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Ultrasensitive DNAzyme-based electrochemical biosensor for Pb^{2+} based on FcHT-mediated biocatalytic amplification

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A ultrasensitive DNAzyme-based electrochemical biosensor for Pb^{2+} was developed by simultaneously modifying with GR-5 DNAzyme-substrate complex and 6-(ferroceney) hexanethiol (FcHT, a electron mediator) on Au electrode. Horseradish peroxide (HRP) was employed as an electrochemical label. The ultrasensitive detection was achieved by the FcHT-mediated biocatalytic cycle between HRP and hydrogen peroxide (H_2O_2). The electrochemical biosensor was characterized using X-ray photoelectron spectroscopy (XPS) and electrochemical impedance spectroscopy (EIS). Cylic voltammetry was used to monitor the increased electrocatalyzed reduction of H_2O_2 by HRP. The linear range and the detection limit were determined to be 0.1 nM to 1 μM and 0.03 nM (S/N=3), respectively. The validity of the proposed biosensor was tested in Pb^{2+}-spiked water samples, and the results agreed well with those obtained by the conventional ICP-MS method.

Keywords: Electrochemical biosensor; Pb^{2+}; DNAzyme; 6-(ferroceney) hexanethiol (FcHT), signal amplification

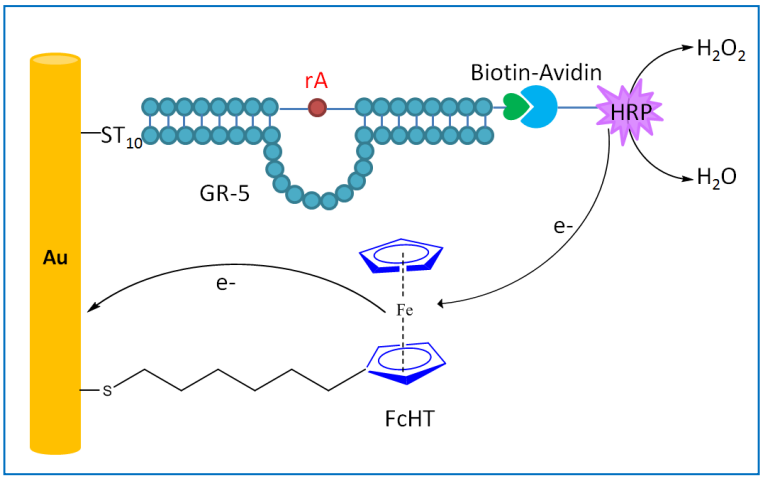
1. INTRODUCTION

The development of highly sensitive detection of Pb^{2+}, a well-known and nondegradable pollutant, is essential in environmental monitoring and food safety [1-2]. Conventional detection methods of Pb^{2+}, including atomic absorption spectrometry [3], and atomic emission spectrometry [4], are usually complicated, time-consuming tedious, and not suitable for point-of- care applications. To

overcome such drawbacks, some promising analytical techniques for routine and effective monitoring of Pb^{2+} have been developed based on fluorescence [5-10], colorimetry [11-15], electrochemistry [16-18], electrochemiluminescence [19-21], etc. Although the above-mentioned techniques resolved some shortcomings of conventional methods, they also encountered some limitations such as the complex design and unsatisfactory detection performance. Therefore, there is need for developing facile, selective, and sensitive approaches for the Pb^{2+} detection in real samples with low-cost and high accuracy.

DNAzyme-based electrochemical biosensors have attracted more attention because of simplicity of construction, high sensitivity, fast response time, low cost, and point-of-care applications [22-25]. DNAzyme (Metalloenzymes, DNA molecules with enzymatic activities) is usually obtained by using metal ions as template to screen from a large DNA library with a combinatorial process [26-29]. Due to high metal ion selectivity, these DNAzymes have been converted into various sensors for metal ions as recognition elements. Among these DNAzymes, the Pb^{2+}-dependent DNAzymes was initially employed to develop electrochemical sensors for the Pb^{2+} detection based on electroactive labels (*e.g.* ferrocene and methylene blue) [30-31]. The detectable signal mainly derives from the labeled DNAzyme on the working electrode. However, the electroactive label-modified DNAzyme immobilized on the electrode surface is limited, which influences the sensitivity of electrochemical sensors to a certain extent. To improve the sensitivity, various amplification strategies have been proposed for Pb^{2+} detection based on inorganic nanoparticles [32], MOFs [33], and enzymatic recycling [34]. These amplification methods often require time-consuming optimization process and long detection time. Thus, sample and sensitive signal amplification strategies for Pb^{2+} sensing are still highly desired.

