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Electrochemical detection of thrombin by sandwich approach using antibody and aptamer

Yan Kang, Ke-Jun Feng, Ji-Wei Chen, Jian-Hui Jiang *, Guo-Li Shen, Ru-Qin Yu *

State Key Laboratory for Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, PR China

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ABSTRACT

The goal of this work was to introduce a modified electrochemical sandwich model for target protein detection, exploiting antibody as the capturing probe, aptamer as the detection probe and methylene blue as the electrochemical active marker intercalating in the probing aptamer without previous labeling. With appropriate design of the sequence of the aptamer, the aptamer was successfully utilized instead of antibody for obtaining the electrochemical detection. A special immobilization interface consisting of nanogold-chitosan composite film was used to improve the conductivity and performance characteristics of the electrode. The capturing antibody was linked to the glassy carbon electrodes modified with composite film via a linker of glutaraldehyde. Differential pulse voltammetry was performed to produce the response signal. Thrombin was taken as the model target analyte to demonstrate the feasibility of proposed methodology. The sensor shows the linear response for thrombin in the range 1–60 nM with a detection limit of 0.5 nM. The proposed approach provides an alternative approach for sandwich protein assay using aptamers.

1. Introduction

In previous protein analysis, antibodies have been extensively utilized as affinity reagents due to their high selective binding ability to protein. However, shortcomings with their production, stability, and modification have urged the chemical workers to seek alternatives. The aptamer, which belongs to a class of synthetic DNA/RNA oligonucleotides obtained from random sequence nucleic acid libraries by an in vitro evolution process [1,2] SELEX (systematic evolution of ligands by exponential enrichment), could mimic an antibody in protein analysis [3,4]. Compared with antibodies, aptamers possess some outstanding features, including high specificity of binding affinity, nice stabilization, easy synthesis and modification with electrochemical active markers, optical dyes, enzymes and other desired substances. Furthermore, aptamers can reversibly capture and release their target protein. It is facile for the aptamer to transduce the recognition events into detectable signals.

In recent reports, systems for protein detection based on aptamer mostly concentrated on optical [5–12] detection, quartz crystal microbalance [13,14] and electrochemical method [15–21]. So far as the electrochemical detection is concerned, sensors based on molecular aptamer beacon labeled with single electroactive marker [17–20] have been extensively studied. For example, Lai et al. [17] labeled the aptamer beacon with an electro-active marker to realize the function of electronic on–off molecular switch for detection of platelet-derived growth factor. Such detective system is very simple and sensitive, though it requires that the aptamer undergoes conformational changes upon the recognition reaction. The sandwich approach of protein detection has been the focus of the research interest. Except for few kinds of proteins with two aptamer binding sites [21,22], previously reported sandwich performances for protein detection mostly focused on conventional antibody-based immunoassays, with one antibody for immobilization and the other labeled with an enzyme for detection. So far as we know, the modification of antibodies was difficult, high-cost and time-consuming.

To circumvent this difficulty, the present study, we introduced a new sandwich [23,24] electrochemical protein detection system using aptamer as the detection probe and a conventional polyclone antibody as the capturing probe. We selected thrombin as the model target analyte. On contrary to the conventional way to obtain the electrochemical signal by modifying the aptamer with appropriate markers, we directly intercalated MB into the thrombin aptamer to avoid a previous labeling procedure. Electrochemical measuring of the recognition events possesses plenty of advantages, which is stable, simple, cost-effective, and avoids external modification on the biomolecules. Introducing polyclone antibody for capturing target facilitated the competitively binding of aptamer, as there were plenty of binding sites on target for a polyclone antibody except these for the aptamer. Moreover, as MB could easily intercalate into DNA aptamer, employing antibody as the capturing probe could reduce the background signal. Thirdly, it is much easy to obtain a conventional antibody and an aptamer for a target for sandwich assay. This strategy also combined the specificity of antibody and aptamer with a possibility to achieve high specifical detection.

* Corresponding authors. Tel./fax: +86 731 8822577.
E-mail addresses: jianhuijiang@hnu.cn (J.-H. Jiang), rqyu@hnu.cn (R.-Q. Yu).

