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ARTICLE

A simple strategy of signal amplification based on DNA hybridization chain reaction for thrombin detection

XiaoYing Liu,[a] GuangYu Shen,*[b]

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A simple signal amplification strategy based on DNA hybridization chain reaction for the detection of thrombin was developed. Two conjugates based on gold nanoparticles were used. The first conjugates (Ap1-GNP-HRP) were prepared by functionalizing gold nanoparticles with thrombin aptamer 1 (Ap1) and horseradish peroxidase. They were immobilized on the electrode surface through sandwich type reaction. The second conjugates (LDNA-GNP-HRP) were prepared by functionalizing gold nanoparticles with linking DNA (LDNA) and horseradish peroxidase. They were immobilized on the electrode surface through hybridization chain reaction. This two introduction of numerous HRP onto the surface of the electrode resulted in significantly amplified electrochemical signal. Using the proposed strategy, we detected thrombin within the range from 0.001 to 20 nM with a detection limit of 0.6 pM based on S/N = 3. The proposed aptasensor had a good specificity and reproducibility. Moreover, the detection strategy could be easily extended to the detection of other biomolecules.

Introduction

Aptamers, a new class of single-stranded DNA or RNA oligonucleotide selected from random sequence nucleic acid libraries, are very stable and can withstand the repetitive denaturation and renaturation. They are easy to be labeled at almost any desired site without loss of activity[1, 2] and also can be attached to specific regions of targets.[3] Compared with antibodies, aptamers have some advantages, such as simple synthesis, high stability, long-term storage, convenient regeneration.[4] Therefore, extensive efforts have been devoted to making use of aptamers to replace antibodies as a recognition element for the design of biosensors.[5-9] Electrochemical aptasensors, being simple, portable and low-cost, have been widely applied in biochemical analysis.[10-14] A sandwich-type electrochemical aptasensor based on horseradish peroxidase (HRP) amplification, in which the detecting aptamer was labeled by HRP, has been reported by Mir and coworkers.[5] In order to improve the sensitivity of the aptasensor, the detecting aptamer was labeled by nanomaterials such as gold nanoparticles, carbon nanotubes used as a carrier to introduce more HRP to the surface of the electrode.[15-17] Particularly, signal enhancement based on rolling circle amplification (RCA) was developed.[18] In the process of RCA, a long single stranded DNA with repetitious sequence was obtained. The RCA products provided a large number of sites to link signal materials or electroactive indicators.[19,20] However, RCA is complicated and many reagents need to be added. Compared to RCA, hybridization chain reaction (HCR), in which the single-stranded DNA molecule can be programmed to self-assemble into nicked double helices analogous to alternating copolymers,[21] is simple. Recently, an electrochemical aptasensor based on hybridization chain reaction for signal amplification was developed for highly sensitive detection of thrombin.[22] In this strategy, a long biotin-labeled dsDNA was obtained by the recycling of two biotin-

[a] College of Science, Hunan Agriculture University, Changsha 410128, China
[b] College of Chemistry and Chemical Engineering, Hunan University of Arts and Science, Changde 415000, Hunan, China.Email: sgyrab@163.com