Herein, we report a simple and sensitive electrochemical sensor of Pb^{2+} by coupling the amplification capability of 6-(ferrocenyl) hexanethiol (FcHT)-mediated electrocatalyzed hydrogen peroxide reduction with the specific recognition properties of DNAzyme. The fabrication process and assay principle of the proposed sensor is illustrated in Scheme 1. GR-5-substrate complex (denoted as GR-5) and FcTH are first simultaneously immobilized on the surface of the gold electrode via gold-sulfur chemistry. Then, the surface is passivated with bovine serum albumin (BSA) for minimizing the nonspecific adsorption. Finally, the modified electrode is treated with avidin-HRP. The detection of Pb^{2+} is based on the measurements of changes of electrocatalytic reduction of HRP-generated hydrogen peroxide in the absence and presence of Pb^{2+} through the mediating ability of FcTH. The changes of electrocatalytic reduction current are proportional to the Pb^{2+} concentrations. This sensor has been characterized using X-ray photoelectron spectroscopy (XPS), electrochemical impedance spectroscopy (EIS), and quartz crystal microbalance (QCM). Various experimental parameters have been optimized for Pb^{2+} detection. The selectivity, stability, and reproducibility of the sensor have been performed. The proposed sensor has been applied in real water samples and compared with conventional ICP-MS method.



Scheme1. Schematic illustration of the fabrication of electrochemical biosensor and detection principle.

2. EXPERIMENTAL

2.1 Materials and Reagents

6-(ferrocenyl) hexanethiol (FcHT), Tris (2-carboxyethyl)-phosphine hydrochloride (TCEP), bovine serum albumin (BSA), and lead nitrate (Pb $(NO_3)_2 \cdot H_2O$) were purchased from Sigma-Aldrich (U.S.A.) and used as received. Avidin-HRP and hydrogen peroxide were obtained from Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). All synthesized oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA) and purified by HPLC. Their sequences were shown as following:

Pb^{2+} DNAzyme (GR-5): 5′-SHT_{10} ACAGACATCATCTCTGAAGTAGCGCCGCCGTATAGTGAG-3′, its corresponding substrate (Pb-Sub): 5′-CTCACTAT*rA*GGAAGAGATGATGTCTGT-bio-3′.

Other metal ions were purchased from Sinopharm Chemical Reagent (Shanghai, China). Ultrapure water (18.2 MQ) was obtained from a Millipore Milli-Q system.

2.2 Apparatus and Instruments

Electrochemical experiments (CV, EIS) were performed with CHI660D electrochemical workstation (CHI Instruments, shanghai, China). Gold and the modified electrodes were used as working electrode. Ag/AgCl electrode (saturated with KCl) and a Pt wire were employed as the reference electrode and the counter electrode, respectively. QCM experiments were measured on a model CHI420A time-resolved electrochemical quartz crystal microbalance. A 7.995 MHz AT-cut quartz crystal (A = 0.196 cm^2) was used for the QCM experiments. The sensitivity factor was calculated as 1.34 ng/Hz. X-ray photoelectron spectroscopy (XPS) investigation was carried out in PHI-5000C ESCA system (PerkinElmer) with Mg Kα radiation (hv = 1253.6 eV). All binding energies (BEs) were referred to the C1s peak (284.6 eV) arising from surface hydrocarbons (or adventitious hydrocarbon).

2.3 Preparation of electrochemical biosensor

Gold electrodes (2 mm in diameter) were polished with 0.05 μm alumina/water slurry on a polishing cloth to a mirror finish, following by sonication and rinsing with ultrapure water. The polished electrode was subsequently cleaned electrochemically in 0.5 M H_2SO_4 by potential scanning between 0 and 1.6 V until a reproducible cyclic voltammogram was obtained. Finally, the electrode was rinsed with ultrapure water and dried with high-purity nitrogen.

