Anal. Chem. **2008,** *80,* 5110–5117

Multifunctional Label-Free Electrochemical Biosensor Based on an Integrated Aptamer

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Aptamers, which are in vitro selected functional oligonucleotides, have been employed to design novel biosensors (i.e., aptasensors) due to their inherent selectivity, affinity, and their multifarious advantages over traditional recognition elements. In this work, we reported a multifunctional reusable label-free electrochemical biosensor based on an integrated aptamer for parallel detection of adenosine triphosphate (ATP) and α-thrombin, by using electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). A Au electrode as the sensing surface was modified with a part DNA duplex which contained a 5′-thiolated partly complementary strand (PCS) and a mixed aptamer (MBA). The unimolecular MBA contained small-molecule ATP binding aptamer (ABA) and also protein α-thrombin binding aptamer (TBA). Thus, the aptasensor could be used for detection of ATP and α-thrombin both. The detection limit of ATP was 1×10^{-8} M, and its detection range could extend up to 10^{-4} M, whereas the detection limit of α-thrombin was 1×10^{-11} M, and its detection range was from 1×10^{-11} to 1×10^{-7} M. Meanwhile, after detecting α-thrombin, the sensing interface could be used for ATP recognition as well. The aptasensor regeneration could be realized by rehybridizing of the MBA strand with the partly complementary strand immobilized on the Au surface after ATP detection or by treating with a large amount of ATP and then rehybridizing the MBA strand with the partly complementary strand immobilized on the Au surface after α-thrombin detection. The aptasensor fabricated exhibited several advantages such as label-free detection, high sensitivity, regeneration, and multifunctional recognition. It also showed the detectability in biological fluid. Therein it held promising potential for integration of the sensing ability such as the simultaneous detection for multianalysis in the future.

Aptamers, which are novel in vitro selected functional DNA or RNA structures from random-sequence nucleic acids libraries,[1–3] possess high recognition ability to specific targets ranging from small inorganic, organic substances to even proteins or cells.[4–6] Due to their inherent selectivity, affinity, and their multifarious advantages over the traditional recognition elements, aptamers can rival antibodies for molecular recognition and detection.[7]

Recently, a lot of aptamer-based sensing interfaces (i.e., aptasensors) have been developed, including optical transduction,[8] quartz crystal microbalance (QCM),[9] surface plasmon resonance (SPR),[10,11] fluorescence,[12–18] colorimetry,[19,20] electrophoresis,[21] atomic force microscopy (AFM),[22,23] electrochemistry,[24–35] and so on. With the use of the developed aptasensors, the detection of targets from small molecules to proteins and cells has been successfully realized.[4–6] For example, Tan's group has developed a method for the rapid detection of leukemia cells using aptamer-functionalized nanoparticles as the molecular recognition element.[36] Many research groups have engaged in the development of sensitive and selective aptasensors for proteins, making use of the varieties of sensing interfaces mentioned above.[4–6] As for small-molecules assay, aptasensors do possess considerable advantages since it is especially difficult to find a specific recognition element for small molecules.

Up to now, many aptasensors for small molecules have been successfully fabricated, among which the analysis based upon electrochemistry has been extensively studied. Electrochemistry

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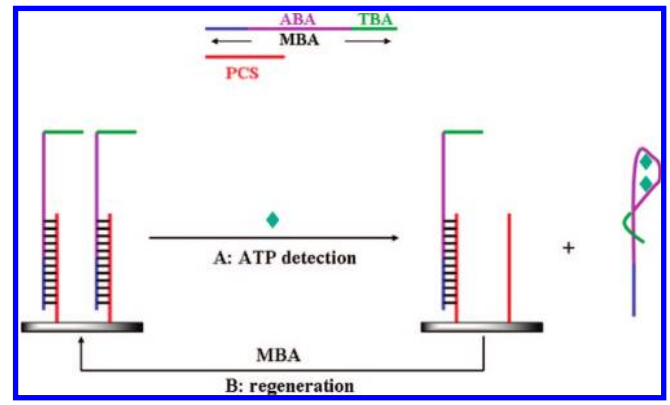
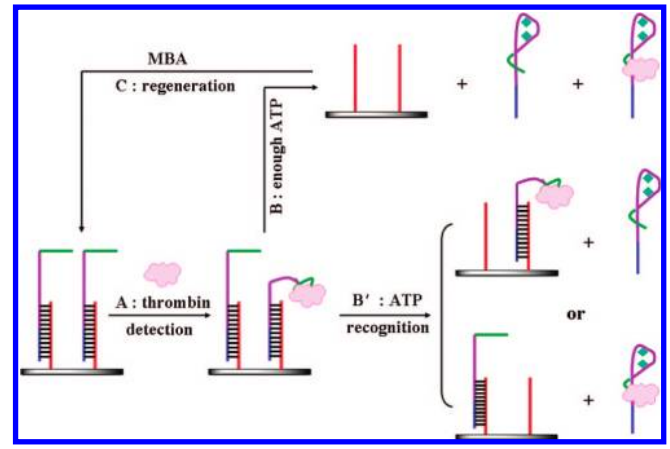
10.1021/ac800303c CCC: $40.75
Published on Web 06/04/2008

