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www.elsevier.com/locate/talanta

PII: S0039-9140(16)30930-4
DOI: http://dx.doi.org/10.1016/j.talanta.2016.11.049
Reference: TAL17078

To appear in: *Talanta*

Received date: 18 August 2016
Revised date: 15 November 2016
Accepted date: 21 November 2016

Cite this article as: Zhaohong Su, Haitao Xu, Xiaolin Xu, Yi Zhang, Yan Ma, Chaorong Li and Qingji Xie, Effective covalent immobilization of quinone and aptamer onto a gold electrode via thiol addition for sensitive and selective protein biosensing, *Talanta,* http://dx.doi.org/10.1016/j.talanta.2016.11.049

Effective covalent immobilization of quinone and aptamer onto a gold electrode via thiol addition for sensitive and selective protein biosensing

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Abstract

Effective covalent immobilization of quinone and aptamer onto a gold electrode via thiol addition (a Michael addition) for sensitive and selective protein (with thrombin as the model) biosensing is reported, with a detection limit down to 20 fM for thrombin. Briefly, the thiol addition reaction of a gold electrode-supported 1,6-hexanedithiol (HDT) with *p*-benzoquinone (BQ) yielded BQ-HDT/Au, and the similar reaction of thiolated thrombin aptamer (TTA) with activated BQ-HDT/Au under 0.3 V led to formation of a gold electrode-supported novel electrochemical probe TTA-BQ-HDT/Au. The thus-prepared TTA-BQ-HDT/Au exhibits a pair of well-defined redox peaks of quinone moiety, and the TTA-thrombin interaction can

sensitively decrease the electrochemical signal. Herein the thiol addition acts as an effective and convenient binding protocols for aptasensing, and a new method (electrochemical conversion of Michael addition complex for signal generation) for the fabrication of biosensor is presented. The cyclic voltammetry (CV) was used to characterize the film properties. In addition, the proposed amperometric aptasensor exhibits good sensitivity, selectivity, and reproducibility. The aptasensor also has acceptable recovery for detection in complex protein sample.

Keywords: Protein biosensing; thiol addition; 1,6-hexanedithiol; quinone; thiolated aptamer

Introduction

Detecting proteins using biosensors in a cost-effective, sensitive and rapid manner is a crucial issue in fundamental research and clinical applications [1-3]. Because of their portability, inexpensiveness, simple instrumentation, high sensitivity and fast response, the electrochemical methods have attracted substantial attention in the development of aptasensors [4-10]. In general, the electrochemical aptasensors were fabricated by employing redox-labeled aptamer [4, 11, 12] or label-free impedance strategy [13, 14]. However, it is not easy to obtain the redox-labeled aptamer, because the existing methods of covalent labeling of the aptamer require complicated and labor-intensive labeling procedures. Therefore, it is still an important and active research topic for developing sensitive, convenient, and cost-effective labeling protocols for aptasensing. Among various aptasensors that utilize differernt signal transduction techniques such as electrochemistry [15, 16], optics [17], and atomic

force microscopy [18], electrochemical aptasensors have exhibited great promise in protein detection with high sensitivity, selectivity, and cost-effectiveness.

The thiols and thiolated chemicals are highly concerned in surface chemistry and biosensors [19, 20]. Interestingly, the redox-active quinone has an alkenes-like electron-conjugate structure can interact with amines for proteins [21, 22] and other molecular [23, 24] immobilization or interact with thiols for proteins [25], thiolated DNA [26] and other molecular [24] immobilization by Michael addition reaction [21-28]. In view of above compelling merits, we were prompted to fabricate gold surface-attached quinone and thiolated aptamer via thiol addition (a Michael addition) that is electrochemically measurable based on TTA-BQ-HDT/Au. To our knowledge, the thiol addition serves as an effective and convenient method of immobilization of quinone and thiolated aptamer onto a gold electrode, and the quinone acts as a signaling transducer for preparation of sensitive electrochemical aptasensor have hardly been investigated for biosensing applications to date.

