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Sensitive Bifunctional Aptamer-Based Electrochemical Biosensor for Small Molecules and Protein

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In this paper, a bifunctional electrochemical biosensor for highly sensitive detection of small molecule (adenosine) or protein (lysozyme) was developed. Two aptamer units for adenosine and lysozyme were immobilized on the gold electrode by the formation of DNA/DNA duplex. The detection of adenosine or lysozyme could be carried out by virtue of switching structures of aptamers from DNA/DNA duplex to DNA/target complex. The change of the interfacial feature of the electrode was characterized by cyclic voltammertic (CV) response of surface-bound $[Ru(NH_3)_6]^{3+}$. On the other hand, DNA functionalized Au nanoparticles (DNA-AuNPs) were used to enhance the sensitivity of the aptasensor because DNA-AuNPs modified interface could load more $[Ru(NH_3)_6]^{3+}$ cations. Thus, the assembly of two aptamer-contained DNA strands integrated with the DNA−AuNPs amplification not only improves the sensitivity of the electrochemical aptasensor but also presents a simple and general model for bifunctional aptasensor. The proposed aptasensor has low detection limit (0.02 nM for adenosine and 0.01 μg mL^{-1} for lysozyme) and exhibits several advantages such as high sensitivity and bifunctional recognition.

Aptamers, reported for the first time in 1990,[1−3] are artificial oligonucleic acids in vitro selected through SELEX (systematic evolution of ligands by exponential enrichment). Aptamers have high affinity and specificity to a wide range of target molecules, including drugs, proteins and other organic or inorganic molecules. Furthermore, the structural switching property of aptamers as well as facile combination with amplification technologies provides a broad prospect for developing novel sensing protocols, which is fascinating for growing number of analysts. Because of their advantages,[2−5] aptamers as alternatives to antibodies become molecular recognition elements in biosensor application. In recent years, on the basis of aptamers, many biosensors for small molecules (e.g., cocaine or adenosine),[6,7] or proteins (e.g., thrombin or lysozyme)[8,9] have been developed because the detection and quantification of small molecules or proteins play essential roles in fundamental research and clinical applications.[10] However, such an aptasensor can only be used to detect one target. Therefore, efforts are being made to develop aptamer-based biosensors for parallel analysis of targets by using a bifunctional nucleic acid. For example, Elbaz et al. reported on the use of a blocked bifunctional nucleic acid that consisted of the cocaine and adenosine 5′-monophosphate (AMP) aptamers, this complex act as an active assembly for the separate analysis of cocaine or AMP.[11] And the detection limits of cocaine and AMP by Faradaic impedance spectroscopy were 5×10^{-6} M and 1×10^{-5} M, respectively. Du et al. fabricated a multifunctional label-free electrochemical biosensor based on an integrated aptamer for parallel detection of adenosine triphosphate (ATP) and α-thrombin, and the detection limits of ATP and α-thrombin were 1×10^{-8} and 1×10^{-11} M, respectively.[12] Although these aptasensors could implement the parallel detection of target molecules, the detection limits still need to be improved. In addition, these bifunctional aptasensors were on the basis of integrated aptamers (the bifunctional aptamer containing two aptamer units). This strategy is limited for the aptamers with long sequence because DNA with long sequence which is more flexible may be spread on the gold electrode surface and affects the formation efficiency of the aptamer−target complex.[13] Therefore, it is essential to explore a new protocol for the development of a bifunctional aptasensor with higher sensitivity.

Because of the relatively low association constants of aptamers to their target proteins, it is difficult to achieve the ultrasensitive detection of small molecules or proteins by basic aptasensor. Many methods for signal amplification have been introduced, such as

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rolling circle amplification (RCA),[14,15] strand displacement amplification (SDA),[16,17] enzyme label[18−20] and et al. These methods for signal amplification are advantageous to improve the sensitivity of aptasensors, yet disadvantageous because they are complicated, expensive, and their detection conditions are rigorous. Therefore, many researchers have been exploring simple methods for ultrasensitive aptasensors. Recently, the ultrasensitive aptasensors based on aptamer-functionalized metal nanoparticles have been widely and simply developed.[21−23] For example, Polsky et al. have reported a new method to amplify DNA and protein biosensing by employing Pt-nanoparticles labels as catalysts for the reduction of H_2O_2. The sensitivity of the method for analyzing thrombin was ~100-fold improved as compared to the previous aptamer-based thrombin detection protocol.[24] On the basis of the use of AuNPs as amplifying labels, Sharon et al. have also developed electrical impedimetric and field-effect transistor methods for analyzing aptamer−substrate complexes.[25]

