A Sensitive Thrombin Aptasensor Based on Target Circulation Strategy

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A convenient homogeneous electrochemical thrombin sensor based on potential-assisted Au–S deposition and a dual signal amplification strategy was established in this study. Potential-assisted Au–S deposition does not require the modification of the gold electrode, thus eliminating the tedious pre-modification of the electrode. To better amplify the output signal, both ends of the signal hairpin probes were modified with a new electroactive substance, tetraferrocene, which was synthesized by the authors. Thrombin was immediately hybridized with a thiol-modified probe to open the stem-loop structure. After chain hybridization, thrombin was replaced and participated in the next round of the reaction; thus, the cascade amplification of the signal was realized. The hybrid chain formed an Au–S deposition under potential assistance, and the electrochemical signal of tetraferrocene could then be measured through differential pulse voltammetry (DPV) and consequently used for the quantitative detection of target thrombin. In addition, the detection limit of thrombin was as low as 0.06 pmol/L, and the detection of common interfering proteins was highly specific.

Keywords Thrombin, electrochemical, tetraferrocene, catalytic hairpin assembly

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Introduction

Thrombin is a special serine protein in the coagulation system of the body. Research suggests that thrombin can be involved in vascular neogenesis, thrombus formation and tumour growth in a variety of ways.[1] The thrombin content in normal bodies is very low, so it is important to establish a sensitive and simple method for the quantitative detection of ultra-micro thrombin.

Aptamers are a kind of single-stranded DNA or RNA molecule with a certain length that can specifically identify various target molecules (such as small organic molecules, peptides, proteins, *etc.*) and have a high affinity and specificity.[2,3] By combining aptamers and biosensor technologies, a variety of aptamer sensors have been established, such as fluorescence sensors,[4-7] colorimetric sensors,[8,9] quartz crystal microbalance (QCM) sensors,[10] and electrochemical sensors.[11-13] Among them, electrochemical sensors have significant advantages of low cost, simple operation and high sensitivity and have been widely used in thrombin detection.[14] In recent years, a variety of signal amplification methods have been reported to improve the analytical performance of thrombin electrochemical sensors, such as strand displacement amplification (SDA),[15,16] rolling cycle amplification (RCA),[17,18] loop mediated amplification (LAMP),[19] hybrid chain reaction (HCR),[20,21] and catalytic hairpin assembly (CHA).[22] The CHA is a new free-energy driven hairpin DNA assembly method developed on the basis of HCR. The target chain can be replaced to trigger the next round of reaction. Because of its ability to detect cyclic amplification signals, it has been increasingly favored. However, the existing thrombin electrochemical sensor based on CHA usually requires the probe to be fixed on the electrode surface in advance, which is time-consuming, and hybridization reaction occurs in a heterogeneous solid–liquid two-phase system. With DNA on the surface of the electrode, the space steric effect will reduce the hybridization efficiency, and the efficiency of the fixed electrode surface and DNA uncertainty will affect the repeatability and reliability of the detection.[23,24] Herein, we build an electrochemical sensor that makes the CHA reaction occur in a homogeneous solution through potential-assisted Au-S deposition[25,26] for the first time. It successfully avoids the complicated electrode pre-modification process, reduces steric hindrance, and effectively improves combining efficiency.

In electrochemical analysis, the intensity of the electrochemical signal is closely related to the selection of signal markers. Commonly used materials include metal nanomaterials,[27] enzymes[28] and metal organic frames (MOFs).[29] However, these methods usually require expensive materials and complex operations. Compared with the above methods, electroactive substances (such as ferrocene (Fc) and methylene blue (MB))[12,30,31] not only avoid these shortcomings but also have the characteristics of strong reversibility, low regeneration potential and stable oxidation reduction states, which makes them highly suitable for signal amplification. However, ferrocene only produces one electron transfer in a redox reaction, which limits its amplification effect. Therefore, the laboratory synthesized an electroactive substance, tetraferrocene, which can produce more electron transfers at a given time for better signal