In this work, effective covalent immobilization of quinone and aptamer onto a gold electrode via thiol addition for sensitive and selective protein (with thrombin as the model) biosensing is reported. Herein the thiol addition acts as an effective and convenient binding protocols for aptasensing, and a new method (electrochemical conversion of Michael addition complex for signal generation) for the fabrication of biosensor is presented. In addition, the reagents involved in the proposed amperometric aptasensor are commercially available and widely used in biosensing, making the proposed amperometric aptasensor cost-effective. The thus-prepared TTA-BQ-HDT/Au electrode showed well-defined redox peaks of quinone moiety, and the TTA-thrombin interaction can sensitively decrease the electrochemical signal. The

prepared amperometric aptasensor was found to exhibit good analytical properties (sensitivity, low detection limit, and good selectivity and reproducibility) for thrombin detection.

Experimental

Chemical reagents

Human α-thrombin and 6-mercaptohexanol (MCH) were purchased from Sigma. TTA (5'-SH-$(CH_2)_6$-GGTTGGTGTGGTTGG-3') was purchased from Sangon Co., Ltd. (Shanghai, China). BQ was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). HDT was purchased from Alfa Aesar. Human blood sera were obtained from the Hospital of Hunan Normal University. 10 mM phosphate buffer solution (PBS, 10 mM NaH_2PO_4–Na_2HPO_4) containing 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (pH 7.0) was used for binding and rinse, and 0.10 M PBS (pH 7.4, 0.10 M KH_2PO_4–K_2HPO_4 + 0.10 M K_2SO_4) was used for electrochemical experiments. All other chemicals were of analytical grade or better quality. Milli–Q ultrapure water (Millipore, >18 MΩ cm) was used throughout.

Apparatus

All electrochemical experiments were conducted on a CHI660C electrochemical workstation (CH Instrument Co., USA), and a conventional three–electrode electrolytic cell was used. Au disk electrodes with 2.0-mm diameter (0.03 cm^2 area) served as the working electrode, the reference electrode was a KCl–saturated calomel electrode (SCE), and a carbon rod served as the counter electrode. All potentials reported here are cited versus the SCE (vs. SCE). The PH-3B pH meter (Dazhong, Shanghai, China) was used for pH measurement.

Procedures

To remove possible surface contamination, the Au electrodes were cleaned according to the protocol [29, 30]. Briefly, the Au electrodes were carefully polished in 0.5- and 0.05-μm alumina suspensions in sequence. After thoroughly rinse with water, the polished electrodes were ultrasonically treated sequentially in water, ethanol, and water each for 5 min to remove residual alumina powder, respectively. Then, the Au electrodes were treated with Piranha solution (H_2SO_4:H_2O_2, v/v 3:1, highly oxidizing and corrosive, treat with great care to avoid its contact with the skin and eye) for 15 s. Afterward, the Au electrodes were subjected to electrochemical rinse in 0.50 M H_2SO_4 to thoroughly remove impurities, namely, potentiostatic treatments at 2 V for 5 s and at -0.35 V for 10 s, and potential cycling for 6 cycles from -0.35 to 1.55 V at 4 V s^{-1}. The cleaned Au electrode was finally characterized by cyclic voltammetry from -0.35 to 1.55 V in 0.50 M H_2SO_4, which showed a single sharp reduction peak at ca. 0.9 V and multiple overlapping oxidation peaks at potentials ranging from 1.2 to 1.5 V. Afterward, the gold electrode was washed thoroughly with ultrapure water and dried under nitrogen gas.

The preparation of the modified electrode is depicted in Scheme 1. For immobilization of the HDT, The cleaned Au electrode was placed in the 1 mM HDT solution for 3 h to produce a self-assembled monolayer (SAM) of HDT. After treatment with 1 mM MCH for 1 h (optimized time, not plotted), the unmodified region of the electrode was blocked and then 50 μM BQ was coated on the resulting HDT/Au electrode, and the interaction was kept at room temperature for 2 h to obtain the BQ-HDT/Au electrode. Subsequently, the BQ-HDT/Au electrode was activated

for 30 s under 0.3 V in 0.1 M PBS (pH 7.4), then 10 μL of 10 μM TTA (5'-SH-$(CH_2)_6$-GGTTGGTGTGGTTGG-3') solution was placed on the actived BQ-HDT/Au electrode for 2 h to obtain TTA-BQ-HDT/Au electrode. Finally, the resulting electrode was incubated for 2 h with various concentrations of thrombin to obtain the thrombin-TTA-BQ-HDT/Au electrode. After each step, the electrode was rinsed thoroughly with ultrapure water and then dried with a stream of nitrogen.

