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Notes & Tips

# Rational design of a thrombin electrochemical aptasensor by conjugating two DNA aptamers with G-quadruplex halves

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ABSTRACT

A novel strategy for the fabrication of an electrochemical aptasensor is proposed; this strategy has been employed in this work to assay thrombin concentration. Two well-designed oligonucleotides were used as the core element. G-quadruplex–hemin complexes can be formed on the surface of the electrode to give a detectable signal only when thrombin is not bound to the aptamers. The detection limit of the biosensor has been lowered to 10 nM. Moreover, since the electroactive probe is not required to be bound to the oligonucleotide, this strategy may integrate the advantages of being both label-free and cost-effective.



Aptamers are functional nucleic acid or peptide molecules with high specificity and affinity that are comparable to antibodies [1–6]. Their targets include small molecules, proteins, and even cells [7,8]. Aptamers can be easily incorporated into a DNA-based system for detection of various targets.

Aptamer-based analytical methods have been developed for protein detection, including electrochemistry [8], fluorescence detection [9], etc. Electrochemistry has been widely employed to take an important role in protein research because of its sensitivity, selectivity, and simplicity [10–12]. For example, the electrochemical thrombin aptasensor was fabricated by tethering a redox-active substance label, such as methylene blue [11], ferrocene [13], or quantum dots [14], to the terminal of an aptamer nucleic acid and immobilizing the oligonucleotide on an electrode, and the thrombin-binding aptamer undergoes a transition to a G-quadruplex structure after binding with thrombin. Since a target-induced conformational change of the aptamer will put the label close to the surface of the electrode, an electrochemical signal is achieved. In the meantime, despite a large amount of success in such labeled methods, label-free strategies are highly needed to increase simplicity and cost-effectiveness.

We described here a label-free, selective, and sensitive aptamer-based electrochemical assay for protein detection. As a proof of principle, human thrombin and its two aptamers, Apt29 and Apt15, were used in this study, Human thrombin is a coagulation protein in the bloodstream that converts soluble fibrinogen into insoluble stands of fibrin as well as catalyzing many other coagulation-related reactions [15–20]. In our strategy, we assay the thrombin concentration by using an electrochemical technique with G-quadruplex–hemin complexes as signal transduction probes. Hemin and its two split G-quadruplex halves can form the G-quadruplex–hemin complex in the presence of potassium ion [8]. Specifically, G-quadruplex–hemin complexes can be formed on the surface of an electrode to give a detectable signal only when the thrombin has not bound to the aptamers. This method to assay thrombin concentration has the advantages of being sensitive, label-free, and convenient, which may give it great potential for application in thrombin detection as well as clinical diagnosis in the future.

## Experimental section

### Chemicals

Thrombin, hemin, and 6-mercapto-1-hexanol (MCH) were obtained from Sigma. Oligonucleotides (Guaranteed Oligos, HPLC-purified) were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). Other chemicals were all of analytical grade. All the reagents are stable at room temperature and 4 °C. All solutions were prepared with double-distilled water ($ddH_2O$), which was purified with a Milli-Q purification system (Millipore, Billerica, MA, USA) to a specific resistance of >18 MΩ cm and stored at 4 °C.

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Two strands of oligonucleotides were adopted in our experiments. The sequences are as follows: DNA1, 5′-AGGGACGGGATTTTT*GGTTGGTGTGGTTGG*TTTTTTTTT-$(CH_2)_6$-SH-3′, and DNA2, 5′-**AGTCCGTGGTAGGGCAGGTTGGGGTGACT**TTTTTTGTGGAGGGT-3′.

DNA1 and DNA2 contain three functional regions: (1) the underlined sequence is a split aptamer targeting hemin; (2) the italic sequence is the thrombin Apt15 aptamer; (3) the sequence shown in bold is the thrombin Apt29 aptamer. The stock solutions of these two oligonucleotides were prepared with final concentrations of 1 μM (DNA1) and 200 nM (DNA2) in Tris buffer (pH 8.0, 10 mM Tris, 1 mM EDTA, 10 mM TCEP). And, the stock solution of hemin (2.0 mM) was prepared in NaOH (0.01 M) and diluted to the desired concentration with 40KT buffer (pH 6.2, 100 mM Tris, 50 mM Mes, 40 mM KCl, 0.05% Triton X-100, 1% (v/v) dimethyl sulfoxide).