On the other hand, there are different analytical technologies for aptasensor, such as quartz crystal microbalance,[26] surface plasma resonance,[27] fluorescence,[28−31] atomic force microscopy,[32,33] electrochemistry,[12,34,35] and colorimetry.[36,37] Because of their high sensitivity, simple instrumentation, portability, and inexpensiveness,[12,38−41] the electrochemical methods have attracted substantial attention in the development of aptasensors. In general, the electrochemical aptasensors were fabricated by employing redox-labeled aptamers[12,42,43] or label-free impedance strategy.[44,45] However, it can not be neglected that it is not easy to obtain the redox-label aptamer, and there are challenges of the label-free impedance for protein biosensing.[46] Therefore, it is necessary to employ a simple and credible method for the aptasensor. Recently, some aptasensors were fabricated by employing $[Ru(NH_3)_6]^{3+}$ as the signaling transducer because the electroactive complex $[Ru(NH_3)_6]^{3+}$ could bind to anionic phosphates of DNA strands completely through electrostatic interactions, and exhibited excellent reversibility of its redox reaction.[8,21,39]

Here, a sensitive bifunctional aptamer-based electrochemical biosensor for small molecules and proteins was fabricated simply. Adenosine and lysozyme were used as the models of small molecules and proteins, respectively. Correspondingly, two DNA strands, adenosine aptamer-contained DNA and lysozyme aptamer, were used as the capture DNA and linker DNA, respectively. Accodring to the reported sequences of adenosine and lysozyme aptamers,[8,21] we designed adenosine aptamer-contained DNA to be complementary with the lysozyme aptamer to form the DNA/DNA duplex between adenosine aptamer-contained DNA and lysozyme aptamer. The electroactive complex $[Ru(NH_3)_6]^{3+}$ served as a signaling transducer. On the other hand, to improve the sensitivity of the biosensor, the short DNA functionalized gold nanoparticles (AuNPs) were used as the signal amplifier.[21,47] The resulted bifunctional electrochemical aptasensor shows high sensitivity, lysozyme and adenosine could be specifically detected at a concentration down to 0.01 μg mL^{-1} and 0.02 nM, respectively.

EXPERIMENTAL SECTION

Materials. All oligonucletides were purchased form Sangon Biotechnology Co., Ltd. (Shanghai, China), and sequences of all oligonucleotides are listed as follows: capture DNA(S1), 5′-HS-$(CH_2)_6$-ACC TGG GGG AGT ATT GCG GAG GAA GGT **CTA AGT AAC TCT**-3′; linker DNA(S2), 5′-*ATC TAC GAA TTC ATC* AGG GCT AAA GAG TGC **AGA GTT ACT TAG**-3′; DNA3, 5′-*GAT GAA TTC GTA GAT*-$(CH_2)_6$-HS-3′. DNA(S1) is a 39-base sequence that contains adenosine aptamer (normal font), as well as complementary sequence (bold) to DNA(S2). DNA(S2) is the lysozyme aptamer, and the sequence (italic) is complementary to the DNA3 sequence. Adenosine, cytidine, uridine, lysozyme, cytochrome *c*, and Tween-20 were from Bio Basic Inc. (Canada) and used without further purification. 6-Mercaptohexanol (MCH) and hexaaminеruthenium(III) chloride ($[Ru(NH_3)_6]^{3+}$) were from Sigma (St. Louis, MO). Tris(hydroxymethyl)aminomethane (Tris) was obtained from Oumay Biotech Co. Ltd. (Changsha, China). All other chemicals

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were of analytical grade. All samples and buffer solutions were prepared using ultrapure water obtained from a Mill-Q water purification system.

Preparation of AuNPs and DNA-Functionalized AuNPs. AuNPs with the diameter of about 13 nm were prepared by the reduction of $HAuCl_4$ in citrate solution according to the literature.[21] Briefly, 5 mL of 38.8 mM sodium citrate was immediately added to 50 mL of 1.0 mM $HAuCl_4$ refluxing solution under stirring, and the mixture was kept boiling for another 15 min. The solution color turned to wine red, indicating the formation of AuNPs. The solution was cooled to room temperature with continuous stirring.