Quantitative determinations of thrombin was performed with differential pulse voltammetry (DPV) between 0 and 0.4 V in 0.1 mol L^{-1} PBS (pH 7.4). The optimal conditions were as follows: amplitude of 0.05 V; pulse width of 0.05 s; sampling width of 0.0167 s; pulse period of 0.2 s.

Results and Discussion

Characterization of the amperometric aptasensor

CV was used to investigate the electrochemical properties of labeled BQ on aptasensor. As shown in Fig. 1, we observed a pair of well-defined redox peaks centering at *ca*. 0.18 V *vs*. SCE during potential cycling of BQ-HDT/Au in 0.1 M PBS (pH 7.4). The peak currents are linear with potential scan rate, proving a facile electrochemical and a surface-controlled redox process of the labeled (surface-confined) BQ. Additionally, in the absence of HDT, the similar redox peaks were not found at the obtained BQ-MCH/Au in the same conditions (not plotted), and the peak currents were negligibly changed after continuous potential cycling (not plotted), indicating stable tethering of BQ on aptasensor via thiol addition.

Fig. 2 shows CV and DPV responses of BQ-HDT/Au (a), TTA-BQ-HDT/Au (b) and thrombin-TTA-BQ-HDT/Au (c) in 0.1 M PBS (pH 7.4). As is seen, the

BQ-HDT/Au electrode shows a pair of well-defined redox peaks, the treatment with MCH on the BQ-HDT/Au electrode leads to a subdued peak currents (not plotted), which demonstrates MCH has been blocked successfully on the electrode. After the interaction between TTA and activated BQ-HDT/Au, the peak currents of BQ-HDT/Au decrease, indicating the occurrence of the thiol adddition reaction of TTA-BQ-HDT. Then the TTA-BQ-HDT/Au electrode was incubated in 1.5 nM thrombin for 2 h, there was a decrease in peak currents, the peak decrease of the thrombin-TTA-BQ-HDT/Au electrode may originate from the bulky thrombin molecules blocking the electrode surface. This implies that thrombin has been bound to the surface of the modified electrode by the aptamer-thrombin interaction. The above findings have been supported by DPV experiments at BQ-HDT/Au, TTA-BQ-HDT/Au and thrombin-TTA-BQ-HDT/Au in 0.1 M PBS (pH 7.4), as shown in Fig. 2.

Electrochemical probing of specific aptamer-protein interactions

The construction and assay principle of the amperometric aptasensor are schematically depicted in Scheme 1. The surface of the Au electrode was first modified with HDT via the self-assembly, and then the unmodified region of the electrode was blocked with MCH (Scheme 1a). For Scheme 1b, the BQ was deposited onto the HDT/Au, the BQ could interact with the HDT/Au via thiol addition, leading to the formation of BQ-HDT/Au. Hereafter, the obtained BQ-HDT/Au was activated for 30 s under 0.3 V in 0.1 M PBS (pH 7.4) (Scheme 1c). For Scheme 1d, the TTA was deposited onto the activated BQ-HDT/Au, the TTA could interact with the activated BQ-HDT/Au via the similar reaction, leading to the formation of TTA-BQ-HDT/Au. Finally, the thrombin introduction and formation of TTA-thrombin

complex decreased the DPV peak current at TTA-BQ-HDT/Au, which gave the thrombin-sensing signals (Scheme 1e). Scheme 1f shows the schematic representation of DPV performance for Scheme 1e. As we know, the assay principle of the proposed amperometric aptasensor is also similar to the quinone hybrids on Au electrodes toward the electrochemical probing of specific carbohydrate-protein recognitions [19].