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In order to improve the conductivity of the electrode, we brought in gold nanoparticles together with the probable improved retainment of the bioactivity of the immobilized antibody. Application of gold nanoparticles for modifying the electrode [25–28] could significantly decrease electron-tunneling distance, facilitating the electron transfer. Chitosan (CHIT) [29–32] is a kind of polysaccharide which is protonated and positively charged in solution. Due to its excellent film-forming ability and richness in amino groups, CHIT has been previously employed to modify the electrode for immobilization antibody [32,33]. Considering the special properties of gold nanoparticles and CHIT, herein, we prepared the nanogold-chitosan composite film [29] for immobilization of thrombin antibody.

2. Experimental

2.1. Materials and reagents

The thrombin aptamer used with additional extended one end with 22-base and the other end with 21-base oligonucleotides (vide infra), 5'-GACAGACGATGTGCTG ACTACTGGTTGGTGAGGTTGGGTAGTCAGCA-CATCGTCTGTC-3', was synthesized according to our design by Takara biotechnology (Dalian, China) Co. Ltd. Purified sheep anti-human thrombin, dissolved in 10 mM HEPES, 0.05 M sodium chloride, pH 7.4, containing 50% glycerol, was purchased from Biodesign (Ohio, USA). Lyophilized human thrombin was purified from human plasma (R&D Systems, Inc). Chitosan (CHIT, 75–85% deacetylation) and $HAuCl_4{\cdot}4H_2O$ were obtained from Sigma. Glutaraldehyde (GA) solution (25%) was obtained from Changsha Chemical Reagents Co. (Changsha, China). Bovine serum albumin (BSA), goat anti-human IgG, MB, potassium ferrocyanide, magnesium chloride and potassium chloride were acquired from commercial vendors. The detection buffer used was 10 mM Tris–HCl (pH7.4, containing 10 mM KCl and 10 mM $MgCl_2$) and rinsing buffer was 10 mM Tris–HCl (pH 7.4, with 10 mM KCl, 10 mM $MgCl_2$ and 0.1 M NaCl). All the reagents were used without further purification and doubly distilled water was used throughout.

2.2. Apparatus

Differential pulse voltammetry (DPV) and cyclic voltammetry(CV) experiments were carried out on a CHI 760B electrochemical workstation (Shanghai Chenhua Apparatus Corporation, China). A conventional three electrode system contained a glassy carbon electrode (GCE) (3.0 mm diameter) as the working electrode, a platinum foil as the auxiliary electrode and a saturated calomel electrode as the reference electrode. Scanning electron micrographs (SEM) of nanogold-chitosan composite films were taken on a JSM-5600LV microscope (JEOL, Ltd. Japan) with the magnification factor of 60,000, accelerating voltage of 20 KV and degree of vacuum of 10^{-5} torr.

2.3. Procedures

2.3.1. Preparation of the nanogold-chitosan composite film

Prior to surface modification, the GC electrode was polished sequentially with 0.3 and 0.05 μm alumina powder followed by ultrasonic cleaning in ethanol and double distilled water. Subsequently, the nanogold-chitosan composite film was prepared by electrochemical deposition. The deposition solution was obtained by mixing proper proportions of CHIT and $HAuCl_4$, and then thoroughly sonicated until a homogenous mixture was formed. Then, the GCE was immersed into the mixture and deposited following a potentiostatic procedure. The applied potential was in the range of −1 to −3 V and was kept for 2–6 min. After deposition, the electrode was removed from the solution and dipped into water for a short time, and then the electrode was dried in air at room temperature overnight.

