Talanta 81 (2010) 63–67

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Aptamer-Au NPs conjugates-accumulated methylene blue for the sensitive electrochemical immunoassay of protein

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ARTICLE INFO

Article history:
Received 20 August 2009
Received in revised form
10 November 2009
Accepted 11 November 2009
Available online 17 November 2009

Keywords:
Au nanoparticles
Aptamer
Sandwich immunoassay
Methylene blue
Electrochemistry

ABSTRACT

In this paper, we combine the advantages of aptamer, nanomaterial and antibody to design an electrochemical sandwich immunoassay for the ultrasensitive detection of human immunoglobulin E (IgE) by using methylene blue (MB) as electrochemical indicator. The sandwich structure is fabricated by using goat anti-human IgE as capturing probe. Aptamer-Au nanoparticles (NPs) conjugates are used both as a sandwich amplification element as well as an accumulation reagent of MB. Once the aptamer-Au NPs conjugates specifically bind to electrode surface, MB molecules are accumulated on its surface by the specific interaction of MB with G base of aptamer-Au NPs conjugates. Therefore, with the increase of human IgE concentration, more aptamer-Au-NPs conjugates are bound, and thus, more MB molecules are accumulated. A good linear relationship is obtained for the detection of human IgE over a range of 1–10,000 ng/ml with a lowest detection limit of 0.52 ng/ml. In addition, by using BSA, human IgA and human IgM as contrast, the excellent specificity of this sensing system for the detection of human IgE is also demonstrated.

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1. Introduction

Aptamer-based protein detection has been receiving increasing attention because of the inherent characteristics of aptamer, such as high association constant with target proteins, easy production and labeling with signal moieties, cost-effectiveness and stability during long-time storage [1]. A variety of aptamer-based detection approaches including surface plasmon resonance [2–4], fluorescence [5,6], chemiluminescence [7–9], quartz crystal microbalance [10–12] and electrochemistry [13–17] have been developed. Once the electrochemical detection is concerned, sensors based on molecular aptamer beacon labeled with single electroactive marker [18–21] have been extensively studied. Such detective system is very simple and sensitive, but the electrochemical signal resulted in the binding of protein is not very strong because each of aptamer is only labeled by one electroactive marker. To amplify electrochemical signal, the sandwich assay has been introduced to develop the high sensitive aptasensor by using aptamer-Au nanoparticles (NPs) conjugates as an amplification reagent [22–26]. Although these developed electrochemical aptasensors possesses high sensitivity, their detecting target mainly focus on proteins bearing two or more aptamer binding sites.

To extend the application of aptamer in the detection of protein, here, we make use of the advantages of aptamers together with nanomaterials and antibody to construct a more general sandwich biosensor for the detection of protein. Human immunoglobulin E (IgE) is used as a model protein to confirm the feasibility of the present sandwich approach because human IgE is important in human allergic responses and its extreme low concentration in human serum [27,28] makes it difficult to be detected by direct electrochemical detection. In the current paper, human IgE is detected with a very low detection limit (~0.52 ng/ml) and a wide detection range (1–10,000 ng/ml). More importantly, this detection method is very easy to be extended to other proteins possessing either one or more binding sites with aptamer by changing proteins and corresponding antibody and aptamer.

2. Experimental

2.1. Materials

Hydrogen tetrachloroaurate(III) ($HAuCl_4$), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), ethanolamine, 3-mercaptopropionic acid (MPA) and 6-mercaptohexan-1-ol (MCH) were purchased from Sigma and used as received. Albumin from bovine serum (BSA), human immunoglobulin M (IgM) and immunoglobulin A (IgA) were ordered from Thermo Scientific (USA). Human IgE and goat anti-human IgE were obtained from Fitzgerald Industries International

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0039-9140/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.talanta.2009.11.035

(USA). Human IgE aptamer [29] was obtained from Integrated DNA Technologies (IDT). The sequence was 5′-SH-C_6-ATC TAC GGG GCA CGT TTA TCC GTC CCT CCT AGT GGC GTG CCC C-3′. Aptamer was dissolved in 50 mM pH 8.0 Tris–HCl buffer containing 138 mM NaCl and 5 mM $MgCl_2$. Protein solutions were prepared by dissolving different proteins in pH 7.4 PBS (10 mM).