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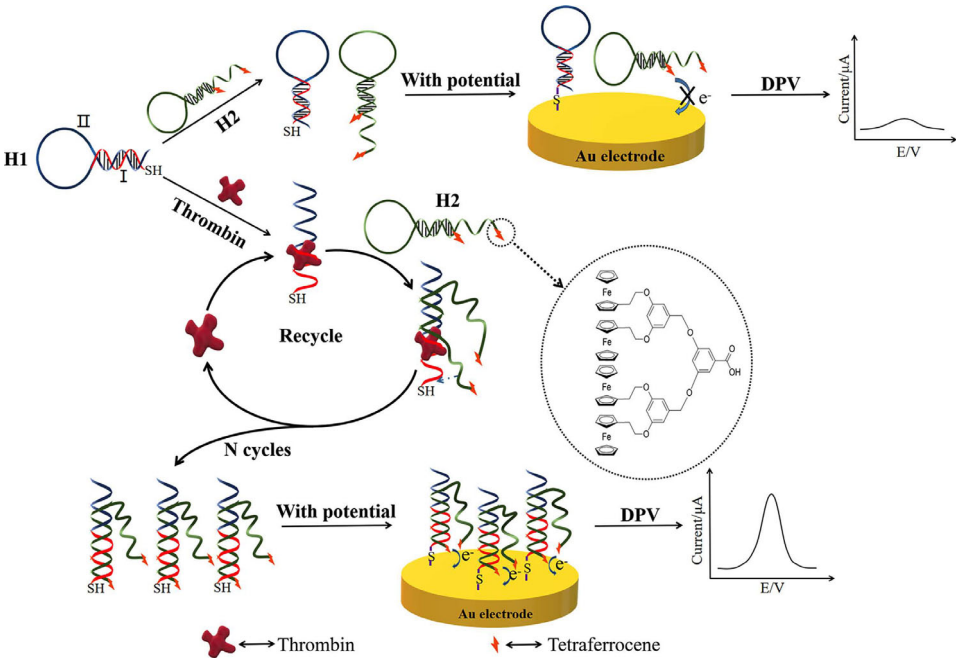

Scheme 1 Schematic diagram of the homogeneous and dual signal amplification strategy based on catalytic hairpin assembly (CHA) and the electroactive substance tetraferrocene.

amplification.

In this paper, based on the principle of electrochemically assisted Au–S deposition, a homogeneous, non-modified electrochemical thrombin sensor with a dual signal amplification strategy of a CHA and tetraferrocene was established. The detection principle is shown in Scheme 1. First, the 3′ end of hairpin probe H1 was marked with thiol, and tetraferrocenes were modified at both ends of hairpin probe H2. In the absence of thrombin, probes H1 and H2 did not hybridize. Although probe H1 could form a stable Au–S bond with the gold electrode under potential assistance, probe H2 was still far from the surface of the electrode; therefore, no electrochemical signal was detected. Once the target thrombin was added, thrombin rapidly hybridized with H1, and the stem-loop structure was opened. H1 exposed the complementary sequence that can open hairpin H2, eventually obtaining the H1/H2 double-chain complex and replacing thrombin to realize the circulation of thrombin and participate in the next CHA reaction. These H1/H2 double-chain complexes formed a stable Au–S bond with the exposed gold electrode surface through potential assistance; then, the tetraferrocene labelled by the hairpin probe H2 5′ end was closer to the gold electrode. At the same time, due to the existence of a large number of unhybrid T bases at the 3′ end of H2, the tetraferrocene fixed at the H2 5′ end was also close to the gold electrode. Compared to our previously published work using tetraferrocene to amplify the signal,[32] this thrombin electrochemical sensor demonstrates higher sensitivity because CHA enables target recycling, which amplifies the signal greatly and in particular increases the detection limit significantly.

Experimental

Reagents and materials

Thrombin, bovine serum albumin (BSA), and immunoglobulin G (IgG) were supplied by Sangon Biotechnology Co., Ltd. (Shanghai, China). Unless otherwise indicated, the required reagents, such as concentrated sulfuric acid (H_2SO_4) and concentrated nitric acid (HNO_3), were analytical-reagent grade and acquired from Dingguo Biotechnology Inc., and all the experimental water was prepared with ultrapure water from Millipore Milli-Q (USA). The oligonucleotide sequences used in this experimental process were purchased from Sangon Biotech Company, Ltd. (Shanghai, China), and their sequences are listed below.

