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ORIGINAL PAPER

Aptamer based label free thrombin assay based on the use of silver nanoparticles incorporated into self-polymerized dopamine

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Abstract

The authors describe an electrochemical aptasensor for thrombin that is based on the use of a glassy carbon electrode (GCE) modified with polydopamine that is loaded with silver nanoparticles (PDA/AgNPs). The use of AgNPs improves the conductivity of the film and increases the surface area of the GCE. PDA was deposited on the GCE via self-polymerization, and the thrombin binding aptamer was grafted onto the PDA-modified GCE by a single step reaction. Residual electrode surface was blocked with 6-mercapto-1-hexanol. On exposure to thrombin, the electrochemical impedance of the modified electrode increases gradually. Response is linear in the 0.1 pM to 5.0 nM thrombin concentration range, and the limit of detection is as low as 36 fM. The method is selective and capable of detecting thrombin in diluted human serum. In our perception, such a GCE modified with AgNP in a PDA matrix may be applied to many other analytes for which appropriate aptamers are available.

Keywords Aptasensor · Polydopamine · Nanocomposites · Chemically modified electrode · Self-polymerization · Electrochemistry · Biosensor · Electrochemical impedance spectroscopy

Introduction

Thrombin (TB) catalyzes the conversion of soluble fibrinogen to become insoluble fibrin that promotes blood coagulation, and is involved in various diseases such as thromboembolic disease, leukemia, and inflammation reactions [1–3]. Many technologies have been developed for TB detection, such as colorimetric [4], surface plasmon resonance [5], fluorescent [6], electrochemiluminescence [7] and electrochemical aptasensor [8]. Among these, electrochemical aptasensor shows significant predominance because of its low cost, flexible design, high sensitivity and good stability [9–11]. However, most of the electrochemical aptasensors are based on enzymes or label signals using the signal amplification method [12–16]. They not only cost too much, but also operate complexly. Different from those methods mentioned above, we developed an enzyme-free and label-free electrochemical aptasensor for the detection of thrombin based on the self-polymerized dopamine (DA).

To construct an electrochemical aptasensor, the choice of electrode substrate material is very critical. DA, as a biomimetic material, contains two functionalities of 3,4-dihydroxy-L-phenylalanine and lysine amino acids which are rich in adhesive proteins that are secreted by mussels and used as bioglue to attach to any surface [17]. Under the conditions of alkaline aqueous solution and oxygen, DA can self-polymerize to form thin adherent polydopamine (PDA) films (Scheme S1A), which contains a large number of hydrophilic hydroxyl (−OH) groups, which can improve hydrophilicity of the material surface. Furthermore, the PDA coating can then serve as a "primer", and the thiol (−SH) or amino ($-NH_2$) modified biomolecules can be easily to covalently bond to

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the PDA film surface via the Michael addition or Schiff base reactions (Scheme S1B). Additionally, PDA shows low cytotoxicity [18–22]. However, PDA film has poor electrical conductivity restricting its application in electrochemical assays.

Silver nanoparticles (AgNPs) possess good electrical conductivity and large specific surface area, and they can immobilize biomolecules via Ag-S bond. In Sileika's work, PDA film was used as the intermediate to prevent the direct interaction of SH-PEG to AgNPs, as it may result in delamination of the silver layer [23]. Moreover, AgNPs embedded in a polymer matrix have been reported to show an excellent active antimicrobial behavior [23]. Considering the fact that PDA can enhance the interfacial chemical multifunction and AgNPs possess high conductivity, the combination of these two materials may be used as ideal electrode substrates for electrochemical biosensor.

Taking advantage of the multifunction of PDA and the conductivity of AgNPs, we report a novel aptasensor for the detection of thrombin using electrochemical impedance spectroscopy (EIS) method based on PDA film embedded with AgNPs, as shown in Scheme 1. Firstly, AgNPs were electrodeposited on a glassy carbon electrode (GCE) through the cyclic voltammetry (CV) technique. Secondly, PDA film was self-polymerized onto AgNP/GCE surface, and then the thrombin aptamer was immobilized via the Michael addition reaction. Thirdly, to avoid possible nonspecific protein adsorption, 6-mercapto-1-hexanol (MCH) was used as blocking agent to cover the active sites of the electrode surface [24]. Finally, the MCH/aptamer/PDA/AgNP modified GCE was used to selectively detect thrombin. EIS technique was employed to investigate the analytical performance, and the results show that the aptasensor exhibits satisfying sensing performance for the detection of TB even in diluted human serum samples.