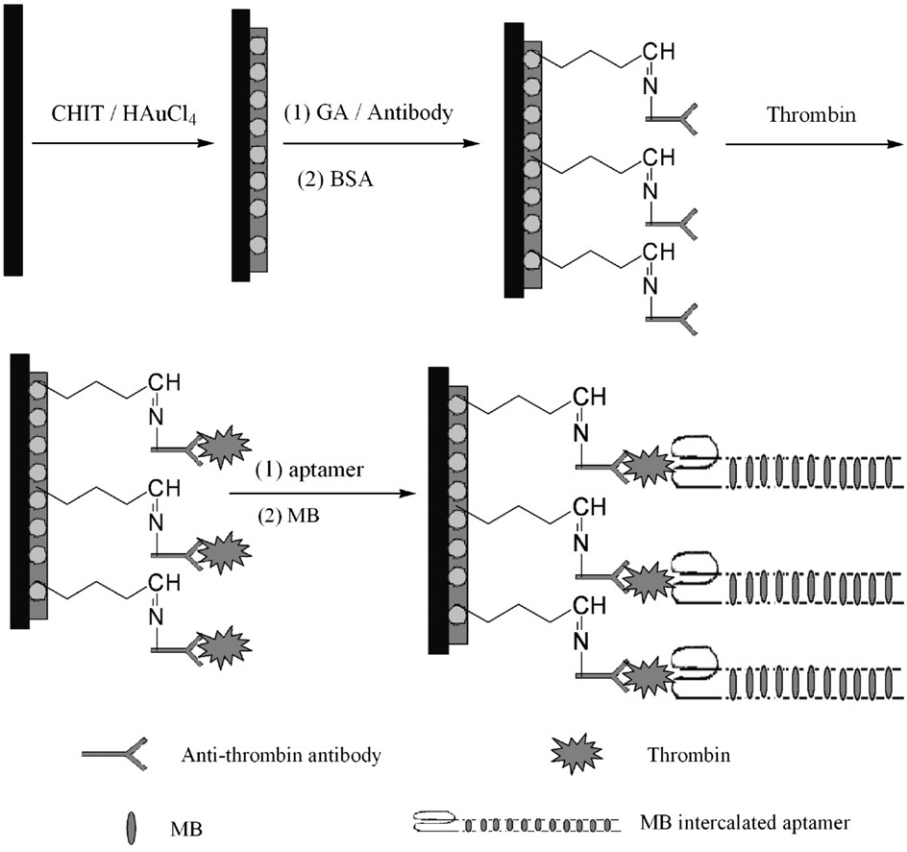

Fig. 1. Schematic representations of the principle for the sensing steps.

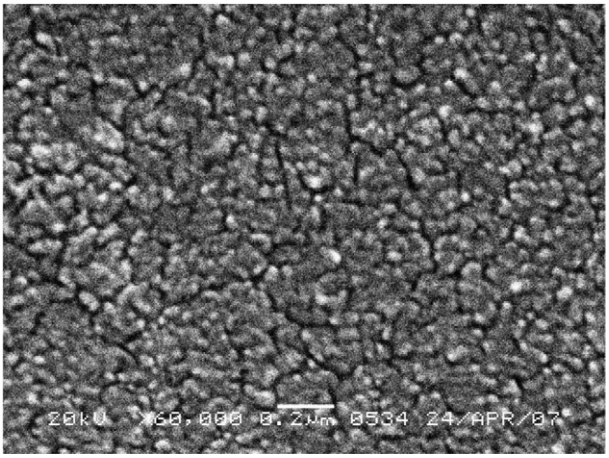

Fig. 2. SEM image of nanogold-chitosan composite film on glassy carbon electrode.

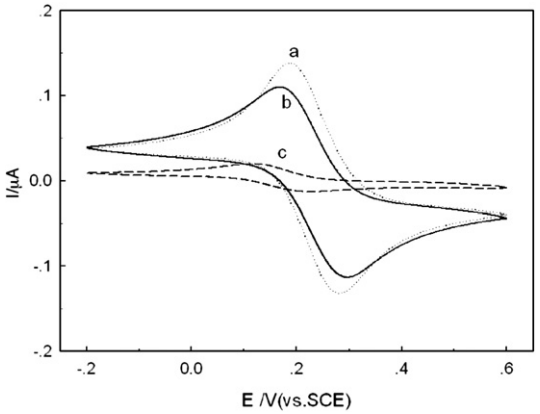

Fig. 4. Cyclic voltammetric curves of nanogold-chitosan/GCE (a); bare GCE (b); antibody/nanogold-chitosan/GCE(c). Supporting electrolyte: 10 mM $[Fe(CN)_6]^{3-}$ containing 0.2 M KCl; Scan rate 100 mV s^{-1}.

2.3.2. Fabrication of the immunosensor

10 μL diluted glutaraldehyde solution (2.5%) was dropped onto the nanogold-chitosan derivatized GCE and kept in the fridge (4 °C) for 1 h. Diluted sheep anti-human thrombin antibody (450 μg/mL) was coupled onto the surface with the cross-linker glutaraldehyde for 1 h at 37 °C. The unreacted aldehyde was blocked with 2% BSA solution at 37 °C for 1 h, followed by rinsing with buffer and double distilled water.

