

Endonuclease Based Hairpin Aptamer Probe for Background Current-eliminated Electrochemical Detection of Thrombin

Songbai Zhang^{1,a*}, Peng Xu^{2,a}, Yuhong Li¹, Xia Hu¹, Jilin Lu¹

¹ College of Chemistry and Materials Engineering, Hunan Province Cooperative Innovation Center for The Construction & Development of Dongting Lake Ecological Economic Zone, Hunan University of Arts and Science, Changde 415000, P. R. China

² Guangdong Institute of Microbiology, Guangzhou 510070, P. R. China

*E-mail: zsb0119@163.com

^aThese two authors contributed equally to this paper

Received: 27 October 2018 / Accepted: 29 December 2018 / Published: 7 February 2019

In this contribution, *Hpa* II restriction endonuclease is used for the first time to design endonuclease based hairpin aptamer probe and develop background current eliminated electrochemical aptasensor for sensitive signal-on detection of protein biomarker using thrombin as the model target. The hairpin aptamer probe consists of two functional domains: the aptamer sequence for target thrombin and the recognition sequence for *Hpa* II endonuclease. In the absence of target, the ferrocene labeled aptamer probe folds into hairpin structure with a palindromic recognition site which can be digested by *Hpa* II endonuclease, resulting in thorough removal of electro-active ferrocene from the electrode surface and complete elimination of background current for blank sample. While in the presence of target, the aptamer probe changes its conformation when binding with thrombin and dissociates the palindrome structure, resulting in obvious peak current. By eliminating the background current signal instead of amplifying the detection signal, the proposed electrochemical aptasensor reveals an excellent sensitivity towards target thrombin with a low detection limit of 2.67 pM. The sensing system is cost-efficient and easy controllable because only one oligonucleotide probe is involved. Moreover, the design principle of endonuclease based hairpin aptamer probe is easy adapted to other analytes.

Keywords: endonuclease; aptamer; electrochemical; thrombin; *Hpa* II

1. INTRODUCTION

As a major class of biomarkers, proteins are important and ubiquitous in all living organisms [1]. They play a vital role in many biological activities such as immune interaction, maintenance of body tissue, transportation and storage of certain molecules, and regulation of hormones [2]. The elevated or depressed protein expression level in serum, tissue, or saliva has held as a great promise as

important indicators of biological status and have played essential roles in clinical prognosis and diagnosis [3]. Particularly, catastrophic diseases such as cancer or Alzheimer's disease are often tightly associated with abnormal expression of unique proteins [4]. For example, thrombin, the main protease of coagulation cascade, is generated from inactive zymogen prothrombin and exhibits perfect procoagulant properties by converting soluble fibrinogen into insoluble fibrin [5–8]. It can also catalyze many coagulation related reactions [9] and act as a hormone to regulate platelet aggregation, endothelia cell activation, and other important responses in vascular biology [5,10]. Namely, abnormal concentration of thrombin is closely associated with various diseases such as cancers [6,11]. Therefore, the development of sensitive, cost-effective, reliable, and specific strategies for detecting and quantifying these disease-related protein biomarkers is of significant importance in researches.

Clinical diagnostic methods for protein biomarkers are traditionally based on immunological assays. However, the immune assay is faced with several considerable challenges such as long synthesis period *in vivo* and poor preserving stability [1,8]. In recent years, the development of aptamer technique provided a new candidate for detection of protein markers. Aptamers are artificially selected single stranded DNA or RNA oligonucleotides with many unique properties, such as high specificity and affinity with their targets, good stability, simple synthesis and easy modification [12–14]. Therefore, aptamers have been widely used as molecular recognition element in various biosensing strategy, including colorimetric [15–17], fluorescent [18–20], electrochemiluminescent [21–23], surface plasmon resonance [5,24], surface enhanced Raman spectroscopy [25,26], electrochemical [6,8,27,28] methods and so on. Compared to other detection approaches, aptamer-based electrochemical biosensors attracted much attention due to their remarkable superiorities, such as high sensitivity, cheap apparatus, easy pretreatment, low sample volume and adapted to miniaturization [8,29].

According to reported papers, however, many electrochemical aptasensors often encounter with the problem of high background current for not only the signal-off type but also the signal-on type due to the fact that most aptamer-involved electrochemical sensing strategies are based on structure switching of aptamer probe. Using thrombin detection as an example, Plaxco group reported a reagentless signal-on electronic, aptamer-based sensor via target-induced strand displacement [30]. A methylene blue (MB) tagged oligonucleotide hybridizes with thrombin-binding aptamer, forming rigid duplex DNA which prevents the methylene blue tag from approaching the electrode surface and suppresses the Faradaic currents. Thrombin binding stabilizes the G-quadruplex conformation of the aptamer, liberating the 5' end of MB labeled oligonucleotide and allowing the MB tag to collide with the electrode surface which produces a detectable Faradaic current. As a result, a simple signal-on electrochemical aptasensor was achieved. Almost at the same time, Radi reported a signal-on electrochemical molecular beacon aptasensor for thrombin detection with simpler design [31]. Aptamer probe with redox-active ferrocene moiety and thiol group was immobilized on the electrode. Upon binding with target thrombin, the aptamer probe changed its conformation from coli-like configuration to quadruplex structure, generating an increased current. Though both these electrochemical aptasensing strategies possess a signal-on sensing mechanism, the blank sample without target thrombin still showed an obvious background peak current, which influenced the detection sensitivity of the aptasensor to some extent and only nM-level detection limit are achieved.