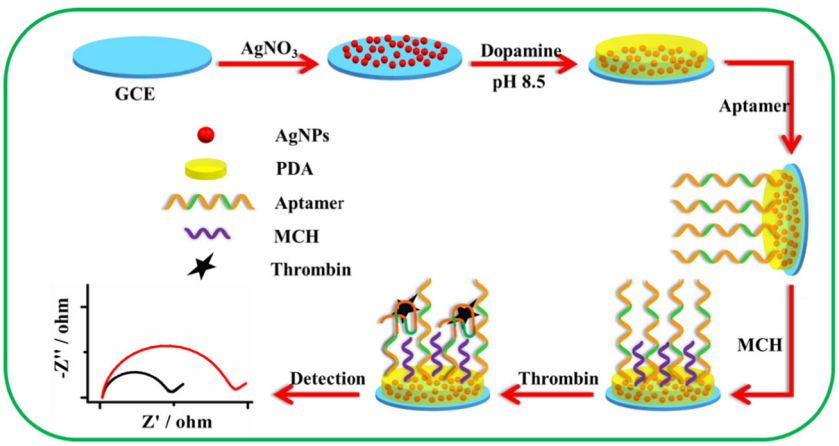
Experimental

Materials and apparatus

Thrombin aptamer with the sequence of 5'-SH-$(CH_2)_6$-GGT TGG TGT GGT TGG-3′ was purchased from Sangon Biotech Co., Ltd. (Shanghai, China, http://www.sangon.com/). Thrombin, dopamine, 6-mercapto-1-hexanol (MCH), lysozyme (Lys), bovine serum albumin (BSA), immunoglobulin G (IgG), and human serum albumin (HSA), were purchased from Adamas Reagent, Ltd. (Shanghai, China, http://www.adamas-beta.com/home!home.action).

50 mM Tris–HCl buffer (pH 7.4) containing 140 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$ was used as a binding buffer. Phosphate buffered saline (PBS) (pH 7.4, 10 mM) containing 5 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ was used as the working buffer. All other reagents were of analytical grade, Milli-Q water (18.2 MΩ.cm) was used to prepare all solutions.

Electrochemical measurements were performed with the CHI650E electrochemical workstation (CH Instruments, China, http://www.chinstr.com/) with a conventional three-electrode system, where a platinum wire as counter electrode, a silver/silver chloride (Ag/AgCl, 3 M KCl) as reference electrode, and GCE (diameter 3.0 mm) as working electrode. Static water contact angle measurement was carried out using a JC2000D Instrument (Shanghai, China, http://www.powereach.com/). X-ray photoelectron spectroscopy (XPS) measurement was carried out by using a Thermo-VG Scientific ESCALAB 250Xi spectrometer and with Al Kα X-ray (15 kV) as the light source (Thermoelectricity Instruments, US http://www.thermofisher.com/cn/zh/home.html). Scanning electron microscope (SEM) with the JEOL JSM-7500F (JEOL Ltd., Japan https://www.jeol.co.jp/en/products/list_sem.html) was used to characterize morphologies of different nanostructures.



