Biosensors and Bioelectronics 27 (2011) 178–182

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

An electrochemical alkaline phosphatase biosensor fabricated with two DNA probes coupled with λ exonuclease

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ARTICLE INFO

Article history:
Received 27 March 2011
Received in revised form 24 May 2011
Accepted 28 June 2011
Available online 7 July 2011

Keywords:
Alkaline phosphatase
λ exonuclease
Electrochemical biosensor
Dephosphorylation
DNA probes

ABSTRACT

In this work we have developed a novel electrochemical biosensor for the detection of alkaline phosphatase (AP) by the use of two complementary DNA probes (DNA 1 and DNA 2) coupled with λ exonuclease (λ exo). Firstly, the 5′-phosphoryl end of DNA 1 is dephosphorylated by AP. Then DNA 1 hybridizes with DNA 2, previously modified on a gold electrode surface. In this double-strand DNA, DNA 2 strand will be promptly cleaved by λ exo with its phosphoryl at the 5′ end. After the DNA 2 strand is completely digested, DNA 1 will be released from the double strands and then hybridizes with another DNA 2 strand on the electrode surface, thus the cycle of the release of DNA 1 and the digestion of DNA 2 continues. Since the DNA probes may absorb hexaammineruthenium(III) chloride, the electrochemical species, and the removal of the DNA 2 strand from the electrode surface will result in the decrease of the detected electrochemical signal, which is initially activated by AP, an electrochemical biosensor to assay the activity of AP is proposed in this work. This method may have a linear detection range from 1 to 20 unit/mL with a detection limit of 0.1 unit/mL, and the detection of the enzymatic activity in complex biological fluids can also be realized.

1. Introduction

Alkaline phosphatase (AP) is an enzyme commonly used in clinical assays. It is often used as biomarkers in the diagnosis of many diseases since it is capable of catalyzing the hydrolysis of a series of phosphate compounds with a broad range of substrates (Giusti and Gakis, 1971). AP exists in leukemia (Neumann et al., 1971, 1976), lymphomas of B cell origin (Nanba et al., 1977) and lymphoblastoid B cell lines, so it is a specific marker for B cell activation (Garciarozas et al., 1982; Burg and Feldbush, 1989). The level of AP rises when renal, intestinal or placental damage occurs, while diseases of the skeletal system such as Paget's disease, osteomalacia, fractures and malignant tumors, may cause the increase of AP in serum (Drexler and Gignac, 1994). Therefore, detecting AP sensitively and selectively is highly required in many diagnostic and clinical assays.

Up to now, some methods have been proposed for the assay of AP with the techniques such as chemiluminescence (Ximenes et al., 1999), colorimetry (Grates et al., 2003; Wei et al., 2008), fluorescence (Chen et al., 2010; Fenoll et al., 2002; Freeman et al., 2010; Liu et al., 2009, 2010) and surface-enhanced Raman spectroscopy (Ruan et al., 2006). Meanwhile, new approaches by using electrochemical techniques have also been reported (Ruan and Li, 2001; Sun et al., 2006; Ito et al., 2000; Hassan et al., 2009; Wang et al., 2009). For example, Santiago et al. (2010) have proposed a microsystem with highly sensitive response to the oxidation of p-aminophenol, which can be translated in a very sensitive electrochemical detection of AP. Fanjul-Bolado et al. have made use of a substrate of AP, 3-indoxyl phosphate, to produce a compound that is able to reduce silver ions into a metallic deposit by AP. The deposited silver can be further electrochemically stripped into solution and measured by anodic stripping voltammetry (Fanjul-Bolado et al., 2007). Nevertheless, while some of the reported methods require complicated and expensive instrumentation like fluorescence spectrophotometer, others can only obtain relatively weak signals like Raman spectroscopy, thus they may not meet the requirements of sensitivity and stability. Considering the importance of AP detection, and its wide range of applications in fields like pathogen detection and environmental monitoring, more methods should be developed.