In order to improve the detection sensitivity of electrochemical aptasensors, many scientists focus on enhancing the signal by using different signal amplification approaches, including various nanomaterials [32–34], enzymatic catalysis [32,35], recycling amplification [6,36–38] and even cascade signal amplification with combination of several kinds of means [39,40]. However, these complicated amplification means increasing the complexity of the sensor fabrication as well as the cost, which bring the disadvantage of less practicability. Some researchers tried to enhance the detection sensitivity of electrochemical biosensors by reducing the background current of blank sample. For example, Jiang reported a label-free aptasensor for thrombin detection based on background reduction and direct electron transfer of hemin [41]. Exonuclease I was used to cut the aptamer probe in the absence of target thrombin and minimize the background current noise of blank sample. However, it seems that the background noise cannot be suppressed completely since there is still a observable current for the blank sample. Our group have employed *EcoR* I restriction endonuclease to develop electrochemical aptasensors [8,29]. Compared with exonuclease, restriction endonuclease exhibit much better efficiency in background current suppression and much better analytical performance of the electrochemical aptasensor due to their high digestion efficiency and high fidelity of sequence-specific cleavage.

Herein, a hairpin aptamer probe is designed to develop a background current-eliminated electrochemical aptasensor based on *Hpa* II restriction endonuclease for the first time for sensitive detection of protein markers using thrombin as the model analyte. A specific recognition site for *Hpa* II restriction endonuclease was skillfully integrated into a ferrocene modified thiolated aptamer for thrombin, endowing the designed aptamer probe with two functional domains: recognition element for target molecules and cleavage site for restriction endonuclease. The aptamer probe folds into hairpin conformation in the absence of target molecules, and a palindrome structure is formed in the stem of the hairpin, which can be cut off after treating with *Hpa* II endonuclease. As a result, the background current for blank sample is complete eliminated since the ferrocene labeled moiety is thoroughly removed from the electrode surface after digestion. While binding with thrombin target molecules, the aptamer probe changes its structure to form a G-quadruplex conformation. The hairpin structure and the cleavable palindrome are dissociated, while the aptamer probe is intact and still close to electrode, resulting in significant detection current. By such delicate design, not only the background peak current corresponding to blank sample can be sufficiently suppressed but also a signal-on response mechanism is achieved. The proposed electrochemical aptasensor showed good sensitivity, selectivity, reproducibility and could be used for thrombin detection in real samples with satisfactory results.

2. EXPERIMENTAL

2.1 Chemicals and materials

Hpa II restriction endonuclease set (including *Hpa* II restriction endonuclease and 10× buffer) was bought from Fermentas. Thrombin and PDGF-BB were purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China). IgG, HSA, BSA and Tris were all obtained from Dingguo Biotechnology

Co., Ltd. (Changsha, China). 0.1M buffer contained 50 mM Tris-HCl (pH 7.8) and 100 mM NaCl was used as the oligonucleotide stock and dilution buffer. 100 mM NaClO₄ was used as the ac voltammetry (ACV) measuring solution. Millipore water (resistance >18MΩ·cm) was used in the experiments. All other chemicals were of analytical-reagent grade.

The aptamer probe used in this study was synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China) with a sequence as follows:



The aptamer probe is labeled with thiol group and ferrocene tag in the 5' and 3' terminal, respectively. The underline portion is the original aptamer sequence of thrombin, while the italic part is the specific recognition site of *Hpa* II restriction endonuclease with a CCGG palindrome sequence.

2.2 Apparatus

The CHI 660E electrochemical workstation (Chenhua Instruments, China) with a homemade detection cell and a conventional three-electrode system (gold electrode of interest as working electrode, platinum foil as counter electrode and KCl saturated calomel electrode as reference electrode) was used for all electrochemical measurements. All potentials measurement were referenced to the saturated calomel electrode (SCE) reference electrode.

2.3 Sensing interface fabrication

The gold electrode was burnished on suede to mirror smoothness with alumina slurries followed by rinsing with Millipore water, absolute alcohol and Millipore water in ultrasonic bath for 5 min, respectively. Then the gold electrode was soaked in piranha solution for 20 min. After rinsing with Millipore water, the electrode was further cleaned by cycling the potential in 0.1 M H₂SO₄ solution to obtain a repetitive cyclic voltammogram. After rinsing with Millipore water, 20 μL of aptamer probe was pipetted onto the freshly prepared gold electrode and incubated for 2 h, followed by rinsing with Millipore water again to remove the unbound probes. Then, 20 μL of 1 mM 6-mercaptohexanol was dropped on the electrode and incubated for 15 min to block the non-specific absorption sites. After rinsing with Millipore water, the electrode was ready for detection of target protein biomarker.

2.4 Electrochemical characterization and target protein detection

AC impedance was performed for characterization of fabrication and detection process of the proposed aptasensor. The impedance measurements were carried out in 5 mL of 10 mM PBS (pH 7.4) containing 5 mM [Fe(CN)₆]³⁻/[Fe(CN)₆]⁴⁻ and 0.1 M NaCl at the formal potential of the system ($E^0 = 240$ mV) using alternating voltage of 5 mV and a frequency range from 1 Hz to 100 KHz.