Thiolated hairpin probe (H1) containing thrombin aptamer sequences:

5′-ACACCAACCTTTTCGTTTTGGTTGGTGTGGTTGGA-ATTTTTT-$(CH_2)_6$-SH-3′.

The connection of the probes to the tetraferrocene was accomplished with the help of the Sangon Biotech Company. The probe sequences of H2, H2-1 and H2-2 are shown as follows.

Signal hairpin probe (H2):

5′-tetraferrocene-ACCTTTTCGTTTTGACCACACCAACC-AAAACGAAAAGGTTGGTGTTTCATTTTTTTTTTTTTT-TTTTTTT-tetraferrocene-3′.

Signal hairpin probe (H2-1):

5′-tetraferrocene-ACCTTTTCGTTTTGACCACACCAACC-AAAACGAAAAGGTTGGTGTTTCATTTTTTTTTTTTTT-TTTTTTT-3′.

Fluorescence hairpin probe (H2-2):

5′-FAM-ACCTTTTCGTTTTGACCACACCAACCAAAAC-GAAAAGGT-DABCYL-TGGTGTTTCATTTTTTTTTTTT-TTTTTTTTT-3′.

To reduce the formation of the S–S bonds and obtain the SH terminal groups, the above probes were treated with 10 mM Tris (2-carboxyethyl) phosphine hydrochloride for 1 h, and then a Sephadex G25 column (NAP-10, Pharmaci a Biotech) was used to purify the treated probe. Subsequently, the concentration of the probe was measured spectrophotometrically at 260 nm, followed by storage at 4°C for later use.

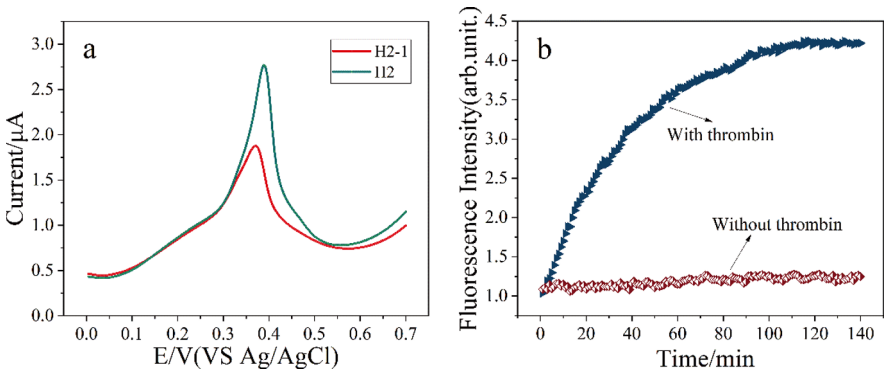

Fig. 1 (a) Comparison of DPV responses of H1 hybridized with different H2 under 1.5×10^{-9} mol/L thrombin. (b) Changes in fluorescence intensity in 140 min of (red) H1 and H2-2 incubated without thrombin (blue) H1 and H2-2 incubated with thrombin (1.2×10^{-11} mol/L).

Apparatus

All the electrochemical experiments were measured on an AutoLab electrochemical workstation (Metrohm Instruments Co., Swiss) with a three-electrode system: a gold electrode (diameter of 2.0 mm) as the working electrode, a platinum wire as the counter electrode and Ag/AgCl electrode (saturated in KCl) as the reference electrode at room temperature (25°C). The pH values of the reagents used in the experiment were determined by a pH meter (PH5520, HeBei Create Instrument Technology Co., Ltd, China). An F-4500 spectrophotometer (Hitachi, Japan) and Varioskan LUX microplate reader (Thermo Scientific, America) were used.