In this work, we have proposed a novel electrochemical method to detect AP by using two DNA probes and λ exonuclease (λ exo). Different from most of the previous approaches, this method makes use of enzyme-mediated signal amplification technique (Hsieh

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doi:10.1016/j.bios.2011.06.047

et al., 2010; Goodrich et al., 2004; Song and Zhao, 2009), thus high sensitivity and selectivity can be achieved. Furthermore, not only an enzyme, λ exo, a highly processive 5′–3′ exonuclease that degrades one DNA strand with a phosphate moiety at the 5′ end in the double-stranded DNA (Hsieh et al., 2010; Mitsis and Kwagh, 1999; van Oijen et al., 2003; Subramanian et al., 2003), is employed for signal amplification, but two DNA probes (DNA 1 and DNA 2) are also used for easy and simple assay of the AP activity with electrochemical technique. Firstly, AP dephosphorylates the 5′-phosphoryl end of DNA 1. Then, the dephosphorylated DNA 1 hybridizes with DNA 2, which has been previously modified on an electrode surface. Since only the DNA 2 strand containing 5′-phosphoryl end in the double-strand DNA can be cleaved by λ exo, DNA 1 will be released from the double-strand DNA after the completely digestion of DNA 2. Consequently, with the continuous removal of DNA 2 from the electrode surface, the loaded electrochemical species, hexaammineruthenium(III) chloride ($[Ru(NH_3)_6]^{3+}$) bound to the DNA probes via the electrostatic interaction (Sassolas et al., 2008; Zhang et al., 2006; Steel et al., 1998; Lao et al., 2005), will be decreased. Since the whole process is activated by the enzyme AP, an electrochemical method to assay AP activity is proposed. Compared with the previous reports, this method may not only have the advantages of electrochemical techniques, such as simplicity, convenient operation and inexpensive cost, but also make use of the digestion cycles coupled by λ exo to amplify the signal, thus the sensitivity of this biosensor can be further enhanced.

2. Experimental

2.1. Materials and chemicals

Fetal calf serum (FCS) was purchased from Shanghai Branch micro-biological. Ethylenediaminetetraacetic acid (EDTA), mercaptohexanol (MCH), $[Ru(NH_3)_6]^{3+}$, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), sodium orthovanadate (Na_3VO_4), bovine serum albumin (BSA), human serum albumin (HSA) and hemoglobin (Hb) were purchased from Sigma. AP and λ exo were obtained from New England Biolabs (Beijing) Ltd. All the other chemicals were of analytical grade and used as received.

The two oligonucleotides named as DNA 1 and DNA 2 were synthesized and purified by Shanghai Invitrogen Biotechnology Co., Ltd. The concentrations were quantified by OD260 based on their individual absorption coefficients. Both DNA 1 and DNA 2 contained 5′-phosphoryl ends and DNA 2 was further thiol-modified at 3′ end. The sequences of them were complementary and were listed as follows:

DNA 1: 5′-CGCTGTCCAGCCTGTGCCCGGC -3′
DNA 2: 5′-GCCGGGCACAGGCTGGACAGCGTTTTTTTT-3′

The 8 thymidines in DNA 2 could make the oligonucleotide molecules longer so as to reduce the steric hindrance between the electrode and λ exo bound with the double-strand DNA.

The buffer solutions employed in this work were as follows. DNA immobilization buffer: 10 mM Tris–HCl, 1 mM EDTA, 10 mM TCEP, and 0.1 M NaCl (pH 7.4). Reaction buffer for AP: 50 mM Tris–HCl (pH 9.0) containing 1 mM $MgCl_2$. Reaction buffer for λ exo: 67 mM glycine–KOH, 2.5 mM $MgCl_2$ and 50 μg/mL BSA (pH 9.4). All solutions were prepared with doubly distilled water, which was purified with a Milli-Q purification system (Barnstead) to a specific resistance of >18 MΩ cm.