The DNA3-functionalized AuNPs (DNA3-AuNPs) were prepared according to the procedure reported in the literature.[21,47] Briefly, a solution of AuNPs (1.5 mL) was mixed with the thiolated DNA3 (1 OD) overnight and then slowly brought to 0.1 M NaCl, 10 mM phosphate buffer (pH 7.0) and allowed to be incubated for 40 h. Subsequently, the DNA3-AuNPs conjugate was collected by centrifugation at 16 000 rpm for 30 min. The red precipitate was washed, centrifuged, and dispersed in 1.5 mL of 25 mM Tris-HCl buffer solution containing 0.3 M NaCl (pH 8.2).

Preparation of the Modified Electrodes. Prior to aptamer immobilization, the gold electrode was polished sequentially with 1.0 and 0.3 μm alumina slurry, followed by ultrasonic cleaning in ethanol and ultrapure water. Subsequently, the gold electrode was cleaned in piranha solution (v/v 3:1 H_2SO_4/H_2O_2) (**Caution:** piranha solution reacts violently with many organic materials and should be handled with great care), followed by treatment of the electrode with nitric acid. Afterward, the gold electrode was washed thoroughly with copious amounts of ultrapure water and dried under nitrogen gas. The thiol-terminated DNA(S1) was immobilized on the cleaned gold substrate by placing a 10 μL droplet of 1.0 μM DNA(S1) solution (in 10 mM Tris-HCl buffer, 1 M NaCl, pH 7.4) for ~16 h and producing a self-assembled monolayer (SAM) of DNA(S1). After modification, the gold electrodes were incubated for 1 h in 1 mM 6-mercaptohexanol (MCH) solution to remove nonspecific DNA adsorption on the gold surface. The electrodes were rinsed thoroughly with ultrapure water and dried with a stream of nitrogen. Then a 10 μL droplet of 1.0 μM DNA(S2) solution (in 10 mM Tris-HCl buffer, 1 M NaCl, pH 7.4) was placed on the resulted Au/DNA(S1)/MCH electrode for 2 h to obtain the Au/DNA(S1)/MCH/DNA(S2) electrode by hybridization between DNA(S1) and DNA(S2). For signal amplification, the DNA3-AuNPs solution (20 μL) was placed on the Au/DNA(S1)/MCH/DNA(S2) electrode for 2 h to carry out the hybridization between DNA(S2) and DNA3 and obtain the Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs electrode. After every step, electrodes were extensively rinsed with washing buffer (10 mM Tris-HCl, pH 7.4) and dried under a stream of nitrogen prior to electrochemical characterization. For each step of immobilization, the electrochemical impedance measurements were performed in the presence of equimolar $[Fe(CN)_6]^{3-/4-}$ as redox probe.

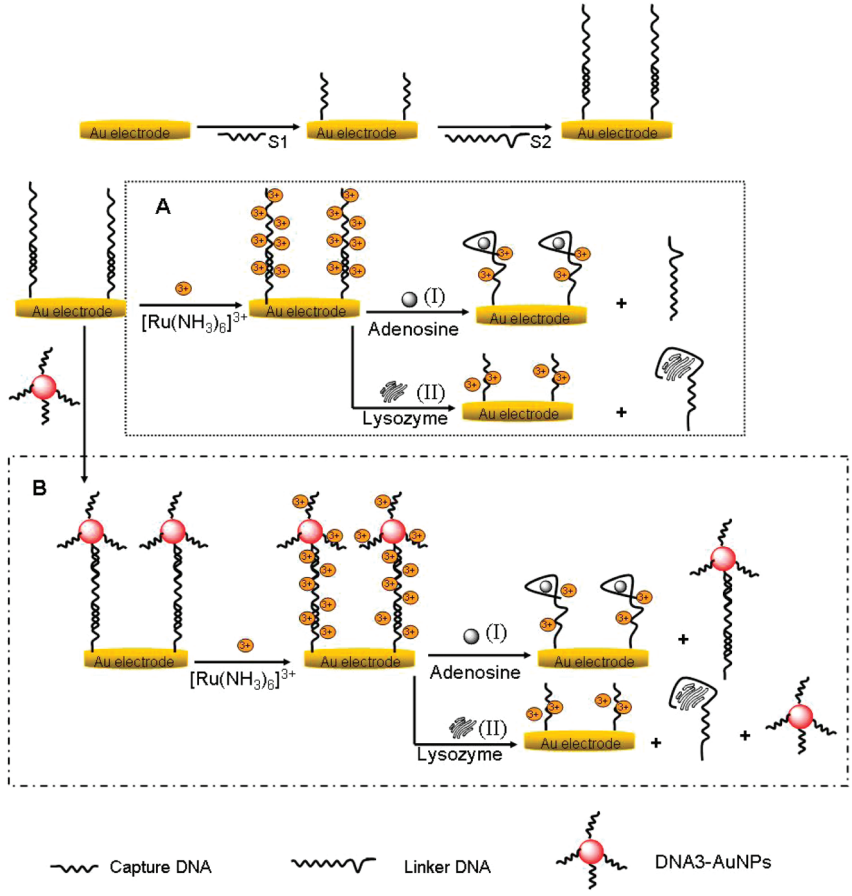
Apparatus and Measurement Procedure. All electrochemical characterizations were carried out on a CHI660A electrochemical workstation (Chenhua Instrument Company of Shanghai, China) with a conventional three-electrode cell. A bare gold electrode (2 mm in diameter) was used as the working electrode. A saturated calomel electrode (SCE) and a platinum wire were used as the reference and counter electrodes, respectively. All the potentials in this paper were in respect to SCE. Supporting electrolyte, buffer, was deoxygenated via purging with nitrogen gas for 15 min prior to measurements. All experiments were carried out at laboratory ambient temperature.

