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An immobilization free DNAzyme based electrochemical biosensor for lead determination

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DNAzyme based electrochemical biosensors have the characteristics of high sensitivity and selectivity, but traditional DNAzyme based electrochemical biosensors need the immobilization of DNAzyme on the electrode surface first, and the procedures are time consuming and tedious, which limit their real application. In this study, a simple but sensitive immobilization free DNAzyme based electrochemical biosensor has been proposed and lead has been chosen as a model target because of the severe effects of lead toxicity. The different diffusivity and electrostatic repulsion between long and short DNA on the negatively charged ITO electrode can be used to discriminate the short and long DNA. Lead dependent DNAzyme was hybridized with its substrate (which was modified with methylene blue at the 3′ terminal) beforehand. Since the DNAzyme/substrate complex contains a large negative charge, it cannot diffuse easily to the negatively charged ITO electrode surface and little electrochemical signal has been detected. The presence of lead would trigger the cleavage of the DNAzyme/substrate complex and cause the release of a methylene blue-labeled short-oligonucleotide into the solution. The methylene blue-labeled short-oligonucleotide can diffuse easily to the surface of the negatively charged ITO electrode and results in the enhanced electrochemical response being detected. Each lead can cleave a lot of DNAzyme/substrate complex to realize signal amplification. The enhanced electrochemical signal has a linear relationship with the Pb^{2+} concentration in the range of 0.05–1 μM with a detection limit of 0.018 μM (S/N = 3). The proposed biosensor has been applied to detect Pb^{2+} in water samples with satisfactory results.

1. Introduction

DNAzymes, DNA sequences with catalytic activities, are isolated through *in vitro* selection.[1] DNAzymes have inherent characteristics such as specificity, versatility, stability, ease of synthesis and modification, due to which they have been applied in various fields including clinical diagnosis,[2] environmental monitoring,[3] biosensing[4] and cell imaging.[5] The electrochemical technique has the advantages of having low cost and high sensitivity, and can be applied in on-field detection easily; many selective and sensitive DNAzyme based electrochemical biosensors have been developed for versatile targets.[6–10] But, nearly all DNAzyme based electrochemical biosensors developed until now need the immobilization of DNAzyme on the electrode surface first. The procedures are tedious and time-consuming. Furthermore, the hybridization between the immobilized DNAzyme and the other DNA occurred on the solution–electrode interface, this strategy suffers from low efficiency because of the spatial hindrance effect of the electrode surface. Third, the immobilized DNAzyme may release from the electrode surface during the long-time storage, which affects the stability and reproducibility of the biosensor. It is necessary to develop an immobilization free system to overcome these drawbacks.

Lead-dependent DNAzymes have been paid much attention since lead can cause many side effects to human health.[11] Many sensitive DNAzyme based electrochemical biosensors for lead determination have been proposed. Xiao *et al.* proposed an electrochemical sensor of parts-per-billion lead based on an electrode-bound DNAzyme assembly,[6] where the double-stranded DNA containing the signal probe must be immobilized on the electrode surface first, then the DNAzyme cleaves the substrate by the addition of Pb^{2+}; as a result, the MB transfers electrons to the electrode. Shen *et al.* showed an electrochemical method based on catalytic reactions of a DNAzyme upon its binding to Pb^{2+} and the amplification of DNA–Au bio-bar codes.[12] Pelossof *et al.* developed an electrochemical sensing platform for lead based on the amplification mechanism of the immobilization of the sensing complex on Au NPs and the electronic coupling between the NPs and the surface plasmon wave.[13] Yang *et al.* reported an electrochemical

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DNAzyme sensor for Pb^{2+} by using Pb^{2+}-specific DNAzyme functionalized gold nanoparticles (AuNPs).[14] But all these sensors need DNA immobilization on the electrode surface.