2.2. Apparatus

Electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were carried out with an Autolab PGSTAT12. A normal three-electrode configuration consisting of a modified gold working electrode (1.6 mm in diameter), an Ag/AgCl as reference electrode, and a platinum wire as auxiliary electrode was used. CV was performed in 10 mM PBS buffer which was purged with highly purified nitrogen for at least 20 min. The voltage range was from −0.2 V to 0.5 V, and scan rate was 0.1 V/s. DPV was also performed in 10 mM PBS buffer (including 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$). The parameters applied were: 50 ms modulation time, 0.1 s interval time, 25 mV modulation amplitude, 5 mV step potential and voltage range from −0.5 V to 0.1 V. For EIS detection, an oscillation potential of 10 mV over the frequency range of 10 kHz to 0.1 Hz was done in the solution of 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (in 10 mM PBS buffer). All the measurements were carried out at room temperature.

2.3. Synthesis and modification of Au NPs

Au NPs stabilized with citrate were synthesized according to the literature procedure [30]. Human IgE aptamer-Au NPs conjugates were prepared according to our previous work with minor modification [31,32]. In brief, 138.83 μL of 15.1 μM aptamer was added in 10 ml of Au NPs solution and incubated for 16 h. The modified Au NPs were centrifuged at 14,000 × *g* for 25 min twice to remove the free aptamer. Then, the prepared Au NPs were dispersed in 10 ml of buffer containing 27.6 mM NaCl, 10 mM Tris–HCl, 1 mM $MgCl_2$, pH 8.0. After that, MCH was added to the prepared aptamer-Au NPs conjugates to give a final MCH concentration of ∼10 μM. The time of MCH treatment was 0.5 h at room temperature and the mixture was stored at 4 °C for 8 h. Then, a similar centrifugal procedure was used to collect the aptamer-Au NPs conjugates. At last, the aptamer-Au NPs conjugates were dispersed in buffer containing 27.6 mM NaCl, 10 mM Tris–HCl, 1 mM $MgCl_2$, pH 8.0, again. The number of aptamer on each Au NPs was about ∼34, which had been confirmed in our previous work [33].

2.4. Fabrication of the sensing system

The gold electrode was first polished with 1.0 μm, 0.3 μm α-Al_2O_3 and washed ultrasonically with pure water, followed by electrochemically cleaning in 0.1 M H_2SO_4 by potential scanning between −0.2 V and 1.5 V until a reproducible cyclic voltammogram was obtained. And then, the gold electrode was dipped in a 100 μM MPA in absolute ethanol for 12 h. After washing with ethanol and water, the gold electrode was immersed in a mixing solution of 200 mmol/L EDC and 50 mmol/L NHS in PBS buffer for 0.5 h. After rinsing, goat anti-human IgE solution in PBS (100 μg/ml) was added and incubated on the gold electrode for 12 h at 4 °C. Then, the resulting electrode was immersed into 1 mol/L ethanolamine solution for 1 h to deactivate and block the unreacted esters and prevent electrostatic binding of proteins or MB. After rinsed thoroughly with water, 10 μl human IgE with different concentrations in PBS buffer were covered to the electrode surface for 30 min. After that, the electrode was further immersed into aptamer-Au NPs conjugates solution for 30 min. Then, the modified electrode was dipped in 100 μM MB solution for 30 min. At last, the oxidation peak current of the accumulated MB was measured by using DPV.

3. Results and discussion

3.1. The detection principle of electrochemical immunoassay

Fig. 1 presents the detailed detection process of the electrochemical immunosensor for human IgE. Goat anti-human IgE is first immobilized onto the surface of gold electrode as a sensing surface by carboxyl activation strategy. When human IgE with different concentrations is added, human IgE will bind with its antibody by the specific interaction. It should be pointed out that the binding site for goat anti-human IgE with human IgE and the binding site for aptamer with human IgE are different [3]. Thus, after human IgE binds to anti-human IgE immobilized on the electrode surface, it can still bind with aptamer in the aptamer-Au NPs conjugates and adsorb aptamer-Au NPs conjugates to the electrode surface. The specific binding of aptamer-Au NPs conjugates with human IgE immobilized on the electrode surface enable the accumulation of MB on the electrode surface by the interaction of MB with G bases in aptamer-Au NPs conjugates. Therefore, an amplified electrochemical signal of MB can be observed. The current electrochemical sensing system is different in several ways. First, using antibody for capturing the target facilitates the sandwich binding of aptamer because the binding sites of aptamer are different from that of antibody for the target. Furthermore, as MB could specifically bind with G bases in aptamer, employing antibody as the capturing probe could reduce the background signal of MB on the electrode. Secondly, aptamer-Au NPs conjugates can greatly enhance the adsorption amount of MB on the electrode surface because there are a large number of G bases in aptamer-Au NPs conjugates, which increases the sensitivity of the electrochemical biosensor. Lastly, the scheme provides a potential possibility for detecting other proteins with sandwich manner by MB with the help of aptamer.