2.2. Dephosphorylation of DNA 1 by AP

AP stock solution was firstly diluted to a series of concentrations from 1 to 50 unit/mL by using its reaction buffer. Then, the above AP solutions were incubated with 0.2 μM DNA 1 at 37 °C for 1 h. During this process, 5′-phosphoryl end of DNA 1 was removed. Finally, the solutions were heated at 95 °C for 5 min to inactivate AP.

2.3. Preparation of DNA 2 modified electrode

DNA 2 was immobilized onto a gold electrode surface via gold–sulfur chemistry. Firstly, the gold electrode (3 mm diameter) was soaked in piranha solution (98% H_2SO_4:30% H_2O_2 = 3:1) for 5 min *(Caution: Piranha solution dangerously attacks organic matter!)* in order to remove the adsorbed material. Then, it was rinsed with double-distilled water. After that, the electrode was polished carefully to a mirror-like surface with P3000 silicon carbide paper and 1 μm, 0.3 μm, 0.05 μm alumina slurry, respectively. Finally, the electrode was sonicated for 5 min in both ethanol and water to eliminate the residual alumina powder. The pre-treated gold electrode should be further soaked in nitric acid (50%) for 30 min, followed by being electrochemically cleaned with cyclic voltammetry, scanning from 0 to 1.6 V for 20 cycles in 0.5 M H_2SO_4 to remove any remaining impurities on the electrode. After being dried with nitrogen, the electrode was incubated with 0.2 μM DNA 2 for 16 h, followed by a 1 h treatment with an aqueous solution of 1 mM spacer thiol molecules, MCH (Meng et al., 2009). The electrode was then further rinsed with pure water and dried again with nitrogen.

2.4. DNA 2 digestion by λ exonuclease

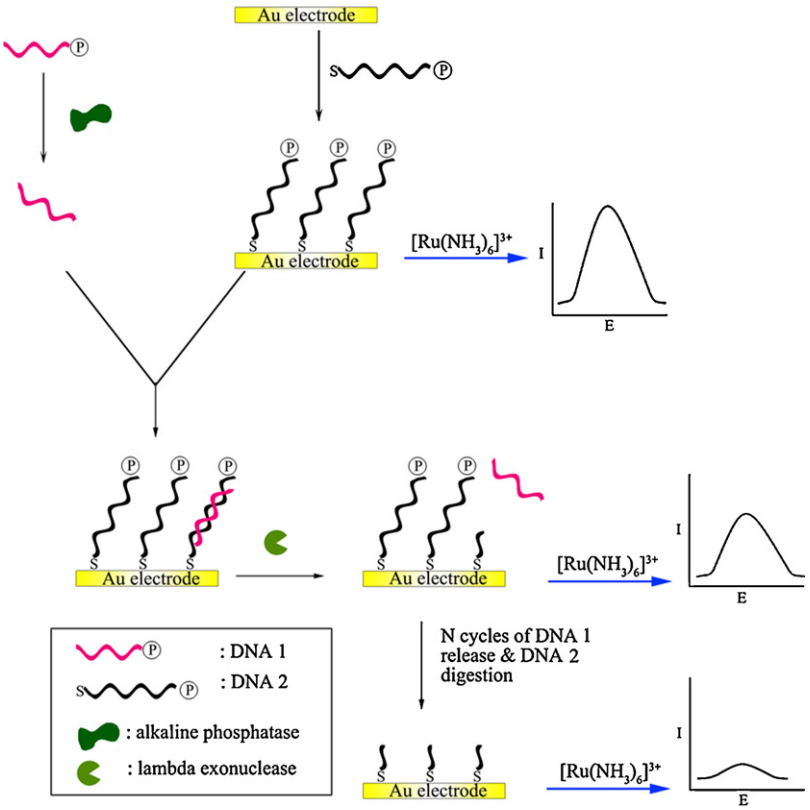
30 μL dephosphorylated DNA 1 solution was firstly mixed with 1 μL λ exo of 0.5 unit/mL and 269 μL reaction buffer for λ exo. Then, the DNA 2 modified electrode was immersed into the above solution at 37 °C. After a dephosphorylated DNA 1 strand hybridized with DNA 2, λ exo would bind with the double strand DNA and cleave DNA 2 with the 5′-phosphoryl end. DNA 1 was then released after the complete cleavage of DNA 2, and it might further hybridize with another DNA 2 strand, thus another cycle of DNA 2 digestion was achieved. After 10 min reaction, the electrode was treated by 1 mM MCH for another 1 h to make the oligonucleotides in good order.