2.3.3. Electrochemical detection of thrombin

Fig. 1 illustrates the scheme of the sandwich approach using antibody and aptamer adopted in this study. Once the recognition layer was built up, different samples of thrombin solution at various concentrations was dropped onto the sensor surface, followed by incubated at 37 °C for 1 h, and then 170 nM heat-treated aptamer was added and incubated for another hour under the same condition. After each step, the electrode surface was washed with rinsing buffer under stirring for 10 min. At last, the MB was accumulated on the aptamer by immersing in 10 mM Tris–HCl buffer containing 20 μM MB for 15 min under stirring. Unbound MB was removed by washing the electrode with rinsing buffer for several times. The electrochemical signals were obtained at room temperature in detection buffer by using DPV with amplitude of 20 mV and pulse width of 0.05 s.

3. Results and discussion

3.1. Optimization and characterization of the nanogold-chitosan composite film

In this work, we have yielded a well-behaved surface by depositing the mixture of CHIT and $HAuCl_4$ [29] for antibody immobilization.

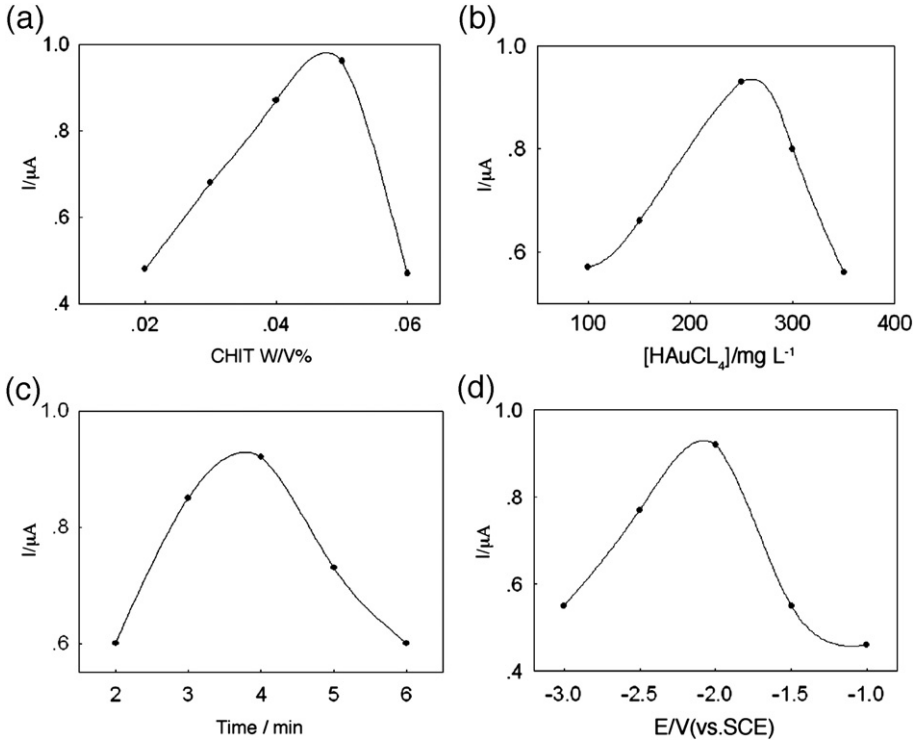

Fig. 3. (a) Effect of CHIT concentration on the sensor to 60 nM thrombin in Tris–HCl buffer. Electrochemical deposition conditions: $HAuCl_4$ concentration 250 mg L^{-1}, electrochemical deposition time 240 s, applied potential −2 V; (b) Effect of $HAuCl_4$ concentration. Electrochemical deposition conditions: the same as (a) with 0.05% CHIT; (c) Effect of deposition time. Electrochemical deposition conditions: the same as (b) with 250 mg L^{-1} $HAuCl_4$; (d) Effect of applied potential. Electrochemical deposition conditions: the same as (c) with electrochemical deposition time of 240 s.