For the detection of adenosine or lysozyme, the modified gold electrodes were immersed in the adenosine solution (25 mM Tris-acetate buffer, 0.3 M NaCl, pH 8.2) or lysozyme solution (50 mM Tris-HCl buffer, 0.1 M NaCl, pH 7.4) with various concentrations and kept for 90 min, followed by a 20 min washing in wash buffer (20 mM Tris-HCl + 0.1 M NaCl + 5 mM $MgCl_2$ + 1.0%(v/v) Tween-20 at pH 7.4) to remove nonspecifically bound adenosine or lysozyme.[8,21] According to the literature,[8] 5.0 μM $[Ru(NH_3)_6]^{3+}$ was suitable for the experiments because it could ensure saturation of the DNA-modified surface with the redox-active complex. Cyclic voltammetry was performed at a scan rate of 500 mV s^{-1} in 4 mL of degassed 10 mM Tris-HCl solution (pH 7.4) containing 5.0 μM $[Ru(NH_3)]_6^{3+}$ (after incubation in the same solution for 15 min).

RESULTS AND DISCUSSION

Design Strategy of the Aptasensor. An electrochemical sensing interface for highly sensitive detection of small molecule (adenosine) or protein (lysozyme) was proposed. The system contains capture DNA(S1), linker DNA(S2), and the DNA3-AuNPs. The assay protocol of the electrochemical aptasensor was depicted schematically in Scheme 1. The surface of the gold electrode was first modified with the thiolated DNA(S1) via the self-assembly. The bold part of the lysozyme aptamer hybridized to the bold part of the capture DNA(S1). The sensing interface was ready for the detection of adenosine or lysozyme. For Scheme 1A, the DNA-modified electrode was immersed into Tris-HCl buffer solution containing $[Ru(NH_3)_6]^{3+}$, the negatively charged phosphate backbone of DNA could interact with the redox-active cations ($[Ru(NH_3)_6]^{3+}$), leading to the adsorption of $[Ru(NH_3)_6]^{3+}$ on the DNA-modified electrode. When adenosine introduced, the adenosine aptamer-contained DNA(S1) preferred to form the aptamer−adenosine complex, which resulted in the dehybridization and the lysozyme aptamer released into the solution (Scheme 1A-I). When lysozyme introduced, the aptamer−lysozyme complex formed, which also made the dehybridization between DNA(S1) and the lysozyme aptamer, and then the aptamer−lysozyme complex released into solution (Scheme 1A-II). In either case, it was expected that the peak currents of $[Ru(NH_3)_6]^{3+}$ at the DNA-modified electrode would decrease. On the other hand, to improve the sensitivity of the sensing interface, DNA3-AuNPs was employed to hybridize to the lysozyme aptamer, as shown in Scheme 1B. Since DNA3-AuNPs modified interface could load more redox-active cations ($[Ru(NH_3)_6]^{3+}$) on the DNA-modified electrode, the introduction of the adenosine (Scheme 1B−I) or lysozyme (Scheme 1B−II) would remove more $[Ru(NH_3)_6]^{3+}$ from the electrode, offering a significant amplification for the detection of adenosine or lysozyme. The design is to fabricate a bifunctional aptamer-based biosensor by relying on the structure-switching properties of aptamers binding to their target molecules.[48−50] Because two aptamer units are not integrated on one DNA strand, there