methods will play an important role in this field due to their significant advantages such as simplicity, high sensitivity, low cost, and high stability. Baker et al. have developed an electrochemical aptamer-based sensing platform for rapid and sensitive detection of small-molecule cocaine in biological fluids.[24] Though their sensor could be regenerated via a brief washing at room temperature, the aptamer itself was labeled with a redox probe methylene blue. Such labeling bears the disadvantages that the labeling process makes the experiments relatively complex and expensive and also affects the binding affinity between the targets and their aptamers to a certain degree. Fan's group has reported a similar electrochemical aptasensor for detection of nanomolar adenosine triphosphate (ATP) with high sensitivity and selectivity.[34] Wu et al. recently reported a reusable electrochemical sensing platform for highly sensitive detection of small-molecule adenosine based on structure-switching signaling aptamers.[31] Their sensing aptamers needed to be tagged with the redox probe ferrocene. A label-free reagentless aptasensor for the selective analysis of small-molecule adenosine has been reported by Willner's group.[37] Their design was based on a reagentless aptamer/partly complementary strand aptamer/target sensing interface using an ion-selective field-effect transistor (ISFET) for measurement where the aptamer contained-strands were immobilized on the gate surface and further hybridized with the partly complementary strands. We also immobilized the partly complementary strands on the electrode surface, which were then hybridized with the aptamer contained-strands. The aptasensors provide not only higher sensitivity of detection but also an easily reusable sensing platform.[38] Very recently Elbaz et al. realized the parallel electrochemical analysis of two analytes in solutions or on surfaces by using a bifunctional aptamer.[39] Besides the analytical significance, their study suggested future possibilities to tailor bioelectronic logic gate systems.[39]

Scheme 1. Schematic Routine for ATP Detection (A) and the Sensor Regeneration (B)



Scheme 2. Schematic Routine for α-Thrombin Detection (A), the Sensor Regeneration (C), and Multifunctional Recognition of ATP (B and B′)



Though all the electrochemical aptasensors developed could realize the small-molecule detection successfully, the fabrication of a small-molecule aptasensing platform owning the advantages of being highly sensitive, label-free, and reusable is still a challenge for analytical scientists. Electrochemical impedance spectroscopy (EIS) is a rapidly developing electrochemical technique, and it has been employed to design aptasensing interfaces for its advantages such as sensitivity, low cost, convenience, and being label-free.[25,28,29,32,33,35,37–39] Though EIS aptasensors for small molecules have been reported,[37–39] however, the studies were focused on protein detection because most proteins have high molecular weight or high-density charges and are able to affect the electrode surface easily.[25,28,29,32,33,35]

Here we report an EIS aptasensor for small-molecule ATP that could meet the merits of being highly sensitive, label-free, and reusable (Schemes 1 and 2). In comparison with our previous work, a part duplex composed of an aptamer contained-strand and a partly complementary strand is employed as the sensing element (Figure 1B). The interesting improvement is that the aptamer contained-strand is designed as a unimolecular DNA strand (referred to as MBA) containing both ATP binding aptamer (ABA) and α-thrombin binding aptamer (TBA), for multiuse of small-molecule and protein analysis. The TBA embedded in the MBA, first, extends the aptamer contained-strand and results in amplified

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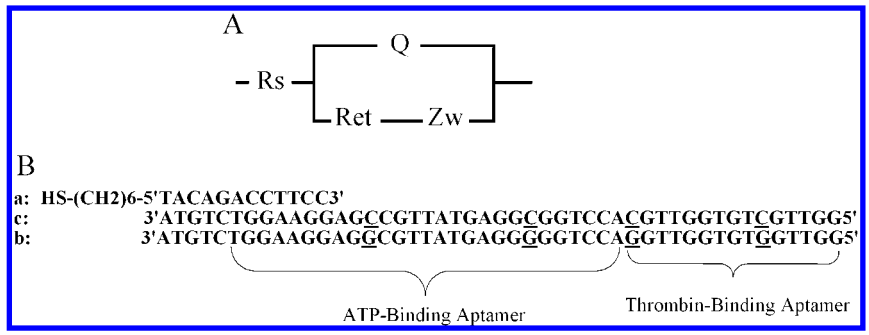

Figure 1. (A) Circuit for the EIS. (B) Sequences of all oligonucleotides: (a) PCS sequence; (b) MBA sequence; (c) mismatch aptamer sequence.

signals. Thus, highly sensitive ATP detection is realized with a detection limit of 1×10^{-8} M. The sensor is easily regenerated by rehybridizing the MBA strand with the partly complementary strand immobilized (Scheme 1). Second, the designed aptasensor is also used for α-thrombin detection. After this process, the sensor could still be directly used for ATP recognition and the sensing sensitivity for it could be promisingly further amplified due to the huge protein binding on the end of MBA. Similarly, when such detections (both for α-thrombin and ATP) are finished, the regeneration would also be realized by displacing the MBA strand via the interaction with a large amount of ATP and the rehybridization process (Scheme 2). Third, the aptasensor has a promising potential detectability in real samples, which has been testified in 1% pretreated human plasma. In a word, what make the sensor interesting and feasible are the amplification and regeneration methods. On the one hand, α-thrombin or α-thrombin/aptamer is for ATP detection amplification. On the other hand, ATP is for the sensing surface regeneration after α-thrombin and ATP detection. Thus, the two targets could benefit each other, which provides more applications of aptamer in sensor design.

