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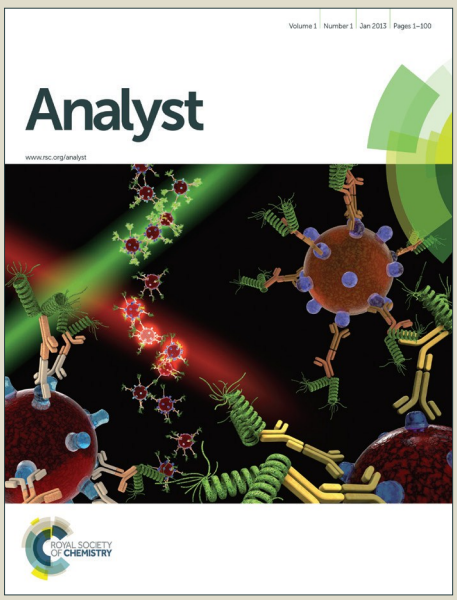

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Abstract

DNAzyme based electrochemical biosensor has the characters of high sensitivity and selectivity, but traditional DNAzyme based electrochemical biosensor need the immobilization of DNAzyme on the electrode surface first, the procedures are time consuming and tedious, which limit its real application. In this study, a simple but sensitive immobilization free DNAzyme based electrochemical biosensor had been proposed and lead had been chosen as a model target becuse of the sever effects of the lead toxicity. The different diffusivity and electrostatic repulsion between long and short DNA on the negatively changed ITO electrode can be used to discriminate the short and long DNA. Lead dependent DNAzyme had been hybridized with its substrate(which had been modified with a methylene blue at 3'terminal) beforehand. Since the DNAzyme/substrate complex contains large negative charge, which can not diffuse easily to the negative charged ITO electrode surface and little electrochemical signal has been detected. The prescence of lead would trigger the cleavage of DNAzyme/substrate complex and cause the releasing of methylene blue-labeled short-oligonucleotide into the solution. The methylene blue-labeled short-oligonucleotide can diffuse easily to the surface of the negative charged ITO electrode and results in the enhanced electrochemical response 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 had been applied to detect Pb^{2+} in water samples with satisfied results.

Keywords: immobilization free; DNAzyme; electrochemistry; lead; ITO electrode.

1. Introduction

DNAzymes, DNA sequences with catalytic activities, are isolated through in vitro selection[1]. DNAzyme has the inherent characters such as specificity, versatility, stability, ease of synthesis and modification, which has been applied in various fields including clinical diagnosis[2], environmental monitoring[3], biosensing[4] and cell imaging[5]. Electrochemical technique has the advantages of low-cost, high sensitivity and can be applied in on-field detection easily; many selective and sensitive DNAzyme based electrochemical biosensors had been developed for versatile targets[6-10]. But until now, nearly all DNAzyme based electrochemical biosensors 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 the 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 DNAzyme has been paid much attention since lead can cause many side effects to the human health[11]. Many sensitive DNAzyme based electrochemical biosensors for lead determination had proposed. Xiao et al. proposed a 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 to transfer electrons to the

electrode. Shen et al. showed an electrochemical method base 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 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 electrodes suface.

DNA contains negative charge due to the negative charge phosphate component[15]. Early report showed that ITO electrode surface can be negatively charged after simple treatment[16]. Since short DNA contains little negative charge while long DNA contains big negative charge, so there has difference electrostatic repulsion between the short DNA and long DNA with the negatively charged surface of ITO electrode. Short DNA can diffuse easily to the negatively charged surface, while long DNA cannot reach the surface easily. This character had been applied to discriminate the short DNA and long DNA with high efficiency, many immobilization free electrochemical biosensors had 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 base 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 of exonuclease III-aided autocatalytic two target recycling strategys[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-bases mismatch[22].These studies showed the convenient of the biosensors based on the negative charged ITO electrode. But till

now, the targets for these immobilization free biosensors are mostly focus on DNAs. It is necessary to find out some way to expand the application of ITO electrode based immobilization free technology.