2.5. Electrochemical measurements

Electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV), and chronocoulometry (CC) were performed on an electrochemical analyzer (CHI660B, CH Instruments) at room temperature by using three-electrode system consisting of the modified gold electrode as the working electrode, a saturated calomel reference electrode (SCE) and a platinum auxiliary electrode. The test solution for EIS was 1 M KNO_3 containing 5 mM $Fe(CN)_6^{3-/4-}$. For CV and CC experiments, it was 10 mM Tris–HCl solutions (pH 7.4) containing 50 μM $[Ru(NH_3)_6]^{3+}$. The scan rate was 100 mV/s for CV, and the pulse period was 250 ms for CC. For EIS, the experiments were performed upon application of the biasing potential 0.259 V, applying 5 mV amplitude in the frequency range of 0.1 Hz–100 kHz.

2.6. Inhibition of AP in the assay

Our proposed approach has been further used to evaluate the inhibition of the enzyme. It was conducted as follows: 50 unit/mL AP was firstly mixed with 5 mM Na_3VO_4 as a kind of inhibitor. DNA 1 was then incubated with the above mixed solution instead of pure AP solution. After the other same experimental steps were performed as those for AP assays, the inhibition effect of Na_3VO_4 can be tested.



Scheme 1. Schematic illustration of the electrochemical detection of AP by two DNA probes coupled with λ exonuclease.

2.7. Interference of the AP detection by other proteins

To examine the specificity of the AP detection, BSA, HSA and Hb were separately employed in this study to replace AP in the first step of the assay to dephosphorylate DNA 1.

2.8. Detection of AP in biological fluids

Complex biological fluids were also introduced in this work. AP samples in biological fluids were prepared by adding 5 unit/mL and 50 unit/mL AP to 1% fetal calf serum, respectively. The mixture solutions instead of pure AP solution were then used to dephosphorylate DNA 1 in the first step of the assay.

3. Results and discussion

The strategy to electrochemically detect AP in this work is illustrated in Scheme 1. Firstly, the 5′-phosphoryl end of DNA 1 is dephosphorylated by AP. After that, a gold electrode modified with DNA 2 via gold–sulfur chemistry is immersed in the mixed solution of dephosphorylated DNA 1 and λ exo, first to make the hybridization of DNA 1 with DNA 2, then to cleave the DNA 2 strand with 5′-phosphoryl end in the double-strand DNA by λ exo. When one DNA 2 strand is digested completely, the dephosphorylated DNA 1 can be released from the double-strand DNA and hybridizes with another DNA 2 strand, which starts a new DNA 2 cleavage cycle. Therefore, the number of DNA 2 will be decreased greatly during the cycles, and more AP, which dephosphorylates more DNA 1, will make more DNA 2 to be digested in a specific reaction duration. Since low density of $[Ru(NH_3)_6]^{3+}$ bound to DNA 2 on the electrode surface will give a decreased electrochemical signal, which is relevant to the concentration of AP, a sensitive biosensor for AP detection can be developed.