Scheme 1 Schematic representation of the biosensor fabrication process

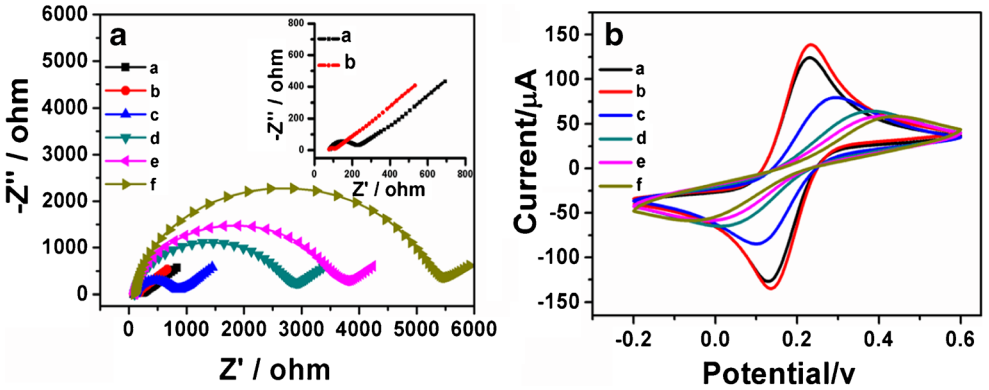


Fig. 1 EIS (**a**) and CV curves (**b**) of various modified electrodes in PBS (10 mM, pH 7.4) containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl. EIS measurement conditions: frequency range from 1 to 100,000 Hz; amplitude of the applied sine at 5.0 mV; current potential of 0.20 V. CV conditions: scanning range from −0.2 to 0.6 V with scan rate of 0.10 V/s. (a) GCE; (b) AgNP/GCE; (c) PDA/AgNP/GCE; (d) aptamer/PDA/AgNP/GCE; (e) MCH/aptamer/PDA/AgNP/GCE; (f) TB/MCH/aptamer/PDA/AgNP/GCE

Sensing interface fabrication

GCE was polished with 1.0 μm, 300 nm, and 50 nm alumina slurries in sequence, followed with ultrasonically washing in Millipore water, ethanol and Millipore water for about 3 min, respectively. The pretreated GCE was immersed in 10 mM $AgNO_3$ solution containing 0.1 M KNO_3 to electrodeposit AgNPs by cyclic sweeping in the potential range of −1.7 to 0.5 V at 100 mV s^{-1} for 4 segments. Then, the GCE was immersed into 2.0 mg mL^{-1} of dopamine solution (dissolved with Tris–HCl buffer, 10 mM, pH 8.5) for 1 h at room temperature. In order to immobilize aptamer, the modified GCE was rinsed with water and Tris–HCl buffer (50 mM, pH 8.5), and subsequently the modified electrode was incubated in 60 μL solution (50 mM Tris buffer) containing 1.0 μM aptamer for overnight incubation at ambient temperature. Aptamer was introduced onto the GCE surface via the Michael addition reaction. The Tris–HCl buffer was fully degassed with nitrogen before use to prevent the oxidation of thiol groups of aptamer into disulfide bonds. After thorough rinsing with water, the aptamer/PDA/AgNP/GCE was incubated with 1.0 mM of MCH solution (50 mM Tris–HCl buffer) for 60 min, and the small sized MCH was also introduced onto the GCE surface via the Michael addition reaction to block the remaining active sites of PDA film.

Electrochemical measurements

EIS is a widely used technique to survey changes happened at biosensor surfaces, and it can convert the complex bio-recognition process into measurable electrochemical signals. What's more, compared with the complicated and expensive labeled assays, the label-free EIS technique can significantly save time and reduce costs [26]. For the bio-sensing application, the modified electrode was incubated with 100 mL different concentration of thrombin solution. (10 mM PBS, pH 7.4) for 45 min at 37 °C. Using EIS technique, the impedance changes after thrombin binding were recorded as response signals. EIS measurements were recorded in PBS (10 mM, pH 7.4) containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ solution and 0.1 M KCl. The direct current potential was set at 0.20 V, the frequency range was from 0.1 to 10^5 Hz, and the amplitude of the applied sine wave was 5 mV.

Results and discussion

Choice of materials

PDA film embedded with AgNPs was prepared and used as the substrate for the construction of the aptasensor. AgNPs