For thrombin detection, 20 μL of thrombin solution with a certain concentration was dropped on the freshly prepared electrode. After incubating at 37 °C for 80 min, 20 μL of 20 U/mL *Hpa* II

restriction endonuclease solution was pipetted on the resulting electrode and the digestion reaction was maintained at 37 °C for 90 min. Then the electrode was rinsed with Millipore water and ready for electrochemical measurements. AC voltammogram (ACV) measurements were carried out to evaluate the response characteristics of proposed aptasensor in 5 mL of 100 mM NaClO₄ solution from 0.0 to 0.6 V (versus SCE) at a specific frequency. The peak current intensity at 0.26 V is employed to measure target amount in sample and to evaluate the analytical performances of the proposed aptasensor.

2.5 Thrombin detection in serum and plasma sample

For thrombin detection in serum matrix, the obtained serum from local hospital was diluted by 10 times with Tris-HCl dilution buffer. Then different concentrations of standard thrombin were added into the diluted serum and ready for detection under the experimental conditions and processes described above. For the assay of endogenous thrombin in plasma sample, fibrinogen in plasma was first precipitated to avoid sample clotting by selective precipitant of ammonium sulfate. Briefly, 0.25 mL of plasma was mixed with 1.25 mL of 2 M ammonium sulfate and 1.0 mL of 0.1 M NaCl. After 4 minutes, the mixture was centrifuged and the supernatant was reserved, in which thrombin is present in the form of its precursor, prothrombin. Then, 200 uL of 0.25% trypsin and 1.0 M Calcium ion were added into the obtained supernatant (fibrinogen-free plasma). The analyte of thrombin was generated from prothrombin via proteolytic processing and detected by the proposed aptasensor.

3. RESULTS AND DISCUSSION

3.1 Design of hairpin aptamer probe and signal improvement

Generally, high signal-to-noise ratio means high sensitivity for a sensor. However, many reported aptamer-based electrochemical sensing strategies are accompanied by high background current for blank sample even for signal-on type of sensors, which influenced the detection sensitivity of the sensor to some extent. Many researchers tried to improve the detection sensitivity by different kinds of signal amplification means. Unfortunately, these complicated amplification approaches often brought difficult design and multiple procedures, which are prone to be affected by stochastic factors. Biosensors which are adapt to practical applications should possess the features of simple design and easy operation. Motivated by such considerations, we designed a restriction exonuclease based hairpin aptamer probe and proposed an electrochemical aptasensor in the present contribution to suppress background current and to improve detection performances. The design of the hairpin aptamer probe and the sensing mechanism of the proposed aptasensor are shown in Figure 1. The sequence in red is the original aptamer for thrombin, which contains 15 bases with the sequence of GGTGGTGTGGTTGG. By skillfully extending the 5' end and 3' end of the thrombin aptamer with 5 bases and 7 bases, respectively, the aptamer probe forms a stable hairpin structure in the absence of target thrombin. As shown in panel a of Figure 1, a seven-pair-bases stem containing a cleavable

palindrome structure with the sequence of CCGG in the blue dotted rectangle is formed as predicted by 'mfold' software, which can be specifically recognized by *Hpa* II restriction endonuclease and cut off between two cytosine (the green folded line shows the cutting site). Only four base pairs are remained after efficient digestion (panel b), which is not stable at room temperature. As a result, the cleavage products along with the ferrocene tags are easily moved away from the electrode and no peak current is observed (panel d), indicating thorough elimination of background current for blank sample. In the presence of target thrombin, the aptamer probe specifically binds with thrombin and changes its structure from hairpin to G-quadruplex (panel c), resulting in dissociation of palindrome-structured recognition site for *Hpa* II endonuclease. Accordingly, the aptamer probes maintain intact and close to the electrode surface, and a high peak current which is directly related to the concentration of thrombin target protein could be detected (panel e). By this approach, not only a background current eliminated electrochemical aptasensing system with high signal-to-noise ratio is developed but also a signal-on signaling mechanism is achieved, which improves the detection sensitivity and avoids the drawbacks of high background current for signal-off types of biosensors. What's more, only one oligonucleotide sequence is involved in sensor fabrication, endowing the sensing system with unique characteristics such as simple design, easy control, timesaving operation and great potential in practical application.

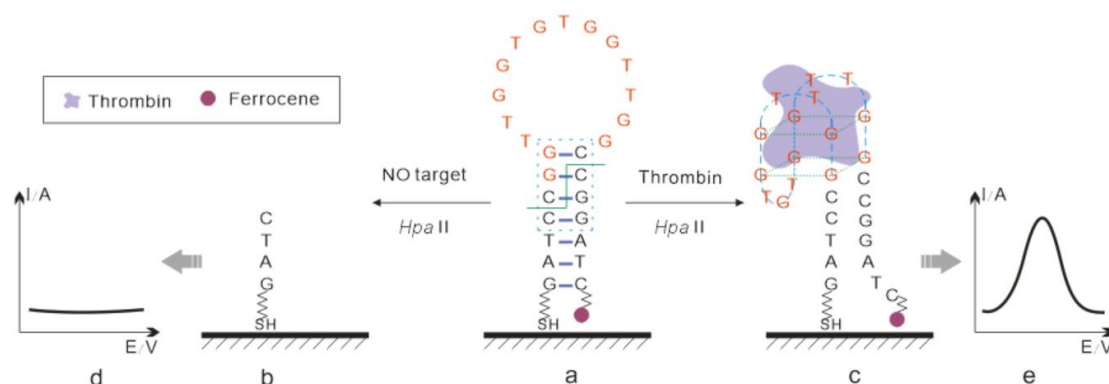


Figure 1. Design of hairpin aptamer probe and detection principle of the electrochemical aptasensor: (a) the predicted secondary structure of the hairpin aptamer probe without target; (b) residual sequence after digestion; (c) structure switching after exposure to target thrombin; (d) ACV measurement without target shows complete eliminated background peak current; (e) ACV measurement with target thrombin shows high detection peak current.