*Preparation of working electrode*

A gold electrode was first cleaned with freshly prepared piranha solution (1:3 mixture of 30% $H_2O_2$ and concentrated $H_2SO_4$) for 5 min and rinsed thoroughly with double-distilled water. Then it was polished carefully with rough and fine sandpaper and subsequently alumina slurries (1.0, 0.3, and 0.05 mm), followed by sonication in absolute alcohol and $ddH_2O$ for 5 min each. Finally, the electrode was electrochemically cleaned in 0.5 M $H_2SO_4$, until a stable cyclic voltammogram was obtained, and dried with purified nitrogen.

After the pretreatment, the freshly cleaned gold electrode was immediately immersed in the stock solution of DNA1 at 4 °C for 12 h to form a self-assembled monolayer of DNA1 on the surface of the electrode. Afterward, the electrode was reacted with 1 mM MCH in an aqueous solution for 1 h to fill the pinholes in the monolayer with MCH. Thus, the working electrode was prepared and was ready to be used for the analysis of the enzymatic activity of thrombin.

*Enzyme assay*

The above-prepared working electrode was treated with thrombin solution (pH 7.4, 20 mM Tris–HCl, 5 mM KCl, 1 mM $MgCl_2$, 0.1 M NaCl, 5 mM $CaCl_2$, and 0.02% Tween 20) for 30 min. DNA2 was then modified to the surface of the electrode by combining with thrombin followed by thorough washing with double-distilled water. Then the electrode was kept in 40KT buffer for 30 min to allow proper unfolding of DNA1 and DNA2. After that, the electrode was immersed in a solution containing 100 μM hemin for 30 min so that hemin, the electrochemical probe, might well interact with the G-quadruplex halves. Thus, the electrochemical signal used to assay the activity of thrombin was achieved.

*Electrochemical measurements*

Electrochemical measurements were carried out with a CHI660C Potentiostat (CH Instruments). The commonly used three-electrode configuration was employed for the electrochemical measurements. A saturated calomel electrode served as the reference and a platinum wire as the counterelectrode. A 20 mM Hepes buffer (pH 8) containing 20 mM KCl was adopted as the electrolyte. Before the measurement, the buffer solution should be first bubbled thoroughly with highly pure nitrogen for 40 min.

## Results and discussion

*Scheme of the strategy*

Scheme 1 illustrates the strategy in this work to fabricate the thrombin aptasensor. In the presence of thrombin, the thrombin aptamer will form a thrombin–aptamer complex, which will be able to keep the two fragmented G-quadruplex halves regions at a distance from each other. Thus, further addition of hemin in the presence of potassium ions will not lead to the formation of a recombined G-quadruplex and thus loss of the electrochemical response. Therefore, in the presence of thrombin, the electroactive label, hemin, will be suppressed with a reduced electrochemical output, which forms the basis for thrombin sensing.

*Characterization of the modified electrode with the electrochemical method*

Fig. 1 shows the electrochemical characterization of the surface modification of the gold electrode. When the DNA1/DNA2/gold electrode was directly treated with hemin (0 nM thrombin), the acceptance of hemin by the G-quadruplex halves resulted in a large peak current. Otherwise, when thrombin was present, it blocked the binding of hemin with the G-quadruplex halves, so a lower amount of hemin was loaded onto the electrode surface and only a small peak could be observed. So, the differential pulse voltammetric (DPV) signals were negatively correlated with the quantity of surface-confined thrombin, and the quantity of both hemin and thrombin on the surface of the gold electrode put up a relation of ebb and flow of the peak current. In addition, the DPV responses of the DNA1-modified gold electrode (control 2 in Fig. 1) and the response of the DNA1/DNA2/hemin-modified gold electrode without potassium ions (control 1 in Fig. 1) were minimal and could not be distinguished. Therefore, we conclude that this sensor leads to minimal nonspecific binding of hemin. So, the electrochemical signal may be used to assay the enzymatic activity of thrombin.