EXPERIMENTAL SECTION

Chemicals and Materials. Oligonucleotide containing specific sequence (MBA, 5′ GGTTGGTGTGGTTGGACCTGGGGGAGTATTGCGGAGGAAGGTCTGTA 3′) and the mismatch aptamer sequence (MAS, 5′ GGTTGCTGTGGTTGCACCTGGCGGAGTATTGCCGAGGAAGGTCTGTA 3′) were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). HPLC-purified oligonucletide, the part complementary strand (PCS), 5′HS−$(CH_2)_6$−TACAGACCTTCC 3′, was designed according to the literature[37,38] and synthesized by the TaKaRa Biotechnology Co. Ltd. (Dalian, China). The concentrations of oligonucleotides were determined using the 260 nm UV absorbance and the corresponding extinction coefficient. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Bio Basic Inc. (Markham Ontario, Canada). ATP, cytidine triphosphate (CTP), guanosine triphosphate (GTP), β-thrombin, and γ-thrombin were all purchased from Haematologic Technologies Inc. (Vermont). α-Thrombin and bovine serum albumin (BSA) were purchased from Sigma (Missouri). 2-Mecaptoethanol (MCE) was obtained from Fluka (Buchs, Switzerland). Tris(hydroxymethyl)aminomethane (Tris) was bought from Shanghai Chemical Reagent Company (Shanghai, China). MCE was dissolved in the Tris−HAc buffer, and different concentrations of α-thrombin, ATP, BSA, GTP, and CTP were prepared in the Tris−HCl buffer. All other chemicals were of analytical grade. The DNA, nucleotide, and protein solutions were stored at 4 °C before use. All stock and buffer solutions were prepared using autoclaved double-deionized water.

Instrumentation. Cyclic voltammetry (CV) and EIS were performed on an Autolab PGSTAT30 (Utrecht, The Netherlands, controlled by GPES4 and Fra software). Chronocoulometry was performed with a model CH Instrument 832B electrochemical workstation (Shanghai Chenhua Equipments, China). A conventional three-electrode system, with a Au electrode (1.2 mm in diameter) as working electrode, a Ag−AgCl reference electrode, and a platinum wire as counter electrode was used. The cell was housed in a homemade Faraday cage to reduce stray electrical noise. All the measurements with the Autolab were carried out at room temperature (~28−30 °C). EIS was performed under an oscillation potential of 5 mV over the frequency range of 10 kHz to 0.1 Hz. The amplitude of the alternate voltage is 5 mV. All the measurements (EIS, CV) were performed in the solution of 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ (in 67 mM PBS buffer, pH = 7.0). SPR measurements were carried out for characterizing the fabrication of the sensing interface using an Autolab SPR instrument (Eco Chemie BV, The Netherlands). The measured $\Delta\theta$ values correspond to the amount of adsorbed material with a mass sensitivity factor of 120 mDeg per 100 ng/cm^2.

Electrode Cleaning and Pretreatment. The Au electrode was polished with 1.0 μm, 0.3 μm α-Al_2O_3 and then washed ultrasonically with pure water for three times successively, followed by electrochemically cleaning in 0.1 M H_2SO_4 by potential scanning between −0.2 to 1.6 V until a reproducible cyclic voltammogram was obtained. Then it was rinsed with a copious amount of pure water and finally blown dry with nitrogen before assembly.

Fabrication of the Sensing Interface. The pretreatment of the part DNA duplex (PCS−MBA) was followed by these steps. The mixture of 1.9 μM PCS (in 25 mM Tris−HCl, 300 mM NaCl, 30 μM TCEP, pH = 8.2) and 2.2 μM MBA (in 25 mM Tris−HCl, 300 mM NaCl, pH = 8.2), was heated and kept at 90 °C for 10 min, and then the solution was slowly cooled to room temperature and kept over 30 min. The sensing interface (Au/PCS−MBA/MCE) was prepared by placing 20 μL of freshly prepared PCS−MBA solution on the Au electrode held upside-down, covering the end of the electrode with a plastic cap to prevent the solution from evaporation. The assembly was kept 16 h at room temperature and then rinsed with washing buffer (10 mM Tris−HAc, pH = 7.41) and pure water for several times. Then

the electrode was dried in a nitrogen stream, after which the interface was covered with 5 μL of MCE (10 mM Tris−HAc, pH = 7.41) and kept at room temperature for 1 h, followed by rinsing with washing buffer and pure water. The sensor was obtained after dried with nitrogen.

Electrochemical Detection of ATP and Regeneration of the Sensing Interface. As shown in Scheme 1A, the new as-prepared sensing interface was immersed in series of ATP (dissolved in 25 mM Tris−HCl, 100 mM NaCl, pH = 8.2) solutions with different concentrations for 1.5 h. Before measurement, the sensing interface was rinsed with washing buffer and pure water for several times and dried with nitrogen. After being treated with ATP, the used sensing interface could be regenerated easily by rehybridization (Scheme 1B). An amount of 15 μL of PCS was placed on the electrode surface, and the hybridization reaction was allowed to proceed for 4 h. A control experiment was also carried out. The sensing interface was immersed in mixed solution of 0.1 mM GTP and 0.1 mM CTP (in 25 mM Tris−HCl, 100 mM NaCl, pH = 8.2) for 2 h.

EIS Detection of α-Thrombin, Recognition of ATP, and Regeneration of the Sensing Interface. For α-thrombin detection, the sensor was immersed in α-thrombin solutions (in 20 mM Tris−HCl, 140 mM NaCl, 5 mM KCl, pH = 7.4) for 1 h (Scheme 2A). After detection of α-thrombin by EIS measurement, the sensing interface was allowed to recognize ATP as well, i.e., the sensing interface was immersed in ATP solutions to recognize the subsistent ATP (Scheme 2B′). Likewise, the prepared electrode was dipped into 100 nM BSA, 20 nM β-thrombin, and 20 nM γ-thrombin (in 20 mM Tris−HCl, 140 mM NaCl, 5 mM KCl, pH = 7.4) solution, respectively, to react as a control measurement. A copious washing buffer and pure water were used for clean the sensing interface before successive measurements.