3.2 Proof of principle by control experiments

The proof of principle of the proposed sensing strategy was confirmed by control experiments as shown in Figure 2. Target sample with a high concentration of 1.0 $\mu\text{g/mL}$ thrombin induced an obvious peak current of about 122 nA (solid line), which confirmed the hypothesis that the aptameric probe changed its structure to bind with target molecules and dissociated the cleavable palindrome recognition site for *Hpa* II endonuclease. The ACV current peak was consistent with previous reference [8], which meant that the electroactive ferrocene moiety was unsusceptible to the digestion reaction by *Hpa* II endonuclease. It should be noted that the peak current intensity induced by

thrombin with a high concentration is only a little lower than the freshly prepared sensing interface with a peak current intensity of about 138 nA. That might be attributed to the fact that the hairpin-structured aptamer probe and mixed self-assembled layer of hairpin probe and 6-mercaptohexanol provided sufficient space for binding with target protein. The other possible reason is that the ferrocene moieties labeled on the reacted aptamer probe are still as close to the electrode surface as the hairpin aptamer probes after binding with thrombin. On the contrary, almost no peak current of ferrocene was observed for blank sample (dotted line) by AC voltammetry measurements, indicating that the specific palindrome-structured recognition site for *Hpa* II endonuclease was successfully formed and thoroughly digested by enzyme digestion reaction. Thus, a signal-on sensing mechanism with eliminated background current and high signal-to-noise ratio was successfully achieved.

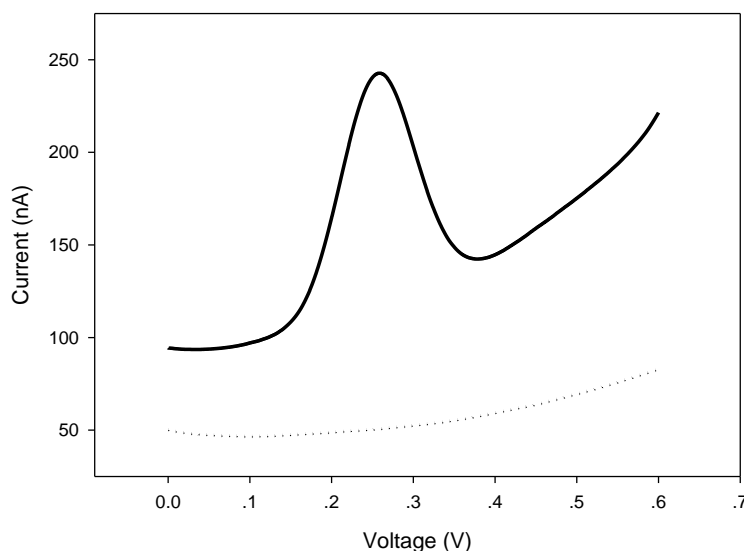


Figure 2. Control experiments for electrochemical response of the aptasensor without target (dotted line) and with 1.0 $\mu\text{g/mL}$ thrombin (solid line). All ACV measurements were performed in 5 mL of 100 mM NaClO_4 solution.

3.3 Electrochemical characterization of sensing interface fabrication and target detection process

Impedance spectroscopy of electroactive species in conducting aqueous solution is a helpful method for electrochemical characterization of the modified gold electrode. To monitor the sensor preparation process and further verify the feasibility of hairpin aptamer probe as well as the present sensing system, impedance spectroscopy of a fairly reversible redox couple $[\text{Fe}(\text{CN})_6^{3-}]/[\text{Fe}(\text{CN})_6^{4-}]$ for each step of sensor preparation and thrombin detection were performed as shown in Figure 3A. The bare gold electrode showed only a small impedance (line a), indicating the treated electrode had good electrochemical conductivity. After self-assembling with aptamer probe, the electrode exhibited an obvious increase of electrochemical impedance as shown in line b since a rigid and well-ordered assembly monolayer of hairpin probe was probably formed. Accordingly, electron transfer of the redox couple was hindered to some extent by the negatively charged phosphate backbone of the hairpin probe. The subsequent self-assembly of mercaptohexanol blocked the unreacted sites of gold electrode

and formed a mixed monolayer, resulting in further increase of impedance as shown in line c. Moreover, the obtained monolayer could provide adequate interstitial space for binding with target thrombin, resulting in further prevention of electroactive molecule from getting close to the electrode and a significant increment of impedance as seen in line d. However, an obvious decrease of electrochemical impedance was found (line e) after enzyme digestion by *Hpa* II restriction endonuclease. Obviously, this experimental phenomenon directly demonstrated the sensing mechanism from two aspects. First, the palindrome structure of unreacted hairpin probe can be cut off by *Hpa* II restrict endonuclease, inducing removing of the negative charged digestion products from the electrode. As a result, the electron transfer ability increased and the electrochemical impedance decreased accordingly. Second, since a portion of hairpin probes had reacted with target thrombin, the changed structure would hinder the recognition and digestion reaction by *Hpa* II restriction endonuclease. These reacted part of aptameric probes were maintained on the electrode, so the observed electrochemical impedance after digestion was still higher than line c. Cyclic voltammetry was also conducted to characterize the fabrication and detection process of the proposed aptasensor. It is well known that the lower the impedance, the higher the current. As shown in Figure 3B, the changing trend of current of the gold electrode at different stage was in good consistent with impedance. Distinctly, these characteristic experiments directly demonstrated the success in the design of the sensing strategy.