3.2. The fabrication and characterization of sensing interface

To characterize the modified electrode, we utilize CV and EIS to validate the fabrication of the sensing interface. For the modified electrode, $[Fe(CN)_6]^{4-/3-}$ anions is used as a redox probe to detect the interfacial electron-transfer kinetics of electrode after each of modification steps. The results are shown in Fig. 2A. The bare Au electrode shows a very large current of $[Fe(CN)_6]^{4-/3-}$. After assembling MPA, CV response slightly decreases due to the decrease electron-transfer capability of the modified electrode. However, CV response obviously decreases after goat anti-human IgE is immobilized. This is because most of the electrode surface is covered by goat anti-human IgE, which greatly block the electron-transfer efficiency of $[Fe(CN)_6]^{4-/3-}$ at solid/liquid interface. After immersing the sensing interface in 10 μg human IgE solution for 10 min, we can see the CV response further decreases and the peak-to-peak separation increases. It means human IgE is adsorbed onto the electrode surface, although the change of CV signal is small. In order to confirm the sensing interface is constructed successfully, we further utilize EIS to monitor the impedance change of electrode after each of modification steps is applied. The results are shown in Fig. 2B. The inset of Fig. 2B shows the circuit that includes the commonly existed electrolyte resistance (*R*s), constant phase element (*Q*), Warburg impedance (*Z*w) and the electron-transfer resistance (*R*et/*R*ct). From Fig. 2B, we can see the bare Au electrode shows a very small semicircle domain which is represented as the *R*et. While for the MPA monolayer electrode, the response of the equimolar $[Fe(CN)_6]^{4-/3-}$ anions are retarded, leading to decreased