Prior to the modification, a volume of 100 μL of the mixture of the thiolated GR-5 DNAzyme and the biotin-labeled substrate (1μM, in 50 mM pH 8.2 Tris-acetate buffer containing 500 mM NaCl) was heated 90 ℃ for 5 min and then cooled down to room temperature. Next, the complex was activated by the addition of 5 μL of 1 mM TCEP to reduce disulfide bonds. 5μL of the mixed solution of complex and FcHT (20:1) was dropped on the working electrode and incubated overnight at 4 ℃ in 100% humidity. The resulting electrode was denoted as Au/ FcTH /GR-5.

Then, 3 μL of 2μM HRP-conjugated Streptavidin was pipetted onto the modified electrode and incubated for 2 h. After rinsing with 50 mM Tris-acetat buffer (pH7.4), the working electrode was passivated with 1% BSA to remove nonspecific adsorption sites. The modified electrode was denoted as Au/FcHT /GR-5 /HRP and used as the electrochemical sensor for Pb^{2+}.

As a control electrode, Au/GR-5/HRP was modified with the same procedure except for FcHT.

2.4 Electrochemical measurement of Pb^{2+}

5μL of Pb^{2+} at various concentrations or samples was dropped onto the modified working electrode and incubated at room temperature for 15 min. After washing with 50 mM Tris-acetat buffer (pH7.4), the electrode was immersed in 5 mL 50 mM Tris-acetate buffer (500 mM NaCl, pH 8.2) containing 6 mM H_2O_2, cyclic voltammetric measurements were then performed in the potential range of 0~-0.25 V at a scan rate of 10 mV/s.

3. RESULTS AND DISCUSSION

3.1 Characterization of Au/FcHT/GR-5 and Au/FcHT/GR-5/HRP

The Au/FcHT/GR-5 modified electrode and gold electrode were characterized using X-ray photoelectron spectroscopy (XPS) [35]. Fig. 1(A, B, C) showed the XPS spectra of the Au/FcHT/GR-5 modified surface and gold electrode. The survey spectrum obtained for gold electrode showed two peaks at 83.5 and 87.3 eV, which correspond to Au4$f_{7/2}$ and Au4$f_{5/2}$, respectively (Fig.1A curve a). After modification with Au/FcHT/GR-5, a significant decrease in the spectral intensity of Au element was observed (Fig.1A curve b). Furthermore, the survey spectra obtained for Au/ FcHT/GR-5 showed the characteristic peaks of Fe element (Fe $2p_{3/2,}$ 709.2 eV; Fe $2p_{1/2}$,722 eV) in Fig.1B (curve b) and S element (S 2p, 163.0eV) in Fig. 1C (curve b), which were not observed in the spectrum of only gold electrode (Fig.1 B, C curve a). These results clearly indicated that GR-5 and FcHT were successfully assembled on the surface of gold electrode.

The Au/FcHT/GR-5/HRP was fabricated via the reaction between biotin-labeled GR-5 and Avidin-HRP [38-39]. The Au/FcHT/GR-5/HRP was characterized by using EIS, which provides information on the surface conductivity of various modification steps [25]. Fig. 1D showed the Nyquist plot obtained for the Au, Au/FcHT/GR-5/, and Au/ FcHT/GR-5/HRP recorded in a $Fe(CN)_6^{3-/4-}$ solution. The charge transfer resistance (R_{et}) exhibits the charge transfer kinetics of the $Fe(CN)_6^{3-/4-}$ redox system. The R_{et} value can be estimated from the diameter of the semicircle part at higher frequencies. The R_{et} of bare gold electrode was about 380 Ω, indicating the fine conductivity of the unmodified electrode (curve a). After the immobilization of GR-5 and FcHT, the negatively charged phosphate backbone of GR-5 repelled $Fe(CN)_6^{3-/4-}$, which resulted in a significant semicircle domain (curve b). However, the R_{et} value increased from 1905 Ω to 5206 Ω when HRP was attached on GR-5, which means that the proposed sensing system has been successfully constructed [40-41].