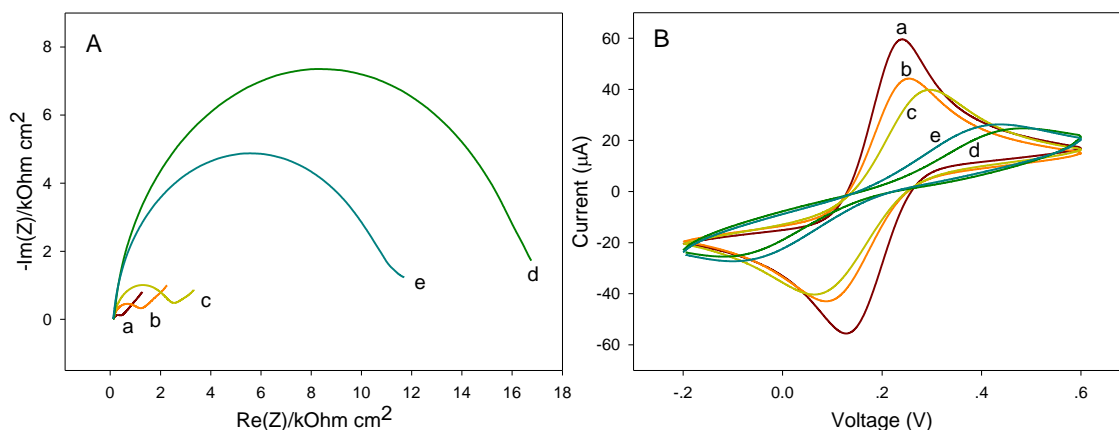


Figure 3. Impedance spectroscopy (A) and cyclic voltammetry (B) characterization of the gold electrode in 10 mM PBS (pH 7.4) containing 5 mM $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ and 0.1 M NaCl at different stages: (a) bare gold electrode; (b) aptamer probe immobilized electrode; (c) mercaptohexanol blocked electrode; (d) 216.5 ng/mL thrombin exposed electrode; (e) *Hpa* II restriction endonuclease digested electrode.

3.4 Influence of assembly time of hairpin probe

In the present work, only one oligonucleotide probe is used for sensor fabrication. The hairpin aptamer probe acts as not only capture probe but also signaling probe with the modification of 5'-SH and 3'-ferrocene. It has been reported that the assembly amount and stability is directly related to the self-assembly period of thiol-group labeled probe [42,43], current response of the electrode for different assembly time of the designed hairpin probe was measured as shown in Figure 4A. The

current intensity is very low when the aptamer probe incubated for only 10 min. That is might attributed to the fact that the assembly event of hairpin aptamer probe is unstable with too short assembly period. The peak current increases with the augment of assembly time and tends stable at about 120 min, indicating the probe self-assembly is saturated and a stable monolayer of hairpin probe is achieved. The results are in agreement with previous references [8][14], indicating that the designed hairpin aptamer probe adopted a similar self-assembly behavior. Thus, the optimum assembly period of 120 min is used in following experiments.