The pre-processing of the electrode

First, the gold electrodes (AuEs) were cleaned by immersing in freshly prepared piranha solution (H_2O_2:H_2SO_4 = 3:7, v/v) for a minimum of 10 min in microcentrifuge tubes, followed by washing with deionized water. Then, the AuEs were polished step-by-step with 0.3 and 0.05 mm alumina powder on a flat pad for 5 min each to obtain mirror-like surfaces; they were sequentially sonicated in deionized water and methanol for 5 min to remove any remaining impurities on the AuE surfaces. The AuEs were then electrochemically cleaned in a fresh deoxy H_2SO_4 solution (0.1 mol/L) with successive scans from 0 to 1.6 V until remarkable voltammetric peaks were obtained. The AuEs were finally dried with a nitrogen stream.

Au–S deposition on the electrode under potential assistance

Firstly, the gold electrode was cleaned at 0.3 V for 5 min, and then the gold electrode was immersed in a target hybrid of 400 μL at 0.4 V for 6 min incubation. Finally, the surface of the gold electrode was thoroughly rinsed with ultra-pure water to remove the weak adsorbed DNA.

Synthesis of 3,5-bis(3,5-bisferrocenethoxybenzyloxy)benzoic acid (9)

The synthetic route of 3,5-bis(3,5-bisferrocenethoxybenzyloxy)-benzoic acid is outlined in Supporting Information.

Electrochemical sensing performance

The sensing assay was performed in 10 mmol/L phosphate buffer (PBS) containing 3.0 mmol/L Mg^{2+}; 10 μL of 0.3 μmol/L probe H1 and 0.4 μmol/L H2 were added to target thrombin, and the mixture solution was stirred for 2 h at 42°C to hybridize the target protein and probe. Then, the H1/H2 double-chain complexes were immobilized on the electrode through Au–S deposition under potential assistance for 6 min. The surface of the gold electrode was washed with 0.1% sodium dodecyl sulfate phosphate buffer (pH 7.3) unless specific adsorption occurred.

The electrochemical measurements were carried out in a 5 mL electrochemical cell with a three-electrode system, and a potential scan was performed by differential pulse voltammetry (DPV). With an increment of 0.001 V, a pulse amplitude of 0.05 V, and a scanning rate of 0.002 V/s, a differential pulse voltammogram in scanning mode was applied in the scanning range of 0 – 0.7 V (*vs.* Ag/AgCl). Finally, the electrochemical signal of tetraferrocene was obtained at a potential of about 0.4 V. Thus, the quantitative analysis of thrombin could be realized *via* the signal intensity.

Results and Discussion

Verification of homogeneous CHA amplification aptasensor

A dual-labelled probe can amplify the signal better than a single-labelled probe. Part I (red) of H1 binds to thrombin and opens the stem ring structure, and part II (blue) contains the CHA starting sequence that can be hybridized with H2. According to the position formed by DNA hybridization, the sequence of H2 was extended through calculation and theoretical simulation so that the tetraferrocene in the H2 3′-end could also be close to the surface of the gold electrode. A hairpin probe H2-1 was designed to visually demonstrate the performance differences of the sensors that marked two tetraferrocenes and marked individual tetraferrocenes. H2-1 was only marked with tetraferrocene at the 5′ end, and the base part was designed in accordance with H2. Thrombin (1.5×10^{-9} mol/L) was added, other conditions were consistent, and DPV signal differences were compared. As shown in Fig. 1a, by comparing the DPV signals of H2 and H2-1, it was found that the signal response of bilaterally modified tetraferrocene increased by 1.5 times.

The CHA reaction induced by thrombin in a homogeneous solution was verified by a fluorescence assay. The probe H1 sequence was unchanged. Probe H2-2 was based on the probe H2 sequence. The stem-loop structure of probe H2-2 caused the fluorescence group FAM to be close to the quenching group DABCYL, and the fluorescence was quenched. The CHA reaction is a cyclic process that can be triggered by a trace of target, so it has obvious time dependence. The temperature was set to 42°C in a microplate reader, and the fluorescence emission wavelength and detection wavelength were set to 490 and