Analytical properties of the proposed amperometric aptasensor for thrombin detection

Fig. 3 shows the calibration curves corresponding to the DPV detection of thrombin based on the changes of the DPV currents at thrombin-TTA-BQ-HDT/Au electrode. From Fig. 3, it can be seen that the ΔI ($\Delta I = I_0 - I_s$, I_0 is the current with no added thrombin, and I_s is the current with added thrombin) values of all electrodes increase with the increase of the concentration of thrombin. On the basis of the thrombin-TTA-BQ-HDT/Au, the linear regression equation of current responses (ΔI in nA) versus thrombin concentrations (c in nM) was $\Delta I = 0.255+27.57c$ (r=0.998), the amperometric aptasensor has a wide detection rang from 50 fM to 1.5 nM. thrombin could be easily detected down to a concentration of 20 fM (S/N=3), which is better than or comparable with those reported in most aptasensor literatures, as listed in Table 1. This demonstrates that we have developed a sensitive amperometric aptasensor for thrombin detection. And herein the thiol addition acts as an effective, convenient and cost-effective binding protocols for protein biosensing. Meanwhile, the detection limit of this amperometric aptasensor is also better or comparable to that of the aptasensor based on other methods, for example, a sensitivity limited of 50 fM was obtained by using impedimetric aptasensor based on carbon nanotubes labeled

with aptamer [15] and 28 fM by using ion-selective microelectrodes [31].

In order to illustrate the selectivity of the aptasensor, the TTA-BQ-HDT/Au electrodes were exposed to a large excess of different proteins. Fig. 4 shows the changes of DPV currents of the TTA-BQ-HDT/Au electrode after incubation with thrombin, human IgG, glucose oxidase, lysozyme, fibrinogen and rabbit IgG, respectively. From Fig. 4, it can be seen that the ΔI values for foreign proteins are much smaller than that for thrombin. This indicates that the nonspecific adsorption of the foreign proteins (human IgG, glucose oxidase, lysozyme, fibrinogen and rabbit IgG) to the base aptamer does not happen obviously, and the effects of these foreign proteins on thrombin detection are no thrombin specifically adsorbed on the aptamer-functionalized electrode surface. These control experiments indicate that the aptasensor is specific to thrombin and possesses high selectivety.

The reproducibility of the aptasensor was investigated at the thrombin concentration of 1.5 nM, and the relative standard deviation for five times is 4.7%. In addition, five freshly prepared modified electrodes have been used for the detection of 1.5 nM thrombin. All five electrodes exhibit similar amperometric response behavior, and the relative standard deviation is 3.2%. This demonstrates that the aptasensor for thrombin detection is highly reproducible.

The analytical application of the proposed aptasensor has been evaluated by the standard addition method. A series of samples were prepared by adding thrombin of different concentrations to human blood serum (obtained from Hospital of Hunan Normal University). The analytical results for thrombin are shown in Table 2. The acceptable recovery and RSD imply that the aptasensor has application potential in complex biological samples.

Conclusions

In summary, A sensitive amperometric aptasensor for thrombin detection has been developed. The preparation of TTA-BQ-HDT/Au is simple and effective, immobilization of quinone and apt by thiol addition. More importantly, the modified electrode exhibits a pair of well-defined redox peaks of quinone moiety, and the TTA-thrombin interaction can sensitively decrease the electrochemical signal, providing sensitive detection of thrombin with a detection limit of 20 fM. In addition, the reagents involved in the proposed amperometric aptasensor are commercially available and widely used in biosensing, making the proposed amperometric aptasensor cost-effective. The amperometric aptasensor based on electroactive quinone moiety and thiol addition could find wide application in the routine detection of proteins with high sensitivity and low detection limit.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (21305039, 21475041, 21175042 and 21075036), the Foundation of Hunan Province (14JJ3097), the Program for Science and Technology Innovative Research Team in Higher Educational Institutions of Hunan Province, the Foundation of Hunan Provincial Education Department for Young Scholar, and the Foundation of Hunan Agricultural University (12YJ05).

