrodes. The obtained results were compared with those measured with fluorescence method (Table 1). It was shown that the values measured by the developed biosensor were in good agreement with the data from fluorescence method, indicating that the biosensor had a good accuracy and had a great potential for practical application for the analysis of thrombin in real clinical samples.

4. Conclusion

The immobilization of the CdSHNs on the aptamer modified gold electrode had a great influence on the detection signal. CdSHNs promoted the electron transfer between gold electrode and $K_3[Fe(CN)_6]$ and facilitated the conformation conversion of the aptamer from the hairpin form to G-quadruplex form after interacting with thrombin, which was in favor of improvement of detection performance. This biosensor showed a wide linear range, good sensitivity, specificity, stability and accuracy which indicated to have a potential value in clinical application.

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