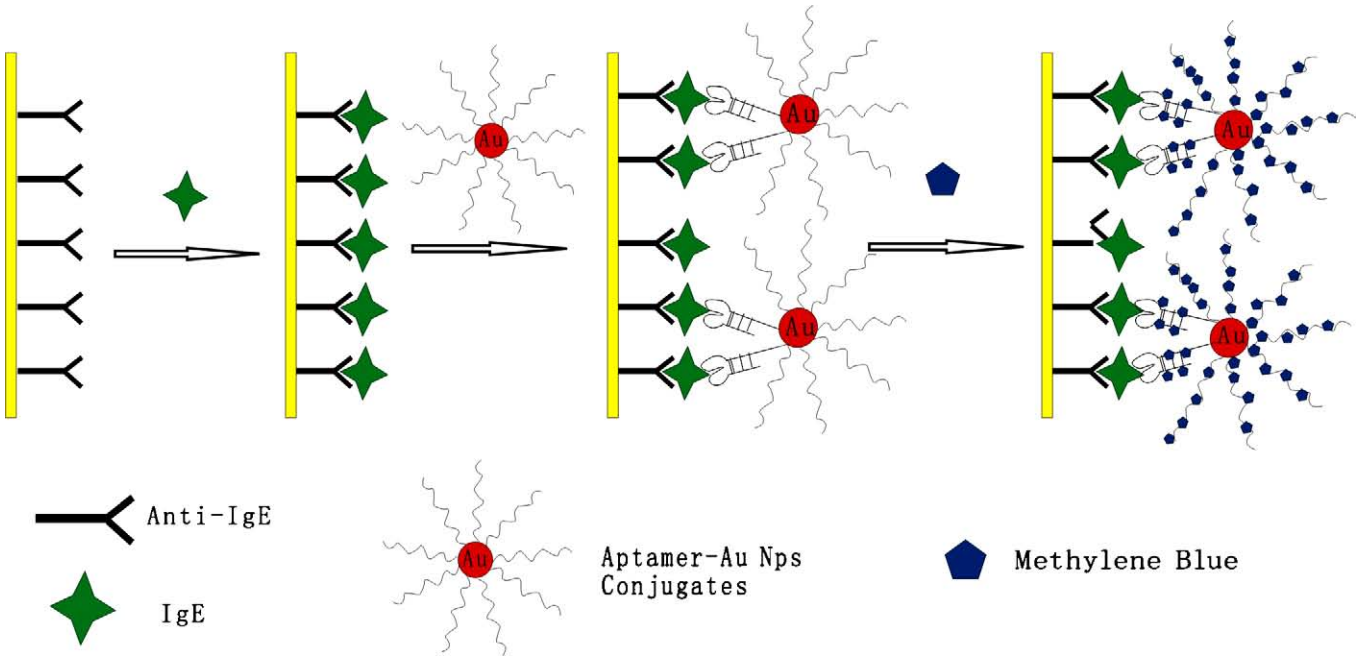

Fig. 1. A schematic representation of the electrochemical sandwich immunoassay.

electron-transfer with large semicircle. After goat anti-human IgE is immobilized, the value of *R*et greatly increase because of the strong block effect of protein for $[Fe(CN)_6]^{4-/3-}$. When the modified electrode was treated with 10 μg human IgE for 10 min, there is a continuous increase of the *R*et. These results above demonstrate the sensing interface is constructed successfully.

3.3. The amplification effect of aptamer-Au NPs conjugates

As well known, MB can specifically bind with G base in ss-DNA [34]. Utilizing these characteristics, many electrochemical sensors for detecting DNA hybridization have been developed. Aptamer, as a kind of ss-DNA with rich of G bases, can undoubtedly absorb lot of MB onto its surface. At the same time, a kind of ss-DNA with special function, aptamer can specifically bind with its target molecules. Based on these facts, it's easy to design an electrochemical biosensor for detecting all kinds of analytes by using aptamer to load MB. In order to increase the loading ability of aptamer for MB, here, we accumulate aptamer with Au NPs. Although previous works have revealed that Au NPs themselves have the effect to amplify electrochemical readout signal. Here, the role of Au NPs is to load more aptamers of human IgE, which will be further used to accumulate MB for electrochemical readout signal. To confirm aptamer-Au NPs conjugates can accumulate MB, we study the electrochemical response of MB at human IgE modified electrode before and after aptamer-Au NPs conjugates is adsorbed. To manifest the amplification effect of aptamer-Au NPs conjugates, we further compare the electrochemical response of MB at aptamer/human IgE/goat anti-human IgE modified electrode and aptamer-Au NPs conjugates/human IgE/goat anti-human IgE modified electrode, respectively. These results are shown in Fig. 3. Curve a in Fig. 3 is the DPV of MB obtained at human IgE modified electrode. A very small oxidized peak of MB is observed. After human IgE modified electrode is immersed into 15.1 μM human IgE aptamer solution for 10 min, we can see the increase of the oxidized peak of MB (curve b in Fig. 3). It means aptamer binding to human IgE can absorb MB to its surface by its G bases. Curve c in Fig. 3 is the DPV of MB after we immerse human IgE modified electrode into aptamer-Au NPs conjugates for 10 min. We can clearly see the oxidized peak of MB is much larger than that value in curve b. Obviously,