For regeneration, the sensing interface after α-thrombin detection was treated with a high concentration of ATP, and it could also be regenerated by rehybridization (Scheme 2, parts B and C). The hybridization reaction proceeded for 4 h. The resulting electrode cleaned with washing buffer and pure water and was ready for the next detection. The regenerated sensing interface could be used to detect ATP and α-thrombin again.

Application of the Aptasensor in Biological Assay. Three kinds of biological fluids were used to confirm the applicability of this aptasensor: human plasma, fetal calf serum, and pretreated human plasma. Pretreatment of human plasma and spiked samples was as follows: healthy human plasma was pretreated with salt solution to avoid the formation of fibrin and the rapid sample clotting according to the previous articles reported by Mascini and co-workers;[40] 500 μL of plasma was treated with 2.5 mL of 2 M ammonium sulfate and 2.0 mL of 0.1 M sodium chloride, which were mixed for 3−4 min, then centrifuged at 10 000 rpm for 10 min, and the supernatant was eluted by Tris−HCl buffer through a gel column (Sephadex G-100) for rapid desalting and buffer exchange. Different concentrations of standard solutions of ATP and α-thrombin were spiked into the diluted pretreatment of 1% plasma to test the performance of the aptasensor. All experiment conditions were the same as the foregoing detection in the ordinary buffer.

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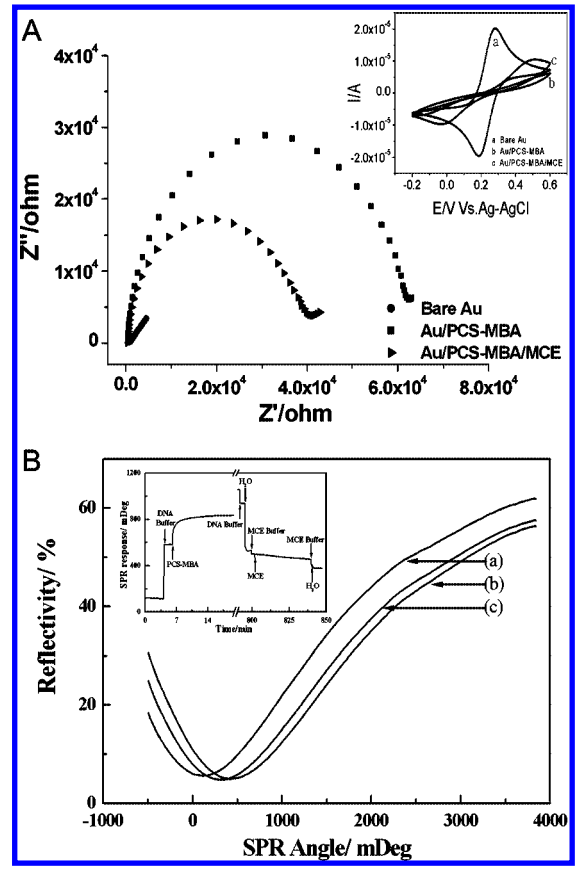

Figure 2. (A) Formation of the self-assembly layer on the Au electrode measured using both electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). The amplitude of the alternate voltage is 5 mV. (B) The angle-resolved SPR curves recorded on the Autolab ESPR instrument during the different steps (bare Au substrate (a), PCS−MBA (b), and blocking-MCE (c)) of the aptasensor immobilization. Inset: stepwise SPR response to the modification of the Au substrate.

RESULTS AND DISCUSSION

Fabrication and Characterization of the Sensing Interface. On the basis of previous articles,[37] a sensing interface was fabricated by functionalizing the Au electrode with part duplex hybridized by PCS and MBA strands (Figure 1B). After that, MCE was used for making the array of DNA on the electrode interface more regularly. Figure 1A shows the circuit that includes the commonly existing electrolyte resistance (Rs), constant phase element (Q), Warburg impedance (Zw), and the electron-transfer resistance (Ret/Rct).[37,38] For the Au/PCS−MBA/MCE electrode, the negatively charged interface repelled the negatively charged probe, $[Fe(CN)_6]^{4-/3-}$ anions, which retarded the interfacial electron-transfer kinetics of the redox probe. The formation process of the self-assembly layer was monitored using both EIS and CV (Figure 2A). The bare Au electrode shows a very small semicircle domain (which represents the Ret) (Figure 2A). However, for the Au/PCS−MBA system, the Ret signals increase obviously, due to the remarkably decreasing electron-transfer efficiency, and the CV response evidently decreases after the sensing interface formed. After MCE assembly, the EIS response

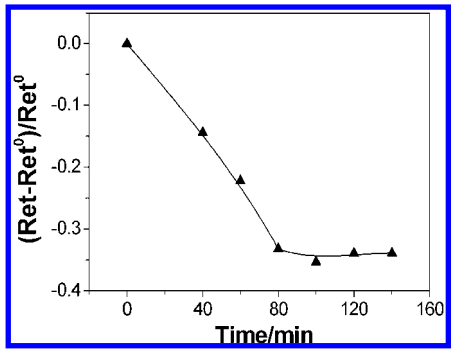

Figure 3. Time-dependent EIS signal response for ATP detection. $(Ret^0 - Ret)/Ret^0$: Ret represented the Ret detected after each target, respectively, and Ret^0 represented the blank Ret—Ret of the Au/PCS−MBA/MCE system.

is reduced compared with the Au/PCS−MBA system, which is consistent with the previous literature.[41] To further characterize the fabrication of the sensing interface and to estimate the coverage of the PCS−MBA duplexes on the Au substrates, the SPR measurement was also carried out. Figure 2B shows the angle-resolved SPR curves for the modification of the gold substrate. Upon the saturation adsorption of the PCS−MBA duplexes and the subsequent MCE regularization on the gold surface, the SPR angle was right-shifted by about 200 mDeg in comparison with the bare gold surface. The PCS−MBA duplexes coverage on the gold electrode was calculated to be about 5×10^{12} molecules/cm^2.