DNA contains negative charge due to the negatively charged phosphate component.[15] An early report showed that the ITO electrode surface can be negatively charged after simple treatment.[16] Since short DNA contains little negative charge while long DNA contains a large negative charge, there is a difference in the electrostatic repulsion between the short DNA and long DNA with the negatively charged surface of the ITO electrode. Short DNA can diffuse easily to the negatively charged surface, while long DNA cannot reach the surface easily. This characteristic has been applied to discriminate the short DNA and long DNA with high efficiency, and many immobilization free electrochemical biosensors have been proposed based on this character also.[17] For example, I.-Ming Hsing *et al.* showed an immobilization-free electrochemical method for Hg^{2+} detection based on exonuclease III activity mediated by Hg^{2+} reshuffling on thymine-rich DNA duplexes.[18] Combined with signal amplification tactics, they also developed two immobilization-free electrochemical sensors for DNA detection.[17,19] Tang *et al.* proposed an electrochemical DNA biosensing platform based on an ingeniously designed exonuclease III-aided autocatalytic two target recycling strategy.[20] Wei *et al.* developed an electrochemical sensor for DNA methylation detection and its inhibitor screening based on the fact that Dpn I can cleave long DNA through the recognition site in the presence of DNA methylation.[21] Recently, we reported an ultraselective homogeneous DNA electrochemical biosensor based on nicking endonuclease signal amplification (NESA), which exhibits a high distinction ability to single-base mismatch and double-base mismatch.[22] These studies showed the convenience of the biosensors based on the negatively charged ITO electrode. But, the targets for these immobilization free biosensors are mostly focused on DNAs. It is necessary to find out some way to expand the application of the ITO electrode based immobilization free technology.

To the best of our knowledge, no method which combines the advantages of DNAzymes and ITO based immobilization free electrochemical biosensors has been reported. In this study, a simple and sensitive DNAzyme based immobilization free electrochemical biosensor for Pb^{2+} detection has been reported, which combines the convenience of ITO based immobilization free biosensors and high specificity of DNAzymes. The established biosensor has high selectivity due to the DNAzyme and ease of operation because it is immobilization free, and has been applied to detect lead in water samples with satisfactory results.

2. Experimental section

2.1 Reagents

DNAzyme and the methylene blue (MB) labeled substrate strand were synthesized by Sangon Inc. (Shanghai, China). Their sequences are shown below: 5′-AAAAAAAAAACCACAC CAACCT-rA-GTGACT-(methylene blue)-3′ (MB labeled substrate); 5′-AGTCATCCGAGCCGGTCGAAAGGTTGGTG GGTTGG-3′ (DNAzyme). Where rA is ribo-adenine. DNA buffers contain 10 mM $MgCl_2$, 50 mM NaCl and 50 mmol L^{-1} Tris-HCl buffer (pH 7.4). Targeted Pb^{2+} buffers are composed of 10 mM $MgCl_2$, 50 mM NaCl and 50 mmol L^{-1} Tris-HCl buffer (pH 7.4). All other chemicals were of analytical grade.

2.2 Electrochemical determination system and ITO electrode preparation

The electrochemical determinations were performed on a CHI660a electrochemical system (CH Instruments, Shanghai, China). An ITO electrode has been used as a working electrode in which the effective working electrode area was 5 mm × 3 mm; two Pt wires have been used as a reference electrode and counter electrode respectively. The potential of the Pt reference electrode in the buffer was determined to be +0.36 V with respect to an Ag/AgCl reference electrode. A negatively charged ITO working electrode surface can be achieved after the treating processes after sonication in an alconox solution (10 g L^{-1} of Alconox of double-distilled water) for 15 min, propan-2-ol for 15 min, and twice in double-distilled water for 15 min in sequence.[23] A 1.5 mL disposable microcentrifuge tube was used as the electrochemical cell.