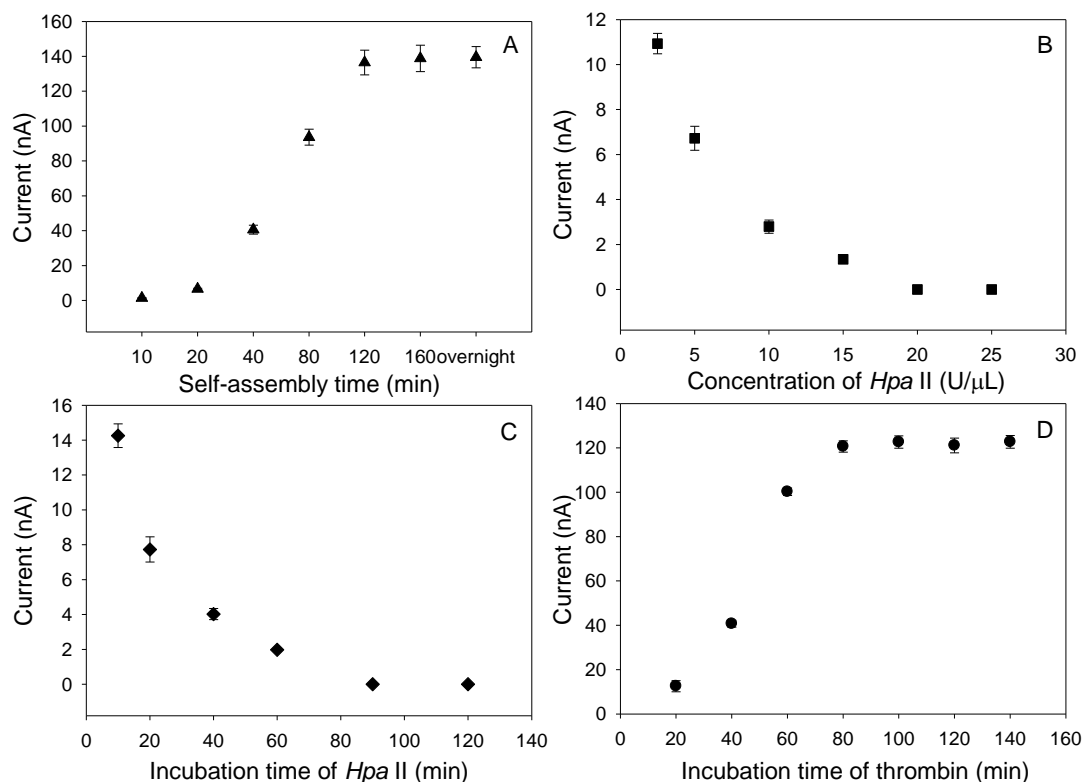


Figure 4. Investigation of experimental conditions: (A) Effect of self-assembly time of hairpin aptamer probe on current response of the aptasensor; (B) Influence of *Hpa* II endonuclease concentration on cleavage efficiency of the palindrome recognition site; (C) Influence of incubation time of *Hpa* II endonuclease on digestion efficiency of recognition site; (D) Effect of incubation time of target protein on current response of sensing system.

3.5 Investigation of *Hpa* II endonuclease concentration

Restriction endonucleases are enzymes that recognize short, specific DNA sequences. They cleave double-stranded DNA at specific sites within or adjacent to their recognition sequences. For example, the *Hpa* II restriction endonuclease has a palindrome recognition site of CCGG, which is usually combined with *M.Sss* I methyltransferase and used in methylation analysis. In the present work, by skillfully designing and combination of recognition sequence of *Hpa* II restriction endonuclease and aptamer sequence of thrombin, the *Hpa* II restriction endonuclease is successfully used for electrochemical aptasensor fabrication for the first time. Since the endonuclease concentration directly

influences the cleavage degree of palindrome recognition site and accordingly influences the sensor performances for thrombin detection, the concentration of *Hpa* II restriction endonuclease should be optimized. Figure 4B depicts the current response of the aptasensor without target for different concentration of *Hpa* II endonuclease. When a low concentration of 2.5 U/mL is used, a peak current of about 11 nA is measured after a sufficient digestion period, which is only 8.0% of that of about 138 nA for freshly fabricated sensing interface. The results mean that 92.0% of the hairpin signaling probes can be efficiently cut off even with a low concentration, indicating that the *Hpa* II restriction endonuclease has a excellent digestion efficiency towards recognition sites. The peak current intensity decreases rapidly as the concentration of *Hpa* II endonuclease further increases. When the concentration reaches a high value of no less than 20 U/mL, the voltage-current curve turns into flat and no current peak is observed, indicating that a relatively high concentration of *Hpa* II endonuclease could induce thorough cleavage of hairpin probe and complete elimination of background peak current for blank sample. In order to improve the detection capability, *Hpa* II restriction endonuclease with a concentration of 20 U/mL is used in the subsequent experiments.

3.6 Effect of incubation time of *Hpa* II endonuclease

Though the *Hpa* II endonuclease has a high digestion efficiency, the incubation time of the restriction endonuclease should also be optimized since different incubation period will influence the cleavage degree of the palindrome structured recognition sequence. The recommended protocol for digestion in the manual of *Hpa* II endonuclease shows that the digestion duration should be more than 1 hour in order to make sure of efficient cleavage in homogeneous solution. In order to save time and make sure all the signaling aptamer probes on the electrode could be digested in the absence of target protein, different incubation periods are investigated as shown in Figure 4C. A peak current of 14.3 nA is detected when the digestion duration maintains for only 10 min, which is only 10.4% of the original current intensity of fresh prepared sensing interface. It means that a 89.6% lost of peak current intensity is obtained under a short digestion period, indicating most of signaling hairpin probes are cleaved and removed from the electrode surface due to the high digestion efficiency of *Hpa* II restriction endonuclease. With the increase of digestion period, the peak current of hairpin probe assembled electrode decreases quickly and zero peak current is observed when the digest time reaches 90 min, indicating that the signaling hairpin probes are thoroughly cut off by *Hpa* II restriction endonuclease. Therefore, a optimum digestion time of 90 min is adopted in the followings experiments.

3.7 Influence of incubation time of thrombin

In the present work, the signal generation is based on structure switching of aptamer probe upon binding with thrombin target molecule, the incubation time of thrombin should be optimized for the binding event. In order to make sure the sufficient reaction between aptamer probe and thrombin, a relatively high concentration (1.0 µg/mL) of thrombin was used to evaluate the influence of incubation time of thrombin on the current response of the aptasensing system. It is worth mentioning that the

investigation of incubation time of thrombin should be evaluated after optimization of other experimental conditions conducted above in order to make sure the total cleavage of aptamer probes and complete elimination of peak current. As shown in Figure 4D, when a short incubation time of 20 min was applied, a very low current intensity of about 13 nA was found since only part of the target molecules bond with the aptamer probe. As the incubation time increased, the current intensity raised correspondingly and stabilized after an incubation period of 80 min, indicating saturated reaction of target thrombin. Thus, 80 min was selected as the sufficient incubation time for target molecules.

3.8 Detection capability of the aptasensing system.

In order to evaluate the detection capability of the hairpin aptamer probe and present aptasensing system, the ACV current responses for different concentrations of thrombin are measured as shown in Figure 5A. A representative AC voltammetry peak at 0.26 V is found and used to calculate the linear detection curve and assess the detection capability. As the augment of thrombin concentration, the measured peak current intensity increases accordingly, indicating that a signal-on mechanism is achieved (Figure 5B). A typical calibration curve between the peak current intensity and the concentration of thrombin is obtained with a linear relationship ranging from 0.68 ng/mL to 216.5 ng/mL as shown in the inset of Figure 5B. The calibration equation is $I = 0.34 C + 11.66$ with a correlation coefficient of 0.9943 (I represents peak current intensity, C represents thrombin concentration). The detection limit of thrombin is estimated as 0.1 ng/mL (about 2.67 pM), at that concentration the target thrombin molecules can induce a tiny peak current which is a little higher than blank sample.