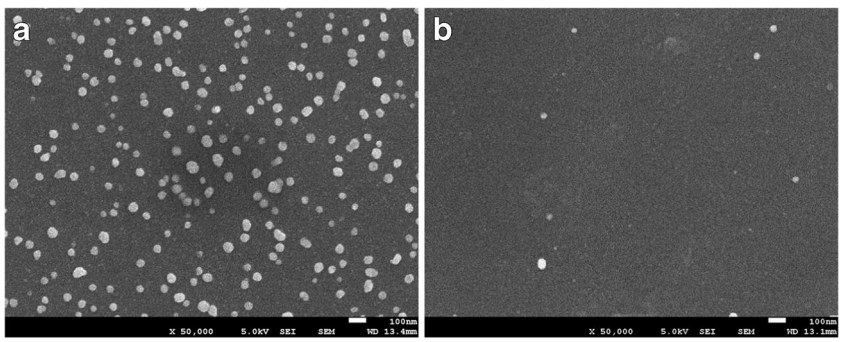


Fig. 2 SEM images of **a** AgNPs and **b** PDA/AgNP modified electrode surfaces

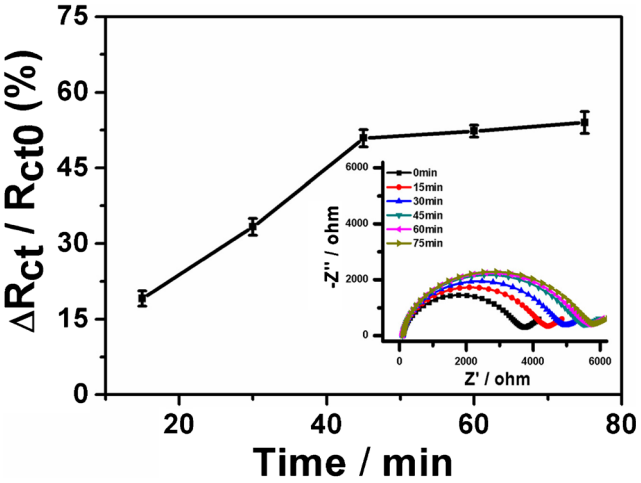


Fig. 3 Optimization of TB incubation time for the aptasensor in 1.0 nM TB containing PBS (10 mM, pH 7.4). Inset: EIS of the aptasensor after incubation in 1.0 nM TB for different time

with good conductivity can be electrochemically prepared on the electrode surface, and they may effectively increase the electrical conductivity of the modified electrode. PDA can simply deposit on the electrode surface through the self-polymerization process, and it can directly immobilize certain molecules through the considerably simple process, the Michael addition or Schiff base reactions, without complicated activation and functionalization steps.

Electrochemical characterization

The semicircle portion in the Nyquist impedance plot reflects the resistance of electron transfer (R_{ct}), which plays a controlling role in the dynamics of the redox probes at the interface of the modified electrode. As shown in Fig. 1a, the fabrication process of the biosensor was monitored using EIS step by step. The bare GCE exhibits a distinct semicircle with the R_{ct} value at about 120 Ω (Fig. 1a curve a), while the R_{ct} of AgNPs modified GCE (Fig. 1a curve b) shows an almost straight line, which distinctly demonstrates the good electron transfer property of AgNPs modified electrode. The R_{ct} increases after the self-polymerization of DA onto the AgNPs modified GCE (Fig. 1a curve c), which may be ascribed to the poor conductivity of PDA that impedes the electron transfer at the interface. Predictably, there will have a sharp increase with R_{ct} when aptamers are immobilized onto the electrode surface, which is because the negatively charged phosphoric acid backbones of aptamer repelled the negative $[Fe(CN)_6]^{3-/4-}$ probe and inhibited the electron transfer (Fig. 1a curve d). With MCH assembled on the PDA film, the R_{ct} increased accordingly (Fig. 1a curve e), indicating successful blocking of the residual active sites of the PDA film with MCH. Interestingly, after incubation with TB (TB binding to its aptamer), the Rct increased significantly (Fig.1a, curve f), which implied that the thrombin further hinders the access of electrons to the modified surface, and this impedance change induced by the binding of TB can be used for the detection of TB. All these changes in R_{ct} suggested that the designed aptasensor was successfully prepared.

CV technique was further used to characterize the fabrication process of the modified electrode. An obvious increase in peak current was observed after AgNPs were modified on the electrode. As expected, the peak currents decreased gradually after modification with PDA, aptamer, MCH and TB in order. These results were in accordance with the EIS characterization.

Hydrophilic property of the interface

The surface wettability of electrode surfaces modified with different materials was characterized by measuring their static water contact angles. As shown in Fig. S1A, the contact angle of the AgNPs modified GCE surface was about 75°. After PDA modification, the contact angle significantly decreased to 43° (Fig. S1B), indicating a remarkable hydrophilicity of the PDA modified surface due to rich −OH in PDA. The electrode surface immobilized with aptamer and MCH became very hydrophilic, and the contact angle was just about 26° (Fig. S1C), which may be ascribed to the presence of MCH with −OH group [25]. Such a hydrophilic surface may help to reduce the nonspecific protein adsorption through the hydrophobic interaction.