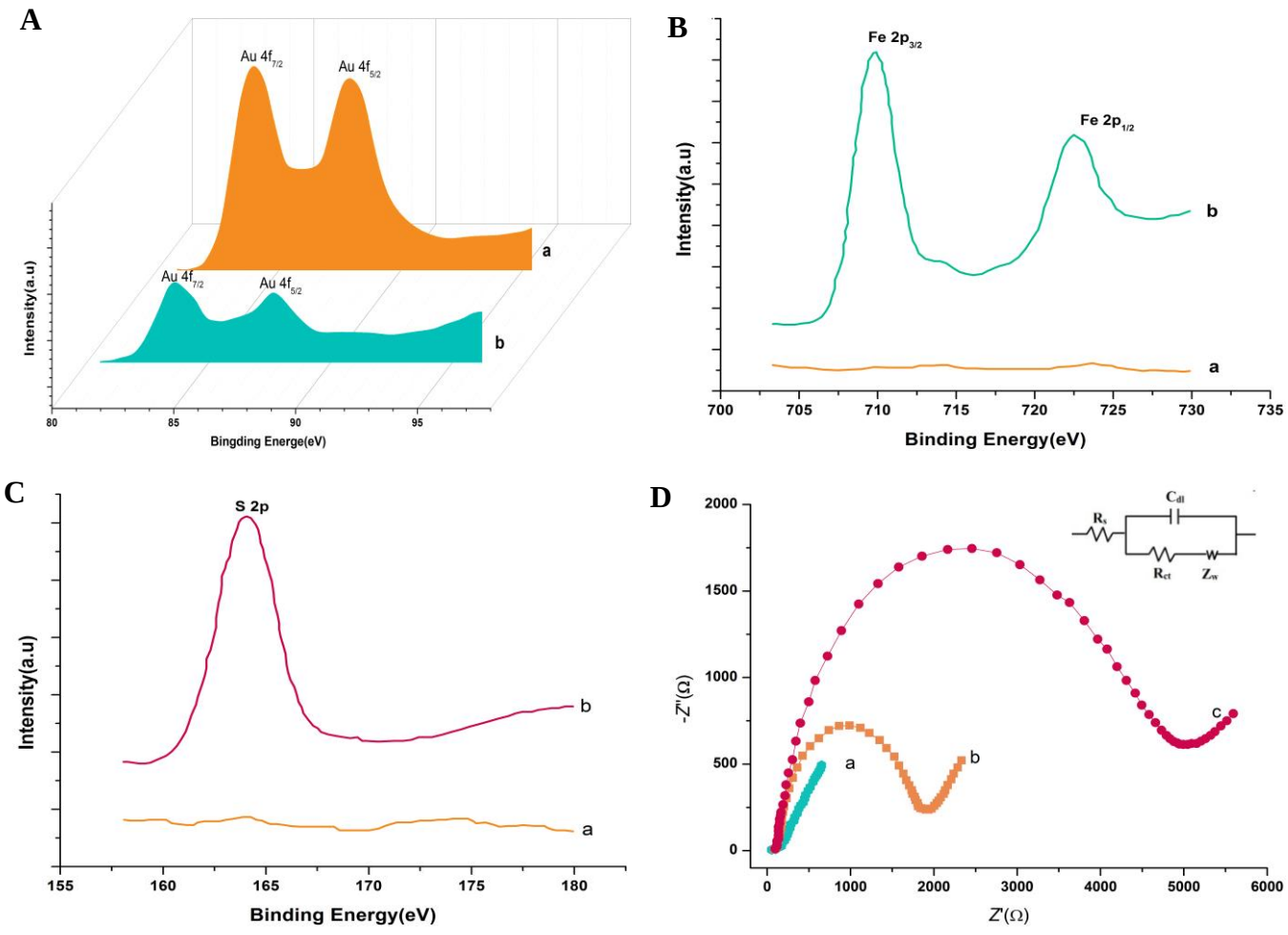


Figure 1. XPS spectrum of (A) Au $4f_{7/2}$ and Au $4f_{5/2}$, (B) Fe $2p_{3/2}$ and Fe $2p_{1/2}$, and (C) S 2p for (a) bare gold electrode; (b) Au/ FcTH /GR-5; (D) EIS analyses of (a) bare gold electrode; (b) Au/FcTH/GR-5; and (c) Au/FcTH/GR-5/HRP. Inset: the general equivalent circuit.