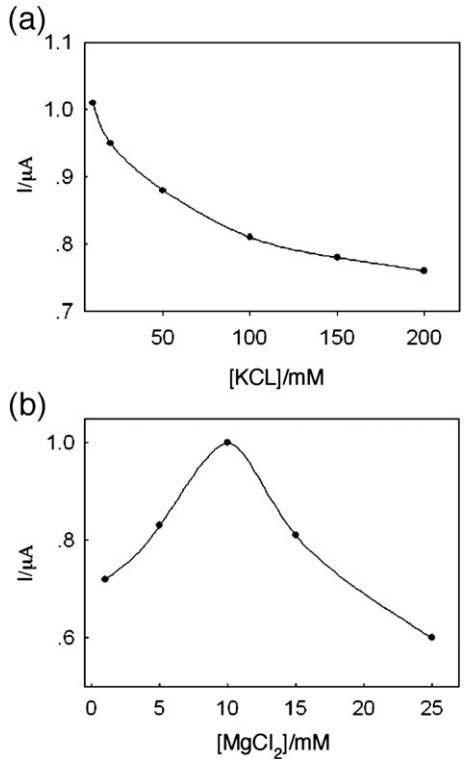

Fig. 5. The electrochemical response of the sensor as a function of KCl (a) and $MgCl_2$ (b) concentration.

During the deposition process, $HAuCl_4$ was reduced to gold nanoparticle and simultaneously deposited to the surface of the electrode along with CHIT. The formed nanogold-chitosan composite film was characterized by SEM. Fig. 2 demonstrates the SEM image of nanogold-chitosan surface. It can be seen that there are plenty of nanopores in the film and most of them are filled with nanoparticles. The gold nanoparticles are distributed evenly and the diameter of the particles mostly distributes around 50 nm.

Optimization of the electrochemical deposition conditions was investigated with respect to different experimental variables. We considered the factors of the concentration of CHIT and $HAuCl_4$, the deposition time and the applied voltage. Fig. 3(a) depicts the effect of different concentrations of CHIT. From this Figure, one notices that the optimum response was obtained with 0.05% CHIT solution. The higher the concentration of CHIT, the thicker the film formed, leading to the decrease of the conductivity of the electrode. On the other hand, insufficient amount of CHIT would result in the unstability of this composite film. Fig. 3(b) represents the effect of $HAuCl_4$, which can strongly affect the size and amount of nanoparticles. With the concentration of $HAuCl_4$ increased, the response increases at first and then reaches the largest signal at the concentration of 250 mg/L. Concerning the effect of the deposition time and applied voltage, we chose 4 min and −2 V respectively. From Fig. 3(c, d), one can see that the maximum signal can be obtained under the above conditions. To further confirm the conductive property of the composite film, we performed CV (Fig. 4) on the bare GCE, nanogold-chitosan modified GCE and antibody linked to the modified GCE. A comparison of these curves demonstrates that surface properties of the electrode have been greatly improved with this composite film.

3.2. The influence of ionic strength on aptamer binding to thrombin

Several articles [34–37] have reported that ionic strength would strongly affect the affinity of aptamer binding to thrombin. Upon aptamer binding to thrombin, in the presence of K^+, Rb^{2+}, Mg^{2+}, Sr^{2+} or Ba^{2+}, the aptamer would incline to fold into stable intramolecular G-quadruplexes [34], which could facilitate the recognition reaction this aptamer binding at various concentrations. In our study, we systematically investigated the effect of K^+ and Mg^{2+}. Fig. 5(a) depicts the effect of different K^+ concentrations. Keeping Mg^{2+} at constant concentration (10 mM), with the increasing amount of K^+, the electrochemical response decreased gradually. This is in accordance to the findings of Vairamani M et al.. Their studies showed that, in the case of low amount of K^+, the aptamer formed an adduct [37] which was prone to binding to thrombin. However, the formed adduct existed in high negatively charged state [37]. In this case, high concentration of cation K^+ could shield the negative charges of adducts, which