Scheme 1. Schematic Routine for Lysozyme or Adenosine Detection without DNA-AuNPs Amplification (A) and with DNA-AuNPs Amplification (B)



are no special requirements on the aptamer part. The design provided in this paper is generally applicable to many aptamers, especially for the aptamers with long sequence.

Characterization of the Modified Electrode. Figure 1 shows the electrochemical impedance results of (a) the bare Au, (b) Au/DNA(S1), (c) Au/DNA(S1)/MCH, (d) Au/DNA(S1)/MCH/DNA(S2), and (e) Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs electrodes. In the Nyquist plots of impedance spectra, a linear section at the lower frequencies is attributable to a diffusion-limited process, and a squeezed semicircle portion observed at higher frequencies would correspond to the electron-transfer limited process. The increase of the diameter of the semicircle reflects the increase of the interfacial charge-transfer resistance (R_{et}).[40,51] It is noted that the bare gold electrode shows a very small semicircle domain (R_{et} = 200 Ω, curve a in Figure 1), indicating a very fast electron-transfer process of $[Fe(CN)_6]^{3-/4-}$.[40] The self-assembly of a negatively charged DNA(S1) on the Au electrode surface effectively repels the $[Fe(CN)_6]^{3-/4-}$ anions and thus leads to an enhanced electron-transfer resistance (R_{et} =1980 Ω, curve b in Figure 1),[52] which demonstrates that thiolated DNA(S1) has been self-assembled successfully on the electrode. It is also found that the assembly of MCH on the DNA(S1)-modified electrode leads to a significant increase in R_{et} (from 1980 to 4200 Ω, curve c in Figure 1). After hybridization with lysozyme aptamer, the value of R_{et} increases to 7250 Ω (curve d in Figure 1) because of the negatively charged phosphate backbone of DNA(S2) repelling $[Fe(CN)_6]^{3-/4-}$ anions from the electrode surface. And then, the hybridization with DNA3-AuNPs was implemented, and there is a large increase in R_{et} (from 7250 Ω to 18060 Ω, curve e in Figure 1). It was caused by the introduction of DNA3-AuNPs, which greatly inhibited the electron-

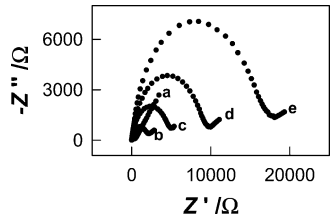


Figure 1. Faradic impedance spectra (Nyquist plots) corresponding to (a) the bare Au, (b) Au/ DNA(S1), (c) Au/DNA(S1)/MCH, (d) Au/DNA(S1)/MCH/DNA(S2), and (e) Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs electrodes in 0.1 M KCl aqueous solution containing 5.0 mM (1:1) $[Fe(CN)_6]^{3-/4-}$ as the redox probe. The impedance spectra were recorded within the range from 100 kHz to 100 mHz at the formal potential of $[Fe(CN)_6]^{3-/4-}$. The amplitude of the alternate voltage was 5 mV.

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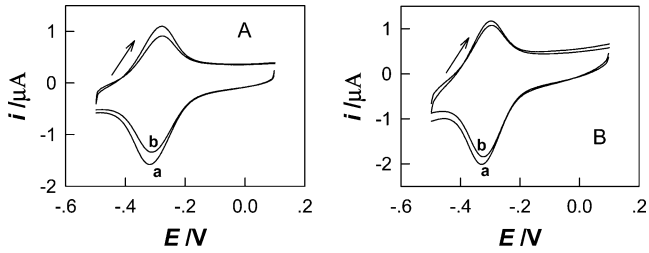

Figure 2. (A) Cyclic voltammograms of 5.0 μM $[Ru(NH_3)_6]^{3+}$ on the Au/DNA(S1)/MCH/DNA(S2) electrode in 10 mM Tris buffer at pH 7.4 before (a) and after (b) incubation with 10 μg mL^{-1} lysozyme. (B) Cyclic voltammograms of 5.0 μM $[Ru(NH_3)_6]^{3+}$ on the Au/DNA(S1)/MCH/DNA(S2) electrode in 10 mM Tris buffer at pH 7.4 before (a) and after (b) incubation with 5 nM adenosine. The scan rate is 500 mV s^{-1}.

transfer of the redox probe on the surface.[21] All these experimental results demonstrate that the sensing interface has been fabricated successfully according to Scheme 1.