labeled hairpin DNAs. Some avidin-labeled HRP can be introduced on the electrode surface via biotin-avidin reaction. But, the labelled biotin at both ends of each hairpin probe might increase the steric hindrance or change the conformation of hairpin probes.[23] Furthermore, the amount of the labelled biotin on each hairpin end is limited, which resulted in limited HRP immobilized on the electrode surface. Additionally, the hairpin probe modification process with indicators is complicated. Other electrochemical aptasensors based on hybridization chain reaction were also reported.[24, 25] Unfortunately, those strategies are very tedious and time-consuming and many reagents and materials need to be used. Herein, we reported a simple signal amplification strategy based on two step enzyme amplification for the detection of thrombin. Sandwich assay, which relies on two different aptamers generated against one target, was designed. Two types gold nanoparticle conjugates were used as signal probes. The first conjugates (Ap1-GNP-HRP) were prepared by functionalizing gold nanoparticles (GNP) with thrombin aptamer 1 (Ap1) and horseradish peroxidase. They were immobilized on the electrode surface through sandwich type reaction, which resulted in the first step signal amplification. The second conjugates (LDNA-GNP-HRP) were prepared by functionalizing gold nanoparticles with linking DNA (LDNA) and horseradish peroxidase. They were immobilized on the electrode surface through hybridization chain reaction. Thus, more enzymes were immobilized on the surface of electrode, which resulted in the second step signal amplification. More interesting, multiple cycles of adding Ap1-GNP-HRP and LDNA-GNP-HRP could further amplify the electrochemical signal. However, the principle of amplification is the same and the process is time-consuming. Thus, our strategy is based on two step enzyme amplification. This strategy has some advantages as following: (1) The fabrication of aptasensor is simple; (2) A great deal of HRP was introduced due to the high surface-to-volume ratio of gold nanoparticles and the use of Ap1-GNP-HRP and LDNA-GNP-HRP conjugates; (3) The further improved detection signal was based on hybridization chain reaction, avoiding the complexity of exonuclease-assisted target recycling amplification. The principle of the aptasensor is illustrated in Fig. 1.

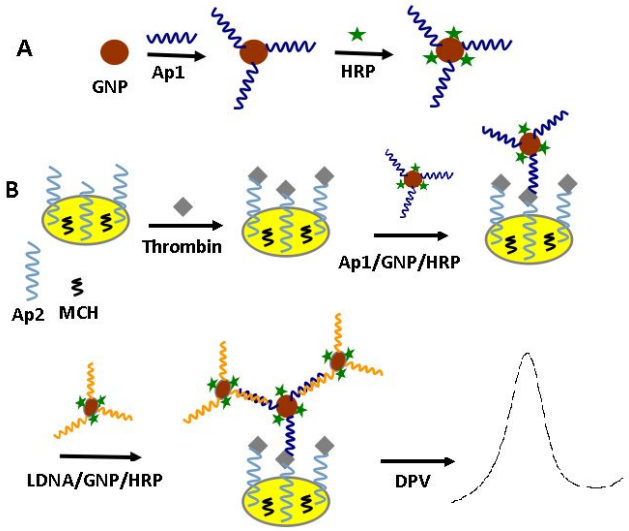

Fig. 1 Schematic diagrams of preparation of gold nanoparticle conjugates (A) and the electrochemical aptasensor (B).

Experimental

Reagents and apparatus

$HAuCl_4$, sodium citrate, 6-mercapto-l-hexanol (MCH), hydroquinone (HQ) and horseradish peroxidase were purchased from Sigma-Aldrich. Human thrombin, bovine serum albumin (BSA), and human immunoglobulin G (IgG) were purchased from Dingguo Biotechnology Inc. (Shanghai, China). Dilute solutions of H_2O_2 were freshly prepared daily. 0.1 M phosphate buffer solution (PBS, pH 7.4) was prepared using Na_2HPO_4 and NaH_2PO_4. All aqueous solutions were prepared with doubly distilled water. The aptamer

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and capture probe used in this study were obtained from Shanghai Sangon Biological Engineering Technology Co., Ltd. and have the following sequences:

Thrombin aptamer1 (Ap1): 5′-SH-(CH2)6-GGT TGG TGT GGT TGG-3′.

Thrombin aptamer2 (Ap2): 5′-SH-AGTCCGTGGTAGGGCAGGTTGG GGTGACT-3′.

Linkig DNA (LDNA): 3′-CCA ACC-ACA-CCA-ACC-(CH2)6-SH-5′.

Preparations

Gold nanoparticles (GNPs) were prepared according to previously reported method.[26] Briefly, a 250 mL aqueous solution of 0.01% HAuCl4 was heated to boiling and vigorously stirred in a 500 mL round-bottom flask, and then 4.5 mL of 1% sodium citrate was added quickly to this solution. The color of the solution turned deep blue within 20 s, and finally changed to wine-red after 60 s. After that, the solution cooled to room temperature with a continuous stirring. The resulting Au colloid solutions were stored in a refrigerator in a dark-colored glass bottle at 4 °C before use.