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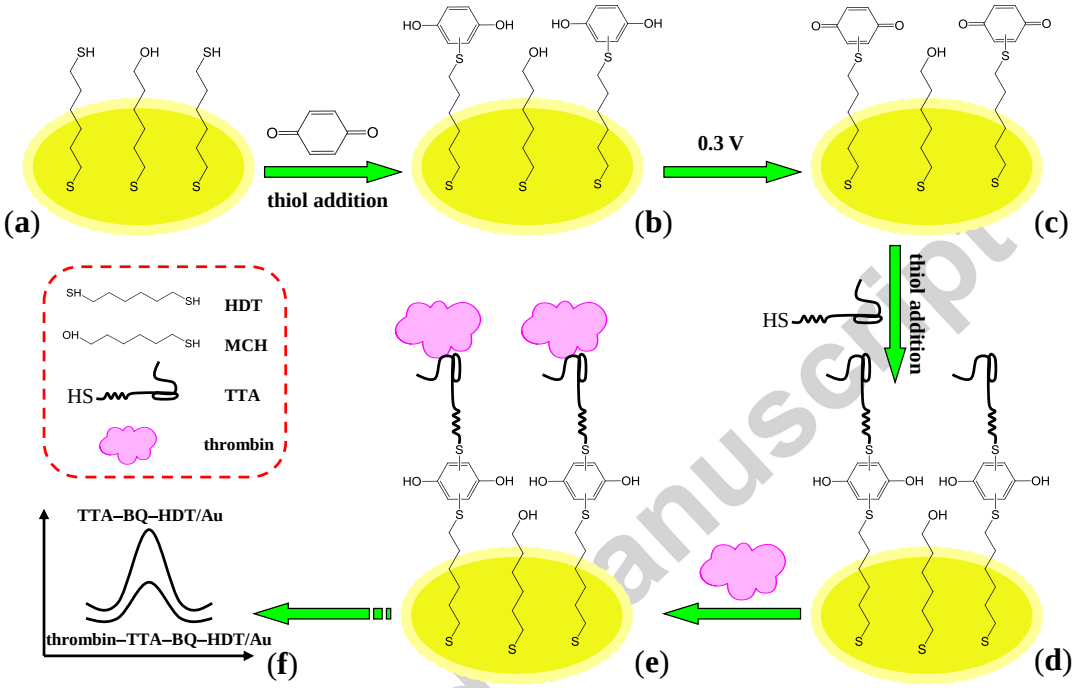

Scheme 1. Illustration of the construction of the aptasensor.

Fig. 1. CV of BQ-HDT/Au at scan rates of 30, 50, 100, 200, 300, 400 and 500 mV s^{-1} in 0.1 M PBS (pH 7.4). Inset: I_p as a function of scan rate.

Fig. 2. CV (A) and DPV (B) responses of BQ-HDT/Au (a), TTA-BQ-HDT/Au (b) and thrombin-TTA-BQ-HDT/Au (c, after addition of 1.5 nM thrombin onto TTA-BQ-HDT/Au and incubated for 2 h) in 0.1 M PBS (pH 7.4). Scan rate: 50 mV s^{-1}.

Fig. 3. DPV curves (A) and the calibration curve (B) of the prepared

TTA-BQ-HDT/Au electrode for thrombin at various concentrations in 0.1 M PBS (pH 7.4). Inset: an enlarged plot for the calibration curve. I_0 is the current with no added thrombin, and I_s is the current with added thrombin.

Fig. 4. The responses of the prepared TTA-BQ-HDT/Au electrode to 1.5 nM thrombin and several other proteins each at 0.5 μM. I_0 is the current with no added protein, and I_s is the current with added protein.