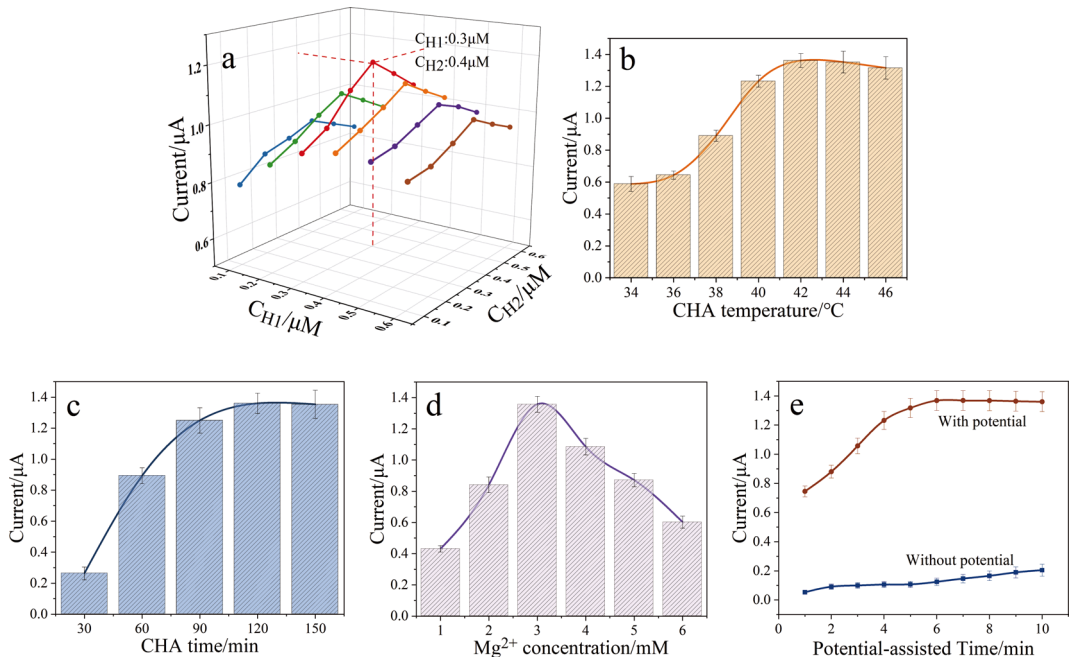

Fig. 2 DPV responses of (a) different hybrid concentrations. (b) Different CHA reaction temperatures. (c) Different CHA reaction times. (d) Different Mg^{2+} concentrations. (e) Different potential-assisted times.

530 nm, respectively. The fluorescence of the mixture was recorded every minute for 140 min, and a scatter plot was drawn (Fig. 1b). In the absence of thrombin, the fluorescence intensity did not change significantly for 140 min when only H1 and H2-2 were incubated, indicating that the CHA reaction did not occur (red curve). When H1, H2-2 and thrombin (1.2×10^{-11} mol/L) were incubated together, the fluorescence intensity increased with time and reached the platform in approximately 120 min, indicating that thrombin could initiate the CHA reaction in a homogeneous solution and that the CHA reaction could be completed in 120 min (blue curve). Fluorescence emission spectroscopy further confirmed that thrombin can induce the CHA reaction in a homogeneous solution (Fig. S2).

Optimization of experimental parameters

To obtain the best sensor performance, we optimized the concentrations of H1 and H2, the CHA reaction temperature and time, the Au–S self-assembly efficiency, the concentration of Mg^{2+} during the detection of thrombin and other experimental parameters according to the sensor's DPV response to thrombin. The concentrations of H1 and H2 were first investigated from 0.1 to 0.6 μmol/L, where the thrombin concentration was 1.2×10^{-12} mol/L and incubated for 90 min. As shown in Fig. 2a, changes in the concentrations of H1 and H2 would cause changes in the value of the current response. The probe with a lower concentration could only bind less thrombin, resulting in fewer formed H1/H2 double-chain complexes, while a higher concentration may increase steric hindrance and decrease the current response. The highest value of the current response occurred when the concentration of H1 was 0.3 μmol/L and that of H2 was 0.4 μmol/L. Thus, 0.3 μmol/L H1 and 0.4 μmol/L H2 were chosen for the sensor fabrication. When thrombin (1.2×10^{-12} mol/L) was incubated with H1 and H2 within the temperature range of 34 – 46°C, the maximum signal response occurred at 42°C (Fig. 2b), which meant that 42°C was the best choice for our next experiment.