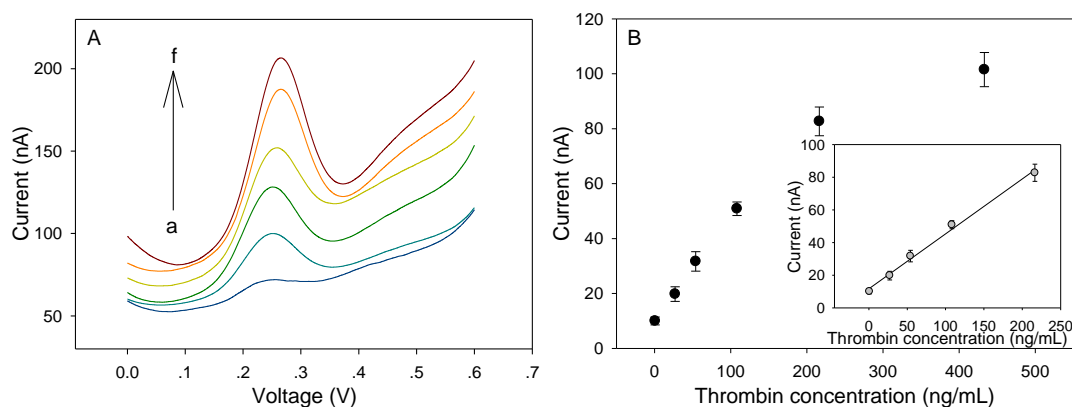


Figure 5. Analytical performances of the electrochemical aptasensor. (A) Line a to f are current response corresponding to 0.68 ng/mL, 27.1 ng/mL, 54.1 ng/mL, 108.3 ng/mL, 216.5 ng/mL and 433.2 ng/mL of thrombin. (B) Peak current intensity for different concentration of thrombin. Inset: the linear relationship between peak current intensity and thrombin concentration. All ACV measurements were performed in 5 mL of 100 mM NaClO₄ solution.

Compared with previous studies (shown in Table 1), the detection limit achieved for thrombin is much lower than reported electrochemical aptasensors that without extra amplification means, such

as electrochemical molecular beacon aptasensor [31], complementary DNA oligonucleotide based electrochemical aptasensor [44] and target-induced strand displacement based electronic aptasensor [30]. Though these aptasensing strategies have simple design and signal-on detection mechanism, the background current for blank sample are inevitable, which influence the detection sensitivity to some extent. Moreover, the achieved detection limit is also superior to or comparable with many signal amplification-involved aptasensing approaches, such as rolling circle amplification based fluorescent aptasensor [45], supersandwich DNA structure amplified electrochemical aptasensor [46], graphene based biosensing platform based on release of DNA probes and rolling circle amplification [47], dual-signal amplification strategy for electrochemiluminescence sandwich aptasensor [23], Y-junction DNA structure based circular signal amplification [6], proximity binding and metal ion-dependent DNAzyme cyclic amplification integrated aptasensor [37] and exonuclease-catalyzed target recycling and enzyme-catalysis [35]. However, these reported sensing strategies need various amplification means or different nanomaterials for signal enhancement, which increase the design difficulty and complexity as well as susceptibility in practical application. Instead of amplifying detection signal, the present sensing strategy improves the detection ability by decreasing noise since the background current is essentially eliminated by restriction endonuclease digestion reaction with high efficiency and fidelity. Moreover, compared with the above-mentioned sensing strategies, only one hairpin aptamer probe is used to develop the aptasensor without extra amplification approaches, endowing the present hairpin aptamer probe and aptasensing system with simple design, easy operation and low cost.

Table 1. Analytical performances of different strategies for thrombin detection.

Method	Strategy	LOD	Reference
Electrochemical	Molecular beacon	0.5 nM	[31]
Electrochemical	Complementary DNA	40 nM	[44]
Electrochemical	Target-induced strand displacement	3 nM	[30]
Fluorescent	Rolling circle amplification	30 pM	[45]
Electrochemical	Supersandwich DNA structure	10 pM	[46]
Fluorescent	Graphene and rolling circle amplification	10 pM	[47]
Electrochemiluminescence	Dual-signal amplification	6.3 pM	[23]
Electrochemical	Y-junction DNA structure	6 pM	[6]
Electrochemical	Proximity binding and DNAzyme	5.6 pM	[37]
Electrochemical	Exonuclease-catalyzed target recycling	1.7 pM	[35]
Electrochemical	Endonuclease based background elimination	2.67 pM	This work

3.9 Specificity and reproducibility of the aptasensing system

Both false positive and false negative signals should be avoided in practical application for a promising biosensor. To evaluate the specificity of the proposed hairpin aptamer probe and aptasensing system, the current responses to other different proteins were measured. Other protein biomarkers and common proteins, namely platelet-derived growth factor-BB, BSA, IgG and HSA, are investigated as shown in Figure 6. The peak current intensity caused by other proteins with concentrations of 10^2 to 10^5 fold higher than thrombin are no more than 5% of the peak current value induced by thrombin with

a concentration of 108.3 ng/mL within the linear detection range. Actually, the intensity of peak currents for other proteins are obtained by manually searching using the electrochemical workstation software because the current peak are too low to observe. It means that a high selectivity for thrombin detection is achieved in the present aptasensing system. These results should be attributed to specific recognition ability of anti-thrombin aptamer and the high fidelity of *Hpa* II restriction endonuclease towards cleavage site.