Ap1-GNP-HRP conjugates were prepared according to the published procedure with a modification.[27] Briefly, 500 µL of 5-fold concentrated GNP was mixed with 5 µL of 100 µM of Ap1 for 24 h, and the solution was centrifuged for 20 min at 12,000 rpm to remove the excess reagents. The Ap1-GNP conjugates were washed with Tris–HCl buffer and re-dispersed in 500 µL of Tris–HCl buffer. For the preparation of the Ap1-GNP-HRP conjugates, 50 µL of 1 mg mL^{-1} HRP solution was added into the 500 µL of Ap1-GNP solution. The mixture was incubated for 1 h. The excess reagents were removed and washed twice by centrifugation for 15 min at 120, 00 rpm. Then the red pellet was redispersed in 500 µL of Tris–HCl buffer containing 2% BSA. LDNA-GNP-HRP conjugates were prepared as the same as above mentioned method. Those prepared conjugates were stored in a refrigerator at 4 °C before use.

Fabrication of aptasensor

Preparation of the aptasensor was carried out as following: A gold electrode (3 mm in diameter) was polished repeatedly with 0.3 and 0.05 µm alumina slurries sequentially, followed by successive sonication in bi-distilled water and ethanol. Prior to the experiment, the gold electrode was cleaned with hot piranha solution (a 3:1 mixture of H_2SO_4 and H_2O_2, v/v) several times, and then continuously scanned over a potential range from −0.3 to +1.5 V in freshly prepared deoxygenated 0.5 M H_2SO_4 until a voltammogram characteristic of a clean gold electrode was established. After the pretreatment, the freshly cleaned gold electrode was immersed for 6 h in a 1.0 µM aptamer (Ap2) solution, and then 2 h in a 1.0 mM MCH solution, resulting in a mixed self-assembled monolayer on the surface of the electrode. After being rinsed with PBS, a 20 µL of different concentration of thrombin was placed on the modified electrode and incubated for 1 h at room temperature. The process was followed by casting 20 µL of Ap1-GNP-HRP conjugates over the electrode and keeping for 1 h at room temperature and then washed with PBS (0.1 M, pH 7.4). After that, the above electrode was incubated with a solution of 20 µL of LDNA-GNP-HRP conjugates for 1 h at room temperature and then washed with PBS (0.1 M, pH 7.4), the aptasensor was obtained.

Measurement protocol

Electrochemical measurements, including cyclic voltammogram (CV) and differential pulse voltammetry (DPV) were carried out with a

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CHI 660 electrochemistry workstation (Shanghai CH Instruments, China). A conventional three-electrode cell consisted of a Pt electrode as counter electrode, a saturated calomel electrode (SCE) as reference electrode, and an Au electrode as working electrode. The cyclic voltammogram (CV) were investigated in 10 mM $K_3Fe(CN)_6/K_4Fe\ (CN)_6$ solution from -0.2 to 0.6 V at a scan rate of 100 mV s^{-1}. Differential pulse voltammetry (DPV) were performed in PBS containing 2 mM of H_2O_2 and 2 mM HQ. The potential range was from −0.6 to 0.6 V, pulse amplitude was 0.05 V, pulse width was 0.05 s, and sample width was 0.02 s.

Results and discussion

GNP, Ap1-GNP-HRP and LDNA-GNP-HRP conjugates were prepared for the fabrication of thrombin aptasensor. They were characterized by TEM. Electrochemical behavior of the electrode modified stepwise was characterized by cyclic voltammogram in 10 mM $K_3Fe(CN)_6/K_4Fe\ (CN)_6$ solution. In addition, the experimental conditions were optimized and the performance of the proposed immunosensor were studied.