2.3 DNA hybridization and cleavage of the hybridization product by Pb^{2+}

Hybridization of the MB labeled substrate and DNAzyme was conducted through the incubation of the substrate (ultimate concentration of 1.0 μM) with DNAzyme (ultimate concentration of 1.5 μM) at 37 °C (which is the generally accepted optimal temperature for hybridization and below the unwinding condition of the DNAzyme–substrate complex (T_m = 56.7 °C)) in 50 μL of 50 mM Tris-HCl buffer (pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl) for 2 h. Then different concentrations of Pb^{2+} were added to the above solution. Cleavage was executed at 37 °C for 120 min to obtain the maximum cleavage of the substrate strand.[6] Then, the differential pulse voltammetry (DPV) signal from the mixture was recorded with a potential interval of 0 to −0.6 V. Each sample was detected five times, and the average value was applied for quantitative analysis.

3. Results and discussion

3.1 Principle of the immobilization free electrochemical biosensor

Fig. 1 illustrates the principle of the proposed immobilization free DNAzyme based electrochemical biosensor. The Pb^{2+}-dependent DNAzyme ("8–17" DNAzyme) employed is a sequence-specific nuclease acting on a single stranded DNA substrate containing a single, sessile ribo-adenine (indicated by the arrow in Fig. 1). The substrate strand was modified with methylene blue (MB) at the 3′ terminal first, and then hybridizes with the DNAzyme to form the DNAzyme/substrate complex. Since the complex contains a large negative charge,

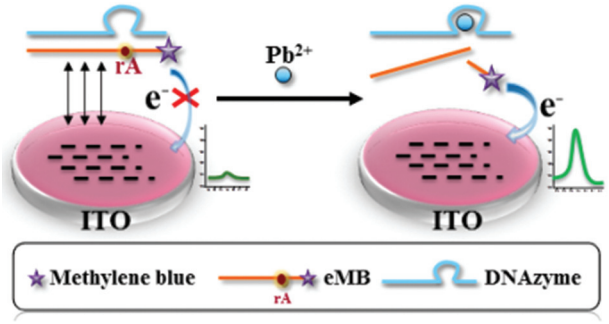

Fig. 1 Mechanism of the proposed immobilization free electrochemical DNAzyme based biosensor for Pb^{2+} determination.

it cannot diffuse easily to the negatively charged ITO electrode surface, so only a weak electrochemical signal can be detected. In the presence of Pb^{2+}, the DNAzyme catalyzes the hydrolytic cleavage of the substrate sequence into two pieces, one of which contains MB has short single stranded DNA (eMB) (only 6-base). And the Pb^{2+} can be applied to trigger the next round of substrate strand cleavage and eT release. Since eMB contains only short DNA, the electrostatic repulsion between the negatively charged ITO working electrode and eT is much little than that of the DNAzyme/substrate complex or substrate, which can diffuse easily to the negatively charged ITO electrode surface and an obvious enhancement of electrochemical signal can be detected. The specificity of the sensor can be ensured by the high selectivity of the DNAzyme. Thus, a sensitive and selective immobilization free sensor can be developed.

A simple assistant experiment has been performed to verify our presumption. The interference from Pb^{2+} was checked first, as shown in Fig. 2, no DPV signal was found (line a) if the solution contained only Pb^{2+}. Little electrochemical response was recorded in the solution which contained the MB labeled substrate (line b) or DNAzyme/substrate complex (line c). The reason lies in the fact that the MB labeled substrate contains a long negatively charged single stranded DNA, which limits its diffusion to the negatively charged ITO electrode surface, so only little signal has been detected. After hybridization with the DNAzyme, the complex contains much more negative charge, so much little signal was detected. But after the addition of Pb^{2+} into the DNAzyme/substrate complex solution, an obvious enhancement of the DPV signal was detected (line d). The reason lies in the fact that Pb^{2+} can activate the DNAzyme and induce the cleavage of the substrate sequence into two pieces including the methylene blue-labeled short-oligonucleotide (eMB). The eMB can diffuse easily to the ITO electrode surface since the electrostatic repulsion between the negatively charged ITO working electrode and the eMB is little, so an enhanced DPV signal can be detected. These results confirmed the feasibility of our principle.