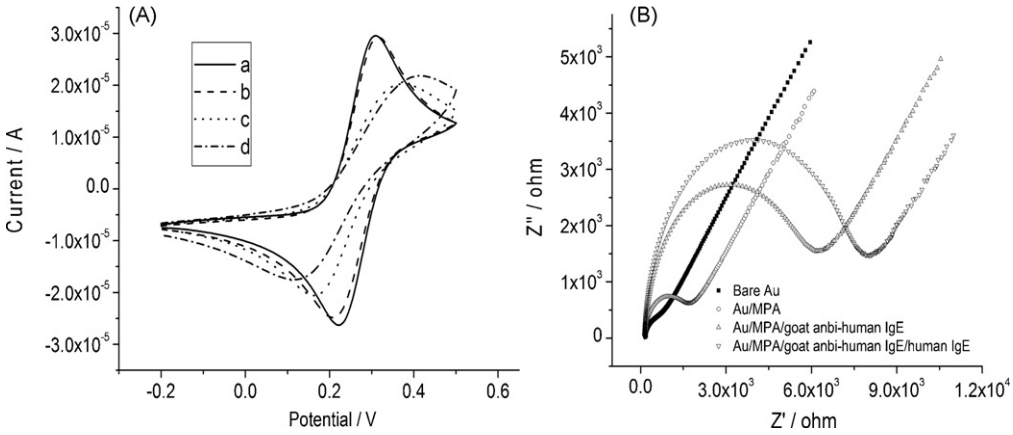

Fig. 2. (A) Characterization of the fabrication of the sensing interface by CV. (a) Bare electrode; (b) MPA; (c) goat anti-human IgE; (d) human IgE. (B) Characterization of the fabrication of the sensing interface by EIS.

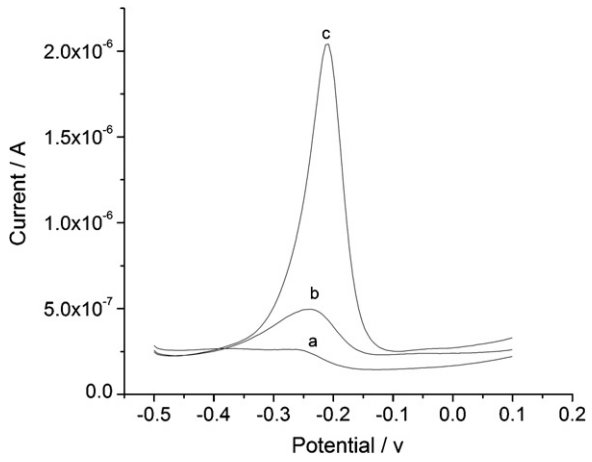

Fig. 3. DPV of MB obtained at (a) human IgE modified electrode; (b) aptamer/human IgE modified electrode; (c) aptamer-Au NPs conjugates modified electrode.

aptamer-Au NPs conjugates accumulate plenty of MB on its surface, which is beneficial to increase the sensitivity of electrochemical biosensor.

3.4. The sensitivity and selectivity of electrochemical biosensor

As shown in Fig. 1, the more human IgE molecules are, the more aptamer-Au NPs conjugates are. Therefore, more electrochemical probes MB can be accumulated onto the surface of aptamer-Au NPs conjugates, and thus larger redox peaks of MB in the CVs can be obtained. As a result, there is a relationship between the peak currents of the MB and the concentration of human IgE. Here, we utilize DPV to detect the change of MB oxidization peak current with the concentration of human IgE. The results are shown in Fig. 4. The concentrations of human IgE are increased in the concentration range of 0 ng/ml, 1 ng/ml, 5 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml, 500 ng/ml, 1000 ng/ml, 2000 ng/ml, 5000 ng/ml, and 10,000 ng/ml. We can see the electrochemical signal of MB is very small in the absence of human IgE. This peak is attributed to the adsorption of MB at gold electrode and protein immobilized on electrode, which will be subtracted as background signal. With the increase of human IgE concentration, well-defined DPV signals of MB are observed at about −0.21 V. The DPV peaks of MB at different concentrations of human IgE have nearly the same peak

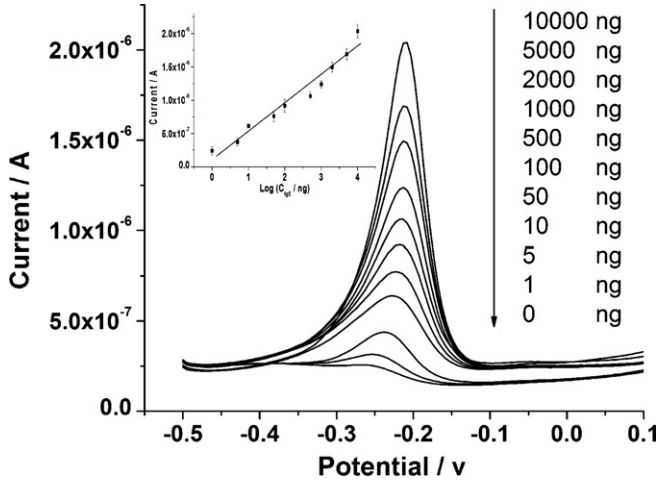

Fig. 4. DPV of MB obtained at the different concentrations of human IgE modified electrode. Inset: The linear relationship between the logarithms of human IgE concentrations and the current of MB oxidization peak.