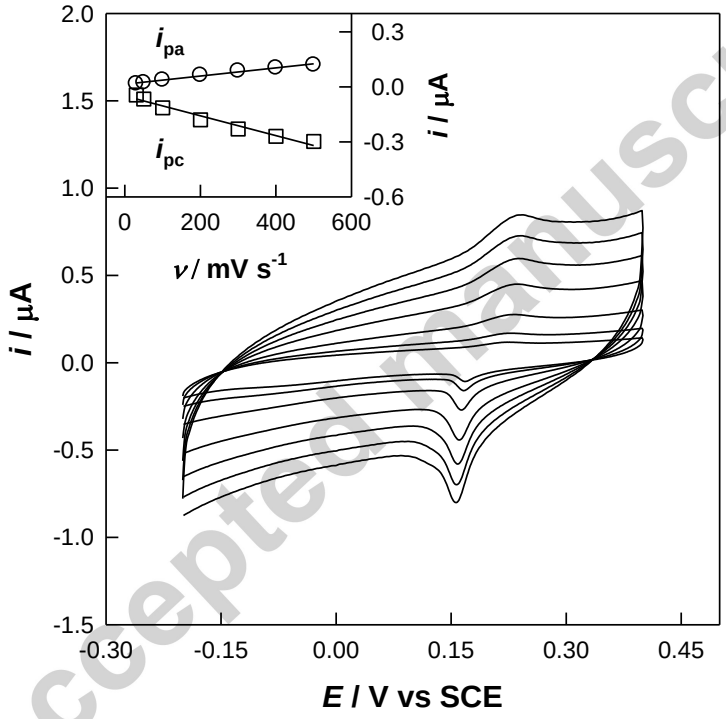

Fig. 1

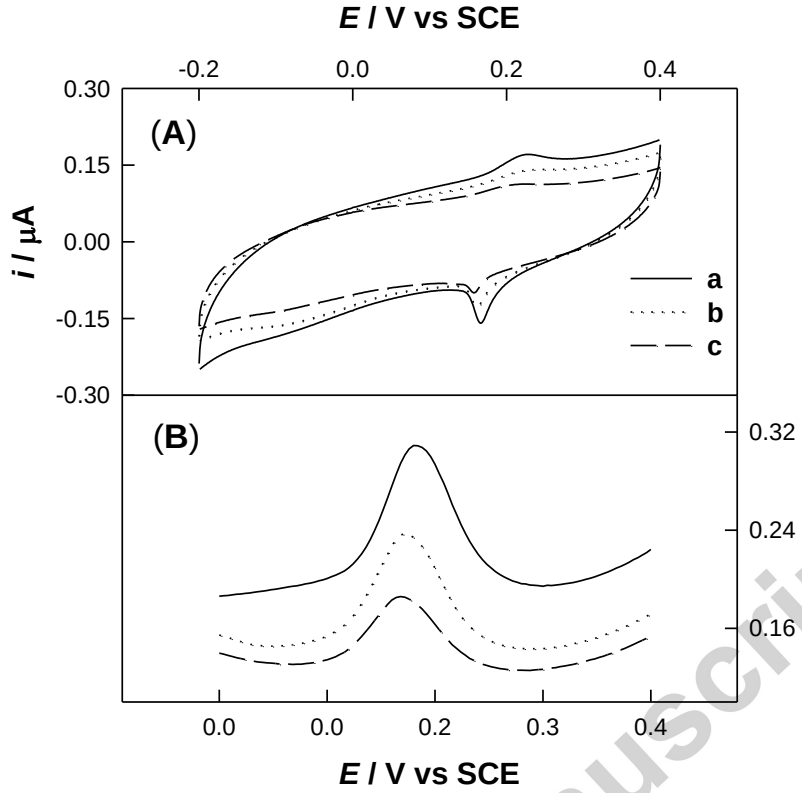

Fig. 2

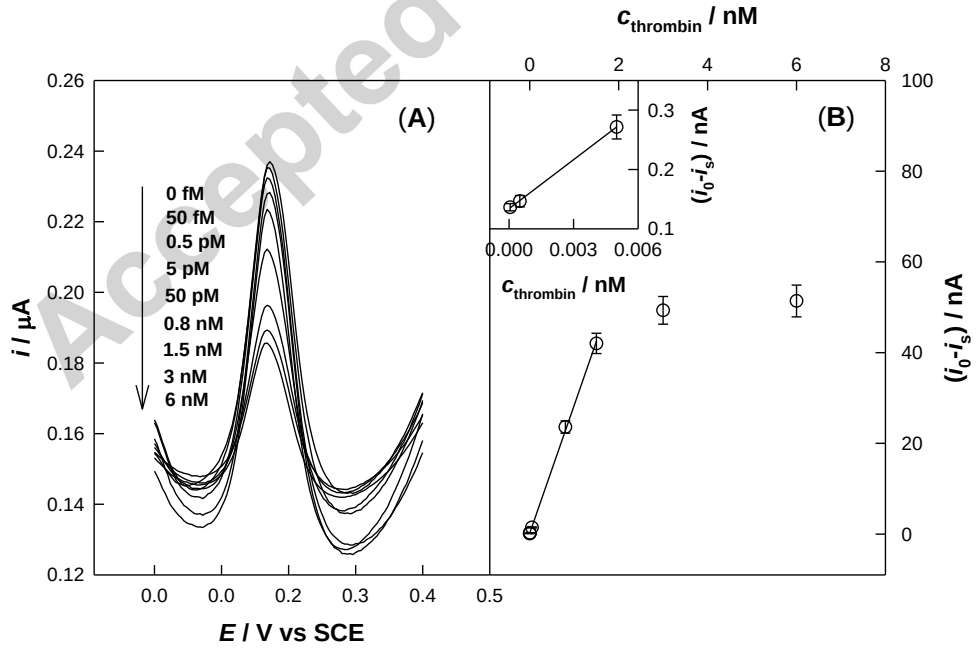

Fig. 3

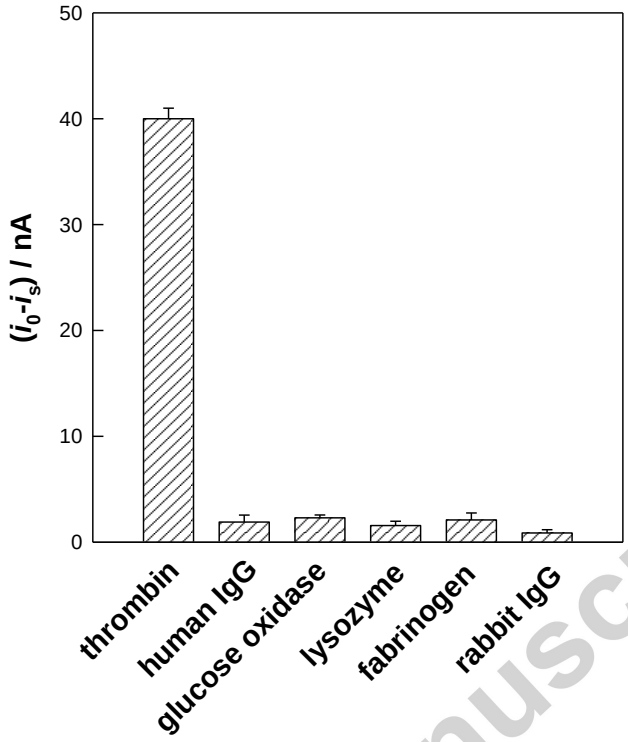

Fig. 4

Table 1. Performance of some typical thrombin electrochemical aptasensors*.

Fabrication	LDR / nmol L^{-1}	LOD / pmol L^{-1}	References

TTA-BQ-HDT/Au	0.00005-1.5	0.02	This work
Apt2/thrombin/apt1-magnetic beads/SPE	-	450	[32]
thrombin/MCH/TBA/Au	-	100	[33]
AuNPs/thrombin/MCE/TBA/Au	0.05-18	20	[34]
SWCNTs@apt II-thrombin-Apt I@MNPs/Au	0.005-7	3	[29]
Apt-thrombin-Antibody/AuNPs-cs/GCE	1-60	500	[35]
Thrombin/apt-MUA/Au	0-50.8	11000	[36]
Fc-TBA-MCE/Au	5-35	500	[37]
AuNPs-Apt/thrombin/MCH/TBA/Au	0.05-35	0.1	[38]
HRP-AuNPs-apt2/thrombin/apt1-AuMNPs/Au	0.0001-60	0.03	[39]

* Apt: aptamer; SPE: screen-printed electrode; MCH: mercaptohexanol; TBA: thrombin-binding aptamer; MCE: 2-mercaptoethanol; MNPs: magnetic nanoparticles; AuNPs: Au nanoparticles; cs: chitosan; MUA: 11-mercaptoundecanoic acid; Fc: ferrocenyl; HRP: horseradish peroxidase; AuMNPs: Au magnetic nanoparticles.

Table 2. Detection of thrombin in the human blood serum substrate (n = 3) using the proposed amperometric aptasensor.

Added / nM	Measured / nM	RSD / %	Recovery / %
0.15	0.14	7.3	93
0.30	0.29	7.8	97
0.50	0.51	8.2	102
1.00	0.96	8.6	96
1.50	1.43	7.9	95

Highlights

- Preparation of electrochemical aptasensor based on thiol addition (a Michael addition) for sensitive and selective protein biosensing.
- Electrochemical conversion of Michael addition complex for signal generation.
- Effective covalent immobilization of quinone and aptamer onto a gold electrode via thiol addition.