*Quantitative measurement of thrombin in buffer solution*

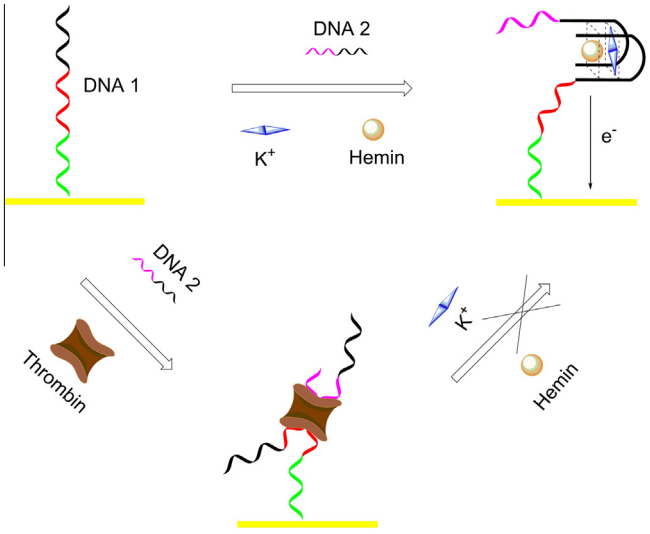
Fig. 1 also shows the DPV waves of the hemin/DNA1/DNA2/gold electrode, which was treated with various concentrations of thrombin. Obviously, the peak currents increase along with decreasing concentration of thrombin. Fig. 2 presents the relationship between the peak currents and the quantity of thrombin. The inset of Fig. 2 reveals that the current is linearly dependent on the thrombin concentration in a range from 0 to 1000 nM ($R^2 = 0.998$). And a detection limit of 10 nM can be obtained, which is lower than that in the report of Rodriguez and Rivas (18 nM) [21]. Thus, the results demonstrate that this aptamer-based electrochemical biosensor works well to assay protein concentration.

*Test of the selectivity of this strategy*

To further test the selectivity of this assay, three other proteins, including bovine serum albumin (BSA), α-fetoprotein (AFP), and carcinoembryonic antigen (CEA), were chosen as possible interfering substances. As shown in Fig. 3, none of the three proteins produced statistically significant loss of electrochemical signal even for concentrations as high as 5 μM. The data in Fig. 3 unambiguously reveal that the assay we designed in this work is highly specific toward the sensing of thrombin.

*Detection of thrombin in human serum*

To be more useful in bioassays, this sensor should be able to tolerate any interference from biological samples (such as serum), which contain ubiquitous endogenous components that produce a high background. Here, we assessed the ability of this electrochemical sensor to detect thrombin in fivefold-diluted human serum. Table 1 shows the experimental results obtained from thrombin-spiked serum samples. The extraction recoveries of adenosine from the serum samples were measured based on the




**Scheme 1.** Schematic illustration of the strategy to fabricate an electrochemical aptasensor to assay thrombin concentration.

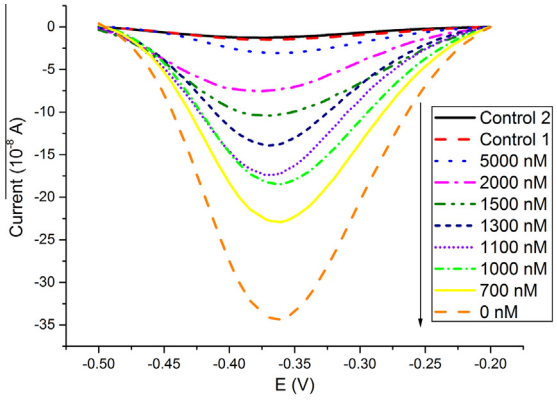



**Fig.1.** Baseline-subtracted DPV response of various concentrations of thrombin by using this sensor (the concentrations are 0, 700, 1000, 1100, 1300, 1500, 2000, and 5000 nM). In addition, DPV responses of control experiments are shown: the response of the DNA1-modified gold electrode (control 2) and the response of the DNA1/DNA2/hemin-modified gold electrode without potassium ions (control 1).