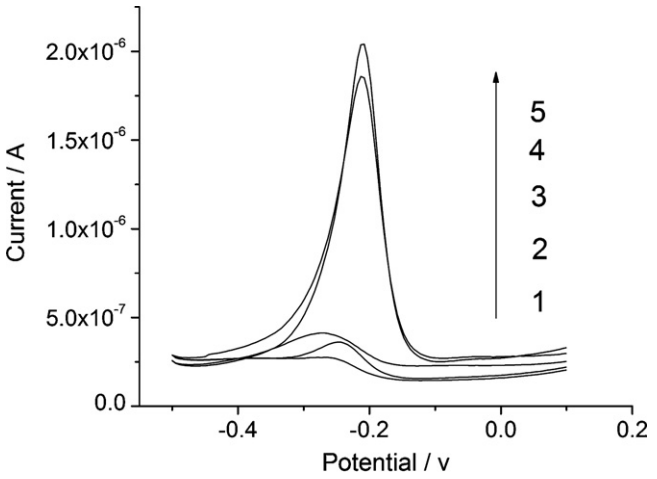

Fig. 5. Specificity analysis of the fabricated electrochemical immunosensor; (1) 0.1 mg/ml human IgA; (2) 0.1 mg/ml human IgM; (3) 0.1 mg/ml BSA; (4) the mixture of 0.1 mg/ml human IgA, 0.1 mg/ml human IgM, 0.1 mg/ml BSA and 10 μg/ml human IgE; (5) pure 10 μg/ml human IgE.

shape and position, which well accord with that of the value of reference [35]. As expected, the DPV peak current of MB increased with an increasing human IgE concentration. After analyzing the change between current of MB oxidization peak and the concentrations of human IgE (shown in the inset of Fig. 4), we find a linear relation between the logarithms of human IgE concentrations and the current of MB oxidization peak over a range of 1–10,000 ng/ml with a correlation factor of 0.976. The regression equation is $y = 4.19 \times 10^{-7}x + 1.17 \times 10^{-7}$ (here, x is the logarithmic concentration of human IgE (ng) and y is the response peak current) and the lowest detection limit of this sensor system was 0.52 ng/ml. This value is comparable with the detection results obtained by other optical and electrochemical aptasensors, such as 1000 ng/ml with direct surface plasmon resonance (SPR) sensor [3], 100 ng/ml with quartz crystal microbalance (QCM) sensor [11], 20 ng/ml with electrochemical sensor [36], 2.07 ng/ml with sandwich SPR sensor [37]. To determine the specificity of the method, we perform the contrast experiments with BSA, human IgA and human IgM (shown in Fig. 5). Curves 1, 2 and 3 are the DPV of MB at the 0.1 mg/ml human IgA, human IgM and BSA-modified gold electrode after all kinds of protein modified electrode is immersed in aptamer-Au NPs conjugates for 10 min, respectively. We can easily see that the electrochemical signal of MB is very small on these protein modified electrode. Curve 5 is the oxidization peak of MB at the pure 10 μg/ml human IgE modified gold electrode after aptamer-Au NPs conjugates are amplified. Obviously, the current is much higher than that of other proteins. Besides that, the electrochemical signal of the mixture of four kinds of proteins with pure IgE is also compared (curve 4 in Fig. 5). A similar electrochemical signal of MB is obtained. A little decrease in the MB electrochemical signal could be explained by the fact that part of binding site of antibody for IgE was blocked by other three kinds of proteins. These results indicate the developed strategy has a sufficient specificity and human IgE could be identified with high selectivity.

4. Conclusions

In summary, we have introduced a modified methodology for the electrochemical detection of protein in sandwich manner by using MB as electrochemical indicator. Utilizing antibody as the capturing probe and using aptamer-Au NPs conjugates both as the sandwich amplification elements and the accumulation reagent of MB, this method has the combined advantage of increasing

the sensitivity of biosensor and extending the format of sandwich immunoassay. This detection system has been successfully used to sensitively detect human IgE with high selectivity. Furthermore, the enhancing effect of aptamer-Au NPs conjugates was also confirmed. These results confirmed that the proposed method can provide an alternative tool for the ultrasensitive detection of various protein targets by electrochemical methods.

Acknowledgement

The authors greatly acknowledge the support of this work by National Science Foundation (EEC-0823974).

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