Figure 2c shows the effect of CHA incubation time intuitively by DPV signal. The current response value increased with time from 30 to 120 min and levelled off after 120 min, indicating an optimal incubation time of 120 min. This was also true for other concentrations of thrombin. At the same time, Mg^{2+} concentration was considered because Mg^{2+} has a significant influence on intermolecular interstem-loop hybridization and intermolecular target-probe hybridization. The signal current reaches the highest value when the Mg^{2+} concentration is 3 mmol/L, so a 3 mmol/L Mg^{2+} concentration is used in the whole detection process (Fig. 2d). After incubating thrombin (1.2×10^{-12} mol/L) with H1 and H2 at 42°C for 120 min, it was difficult to form an Au–S deposition without an applied voltage. In contrast, the Au–S deposition was completed quickly under potential assistance. As shown in Fig. 2e, 6 min was the best auxiliary time.

Sensitivity and selectivity of the aptasensor

After optimization, the sensor was used to detect thrombin at different concentrations. The higher the concentration of thrombin is, the more H1–H2 double-chain complexes are formed, and the stronger the signal produced by tetraferrocene is (Fig. 3a). Through linear regression analysis, the response signal was positively correlated with the logarithm concentration of thrombin in a range from 1.2×10^{-13} to 1.2×10^{-9} mol/L. The regression equation was $y = 0.3428 \log x + 4.7806$ ($R^2 = 0.9941$) (Fig. 3b), and the detection limit of thrombin was 0.06 pmol/L. Compared with other electrochemical thrombin biosensors based on MB or Fc, as shown in Table 1, the developed biosensor has a wider linear range and a higher sensitivity. We believe that this result is mainly due to the high amplification efficiency of tetraferrocene, which can provide more electron transfer than MB or Fc in a redox process. Meanwhile, the CHA reaction occurs in a homogeneous solution, so it has higher hybridization efficiency. Moreover, the electrode does not need pre-modification, which saves

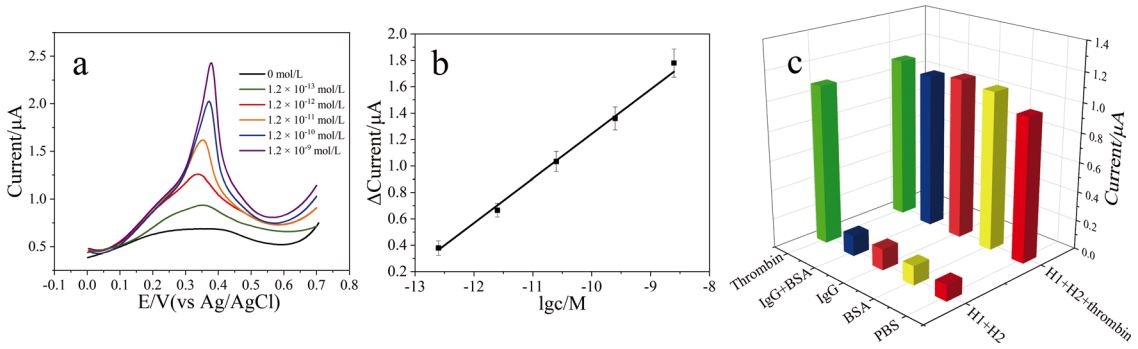

Fig. 3 (a) DPV responses of the sensor to different thrombin concentrations. (b) Calibration curve of thrombin. (c) Specificity of the electrochemical sensing platform for thrombin against PBS, 1.0×10^{-10} mol/L BSA, 1.0×10^{-10} mol/L lgG, 1.0×10^{-10} mol/L BSA + 1.0×10^{-10} mol/L lgG, 1.0×10^{-12} mol/L thrombin.