The PCS−MBA duplexes coverage at the Au electrode surface could also be quantified via an electrochemical approach (i.e., by using chronocoulometry).[42] The result showed that the surface density of PCS−MBA was about 4×10^{12} molecules/cm^2 (see Supporting Information, Figure S1), which is consistent with the SPR technique. All the results indicated that the double-stranded DNA (ds-DNA) was immobilized on the Au electrode successfully.

EIS Detection of ATP and Reproducing of the Sensing Interface. For ATP detection (see Scheme 1A), the as-prepared functional sensing interfaces were immersed in a series of ATP solutions, respectively, at a 30 °C water bath, and a significant Ret decrease was observed. It testifies that ATP could interact with the extended ABA strand embedded in the MBA strand, and draw it away from the electrode surface. For as the density of negative charges on the electrode surface is reduced, the negative charges repelling to the $[Fe(CN)_6]^{4-/3-}$ anions are reduced as well, which leads to the Ret decrease. It is evident that different immersion times might cause different responses of EIS. So the kinetic experiment was done to determine the optimum operation times for ATP detection.[31] The EIS signal response is recorded, and the results are shown in Figure 3. The Ret is decreased significantly when the sensor was immersed in ATP solutions, and it reaches a plateau at about 80 min, which is consistent with other reports.[31] Therefore, 90 min was used to detect ATP in this system. However, there were reports in which the response times for ATP detection were much shorter. The relatively longer response here may be due to the TBA linked to the ABA. It

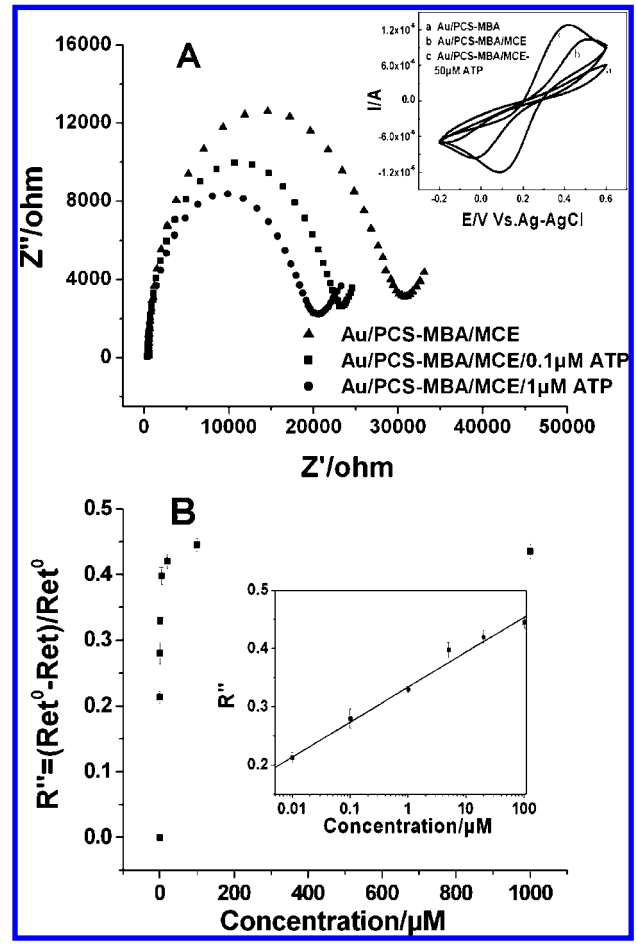

Figure 4. (A) EIS and CV response of the sensing interface to ATP at different concentrations. (B) Response of the sensor to different concentrations of ATP. $R'' = ((Ret^0 - Ret)/Ret^0)$: Ret represented the Ret detected after each target, respectively, and Ret^0 represented the blank Ret—Ret of the Au/PCS−MBA/MCE system. Inset: a liner range from 0.01 to 100 μM. The average of the RSD is 0.01609.

increased the length of the ATP aptamer and thus enhanced the steric hindrance effect during ATP−ABA interaction, leading to a relatively longer response time.

After 90 min of immersion in different concentrations of ATP solutions, ATP−aptamer complex was formed and the ds-DNA was unwound, which led to decrease of Ret in different degrees. As shown in Figure 4A, with increasing concentration of ATP, the EIS signals are decreased obviously. Similarly, the CV responses are enhanced with the immersion in the ATP solutions (Figure 4A). All the experimental phenomena reveal the effective ATP detection. Then a series of ATP solutions from 0.01 μM to 1 mM were investigated. $R'' = ((Ret^0 - Ret)/Ret^0)$ is used for evaluating the Ret response to ATP. As shown in Figure 4B, there is a linearity relationship between R'' and function of the logarithm of the concentration of ATP. And the ATP could be quantified over a concentration range from 0.01 to 100 μM ($R^2 = 0.9946$). Meanwhile a detection limit as low as 0.01 μM is gotten, which is more sensitive than most available impedimetric ATP aptasensors.[37–39] As anticipated, such improved sensitivity based on the amplified signal just results from the TBA strand designed to extend the length of ABA.