Characterizations of gold nanoparticles and the conjugates

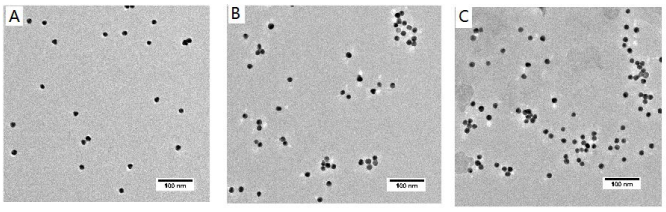

Fig. 2 TEM images of (A) bare GNP, (B) Ap1-GNP-HRP conjugates and (C) LDNA-GNP-HRP conjugates.

GNP, Ap1-GNP-HRP and LDNA-GNP-HRP conjugates were characterized by TEM. The bare GNP were dispersive and the diameter was about 15 nm (Fig. 2A). Compared with bare AuNPs, Ap1-GNP-HRP conjugates (Fig. 2B) and LDNA-GNP-HRP conjugates (Fig. 2C) seem to be in close. It is probably because GNP tend to gather after modified with HRP and Ap1 or LDNA.

Electrochemical characterization of the aptasensor

The cyclic voltammogram of ferricyanide is a valuable and convenient tool to monitor the barrier of the modified electrode. Fig. 3 shows cyclic voltammograms of $Fe(CN)_6^{4-/3-}$ at a different state. As shown in the figure, stepwise modification on the electrode is accompanied by a decrease in the amperometric response of the electrode and an increase in the peak to peak separation between the cathodic and anodic waves of the redox probe. This is consistent with the enhanced electron-transfer barriers introduced upon assembly of these layers.

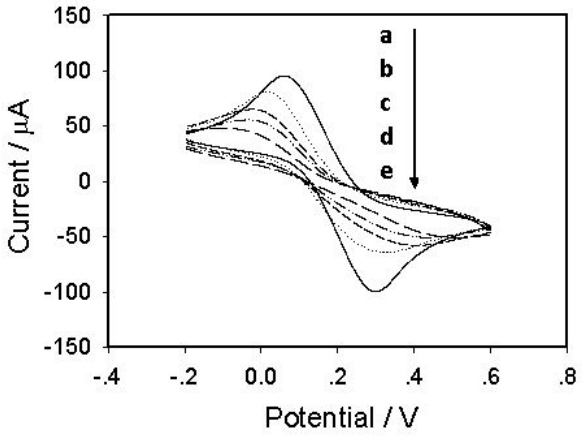

Fig. 3 CVs of different modified electrodes in 10 mM $K_3Fe(CN)_6/K_4Fe\ (CN)_6$ solution at a scan rate of 100 mV s^{-1}: (a) bare Au electrode; (b) Ap2+MH/Au; (c) b incubated with 10 nM thrombin; (d) c incubated with Ap1-GNP-HRP conjugates; (e) d incubated with LDNA-GNP-HRP conjugates.

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Optimization of experimental conditions

The amount of Ap2 immobilized on the electrode surface directly affects the performance of the aptasensor. It can be controlled by Ap2 concentration and immobilization time. As shown in Fig. 4A, it is clearly observed that the peak current is bigger at 1 μM. Therefore, 1 μM of aptamer was chosen for this work. Fig. 4B shows the change in peak current as a function of immobilization time and 6 h was selected for following experiments. In addition, the peak current was also affected by the amount of H_2O_2, a catalytic substrate of HRP. The DPV response signals for thrombin (10 nM) are investigated in PBS containing different concentration of H_2O_2 and 2 mM HQ. As shown in Fig. 4C, the current responses increased with the increasing concentration of H_2O_2 in the range from 0.5 to 2 mM and then reached a platform, which corresponded to the saturated state. Consequently, the optimal concentration of 2 mM H_2O_2 was selected for further experiments.