3.1. Characterization of the gold electrode

EIS is an efficient tool for studying the interface properties of surface-modified electrode (Arya et al., 2007; Lisdat and Schafer, 2008). The impedance spectra contain a semicircle portion at higher frequencies relating to the electron transfer limited process and a linear part at lower frequencies corresponding to diffusion. So, the increase of the semicircle diameter may reflect the increase in the interfacial charge transfer resistance. The results of EIS experiments in this work are shown in Fig. 1. Obviously, the semicircle diameter in the impedance spectra of the MCH modified electrode

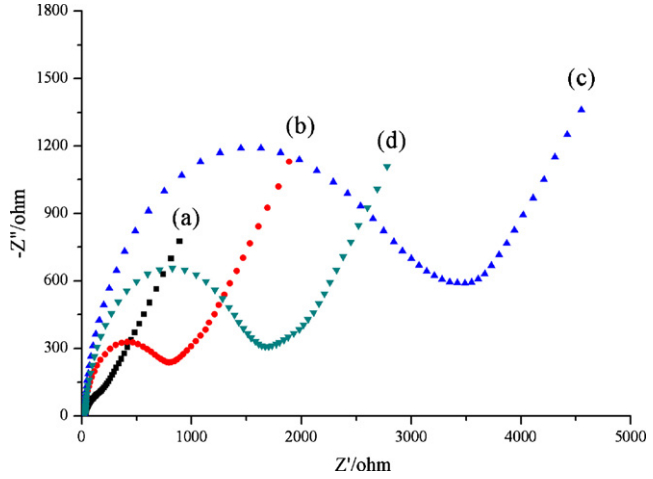


Fig. 1. Nyquist plots corresponding to (a) the bare gold electrode, (b) MCH modified electrode, (c) DNA 2 and MCH modified electrode, (d) DNA 2 and MCH modified electrode after dephosphorylated DNA 1 and λ exonuclease were separately added in the test solution. Buffer: 1 M KNO_3 containing 5 mM $Fe(CN)_6^{3-/4-}$. Biasing potential: 0.259 V. Amplitude: 5 mV. Frequency range: 0.1 Hz–100 kHz.

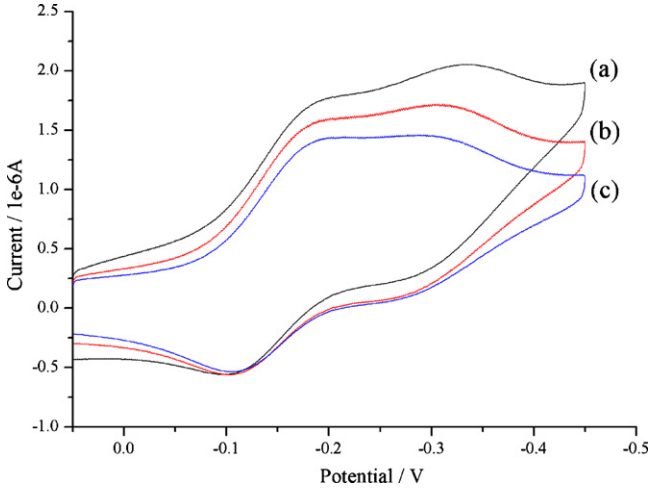


Fig. 2. Cyclic voltammograms obtained at the DNA 2 modified gold electrode after DNA 1, which was pretreated by (a) 0 unit/mL, (b) 5 unit/mL, (c) 50 unit/mL AP, and λ exonuclease were added in the test solution. Scan rate: 100 mV/s.