3.2 Optimization of reaction conditions

The MB labeled substrate plays an important role in the performance of the system since an excessive amount of which can lead to the high background signal while low concentration leads to the weak signal being detected. So the concentrations of the MB labeled substrate were optimized first, DNAzyme (1.5 μM) and Pb^{2+} (1 μM) were mixed with different concentrations of the substrate ranging from 0.2 μM to 2.0 μM. As shown in Fig. 3A, the DPV signals of the system increased with the substrate concentration in the range from 0.2 to 1.0 μM and then reached the saturated condition after 1.0 μM. And if the concentration of the substrate was higher than 1.2 μM, the background signal would grow. Therefore, 1.0 μM substrate was chosen as the optimized condition for the following experiments.

The cleavage time also plays an important role in the performance of the system. As shown in Fig. 3B, the DPV signal increased with the increasing cleavage time first and then reached a plateau after 120 min. The reason may lie in the fact that in short cleavage time, each target can only go through few cycles, so weak signal was detected. In order to make sure

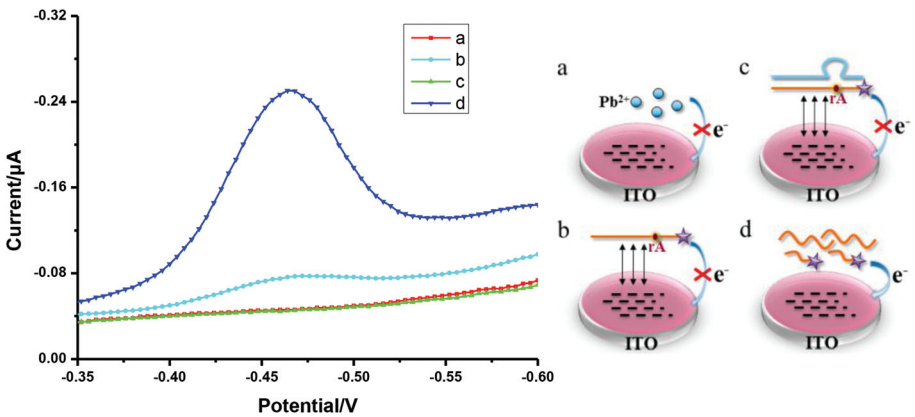

Fig. 2 DPV responses of Pb^{2+} (a), MB labeled substrate (b), MB labeled substrate/DNAzyme complex (c) and MB labeled substrate/DNAzyme complex + Pb^{2+} (d) in Tris-HCl (50 mM, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl) after incubation at 37 °C. [Substrate] = 1 μM, [DNAzyme] = 1.5 μM, [Pb^{2+}] = 1 μM.

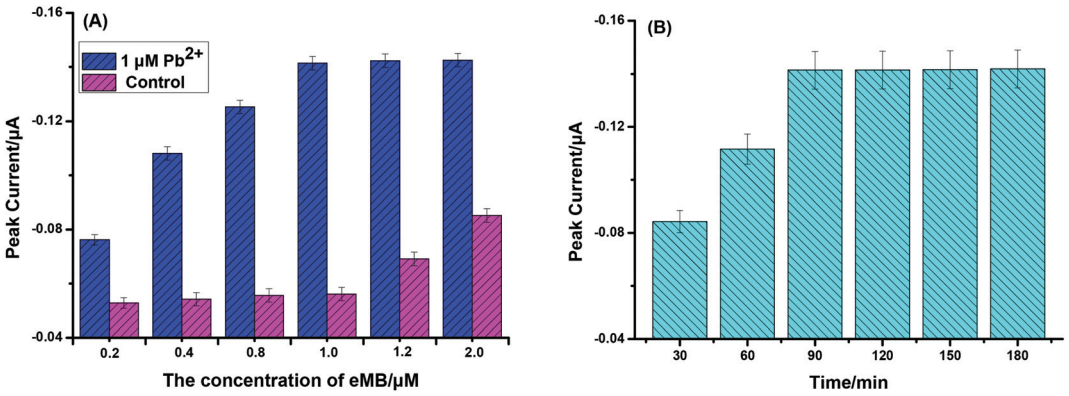

Fig. 3 (A) Effect of the substrate concentration on the detection system. [DNAzyme] = 1.5 μM, [Pb^{2+}] = 1 μM. (B) DPV responses at different cleavage times in Tris-HCl (50 mM, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl) after incubation at 37 °C. [Substrate] = 1 μM, [DNAzyme] = 1.5 μM, [Pb^{2+}] = 1 μM.

that all DNAzyme/substrate complexes were cleaved, 120 min was chosen in this study.