To further testify this phenomenon, chronocoulometry was used to prove the detection of ATP. Shao's group has reported

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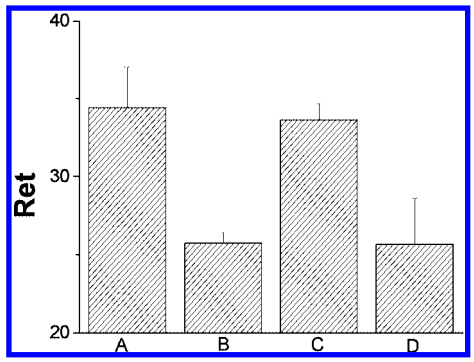

Figure 5. Regeneration of the sensing interface after detecting ATP: (A) never-used sensor; (B) after detecting 50 μM ATP solution; (C) regeneration of the interface; (D) detection of 50 μM ATP for the second time.

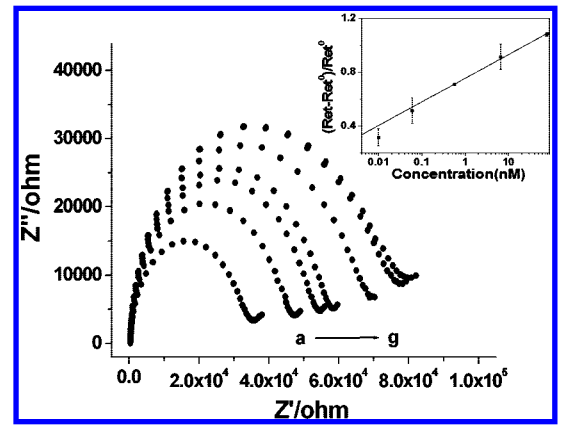

Figure 6. EIS response of the sensing interface to α-thrombin at different concentrations: (a) EIS response of the sensing interface, (b) 0.01 nM α-thrombin, (c) 0.06 nM α-thrombin, (d) 0.60 nM α-thrombin, (e) 6.60 nM α-thrombin, (f) 76.0 nM α-thrombin, and (g) 176.0 nM α-thrombin. Inset: the relative response of the sensor to different concentrations of α-thrombin (from 10^{-11} to 10^{-7} M). A liner detection range is from 0.01 to 76 nM. The average of RSD is 0.03096.

an aptamer sensor for adenosine monophosphate (AMP) by using chronocoulometry.[43] $[Ru(NH_3)_6]^{3+}$ (RuHex) is an electroactive complex, which can bind to DNA strands completely through electrostatic interactions. The RuHex−aptamer−electrode system can be used to generate a significantly more intense signal in chronocoulometry. When ATP existed, a part of MBA could draw away from the electrode with the RuHex, which resulted in a decreasing chronocoulometry signal for the RuHex. And the result is shown in the Supporting Information (Figure S2).

Besides high sensitivity, the sensing interface after detection is easy to regenerate by the rehybridizing process between PCS and MBA (which is also shown in Scheme 1B). Four hours of hybridization was enough, and the regenerated interface could be used to detect ATP again. The second detection result is the same as the first detection of ATP, which is depicted in Figure 5, showing that there is no infection to the next detection after the former measurement. It is proved that the regeneration of the sensing interface is feasible and reasonable.

Parallel Detection of α-Thrombin. As shown in Figure 1B, there was α-thrombin aptamer in the MBA strand. Thus, besides ATP detection, the sensing interface here could be used for α-thrombin detection as well, which is another advantage for this design (Scheme 2A). According to the previous articles, the interaction time of α-thrombin and its aptamer ranged from less than 30 min to more than 1 h.[25,41] In order to make the detection completely, 1 h was adopted in the current experiment.[41] When the sensing interfaces were immersed in different concentrations of α-thrombin solution at a 30 °C water bath, the Ret was increased after 1 h of incubation, which was opposite to the condition of ATP detection (Figure 6). As shown in Figure 6, the obvious increase of EIS signals testifies the formation of α-thrombin−aptamer complex. In this sensing interface, for the ds-DNA could not be separated as the condition with ATP, the α-thrombin−MBA complex formed still remained on the electrode, resulting in an integrated function of blocking of the electrode surface with the bulky protein. And the resistive hydrophobic layer insulated the conductive support; the Ret increased as compared to the ds-DNA modified electrode. In fact with the concentration of α-thrombin increasing, the EIS signal increased obviously. The results are consistent with what we expected, and the principle is similar to that in the previous literatures.[25,28,29,32,35] When the concentration of α-thrombin increased to 170 nM, the EIS signal is no longer increased, indicating the coverage of α-thrombin on the MBA-modified electrode has reached a maximal limit. $(Ret - Ret^0)/Ret^0$ was used to record the relative response of the sensor. The inset of Figure 6 shows a linear detection range between R' and function of the logarithm of α-thrombin concentration from 0.01 to 76 nM ($R^2 = 0.9983$). The method is sensitive, with a detection limit as low as 0.01 nM, which can be compared favorably with most existent impedimetric aptasensors for α-thrombin detection.[28,41] Here because it is a signal-off detection for ATP and a signal-on for α-thrombin, the sensing process interfered when these two targets coexisted in the same sample.