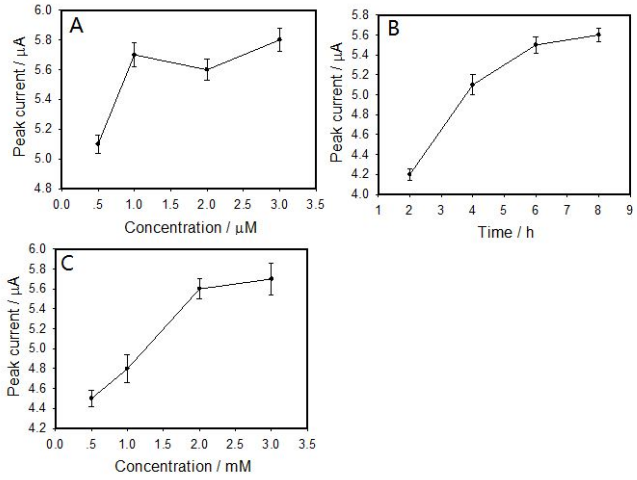

Fig. 4 Effects of the concentration of Ap2 on the DPV peak current (A). Effects of the self-assemble time of Ap2 on the DPV peak current (B). Effects of the concentration of H_2O_2 on the DPV peak current (C). The concentration of thrombin is 10 nM. Error bars represent standard deviation, n=3.

Amplification performance of the proposed aptasensor

In order to evaluate that the LDNA-GNP-HRP conjugates could efficiently amplify the signal response, comparative experiments were performed. Fig. 5 showed the DPV response of the aptasensor before (curve b) and after (curve a) the LDNA-GNP-HRP conjugates were connected to Ap1-GNP-HRP conjugates. As expected, a considerable increased signal was observed (curve a) after the LDNA-GNP-HRP conjugates were immobilized on the electrode surface, indicating the use of LDNA-GNP-HRP conjugates could further amplify the electrochemical signal.

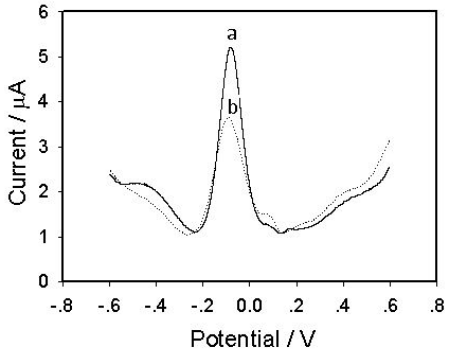

Fig. 5 DPV of aptasensor before (curve b) and after (curbe a) the LDNA-GNP-HRP conjugates were connected to Ap1-GNP-HRP conjugates. The concentration of thrombin is 10 nM. Error bars represent standard deviation, n=3.

Amperometric response and calibration curve

Under the optimal experimental conditions, the detection of thrombin by the proposed aptasensor was carried out in 20 mL 0.1 M PBS (pH7.4) containing 2 mM H_2O_2 and HQ. As can be seen from Fig. 6A, the electrochemical signal increased with increasing concentrations of thrombin. The working curve illustrated in Fig. 6B showed that the peak current of 0.0005 nM deviate far from the

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working curve. A linear relationship between peak current and thrombin concentration was from 0.001 to 20 nM with a detection limit of 0.6 pM based on a signal-to-noise ratio of 3 (S/N = 3, defined as 3 σ , where σ is the standard deviation of the blank solution, n=10). The regression equations of the calibration curve is y=3.914+0.1676x, R^2=0.9936. The performance of proposed immunosensor was compared with other published sensors (Table1).[4,28-32] Table 1 demonstrated that the immunosensor fabricated by us exhibited an acceptable linear range and detection limit.

Tabel 1 Comparison of the proposed immunosensor with other sensors.

Methods	Linearity (nM)	Limit detection	Ref.
Chronocoulometry	0.1-18.5	30 pM	4
Fluorescence enhancemen	1.4-21	0.7 nM	28
Electrochemistry	0-1000	10 nM	29
Electrochemiluminescence	0.001-1.0	0.38 pM	30
Electrochemistry	0.005-100	1.7 pM	31
Electrochemistry	1-60	0.5 nM	32
Electrochemistry	0.001-20	0.6 pM	-

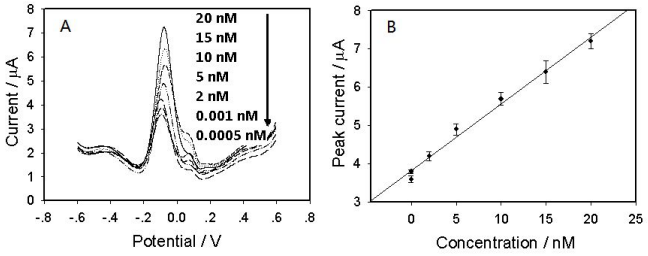

Fig. 6 (A) DPV responses of the aptasensor incubated with thrombin of different concentration in 0.1 M PBS (pH7.4) containing 2 mM H_2O_2 and HQ. (B) The calibration plots of DPV peak current versus the thrombin concentration.