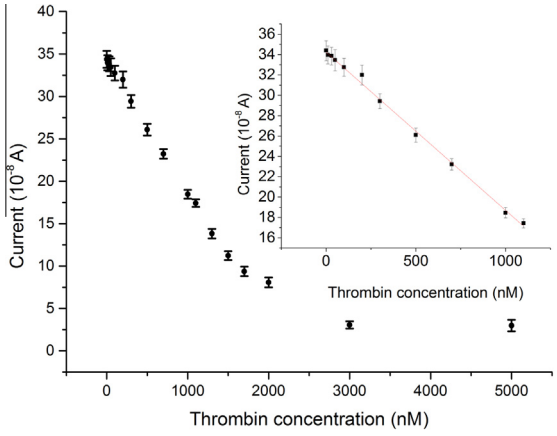



**Fig.2.** The relationship between the DPV peak and the concentration of thrombin. The inset shows the linear range from 0 to 1000 nM. All the data are taken from independent experiments repeated at least three times, and the presented data are the results of averaging.

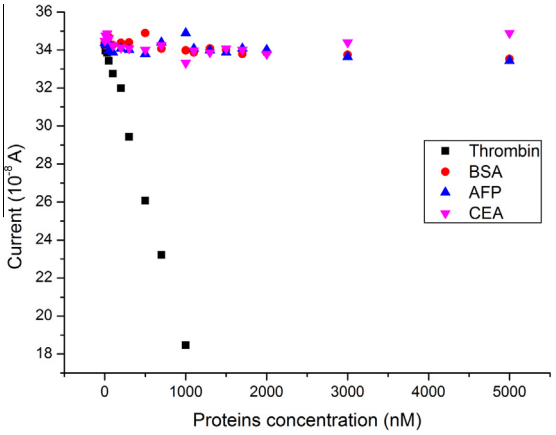



**Fig.3.** DPV response vs concentration curves for the thrombin sensor in the presence of thrombin and other three proteins including BSA, AFP, and CEA.

**Table 1**
Recovery of adenosine in fivefold-diluted human serum.

| Sample | Thrombin added (nM) | Found (nM) | Recovery (%) |
|---|---|---|---|
| 1 | 10.0 | 11.5 | 115.0 |
| 2 | 50.0 | 56.9 | 113.8 |
| 3 | 100.0 | 118.3 | 118.3 |
| 4 | 500.0 | 532.6 | 106.5 |

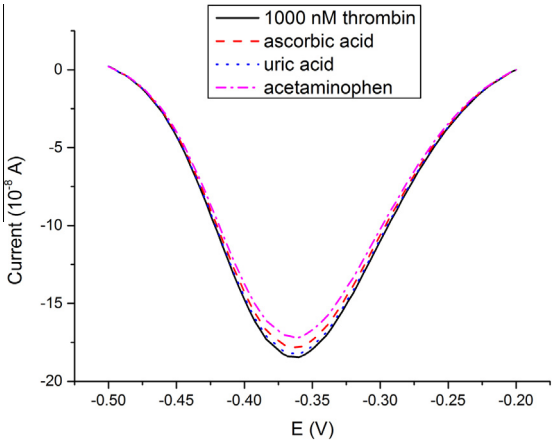



**Fig.4.** The influence of electrode-active materials on the working electrode in fivefold-diluted human serum. The thrombin concentration was 1000 nM. The concentration of the three influencing materials, ascorbic acid, uric acid, and acetaminophen, was 1 mM.

calibration graph using pure adenosine in buffer. The results were higher than those from the pure adenosine concentration in buffer. The high recovery values may be due to the "signal-off" architecture. This signal-off assay might compromise specificity since other environmental stimuli might influence the DPV waves and give "false positive" results. But the recovery values (106.5–118.3%) were less than 120% and were acceptable.

The influence of the electrode-active materials on the working electrode in human serum was also investigated. Three materials, ascorbic acid, uric acid, and acetaminophen, were chosen to be tested in fivefold-diluted human serum that was spiked with 1000 nM thrombin (Fig. 4). The DPV waves show little change for these materials even though their concentration was 1 mM. These results show that interference from electrode-active materials could be overcome.

## Conclusions

This work proposes an elegant label-free electrochemical aptasensor for thrombin detection based on two well-designed oligonucleotides. The elaborate architecture is designed on the surface of a gold electrode. The competition for binding of the aptamers between thrombin and hemin allows the detection of thrombin by measuring the electrochemical signal of hemin. Experimental results show that the electrochemical response is related inversely to the thrombin concentration, with a detection limit of 10 nM. From the view of the protein assay field, the method we propose here may provide a new way to assay the concentration of proteins that have split aptamers or more than one aptamer, such as vascular endothelial growth factor.

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