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A protease-free and signal-on electrochemical biosensor for ultrasensitive detection of lead ion based on GR-5 DNAzyme and catalytic hairpin assembly

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ABSTRACT

A simple protease-free and signal-on electrochemical biosensor based on GR-5 DNAzyme and catalytic hairpin assembly (CHA) for sensitive lead ion (Pb^{2+}) assay was constructed in this work. In the presence of Pb^{2+}, GR-5 DNAzyme specifically reacted with Pb^{2+} and cleaved the substrate strand into two free DNA fragments, and one of the two fragments then opened the hairpin capture DNA (Hc) that was assembled on the Au electrode surface and triggered the CHA reaction. Exploiting the CHA strategy, a large number of the hairpin signal DNA (Hs) labeled with two methylene blues (Mbs) were captured on the Au electrode surface and generated an efficient electron transfer, resulting in the dramatic increase of methylene blue current. In this method, Pb^{2+} could be detected quantitatively in the range of 4×10^{-11}–3×10^{-6} M, and the detection limit was as low as 2.7×10^{-11} M (S/N = 3). Experimental results demonstrated that the biosensor was highly specific for Pb^{2+} and exhibited remarkable improvements of electrochemical analytical performance. This biosensor was also used for the analysis of Pb^{2+} in the serum sample spiked with Pb^{2+}, and the obtained result in good agreement with the correct values. The excellent performance of the biosensor shows its promising potential for the clinical diagnosis of lead poisoning.

1. Introduction

Lead is a well-known highly toxic heavy metal, and its broad application has resulted in extensive environmental contamination and significant public health problems [1–5]. Due to its bioaccumulative and nonbiodegradable nature [6–8], even trace amounts of lead ion can give rise to a range of adverse health outcomes [9–16]. Children are especially susceptible to lead poisoning and are more sensitive to the neurotoxic effects of lead than adults [17,18]. Consequently, early and trace measurement of lead ion content in the body is highly significant.

As the contemporary analytical techniques expanded rapidly, various methods for the analysis of Pb^{2+} have been explored, such as atomic absorption spectrometry (AAS) [19,20], atomic emission spectrometry (AES) [21], inductively coupled plasma mass spectrometry (ICP/MS) [22], and surface plasmon resonance (SPR) [23,24]. They are reliable and accurate for the detection of Pb^{2+}, but many of them suffer from the limitations of the need for cumbersome instruments, sophisticated operation procedures, relative low sensitivity as well as serious mixture interference, all of which severely restrict their practical uses.

Electrochemical biosensors possess the superior features such as good sensitivity, low-cost, simplicity and portability [25], providing a promising platform for the highly effective analysis of Pb^{2+}. To further ameliorate the sensitivity of the detection method, several signal multiplication techniques, such as polymerase chain reaction (PCR) [26], rolling circle amplification (RCA) [27], loop-mediated isothermal amplification (LAMP) [28], enzyme-assisted target recycling (EATR) [29,30], and some DNA machine based technologies [31], have been used in electrochemical biosensors. However, these methods still have many unresolved obstacles, such as time-consuming detection procedure, high assay cost, the need for special thermal cycling equipment, require complicated preparation and the complex process of probe design. Beyond that, many of them employ nucleases, such as endonuclease, polymerase and exonuclease to achieve signal multiplication; however, the enzyme-based amplification aggrandizes the complexity of the analysis procedure and the detection cost. Additionally, false positive and insufficient stability may occur during the

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amplification process. Therefore, a biosensor base on simple, isothermal and protease-free signal amplification strategy biosensor with superior sensitivity is highly desirable.

It is well-known that the nucleic-acid multiplication technique of catalytic hairpin assembly can be obtained by simple mixing of the hairpin shape DNA without the involvement of enzymes [32,33]. CHA is an isothermal and enzyme-free signal multiplication method with negligible background, which is more stable than other multiplication techniques such as the PCR and RCA [34,35]. By a CHA reaction, hundredfold catalytic amplification can be achieved [36]. CHA is not only efficiently amplify the response signal, but also significantly reduce the detection cost and simplify the experimental procedure. Therefore, there have been many reports of biosensors for the assaying of DNA and microRNA that using CHA to amplify the response signal [37–39]. However, nearly all of the previous studies have focused on DNA and RNA determination, with few researches related to ultrasensitive detection of metal ions by CHA. Due to the demand for rapid and portable strategies for the quantification of trace lead ions, a new signal amplification method for highly sensitive and selective analysis of lead ion is still urgently need.

DNAzymes are a sort of DNA molecules selected in vitro with enzymatic catalytic activities that are synthesized by DNA oligonucleotides [40,41]. Compared to other types of biocatalysts, DNAzymes have drew widespread attention recently, due to their low cost, good stability, simple synthesis, easy modification, excellent programmability, and design flexibility [42–46]. DNAzymes are more stable than proteins and can function in relatively severe conditions such as extreme pH and high temperature [47]. Due to these advantages, many DNAzyme-based biosensors have been explored for the analysis of metal ions [48–53]. Among these, the Pb^{2+}-dependent DNAzymes have attracted intense attention. GR-5 and 8–17 are two classical Pb^{2+}-dependent DNAzymes [54]. Compared to the 8-17 DNAzyme-based biosensor, the GR-5 DNAzyme-based biosensor not only displays faster kinetics but also offers a much higher selectivity and even a slightly lower detection limit [52]. Therefore, in our study, a classic Pb^{2+}-dependent GR-5 DNAzyme was selected as the catalytic unit instead of 8-17 DNAzyme.