3.3 Calibration curve and reproducibility of the biosensor

To estimate the analytical performance of the proposed biosensor, the DPV responses of the system containing different concentrations of Pb^{2+} were recorded. As shown in Fig. 4A, the DPV response increased gradually with the increasing Pb^{2+} concentration. This phenomenon is in accord with the fact that higher concentration of Pb^{2+} causes more DNAzyme/substrate complex to be cleaved and more eMB to be produced. The DPV signal has a linear relationship with the Pb^{2+} concentration in the range of 0.05–1 μM (Fig. 4B). The regression equation is:

$$\Delta I/\mu A = 2.5839 \times 10^{-2} + 1.1556 \times 10^{-1} Cx/\mu M, R = 0.9960$$

where ΔI (the difference of the currents detected in the presence and absence of Pb^{2+}) is enhancement of the DPV current, Cx is Pb^{2+} concentration, and R is the regression coefficient. The limit of detection was estimated to be 0.018 μM (S/N = 3). This detection limit is more than sufficient for the routine monitoring of lead levels in food and environmental samples required by the U.S. Food and Drug Administration.[23]

To examine the reproducibility of the biosensor, one ITO working electrode was repeatedly used to detect 5 samples (0.6 μM), and the relative standard deviation (RSD) is 4.13%. And if 5 different ITO working electrodes are used for parallel determination, the RSD is 4.95%. These results indicate that the proposed method has good repeatability.

3.4 Interference assay and sample determination

The selectivity of the proposed sensor has also been studied. The DPV responses after the reaction with Pb^{2+} (1 μM), Cu^{2+} (100 μM), Mn^{2+} (100 μM), Zn^{2+} (100 μM) or Ni^{2+} (100 μM) were shown in Fig. 5. The DPV responses in the presence of other divalent metal ions are nearly the same with the blank solution, but remarkable enhancement was detected in the presence of Pb^{2+}. Therefore, the proposed biosensor has high selectivity to discriminate Pb^{2+} from others.

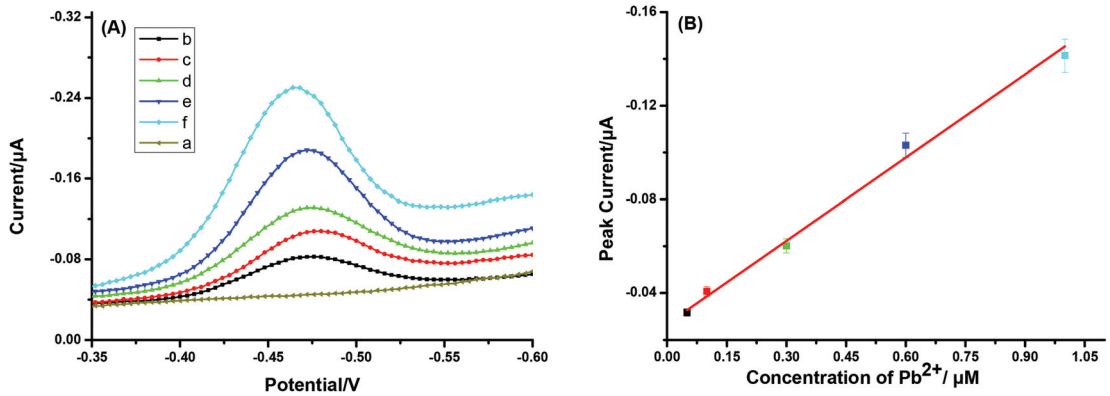

Fig. 4 (A) DPV responses at different concentrations of Pb^{2+} in Tris-HCl (50 mM, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl) after incubation at 37 °C. From a to f: 0 μM, 0.05 μM, 0.1 μM, 0.3 μM, 0.6 μM, 1 μM. (B) DPV peak currents plotted against the concentration of Pb^{2+}. [Substrate] = 1 μM, [DNAzyme] = 1.5μM.