Cyclic Voltammetric Detection of Adenosine and Lysozyme without AuNPs Amplification. First, the detection of adenosine or lysozyme was carried out using $[Ru(NH_3)_6]^{3+}$ as the redox probe without AuNPs amplification, and the assay protocol was depicted in Scheme 1A. Figure 2A shows the electrochemical behavior of $[Ru(NH_3)_6]^{3+}$ at the Au/DNA(S1)/MCH/DNA(S2) electrode before (a) and after (b) binding lysozyme. A pair of well-defined peaks can be observed when the Au/DNA(S1)/MCH/DNA(S2) electrode was immersed in a solution containing $[Ru(NH_3)_6]^{3+}$ (Figure 2A-a).[53] Upon binding lysozyme (Figure 2A-b), the cathodic current of $[Ru(NH_3)_6]^{3+}$ decreases. It results from the reaction between the aptamer and lysozyme taking place and the complex of aptamer−lysozyme separating from the electrode. On the other hand, the sensing interface can also be used to detect adenosine, and the experimental results are shown in Figure 2B. As expected, cathodic current of $[Ru(NH_3)_6]^{3+}$ also decreases upon addition of adenosine. It should be caused by the release of lysozyme aptamer into solution after the formation of aptamer−adenosine complex. Those experimental findings suggest that the designed sensing interface is feasible and effective for the detection of lysozyme or adenosine, and the bifunctional aptasensor for parallel analysis of targets is realized.

To further examine the analytical application of the bifunctional aptasensor, the Au/DNA(S1)/MCH/DNA(S2) electrode was employed to detect various concentrations of lysozyme or adenosine. The inset of Figure 3A shows the cyclic voltammograms of the probe $[Ru(NH_3)_6]^{3+}$ at the Au/DNA(S1)/MCH/DNA(S2) electrode after different concentrations of lysozyme were introduced. Figure 3A represents the relationship between the change in cathodic current (Δi) and lysozyme concentration. It can be seen that the cathodic current of the Au/DNA(S1)/MCH/DNA(S2) electrode decreases continuously as a function of lysozyme concentration, and the Δi value no longer increases significantly as the concentration increases from 10 μg mL^{-1} to higher concentrations. For this sensing interface, lysozyme can be detectable at a concentration as low as 0.1 μg mL^{-1}. On the other hand, Figure 3B shows the relationship between the changes in cathodic current, Δi, and adenosine concentration, and

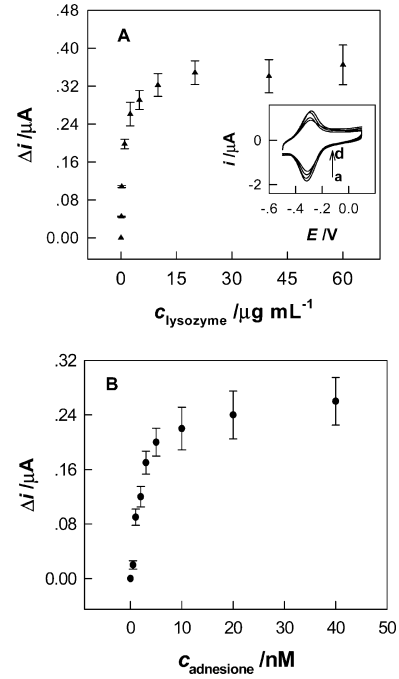

Figure 3. (A) Calibration curve corresponding to the detection of lysozyme based on the changes of the cathodic peak current of $[Ru(NH_3)_6]^{3+}$ at the Au/DNA(S1)/MCH/DNA(S2) electrode. Inset: Cyclic voltammograms of 5.0 μM $[Ru(NH_3)_6]^{3+}$ on the Au/DNA(S1)/MCH/DNA(S2) electrode in 10 mM Tris buffer at pH 7.4 with lysozyme at different concentrations. The scan rate is 500 mV s^{-1}. (B) The calibration curve corresponding to the detection of adenosine based on the changes of the cathodic peak current of $[Ru(NH_3)_6]^{3+}$ at the Au/DNA(S1)/MCH/DNA(S2) electrode.