Specificity and reproducibility of the aptasensor

In order to evaluate the binding specificity of the aptasensor to thrombin, some non-specific proteins such as BSA, human IgG, and HSA mixed with thrombin, respectively, were tested following the same experimental conditions. As shown in Fig. 7, the peak current of the aptasensor corresponding to the addition of bovine serum albumin (50 nM), human IgG (50 nM) or human serum albumin (50 nM) causes insignificant current changes compared with that of the aptasensor corresponding to the thrombin (10 nM). The results demonstrated that the proposed aptasensor was specific to thrombin.

To investigate the reproducibility of the proposed assay method, a sample with a thrombin concentration of 10 nM was successively detected 5 times under the optimized conditions. Acceptable repeatability was observed with a relative standard deviation (R.S.D.) of 5.9%, which indicates that the aptasensor can be constructed and used for analysis with excellent reproducibility.

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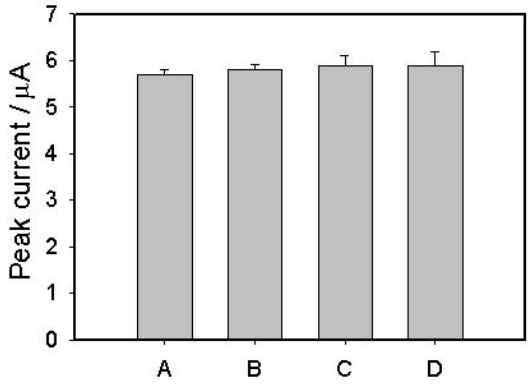

Fig. 7 Peak current of the proposed aptasensor corresponding to 10 nM thrombin (A), mixtures of 10 nM thrombin with 50 nM BSA (B), IgG (C), or HSA (D), respectively. Error bars represent standard deviation, n=3.

Analytical application of the aptasensor

Table 2 Determination of thrombin added in human serum with the proposed aptamer sensor (n=3).

Samples	Added (nM)	Founded(nM)	Relative deviation (%)
1	0.50	0.46	- 8.0
2	1.00	1.07	7.0
3	5.00	4.83	-3.4
4	10.0	9.75	-2.5

In order to evaluate the feasibility of the aptasensor for clinical applications, recovery tests were performed according to the method reported by Zhang.[28] Thrombin is not present in the blood and plasma of healthy subjects when coagulation is not occurring. A series of samples were prepared by adding thrombin of different concentrations into 5-fold-diluted healthy human serum samples. The results obtained by the proposed aptasensor were summarized in Table 2, suggesting that the electrochemical aptasensor has a promising potential application in real biological samples.

Conclusions

In summary, a novel signal amplification strategy based on hybridization chain reaction was developed for thrombin detection. Two types of conjugates containing horseradish peroxidase were prepared. The first conjugates were immobilized onto the electrode surface via specific reaction between aptamer and thrombin, resulting in a detection signal due to horseradish peroxidase. The second conjugates were immobilized onto the electrode surface via hybridization chain reaction between the complementary DNA and the aptamer of the first conjugates. The second conjugates caused a significant signal amplification because more horseradish peroxidase was introduced to the surface of electrode. This novel strategy is simple and avoids the complexity of exonuclease-assisted target recycling amplification. Under the optimal experimental conditions, the detection of range is from 0.001 to 20 nM with a detection limit of 0.6 pM. The aptasensor possesses high sensitivity and good reproducibility. We anticipate that this method can be extended for the quantification of other proteins, and have a potential value in clinical application.

Acknowledgments

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