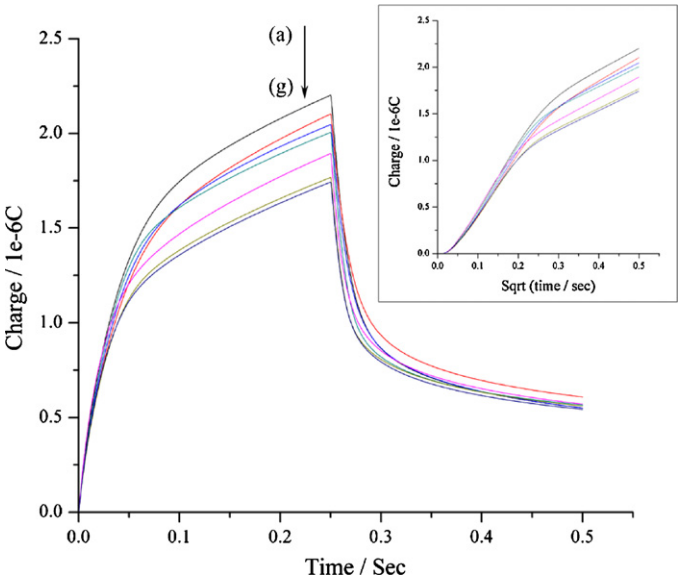


Fig. 3. Chronocoulometry curves for the DNA 2 modified electrodes. Curves (b)–(f) are the cases that 0, 1, 5, 20, 50 unit/mL AP, DNA 1 and λ exo were separately added in the test solution; Curve (g) is the case that another DNA probe with the same sequence as DNA 1 containing no 5′-phosphoryl end is employed. Inset is the chronocoulometry curves of charge versus $t^{1/2}$.

is larger than that of the bare electrode. And a larger semicircle diameter appears after the modification of both DNA 2 and MCH on the electrode, due to the electrostatic repulsion between DNA and $Fe(CN)_6^{3-/4-}$. Nevertheless, since λ exo will cleave the DNA 2 strand hybridized with dephosphorylated DNA 1, which will result in the decrease of immobilized DNA 2 strands on the electrode surface, the semicircle diameter is changed to smaller after the dephosphorylated DNA 1 and λ exo are introduced in the test solution. All these impedance spectra are coherent as is predicted.

3.2. Quantitative analysis of AP by CV

We have employed CV to characterize the electrochemistry of $[Ru(NH_3)_6]^{3+}$ that binds to DNA 2 on the gold electrode surface. From Fig. 2 we can notice there are two pairs of peaks in the cyclic voltammogram, which can be reasonably explained. The $[Ru(NH_3)_6]^{3+}$ molecules diffused to MCH contribute to the peak pair at −0.18 V, while the electroactive species electrostatically bound to the phosphate backbone of DNA contribute to the peak pair at −0.32 V (Steel et al., 1998; Lao et al., 2005). Furthermore, with the increase of AP, more DNA 1 are dephosphorylated, thus more DNA 2 on the electrode will be cleaved, which results in the lower electrochemical signal at −0.32 V.

3.3. Detection of AP by chronocoulometry

It has been reported that $[Ru(NH_3)_6]^{3+}$ can generate significantly more intense signal in CC than in CV (Lao et al., 2005). Therefore, CC technique can provide an accurate approach to measure the redox charges of $[Ru(NH_3)_6]^{3+}$ confined at the electrode surface. Chronocoulometry curves for the DNA 2 modified electrode are shown in Fig. 3. Curves 3b–f are the cases that DNA 1, AP and λ exo are introduced in the test solution. It can be discovered that the redox charges of $[Ru(NH_3)_6]^{3+}$ decrease as the concentration of AP gets higher, which is similar to the results of CV. Curve 3g displays the experimental result using a DNA probe with the same sequence as DNA 1 but containing no 5′ phosphoryl end. This result is nearly the same as the case that 50 unit/mL AP is employed, indicating that 50 unit/mL AP has almost dephosphorylated DNA 1 completely in 1 h. The chronocoulometric curves can be converted to Anson plots by plotting charge versus $t^{1/2}$ (inset in Fig. 3). The linear part of the Anson plot is thus extrapolated back to time zero to obtain the intercept for the plot, which stands for the redox charge of $[Ru(NH_3)_6]^{3+}$. So, the decrease of intercept can be then used to determine the concentration of AP, as is shown in Fig. 4. Inset in Fig. 4 depicts that the decreased redox charge of $[Ru(NH_3)_6]^{3+}$ is linearly related to AP concentration in the range of 1–20 unit/mL with the regression equation being $y = 0.14161 + 0.01387x$, where y is the charge, $\times 10^{-7}$ C; x is the concentration of AP, ×unit/mL; $n = 5$; $r = 0.99033$. The detection limit of the measurements, based on a signal three times the noise level of the background, is calculated to be 0.1 unit/mL. The average coefficient of variation is 4.84%, indicating that the precision of the proposed method is acceptable.