Table 1 Performance comparison of thrombin sensors for different detection methods

Detection method	Amplification strategy	Signal marker	Electrode pre-modified material	Electrode pre-modified time	Response time	Linear range	Detection limit	Ref.
Electrochemistry	MB-DNA	MB	Three-stranded DNA complexes	80 min	90 min	5 pM – 1 nM	1.7 pM	33
Electrochemistry	BMM-powered SPCR	Fc	Hairpin DNA 1 (H1)	2 h	95 min	2 pM – 20 nM	0.76 pM	34
Electrochemistry	AuNPs-MoS2	MB	Aptamer probes labeled with MB	12 h	12 h	0.01 nM – 10 μM	1.2 pM	35
Electrochemistry	MB Fc	MB Fc	Fc-HP	2 h	2 h	3 pM – 30 nM	1.1 pM	36
Electrochemistry	CHA + tetraferrocene	Tetraferrocene	Immobilization-free	0 h	2 h	0.12 pM – 1.2 nM	0.06 pM	This work

operational time.

The selectivity of the proposed aptasensor was investigated using PBS, BSA (0.1 nmol/L), and mouse IgG (0.1 nmol/L) as control proteins (Fig. 3c). According to the figure, only thrombin could cause obvious signal changes, and the other three proteins did not show significant changes. In addition, the mixture of thrombin (1.0 pmol/L) and the probe had a peak current comparable to the peak current in the presence of thrombin at 1.0 pmol/L. These results indicate that the sensor is highly specific to thrombin.

Reproducibility and stability of the aptasensor

Reproducibility is an important index used to evaluate the practicability of an aptasensor. Three concentrations of thrombin (25 pmol/L, 500 pmol/L, and 1 nmol/L) were selected for the experiment, and the corresponding relative standard deviations (RSDs) were 5.14, 4.37, and 4.11%, respectively, indicating that the sensor had acceptable reproducibility. The stability of the aptasensor was also studied. The prepared sensor detected the signal value of 25 pmol/L thrombin as the initial signal. After detection, the gold electrode was removed and polished, and 25 pmol/L thrombin was detected with the gold electrode every 12 h. After 12 h, the signal response value was 98.9% of the original value. The signal response after 72 h was approximately 93.3% of the initial value, indicating that the aptasensor had satisfactory stability. We believe that the excellent stability of the aptasensor stems from the fact that the electrodes used were unmodified, stable, and commercially available gold electrodes.

Detection of thrombin in human serum

Due to the complexity of the human environment *in vivo*, we added different concentrations of thrombin (10, 100, 500, 1000, 1500 pmol/L) into the 10-fold diluted serum for the sample recovery experiment to evaluate the practical application ability of the proposed sensor. According to the results listed in Table 2, the standard recovery of the sensor ranged between 93.27 and 112.87%, and the relative standard deviation ranged between 1.12 and 6.59%. Such satisfactory results indicate that the detection of the sensor in diluted human serum samples is still reliable and has potential application prospects.

Table 2 Recovery results of thrombin in a serum sample with designed sensor

Added/ pM	Found/ average	Rate of recovery, %	RSD, % ($n = 4$)
10	10.1	93.27 – 112.87	1.12
100	99.7	97.79 – 103.61	3.14
500	495.1	99.13 – 101.13	5.08
1000	984.3	99.59 – 100.27	3.53
1500	1472.8	99.72 – 100.52	6.59

Conclusions

In this work, a highly sensitive homogeneous electrochemical sensor for the detection of thrombin was successfully constructed based on Au–S deposition and a dual amplification strategy of a CHA and tetraferrocene. The main advantages of this sensor lie in the integration of potential-assisted Au–S deposition and a

homogeneous strategy. Under optimal conditions, the sensor showed good analytical performance for thrombin detection, with a detection limit as low as 0.06 pmol/L. It is worth noting that the sensor is applicable to real samples, so it has good practical application value and provides a new concept for the highly sensitive detection of thrombin in biomedicine.

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Conflict of Interest

The authors declare that there is no conflict of interest relating to the publication of this article.

Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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