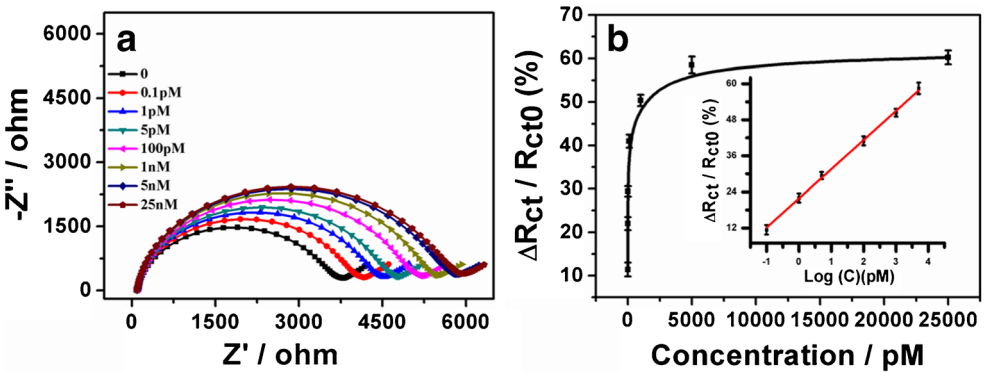


Fig. 4 **a** EIS response of the electrochemical aptasensor for the detection of different concentrations of thrombin (from inner to outer: blank, 0.1 pM, 1.0 pM, 5.0 pM, 100 pM, 1.0 nM, 5.0 nM and 25 nM). **b** Variation of the charge-transfer resistance changes [$\Delta R_{ct}/R_{ct0}$ (%)] at different concentrations of thrombin. Inset: calibration curve of the aptasensor

Table 1 Different methods for thrombin detection

Material	Technique	Liner range(pM)	LOD(pM)	Total time (min)	Ref.
Laser-scribed graphene	DPV	1–100	1	210	10
G-quadruplex/hemin/HRP/AuPd/PPD	DPV	0.1–20,000	0.02	450	26
RNAToggle-25 coated microplate	fluorescence	0.01–25	0.01	750	27
Aptamer-functionalized AuNPs	colorimetry	0.1–100,000	0.02	280	28
AuNP/GO/Aptamer	ECL	10–10,000	6.3	350	29
TiO_2/MWCNT/CHIT/SB	DPV	0.05–10,000	0.001	280	30
Au@GS and DNA-CoPd NPs	amperometry	0.3–54	0.14	480	31
PDA/AgNP and Aptamer	EIS	0.1–5000	0.036	270	This work

XPS characterization

To further confirm the successful preparation of the aptasensor, XPS was utilized to analyze the elemental composition of electrode surface, as is shown in Fig. S2. After the deposition of AgNPs on the glassy carbon surface (Fig. S2A), three peaks appear: Ag_{3d} is characteristic element of the AgNPs, whereas C_{1S} and O_{1S} are characteristic elements of the glassy carbon, which illustrated that AgNPs were successfully electrodeposited. And then after soaking AgNP/GCE into DA solution and self-polymerizing the PDA film on the surface (Fig. S2B), a new peak of N_{1s} appears while the peak for Ag_{3d} decreased significantly, indicating that the surface was successfully coated with a layer of PDA film. Finally, after the immobilization of the MCH/aptamer, two new peaks appeared: One peak appears at 134 eV, representing P_{2p} signal from aptamer (Fig. S2C, right inset), and the other peak appears at 164 eV, corresponding to the S_{2p} signal coming from the –SH group of the MCH and aptamer. Clearly, the XPS characterization indicated that each modification step was successfully achieved.

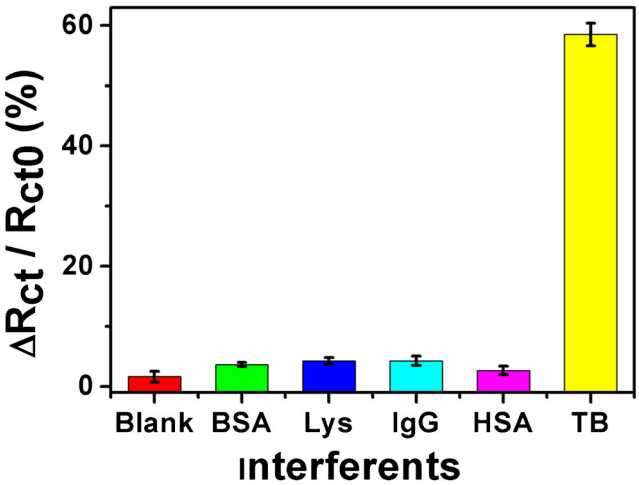


Fig. 5 Detecting specificity of the aptasensor tested in solutions containing 5 nM thrombin, 100 nM BSA, 100 nM lysozyme, 100 nM IgG and 100 nM HSA, respectively