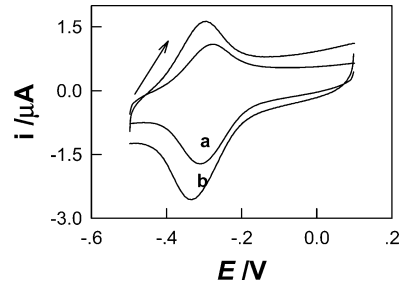

Figure 4. Cyclic voltammograms of 5.0 μM $[Ru(NH_3)_6]^{3+}$ on the Au/DNA(S1)/MCH/DNA(S2) (a) and the Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs (b) electrodes in 10 mM Tris buffer at pH 7.4. The scan rate is 500 mV s^{-1}.

it can be seen that the detectable concentration is down to 0.5 nM. The detection limit of the sensing interface is comparable or lower than that reported in literatures.[12,45,54,55]

Cyclic Voltammetric Detection of Adenosine and Lysozyme Using DNA3-AuNPs as the Amplifier. It is expected that the application of DNA-functionalized AuNPs can further enhance the sensitivity of the present sensing strategy. Therefore, a bifunctional aptasensor with DNA-AuNPs amplification was fabricated. The designed protocol was depicted in Scheme 1B. Figure 4 shows the cyclic voltammograms of the Au/DNA(S1)/MCH/DNA(S2) (a) and Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs (b) elec-

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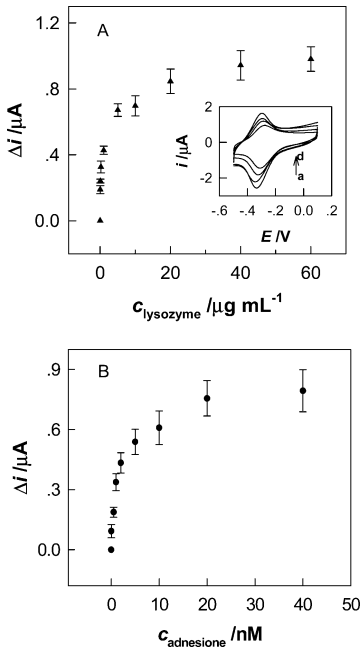

Figure 5. (A) The calibration curve corresponding to the detection of lysozyme based on the changes of the cathodic peak current of $[Ru(NH_3)_6]^{3+}$ at the Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs electrode. The inset: cyclic voltammograms of 5.0 μM $[Ru(NH_3)_6]^{3+}$ on the Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs electrode in 10 mM Tris buffer at pH 7.4 with lysozyme at different concentrations. The scan rate is 500 mV s^{-1}. (B) The calibration curve corresponding to the detection of adenosine based on the changes of the cathodic peak current of $[Ru(NH_3)_6]^{3+}$ at the Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs electrode.

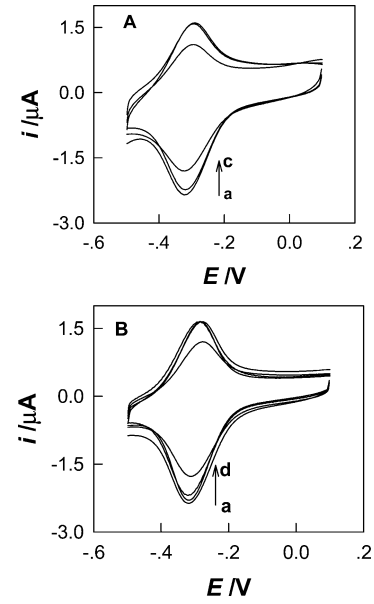

Figure 6. (A) Cyclic voltammograms of 5.0 μM $[Ru(NH_3)_6]^{3+}$ at the Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs electrode in 10 mM Tris buffer at pH 7.4 before (a) and after incubation with 10 μg mL^{-1} cytochrome *c* (b) and 10 μg mL^{-1} lysozyme (c). (B) Cyclic voltammograms of 5.0 μM $[Ru(NH_3)_6]^{3+}$ at the Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs electrode in 10 mM Tris buffer at pH 7.4 before (a) and after incubation with 5 nM cytidine (b), 5 nM uridine (c), and 5 nM adenosine (d). The scan rate is 500 mV s^{-1}.