Chronocoulometry was also used to prove the detection of α-thrombin. Different from the condition for ATP, α-thrombin has negative charges, which makes the density of negative charge on the electrode surface increase. Therefore, as shown in Supporting Information Figure S3, such an electrode surface with more negative charges is apt to attract more RuHex cations, which ultimately leads to a more intense chronocoulometry signal than the condition without α-thrombin. Meanwhile, it could be observed that the slope of the signal line for α-thrombin is a little larger. It illustrates that with the time increasing, the electrode seems to cumulate more charges. The reason is not very clear yet but may be attributed to that α-thrombin is a bulky protein so its absorption of RuHex cations is not very fast. Therefore, the longer time used, the more RuHex cations cumulated, which made the total charges increase by chronocoulometry.

Multifunctional Detection, Regeneration, and Stability of the Sensing Interface. Actually, after the detection of α-thrombin, the sensing interface could still be used to recognize ATP (Scheme 2B′). To testify this point, the sensor was first immersed in a given concentration of α-thrombin solution for 1 h and then immersed in certain amount of ATP solution for 1.5 h. A characteristic phenomenon is showed in Figure 7. The EIS signal increased when detecting of α-thrombin and then decreased when the ATP is present. It indicates that though covered by α-thrombin

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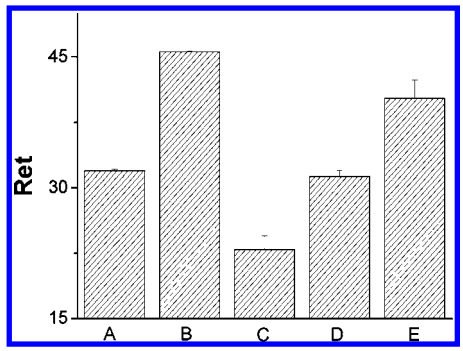

Figure 7. Multifunctional detection and regeneration of the sensing interface: (A) never-used sensor; (B) after being immersed in 5 nM α-thrombin; (C) after detection of α-thrombin, the sensing interface was immersed in 1 mM ATP; (D) regeneration of the interface; (E) detection of 5 nM α-thrombin for the second time.

molecules, the sensing surface could still be used for ATP recognition. In other words, the sensor is employed in two kinds of targets, respectively, which is promising and interesting in fabrication of an easy and multifunctional detection system.

Actually, if the concentration of ATP is high enough, the sensing surface can be directly regenerated after α-thrombin detection (Scheme 2B). Detailedly, at first, the sensing interface covered with α-thrombin was treated with a large amount of ATP to draw off all the MBA strands (including the ones interacting with α-thrombin) on the electrode. And then, the sensing interface was regenerated by rehybridization reaction with MBA again (Scheme 2C). After 4 h of incubation, the hybridization reaction was completely finished and the EIS response reached an equilibrium, which was almost the same value with the one before α-thrombin sensing. It indicates that the MBA already interacted with the protein could also bind with ATP. Then the regenerated sensor is used to detect α-thrombin again, as shown in Figure 7. These results explain why the present sensor possesses the abilities of multifunctionality and regeneration. The sensing interface could be reused a few more than three times. It is found that when the sensing surface is regenerated after more than three times, the MBA strand could not be drawn off by ATP as sensitive as that of three times. That means the sensor could only recognize ATP or α-thrombin but not quantify them using the linear relationship obtained. This might be resulted from the DNA losing its activity by repeating use of the PCS-immobilized sensing surface. It is still a challenge of most existing DNA sensors.

To investigate the stability of the sensing interface, the sensor was stored in pure water at 4 °C over 15 days and then recovered to room temperature slowly. The sensing interface was then used to evaluate its stability. The EIS signal of the conserved sensing interface exhibits the same value as the sensor before cooling treatment. Obviously, the assembled aptasensor achieves a sufficient stability for detection of α-thrombin and ATP both.

Selectivity of the Sensing Interface. ATP, GTP, and CTP, all of these small molecules belong to the nucleoside family; thus, indispensable control experiments were performed, which are shown in Figure 8A. After 2 h of immersion in the mixed solution of 0.1 mM GTP and 0.1 mM CTP, the sensing interface showed hardly any change of Ret. However, 0.1 mM ATP showed an evident Ret change. As compared with α-thrombin detection, there was no remarkable Ret response after the prepared electrode was

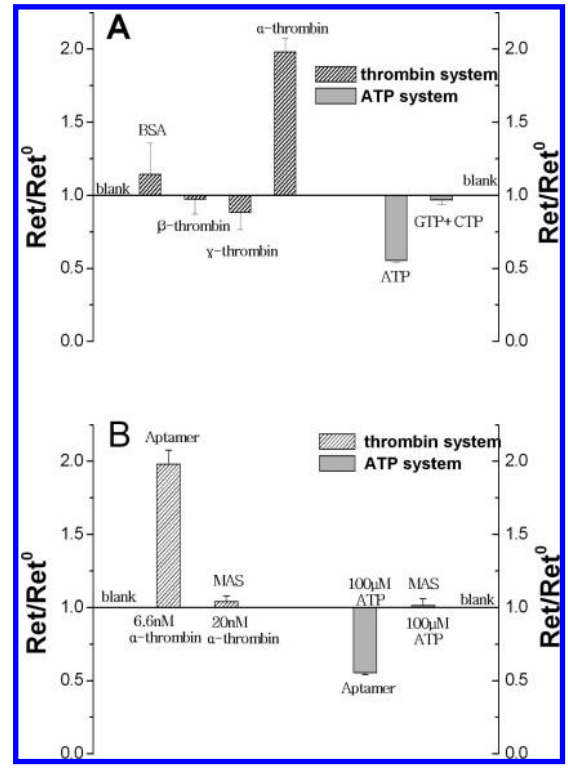

Figure 8. (A) Control experiments for ATP and α-thrombin: BSA, 100 nM; β-thrombin, 20 nM; γ-thrombin, 20 nM; α-thrombin, 6.6 nM; GTP + CTP, 100 μM; ATP, 100 μM. (B) Control experiments for mismatch aptamer. All these two systems showed very little response with MAS.

dipped into 100 nM BSA, 20 nM β-thrombin, and 20 nM γ-thrombin solution, respectively, for 1 h, which are also shown in Figure 8A.