Further, we also investigated the signal amplification efficiency of FcHT-mediated biocatalytic reduction by using cyclic voltammetry in 50 mM Tris-acetate buffer (500 mM NaCl, pH 8.2) containing 6 mM H_2O_2 (Au/GR-5/HRP as a control electrode).

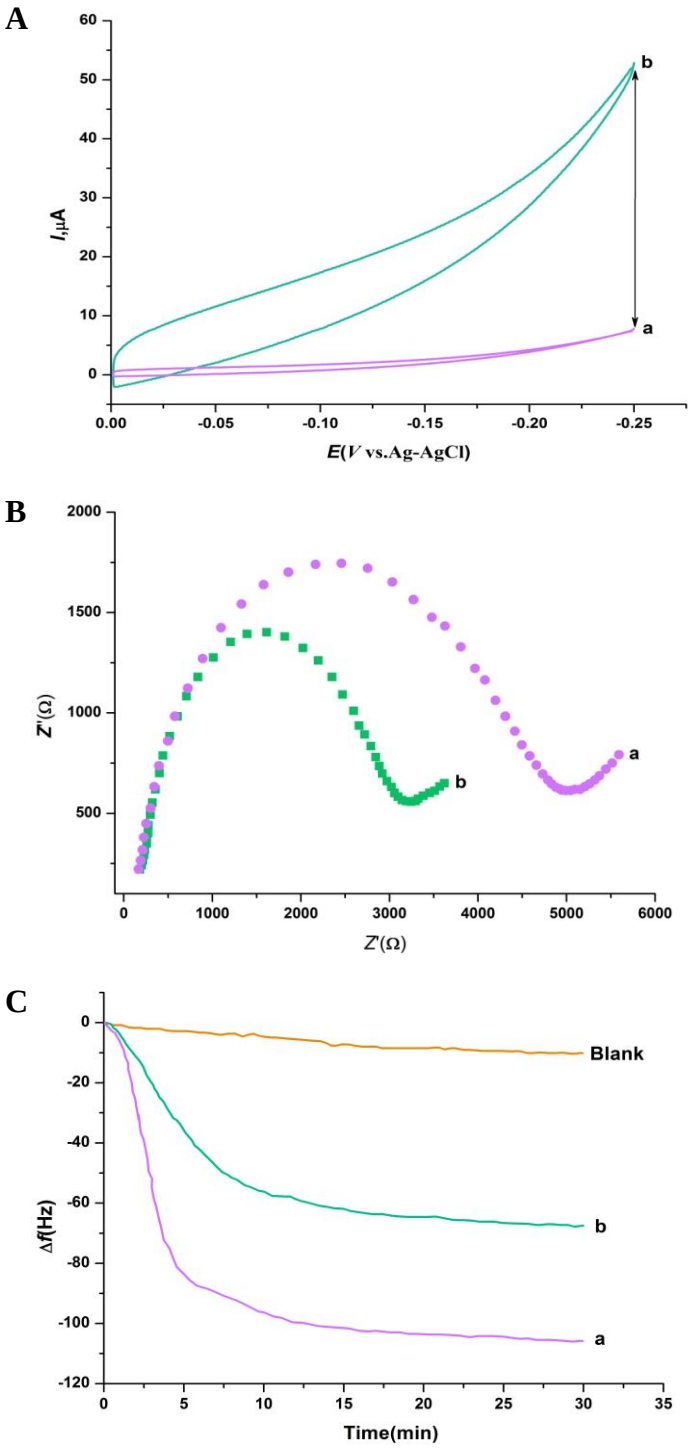


Figure 2. (A) CV responses recorded for (a) Au//GR-5/HRP and (b) Au/FcTH/GR-5/HRP; (B) Electrochemical impedance spectroscopy of the proposed sensor (a) before and (b) after incubation with 500 nM Pb^{2+} ions; (C) QCM analyses of frequency changes of the proposed sensor (a) before and (b) after incubation with 500 nM Pb^{2+} ions.