Measurement reproducibility is another indispensable factor for a successful and applicable biosensor. In order to evaluate the intra- and inter- assay reproducibility of the present aptasensing system, three samples with different concentrations within the linear detection range are investigated. Each sample is measured for five times with the same electrode to obtain the intra-assay reproducibility and with five different electrodes to obtain the inter-assay reproducibility. Relative standard deviations of the five data for each group were used to assess the magnitude of detection reproducibility. The maximum value of intra-assay reproducibility is 7.2%, while that of inter-assay reproducibility is 10.3%, which is a little larger than intra-assay due to the difference of surface areas from electrode to electrode. Nevertheless, the proposed electrochemical aptasensing system could offer an acceptable reproducibility for the detection of thrombin.

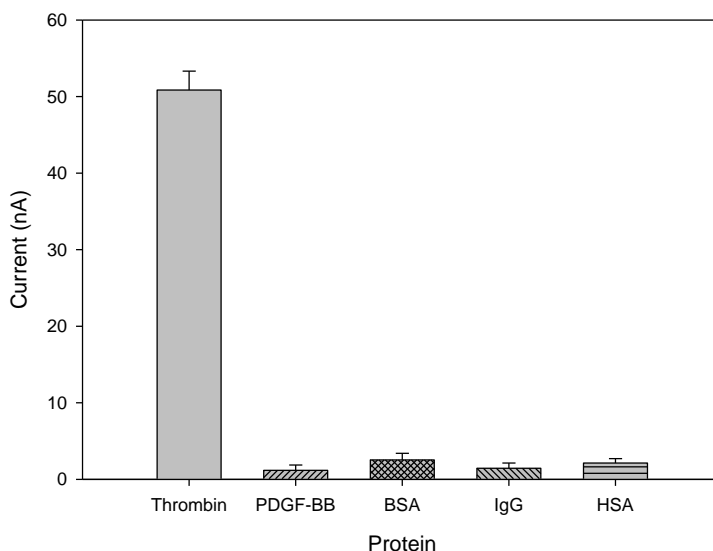


Figure 6. Specificity of the present electrochemical aptasensor. ACV current response to 108.3 ng/mL thrombin compared with other different proteins: 10 μ g/mL PDGF-BB, 10 mg/mL BSA, 1 mg/mL IgG and 1 mg/mL HSA. All ACV measurements were performed in 5 mL of 100 mM NaClO₄ solution.

3.10 Analysis of complex biological samples

Recovery experiments are carried out in human serum to evaluate the practical applicability of the present electrochemical aptasensor. Serum is obtained from whole blood after coagulation, which has similar composition to plasma but does not contain coagulation proteins such as thrombin [48]. By spiking 10-fold diluted human serum solution with different concentrations of thrombin in the linear range, each sample is repeated for three times, and the measured results are shown in Table 2. The

recovery value in the range of 97.8-104.4% is achieved, and the relative standard deviation for thrombin detection in human serum is 7.8-11.2%. To measure the endogenous amount of thrombin in real plasma, fibrinogen was precipitated first to avoid sample clotting [49]. The analyte of thrombin was generated from prothrombin (the precursor of thrombin) by calcium ion activation [50]. Three independent plasma samples with proper dilution were detected by the proposed aptasensor, and the measured concentrations of thrombin were 102.3 nM, 108.6 nM and 96.7 nM, which is in agreement with reported literature [50,51]. These results mean that the present aptasensing system has great potential for accurate analysis of thrombin in complex biological samples.

Table 2. Recovery of thrombin assay in human serum.

Sample	Added thrombin (ng/mL)	Found thrombin (average, ng/mL)	Recovery (%)	RSD (%)
1	216.50	221.82	102.5	9.3
2	54.13	52.93	97.8	7.8
3	0.68	0.71	104.4	11.2

4. CONCLUSION

In summary, we designed a hairpin aptamer probe and developed a restriction endonuclease based electrochemical aptasensing strategy for highly selective detection of thrombin. By integrating aptamer sequence of thrombin and recognition sequence of *Hpa* II restriction endonuclease, a background current eliminated aptasensing system with signal-on mechanism is achieved. Making use of the high efficiency and high fidelity of restriction endonuclease, the proposed aptasensing system could exhibit not only low detection limit but also high selectivity compared with reported strategies. The skillful signaling design, simple sensor fabrication process and excellent analytical performances makes the present electrochemical sensing strategy a competitive alternative for protein biomarkers analysis.

ACKNOWLEDGEMENTS

This study is financially supported by the National Natural Science Foundation of China (Grant No. 21205039), Natural Science Foundation of Hunan Province (Grant No. 13JJ6071), China Postdoctoral Science Foundation (Grant No. 2015M582339), Open Project of State Key Laboratory of Chemo/biosensing and Chemometrics (Grant No. 2016010), Open Fund of Key Laboratory of Chemical Biology and Traditional Chinese Medicine Research (Ministry of Education of China) (Grant No. KLCBTCMR2015-03). National Undergraduate Research and Innovative Experimental Project (Grant No. 201710549005) and Undergraduate Innovation and Entrepreneurship Research Project (Grant No. ZD1702).

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