SEM characterization

Typical SEM images of the AgNPs and PDA/AgNP modified electrode surfaces are shown in Fig. 2. Clearly, the deposited AgNPs with diameters of about 50–60 nm were evenly distributed on the bare GCE surface (Fig. 2a). A uniform and smooth PDA film was obtained when DA was self-polymerized on AgNP/GCE (Fig. 2b), and most AgNPs were embedded in it. The PDA film can increase the stability of the electrodeposited AgNPs, while the presence of AgNPs within the PDA film can increase the conductivity of the PDA film, which is suitable for the construction of electrochemical sensors and biosensors.

Optimization of the incubation time

Figure 3 presents EIS signals of the aptasensor incubated in 1.0 nM TB for different time periods. It can be seen that the EIS signal increased gradually from 15 to 75 min, and reached a relatively stable value at 45 min. Therefore, 45 min was selected as the optimum reaction time of TB to shorten the incubation time and improve the sensitivity of the aptasensor.

Biosensing performance of the aptasensor

To evaluate the analytical performance, the electrochemical aptasensor was incubated in different concentrations of thrombin and measured with an EIS technique under optimal experimental conditions. According to Fig. 4a, the EIS signal

Table 2 The recovery experiments in 1.0% (*V*/V) human serum samples ($n = 3$)

Sample No.	Added (pM)	Found (pM)	Recovery (%)	RSD (%)
1	0.20	0.19	95	8.3
2	1.0	1.01	101	7.5
3	10	10.6	106	5.6
4	100	108	108	3.2
5	1000	940	94	4.6

increases with the addition of the thrombin concentration. The value of [$\Delta R_{ct}/R_{ct0}$ (%)] was linearly related to the logarithm of thrombin concentration from 0.1 pM to 5 nM. The regression equation is $\Delta R_{ct}/R_{ct0}$ (%) = 9.714 logC (pM) + 21.85 (R^2 = 0.9979), and the limit of detection (LOD) is 0.036 pM based on the 3σ calculation. Owing to characteristics of PDA biomimetic materials, and high bio-affinity of the thrombin aptamer to its target, the biosensor showed a very low LOD only through a very direct method without complex signal amplification procedure. The performance of the TB aptasensor we developed was compared with other previously reported aptasensors [10, 26–31], as shown in Table 1. Obviously, the aptasensor showed significant improvement over most of those approaches for thrombin detection.

Selectivity and reproducibility of the aptasensor

In order to test the selectivity of the aptasensor, the biosensor responses to various nonspecific proteins including BSA, Lys, IgG, and HSA were measured. As illustrates in Fig. 5, compared with the R_{ct} change induced by 5.0 nM TB, the fabricated biosensor showed no significant signal response to BSA, Lys, IgG, and HSA, even with a much higher concentration (100 nM, respectively). The reproducibility of the aptasensor was evaluated by using seven independently fabricated aptasensors for the detection of TB with the same concentration of 1.0 pM, and the relative standard deviation (RSD) of the responses of the seven aptasensors was 4.7%.

Assay of TB in serum samples

To further assess the potential applicability of the TB aptasensor in real samples, the standard addition method was employed to carry out recovery experiments for thrombin in 1.0% human serum samples from five healthy volunteers (provided by the 8th People's Hospital of Qingdao, China). It can be seen from Table 2, the recoveries vary from 94% to 108%, and the RSDs are between 3.2% and 8.3%, indicating that the aptasensor is potentially useful for thrombin determination in real samples.

Conclusions

A sensitive and selective electrochemical aptasensor for thrombin detection was successfully fabricated based on the PDA film embedded with AgNPs. Self-polymerized PDA film can act as an ideal electrode substrate material for direct immobilization of biomolecules via the one step Michael addition reaction. The introduction of AgNPs into PDA film remarkably improved the electrical conductivity of the PDA film. The PDA film embedded with AgNPs was proved to be a good substrate for the construction of electrochemical biosensors. The ability of the aptasensor to detect the target directly in biological samples needs to be further improved. It is expected that GCE modified with PDA film embedded with AgNPs may be applied to many other analytes for which appropriate aptamers are available.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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