As shown in Fig. 3A, the CV response of the Au/GR-5/HRP was ~5.8 μA (*E*=-0.25 *vs* Ag/AgCl, curve a), whereas a large increase in CV response (*E*=-0.25 *vs* Ag/AgCl), was observed (~52.8μA, curve b) for Au/FcHT/GR-5/HRP. This is most likely a consequence of the fact HRP could catalyze the reduction of H_2O_2 with the aid of ferrocene mediators, which accelerated the electron transfer process between the HRP's redox site and the electrode, thus resulting in the amplification of the electrochemical signal [42-43].

3.2 Characterization of electrochemical biosensor

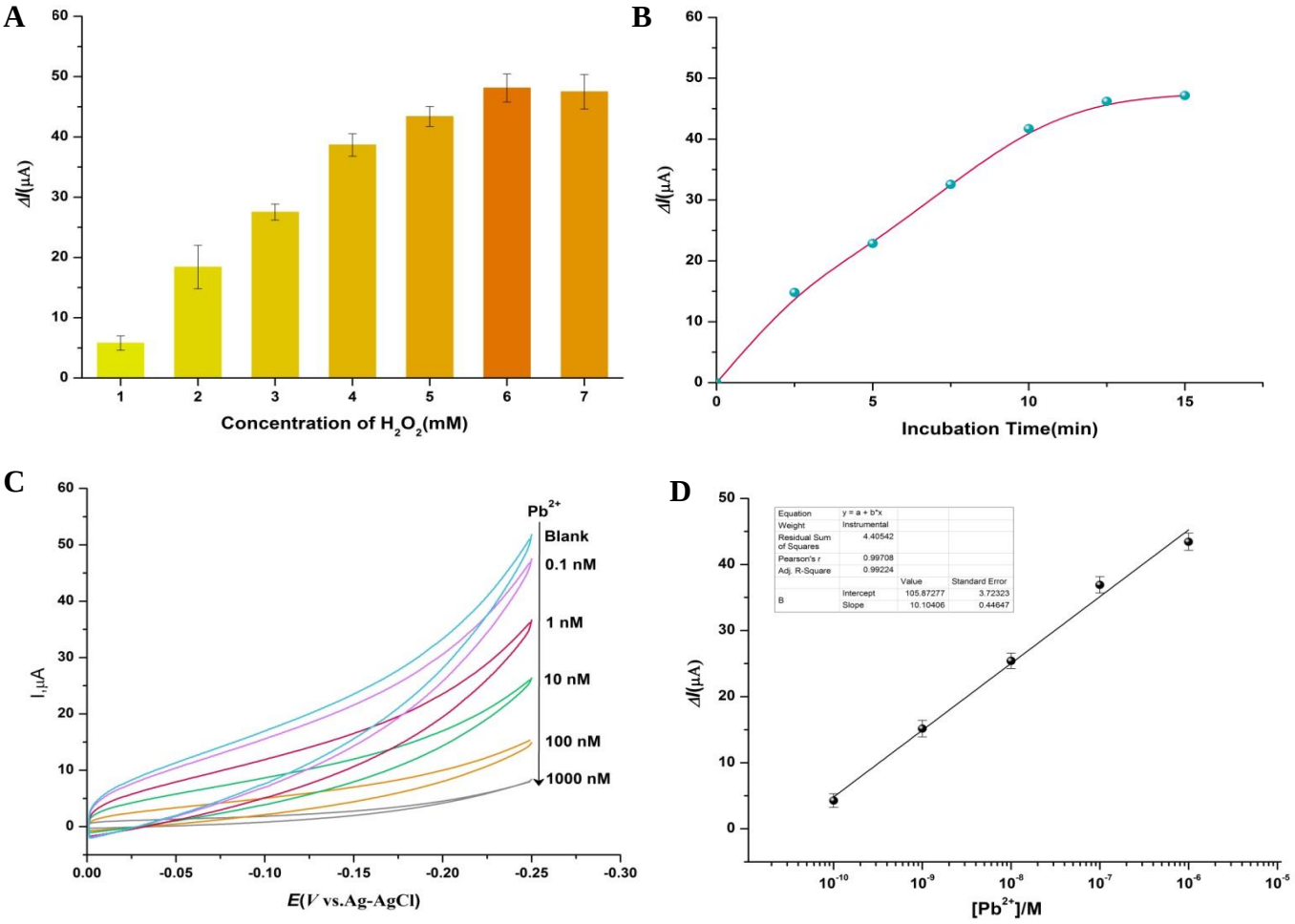


Figure 3. (A) CV responses changes of Au/FcTH/GR-5/HRP in the presence of 1 μM Pb^{2+} at an incubation time of 30 min with various concentrations of H_2O_2; (B) Time-dependent CV responses of Au/FcTH/GR-5/HRP in the presence of 1 μM Pb^{2+} and 6 mM H_2O_2; (C) CV corresponding to the FcTH-mediated bioelectrocatalytic cycle of Au/FcTH/GR-5/HRP in the presence of different concentrations of Pb^{2+}; (D) Semilogarithmic plot of the corresponding calibration curve (*E* =-0.25 V vs Ag/AgCl).

To confirm the cleavage mechanism of our sensor, EIS and QCM techniques were performed for the characterization of Au/FcHT/GR-5/HRP. Fig. 3B showed the impedance changes for Au/FcHT/GR-5/HRP in a 50 mM Tris-acetate buffer (500 mM NaCl, pH 8.2) with (curve b) and

without (curve a) Pb^{2+}(500 nM). In the absence of Pb^{2+}, the R_{et} of Au/FcHT/GR-5/HRP was ~5000 Ω (curve a). When Au/FcHT/GR-5/HRP was incubated with Pb^{2+} for 15 min, the R_{et} of Au/FcHT/GR-5/HRP significantly decreased to be ~3100Ω, which might be ascribed to the fact that the Pb^{2+}-triggered GR-5 cleavage products released from the surface of the modified electrode, thereby resulting in the decrease of R_{et}. The QCM