To further testify the specific binding between the targets and their aptamers, a random oligonucleotide sequence containing two-bases mismatch in ATP aptamer and two-bases mismatch in α-thrombin aptamer (MAS, shown in Figure 1B) was used for the control experiment. All the assembly processes are the same as before except using MAS instead of MBA. The sensing interface was dipped into 0.1 mM ATP and 20 nM α-thrombin solutions, respectively. The results are shown in Figure 8B. In comparison with the Au/PCS−MBA system, the Au/PCS−MAS system has no effect to the ATP and α-thrombin measurement. There is no EIS response in the Au/PCS−MAS system. In these three control experiments, to prevent the nonspecific adsorption of small molecules and protein onto the Au or DNA strands, a copious amount of pure water was used for cleaning the sensing interface. All the results confirm the good selectivity of this multifunctional aptasensor.

Application of the Aptasensor in Biological Assay. It is evidenced that human plasma and fetal calf serum have strong nonspecific adsorption to the sensing interface but not the pretreated human plasma (data not shown). We decide to choose the pretreated human plasma as the biological fluid. In the 1% pretreated human plasma, different concentrations of standard solutions of ATP and α-thrombin are detected by the EIS measurement. The results are shown in Figure 9; the EIS responses at the sensing interface to α-thrombin and ATP of different concentrations in the 1% pretreated biological human

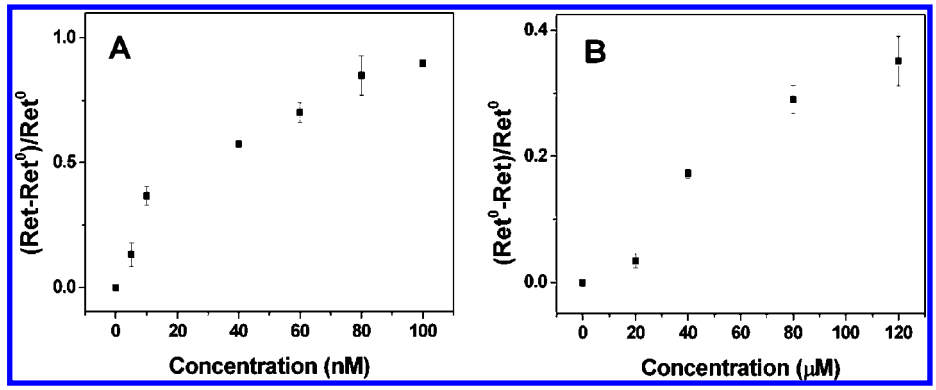

Figure 9. EIS response of the sensing interface to α-thrombin (A) and ATP (B) at different concentrations in the 1% pretreated biological human plasma.

plasma exhibit the same phenomenon to that in ordinary buffer but are less sensitive. The detection of α-thrombin has a remarkable positive response over a concentration range from 20 to 80 nM, while the detection of ATP also has a remarkable positive response over a concentration range from 20 to 120 μM. The results definitely illuminate the potential application of this aptasensor in samples.

CONCLUSION

A multifunctional label-free electrochemical aptasensor for parallel detection both of ATP and α-thrombin is developed using EIS measurement. The detection is based on a part complementary aptamer strands sensing platform. In this measurement, the detection limit of ATP is 1×10^{-8} M, and the detection range extends up to 10^{-4} M. In comparison with ATP, the detection limit of α-thrombin is 1×10^{-11} M, and the detection range is from 1×10^{-11} to 1×10^{-7} M. The as-prepared aptasensor has several advantages. (1) Through the EIS technique, label-free detection is realized which makes the sensing process quite simple and convenient. The aptasensor can detect the targets other than one for two systems. (2) Linked with the TBA strand, the ABA is extended to MBA strand, providing not only an amplified sensitivity of ATP molecule but also the capability for α-thrombin detection. Meanwhile, after detecting α-thrombin, the sensing surface can be used for ATP recognition as well, which displays a promising potential for integration of the sensing ability. (3) When whichever detection is finished, the sensing surface could be directly regenerated by treating with a large amount of ATP and hybridization with MBA strand. Thus, the sensing interface is easily reused. This method really provides a promising way to release the sensing surface from the widely used heating−rehybridizing regenerating methods, in which Au−S may be destroyed.[44] The application of this aptasensor in biological assay shows the sensing interface having a large potential in real samples. (4) The two targets could benefit to each other. It provides another approach of aptasensor design, and it also brings another label-free model of application of aptasensor. However, such fabricated detection system is not suitable for the condition in which multitargets are in one sample. So improvement is still needed by more efforts in the near future. The method using EIS measurement provides a very simple and promising detection technique for both protein and small molecules. It exhibits huge investigation worthiness to other analytes.

ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China, Grant Nos. 20575063, 20675076, and 20735003, and the Chinese Academy of Sciences, KJCX2-YW-H11. Mr. Fu-an Wang is acknowledged for his help of the SPR experiment.

SUPPORTING INFORMATION AVAILABLE

Chronocoulometric characterization of the fabrication of the sensing interface. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review February 13, 2008. Accepted April 30, 2008.

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