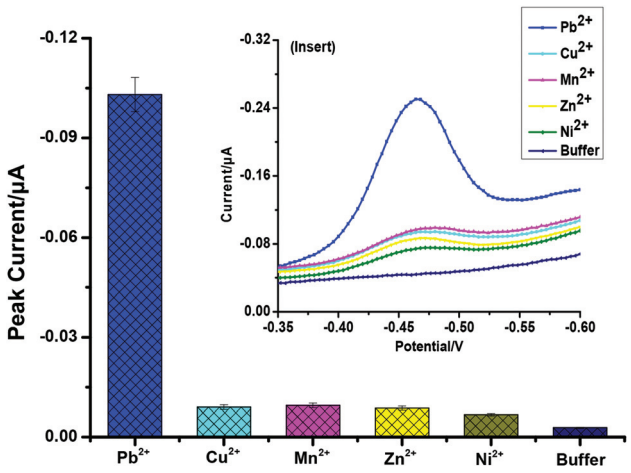

Fig. 5 DPV peak currents with various divalent metal ions in Tris-HCl (50 mM, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl) after incubation at 37 °C. [Substrate] = 1 μM, [DNAzyme] = 1.5 μM, [Pb^{2+}] = 1 μM, [the other interferent ions] = 100 μM. Insert: the corresponding DPV curves.

In order to verify the practical application of the proposed method, the lead concentration in simple samples (water samples collected from Minjiang River and lab tap samples) was detected. The water was filtered to remove the insoluble substance before determination. It is found that the Pb^{2+} content in the tested sample is too low to be probed by the sensor. However, an obvious increase in readout signal was observed if different concentrations of Pb^{2+} were added to the sample. So the samples were spiked with Pb^{2+} with the stock solution at the concentration level of 200, 400, and 600 nM. Table 1 lists the results obtained using both our sensor and inductively coupled plasma mass spectrometry (ICP-MS). On the basis of an *F*-test, the results from our present approach are in good agreement with those obtained using ICP-MS. These results reveal the practicality of using our sensor for the determination of Pb^{2+} ions in environmental samples.

Table 1 Determination of Pb^{2+} in water samples using the proposed method

Sample source	Added/ nM	The proposed method Mean ± SD/ nM (n = 5)	ICP-MS Mean ± SD/ nM (n = 5)	*F*-test between two methods[a]
Tap water	200.0	203.0 ± 2.15	210.7 ± 2.11	1.038
	400.0	413.7 ± 1.61	415.1 ± 1.34	1.444
	600.0	618.1 ± 1.46	613.7 ± 2.21	2.291
River water	200.0	208.1 ± 1.57	211.4 ± 1.69	1.159
	400.0	404.9 ± 2.03	411.5 ± 1.72	1.393
	600.0	615.8 ± 2.18	621.9 ± 3.05	1.957

[a] The *F*-test value is 6.39 at a 95% confidence level.

4. Conclusion

In conclusion, a simple and selective immobilization free solution-phase DNAzyme based electrochemical biosensor for lead determination has been developed. Unlike early reported DNAzyme based electrochemical biosensors which need tedious electrode modification, where the signal probe must be immobilized on the electrode surface, our approach utilized the electrostatic repulsion between the DNA probe and the negative ITO working electrode to achieve the immobilization free solution-phase measurement. The mechanism of the biosensor involves the detection of DNAzyme cofactor target with decent specificity and sensitivity, which may provide a platform for the fabrication of immobilization free electrochemical sensors for other targets since DNAzymes specific for Cu^{2+}, Zn^{2+}, and Co^{2+} have also been obtained.

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