trodes in 10 mM Tris-HCl solution containing 5.0 μM $[Ru(NH_3)_6]^{3+}$. It can be observed that the peak currents of $[Ru(NH_3)_6]^{3+}$ enhance obviously, after the electrode was treated with DNA3-AuNPs. In addition, by integration of the cathodic peak area, the charges of the surface-bound $[Ru(NH_3)_6]^{3+}$ can be obtained for the electrode before (4.80×10^{-7} C) and after (6.81×10^{-7} C) DNA3-AuNPs incubation. It is noted that the integrated charge increases ~42% after DNA3-AuNPs incubation, which quantitatively substantiates the role of DNA3-AuNPs as signal amplifier. Therefore, it is believed that the introduction of DNA3-AuNPs is effective to improve the sensitivity of the bifunctional aptasensor.

The sensitivity of the AuNP-based aptasensor was further investigated according to the above procedure. Figure 5A and B represent the cathodic current response of the sensing system to different concentrations of lysozyme and adenosine, respectively. It can be observed from Figure 5A that lysozyme can be detectable at a concentration as low as 0.01 μg mL^{-1}, which is 10 times lower than that without AuNPs amplification, and also much lower than that of some existed electrochemical aptasensor for lysozyme, for example, 0.2 μg mL^{-1} for the label-free impedance spectroscopy detection,[45] or 0.5 μg mL^{-1} for the voltammetric detection.[8] On the other hand, for the detection of adenosine, a series of adenosine solutions from 0.02 nM to 40 nM were investigated and the results are shown in Figure 5B. There is a linear relationship between Δi and the concentration of adenosine ranging from 0.02 nM to 3 nM ($r = 0.972$). Meanwhile, a detection limit as low as 0.02 nM is gotten, which is much lower than that of the sensing interface without AuNPs (0.5 nM) and some reported electrochemical adenosine aptasensors with the detection limit of 0.18,[21] 0.2,[54] 10,[12] or 20 nM.[55]

Selectivity and Reproducibility. From the practical point, a biosensor is not only sensitive but also specific. The specificity of the bifunctional biosensor has been investigated and the corresponding results are shown in Figure 6. The properties of cytochrome *c* are very similar to those of lysozyme.[56,57] Thus, it served as an excellent control to assess the specificity of the Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs electrode for the detection of lysozyme. As shown in Figure 6A-b, incubation of the Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs electrode with cytochrome *c* (10 μg mL^{-1}) does not produce significant changes in the CV response as compared to the case of lysozyme (Figure 6A-c). On the other hand, cytidine and uridine that belong to the nucleoside family were employed to assess the specificity of the bifunctional aptasensor for the detection of adenosine. It is noted that incubation of the Au/DNA(S1)/MCH/DNA(S2)/DNA3-AuNPs electrode with cytidine (Figure 6B-b) or uridine (Figure 6B-c) does not produce significant changes in the CV response as compared to the case of adenosine (Figure 6B-d). These results demonstrate that the developed strategy has a sufficient specificity to detect lysozyme or adenosine.

The reproducibility of the aptasensor was also investigated. Every five freshly prepared modified electrodes have been used for the detection of adenosine and lysozyme, respectively. All electrodes exhibit similar electrochemical response and the relative standard deviation is 3.5% for adenosine detection, and 4.6% for lysozyme detection. This demonstrates that the bifunc-

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tional aptasensor for the detection of adenosine and lysozyme is highly reproducible.

The long-term stability of the sensing interface is an important issue for the practical implementation of adenosine and lysozyme detection. Therefore, the storage stability of the proposed system has been investigated. The modified electrode, which was stored in double-distilled water at 4 °C for two weeks, could still be used for the measurement of adenosine or lysozyme without significant change of current response. This implies that the proposed aptasensor has a sufficient stability for detection of adenosine or lysozyme.

CONCLUSIONS

A bifunctional electrochemical aptasensor for parallel amplified-analysis of adenosine and lysozyme has been developed by assembly of two aptamer-contained DNA strands and integration with the DNA-AuNPs amplification. With the use of this aptasensor, lysozyme and adenosine could be specifically detected at a concentration down to 0.01 μg mL^{-1} and 0.02 nM, respectively. In addition, the bifunctional electrochemical aptasensor possesses good selectivity, reproducibility, and stability. The bifunctional aptasensor presented in this study would provide a promising platform for electrochemical parallel detection of small molecules and proteins.

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