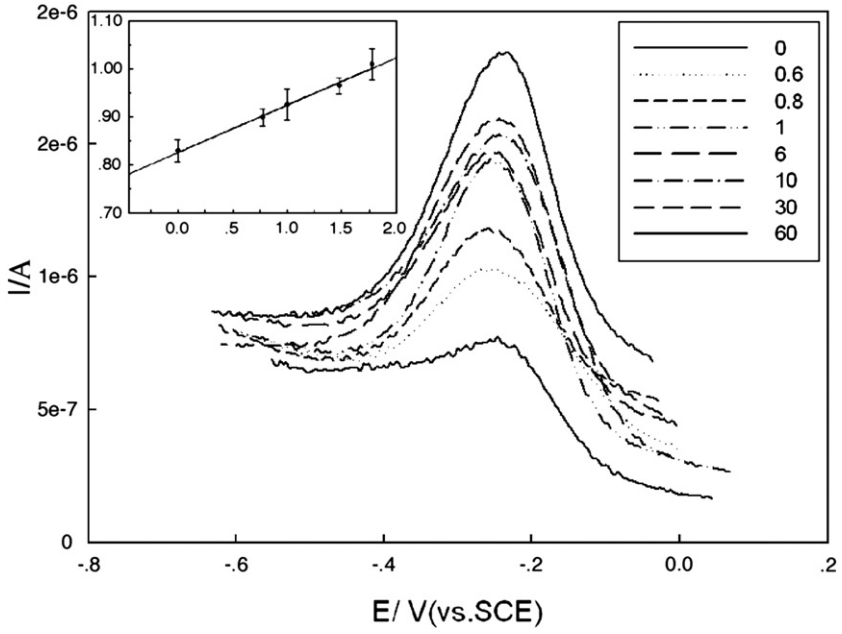

Fig. 6. The sensor response to different concentrations of thrombin in Tris–HCl buffer. Amplitude 20 mV, pulse width 0.05 s. The inset plot is the calibration curve of DPV signal vs logarithm of thrombin concentration.

Table 1
The DPV response of this sensor to different substances

	Blank	BSA	IgG	Thrombin
Concentration (ng mL^{-1})	0	2×10^7	10	5
Peak current (μA)	0.18	0.21	0.22	1.06

probably prevented the approaching of the positively charged MB to the surface of the electrode. A K^+ concentration of 10 mM was selected in our experiment. The effect of divalent ions was also investigated. Divalent ions were required by aptamer to assemble into appropriate three-dimensional conformation. In the case of Mg^{2+} (Fig. 5(b)), a concentration of 10 mM yielded the best response. Lower concentration resulted in decreasing signal, due to lack of bivalent ions to maintain the three-dimensional conformation. Similar results were obtained when excess concentrations of Mg^{2+} (Fig. 5(b)) were used, showing that the aptamer/thrombin interaction becomes infirm when the ionic strength is high.

3.3. Electrochemical detection

In this work, the thrombin aptamer used was designed in such a way that besides the original recognition sequence [38] for thrombin (in bold face), one section of extra extended 22-base oligonucleotides connected with one end and another section of 21-base with the other end of the original sequence (58-mer,5'-GACAGACGATGTGCTGAC-TACT**GGTTGGTGAGGTTGG**GTAGTCA GCACATCGT CTGTC-3'). The extended bases were perfectly complementary to each other to intercalate the electrochemical active marker MB which was widely reported to directly intercalate into single and double strand DNA as an electrochemical active indicator [39–45]. Some article [15] has reported that MB was reduced to leucomethylene blue at a potential of −197 mV, proceeding via two electrons and one proton transfers. The peak potential shifted negatively by around 50 mV after it was accumulated into DNA [46]. However, MB is positively charged, so it can nonspecifically bind to protein or the negatively charged DNA. To circumvent this problem, we blocked the nonspecific site on antibody with 2% BSA. In addition, we thoroughly rinsed the electrode with high ionic strength buffer (10 mM Tris–HCl, pH7.4, containing 10 mM KCl, 10 mM $MgCl_2$ and 0.1 M NaCl). Fig. 6 shows the electrochemical response to different concentrations of thrombin. In this Figure, one can see that the peak current signals increase with the increase of thrombin concentration. Linear relationship between the peak current and the thrombin concentration was observed in the range of 1–60 nM with a correlation factor 0.996. The regression equation is $y=8.27x+0.98$ (here, x is the logarithmic concentration of thrombin (nM) and y is the response peak current) and the detection limit of this sensor system was 0.5 nM.

In order to confirm the specificity of this sensor, control experiments were performed under the optimized experimental conditions. Table 1 shows the peak current responses upon exposure to blank, goat anti-human IgG, BSA and thrombin. The blank response current was around 0.18 μA. A comparison of the response currents shows that this sensor had good recognition selectivity.

4. Conclusions

A modified methodology for electrochemical protein detection in sandwich manner has been developed, utilizing antibody and aptamer as the sandwich elements and MB as the electrochemical active marker. The thrombin was used as the model analyte to demonstrate the feasibility of the proposed methodology as a proof of concept. Having combined both of the specificity of antibody and aptamer, high selectivity of thrombin detection has been achieved. The proposed method can provide an alternative tool for detection of various protein targets.

Acknowledgments

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