To the best of our knowledge, no report which combines the advantages of the DNAzyme and the ITO based immobilization free electrochemical biosensors had been reported. In this study, a simple and sensitive DNAzyme based immobilization free electrochemical biosensor for Pb^{2+} detection had been reported, which combines the convenient of the ITO based immobilization free biosensor and high spectivity of the DNAzyme. The established biosensor has high selectivity due to the DNAzyme and easy operating due to immobilization free, which had been applied to detect the lead in water samples with satisfied 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'-AAAAAAAAAACCACACCAACCT-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 Tris-HCl buffer (pH 7.4). Targeted Pb^{2+} buffers composed of 10 mM $MgCl_2$, 50 mM NaCl and 50 mmol/L 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 had been used as working electrode in which effective working electrode area is 5mm×3mm; two Pt wires had been used as 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 reached after the treating processes after

sonication in an alconox solution (10g/L 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]. 1.5mL disposable microcentrifuge tube has been 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 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 (Tm = 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+} had been added in above solution. Cleavage was executed at 37 °C for 120 min to obtain the maximum cleavage of 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 had been detected five times, the average value had been applied for quantitative analysis.

3. Results and discussion

3.1 Principle of the immobilization free electrochemical biosensor

Fig.1 illustrates the principle of 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 had been modified with a methylene blue (MB) at the 3'terminal first, and then hybridizes with the DNAzyme to form the DNAzyme/substrate complex. Since the complex contain large negative charge, which cannot diffuse easily to the negative charged ITO electrode surface, so only 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 strand DNA (eMB) (only 6-base). And the Pb^{2+} can be applied to trigger the next

round of substrate strand cleavage and eT releasing. Since eMB contains only short DNA, the electrostatic repulsion from the negatively charged ITO working electrode to eT is much little than that of the DNAzyme/substrate complex or substrate, so which can diffuse easily to the negative charged ITO electrode surface and an obvious enhancement electrochemical signal can be detected. The specificity of the sensor can be assured by the high selectivity of the DNAzyme. Thus, a sensitive and selective immobilization free sensor can be developed.

The preferred position for Fig. 1

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

The preferred position for Fig. 2

3.2 Optimization of reaction conditions

MB labeled substrate plays an important role in 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 detected. So the concentrations of MB labeled substrate had been optimized first, DNAzyme (1.5 μM) and Pb^{2+} (1 μM) were mixed with different concentrations of substrate ranged 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 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 importance role in the performance of the system. As shown in Fig.3B, the DPV signal increased with the increasing of cleavage time first and then reached a plateau after 120 min. The reason maybe lies in that in short cleavage time, each target only can go through little cycles, so weak signal had been detected. In order to make sure that all DNAzyme/substrate complexes had been cleaved, 120 min had been chosen in this study.

The preferred position for Fig. 3

3.3 Calibration curve and reproducibility of the biosensor

To estimate the analytical performance of the proposed biosensor, the DPV responses of the system contains different concentration of Pb^{2+} were recorded. As shown in Fig.4A, the DPV response increased gradually with the increasing of Pb^{2+} concentration. This phenomenon is in accord with the fact that higher concentration of Pb^{2+} causing more DNAzyme/substrate complex been cleaved and more eMB 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:

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

Where ΔI (the difference of the currents detected at present and absent 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].

The preferred position for Fig. 4

To examine reproducibility of the biosensor, one ITO working electrode was repeatedly used to detect 5 samples (0.6 μM), the relative standard deviation (RSD) is 4.13%. And if 5 different ITO working electrodes are used for paralleling 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 had been studied also. The DPV responses after 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 had been detected in the presence of Pb^{2+}. Therefore, the proposed biosensor has high selectivity to discriminate Pb^{2+} from other.

The preferred position for Fig. 5

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 sample) had been detected. The water had been 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 had been observed if different concentrations of Pb^{2+} are added into the sample. So the samples were spiked with Pb^{2+} with the stock solution at the concentration level of 200, 400, 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.

The preferred position for Table 1

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 DNA probe and the negative ITO working electrode to achieve the immobilization free solution-phase measurement. The mechanism of biosensor was constructed for 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+}, Co^{2+} have also been obtained.

Acknowledgments

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Figures and Captions

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

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 time 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.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 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 ios] = 100 μM. Insert: the

corresponding DPV curves.