Additionally, based on a recently published work [55], to reduce the nonspecific catalytic hairpin assembly products in the absence of the target, which would result in a wider dynamic linear range and a more sensitive and selective Pb^{2+} assay, this research introduces mismatched base pairs into the breathing sites of the hairpin signal DNA. The mismatched base pairs are shown as the underlined entries in Table S1 (Supplementary material).

Herein, we designed a simple, isothermal, protease-free and signal-on electrochemical biosensor for ultrasensitive determination of Pb^{2+} by using a GR-5 DNAzyme and a catalytic hairpin assembly strategy. We have combined the specific DNAzyme, signal-on amplification strategy and mismatched catalytic hairpin assembly strategy for the first time. Compared to signal-off biosensors, signal-on biosensors can obtain much improved signal, and the background signal observed in the absence of the target is decreased so that the gain of such a sensor, at least in theory, can show an unlimited increase [56]. In the signal-on sensor investigated in this work, mismatched CHA strategy enhances the rate at which the Mb gets close to the electrode, avoids false positives and leads to a current increase. With so many merits, this biosensor shows great selectivity and ultrasensitivity for Pb^{2+} determination. Additionally, this method opens a promising avenue for the detection of other metal ions.

2. Experimental section

2.1. Reagents and chemicals

Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was bought from Sangon Biotechnology Co. (Chongqing, China), 6-Mercapto-1-hexanol (MCH) was bought from Sigma-Aldrich (St. Louis, USA). Lead Standard Solution, Magnesium Standard Solution and the other metal ions Standard Solution were bought from ZONO Biotechnology Co. (Chongqing, China). All other chemical reagents were of analytical purity and were used without any purification or treatment.

Buffers employed in this experiment were as follows: TNaK buffer as the DNAzyme hybridization buffer contained 20 mM Tris-HCL, 140 mM NaCl, 5 mM KCl (pH 7.4). Buffer for CHA reaction: 20 mM Tris-HCl,140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.4). Electrochemical impedance spectroscopy (EIS) buffer: 0.1 M PBS, 10 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl (pH 7.4). Differential pulse voltammetry (DPV) and cyclic voltammetry (CV) buffer: 20 mM Tris–HCl, 140 mM NaCl, and 5 mM $MgCl_2$ (pH 7.4). Ultrapure water (18 MΩ cm^{-1}, Millipore System Inc.) was employed throughout the study. The probe modified with two Mbs was synthesized and purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). All other DNA sequences were provided by Sangon Biotechnology Co. (Chongqing, China) and all oligonucleotide sequences are listed in Table S1.

2.2. Instrumentation

All electrochemical measurements such as EIS, CV and DPV were conducted with an AUTOLAB PGSTAT302 N electrochemical workstation (Metrohm Technology Co. Ltd., Switzerland) at room temperature (RT), which with a conventional three-electrode system composed of a 3-mm-diameter Au electrode as the working electrode, a saturated calomel electrode as the reference electrode and a platinum wire as the auxiliary electrode.

2.3. Preparation of Pb^{2+}-specific DNAzyme

To reduce the background signal and achieve complete hybridization rate of the enzyme strands (ES) and substrate strands (SS), 1 mL enzyme strands (4.8 μM) and 1 mL substrate strands (4 μM) were mixed and heated to 95 °C for 5 min, then slowly cooled down to RT to form Pb^{2+}-specific DNAzyme (The ratio for the hybridization of ES with the SS was 1.2:1). Then, the mixture was gently shaken for 1 h at RT for further hybridization. The obtained hybrid DNA solution was stored at −20 °C for further use.

2.4. Fabrication of biosensor

All hairpin structure DNAs were annealed at 95 °C for 5 min and then gradually cooled down to RT before use. The bare Au electrode was polished with 0.3 and 0.05 μm alumina slurry on a microcloth sequentially and then washed by sonication in ultrapure water, ethanol and ultrapure water for 5 min, in turn. Subsequently, the electrode was soaked in a freshly prepared piranha solution (98% H_2SO_4: 30% H_2O_2 = 3:1, V/V) for 20 min. The piranha solution reacted violently with organic compounds to further clean the gold electrodes, and the electrode was then washed thoroughly with ultrapure water and dried in air. Before assembly on the Au electrodes, a thiolated hairpin capture probe was incubated with 10 mM TCEP buffer (10 mM TCEP, 20 mM Tris-HCl, 140 mM NaCl, 5.0 mM KCl, pH 7.4) for 1 h at room temperature to decrease the disulfide bonds and diluted to 1 μM. Then, 10 μL of 1 μM thiolated hairpin capture DNA was dropped onto the pretreated Au electrode surface and incubated overnight at 4 °C. The modified electrode was then rinsed with ultrapure water to remove the unbound Hc on the surface. Subsequently, the electrode was dipped in 1 mM MCH for 30 min to prevent nonspecific adsorption and obtained a well-aligned DNA monolayer. Then, 4 μL enzyme strand and substrate strand hybrid compounds were reacted with 4 μL Pb^{2+} of different concentrations for 60 min at 37 °C. During this process, Pb^{2+}-specific DNAzyme obtained its cleavage activity, and cleaved the SS into two fragments at the 'rA' site. After adding 2 μL hairpin signal DNA (the ultimate concentration of the Hs was 0.8 μM), the mixture was dropped