3.4. Interference of the AP detection by inhibitor

We have introduced Na_3VO_4 as the inhibitor of AP in this work. By detecting 50 unit/mL AP in the absence and presence of 5 mM Na_3VO_4, we can know that 5 mM Na_3VO_4 may almost completely inhibit the activity of AP (Fig. S1 in supplementary data).

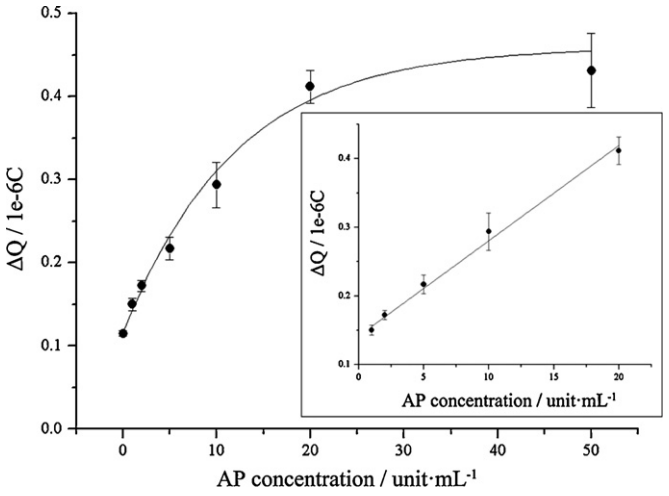


Fig. 4. Calibration curve for the detection of AP. The inset shows a linear relationship between the decreased redox charge of $[Ru(NH_3)_6]^{3+}$ and the concentration of AP from 1 to 20 unit/mL.

3.5. Interference of the AP detection by other proteins

In order to check the selectivity of the proposed biosensor, some other proteins such as HSA, BSA and Hb have been employed as control. Although the concentration of the other proteins are much higher than that of AP, the decreased redox charges of $[Ru(NH_3)_6]^{3+}$ are far less significant as that in the AP assay (Fig. S2 in supplementary data). So, this biosensor may have a good selectivity.

3.6. AP assay in biological fluids

In order to check whether this biosensor can be used in complex systems, samples containing AP in spiked 1% fetal calf serum have been analyzed. Experimental results reveal that the decrease of the redox charge of $[Ru(NH_3)_6]^{3+}$ can be also observed, if the fetal calf serum is employed, and the decrease becomes more significant with the concentration of AP added into the serum (Fig. S3 in supplementary data). So, the proposed method for AP detection might become a promising approach for AP assay.

4. Conclusions

We have herein proposed a novel electrochemical strategy to detect AP by using two DNA probes coupled with λ exo. Although the complementary two DNA probes both have 5′-phosphoryl end, DNA 2 is further thiol-modified at 3′ end so that it can be immobilized on a gold electrode surface to develop an electrochemical biosensor. Furthermore, since the 5′-phosphoryl end of DNA 1 has been dephosphorylated by AP before the performance of the electrochemical detection, only DNA 2 strand in the double-strand DNA can be digested by λ exo to make the cycles of DNA 1 release and another DNA 2 digestion possible, thus highly sensitive detection of AP can be achieved. We have also used the AP inhibitor, other interfering proteins and biological fluids to examine the selectivity of our proposed method and the possibility for application to complex system. The method may also have some other advantages such as simplicity, convenient operation and inexpensive cost. Since AP is an enzyme commonly used in clinical assays, the developed biosensor may have potential applications in the future.

Acknowledgements

This work is supported by the National Science Fund for Distinguished Young Scholars (Grant No. 20925520) and Shanghai Science and Technology Committee (Grant No. 09DZ2271800).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.06.047.

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