Fig. 1

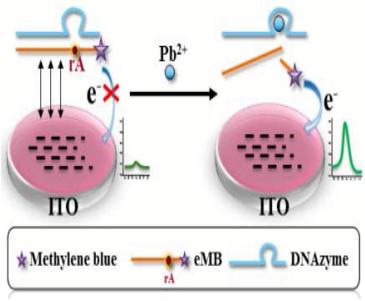

Fig. 2

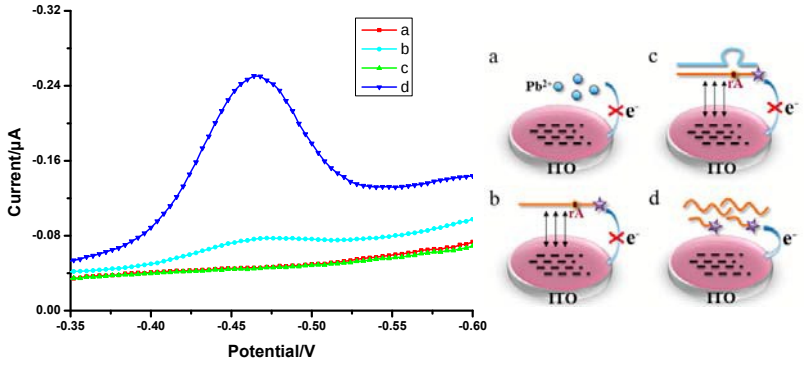

Fig. 3

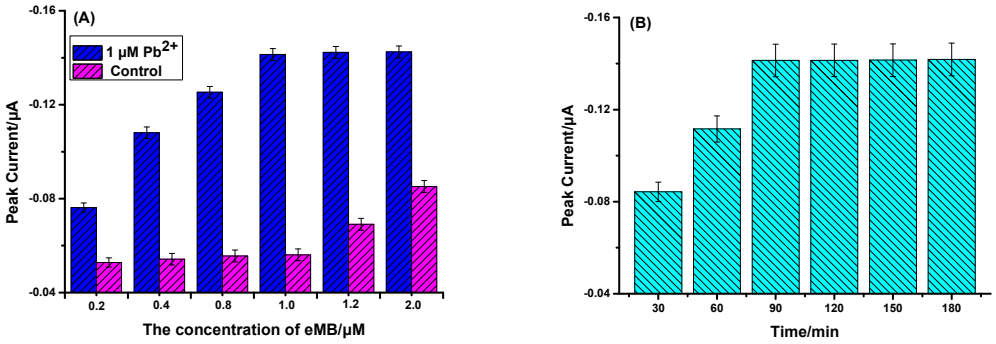

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Fig. 4

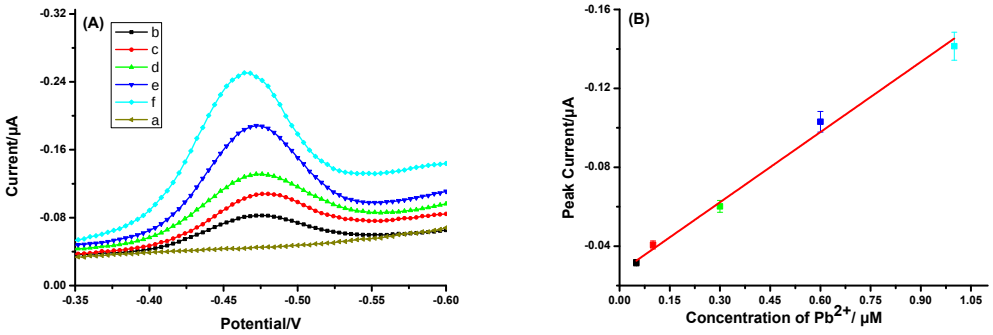

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Fig. 5

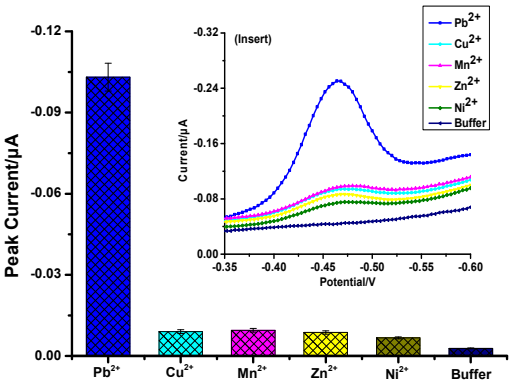

Table 1

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.