experiments could further provide beneficial supports for Pb^{2+}- induced damage to Au/FcHT/GR-5/HRP [44-45]. Fig. 3C showed the frequency-time response obtained for Au/FcHT/GR-5/HRP in the absence (curve a) and presence (curve b) of 500 nM Pb^{2+} ions. The frequency of Au/FcHT/GR-5/HRP decreased gradually and reached a steady state within 30 min. The average frequency change (Δf) was about 95 Hz, which corresponds to an average mass change (Δm) of 127 ng by using a sensitivity factor of 1.34. After challenging with 500 nM Pb^{2+} ions, the frequency of the electrode decrease also attained a steady state with 30 min, and the Δf and Δm were determined to be 57 Hz and 76 ng, respectively. These results clearly indicated that it is feasible for the sensor to detect Pb^{2+} ions.

3.3 Detection of Pb^{2+} with electrochemical biosensor

To obtain high sensitivity for Pb^{2+} detection, we optimized some experimental parameters such as H_2O_2 concentration and reaction time based on the electrocatalytic current change (ΔI, E=-0.25V) ($\Delta I = I - I_0$; I and I_0 represented the currents in the presence and absence of Pb^{2+}, respectively). As shown in Fig. 3A, the best electrochemical response was obtained at 6 mM H_2O_2. Afterward the current change did not significantly increase. Thus, the H_2O_2 concentration was optimized to be 6 mM. Fast detection is an important feature of a simple Pb^{2+} sensor. The effect of reaction time on the current change was then investigated between 0 min and 15 min (Fig. 3B). The electrocatalytic current changes increased with the increasing reaction time and tended to a steady value at 15 min. The reaction time was longer than that of Pb^{2+}-triggered enzymatic cleavage reaction in solution (~5min), which was ascribed to the fact that GR-5 DNAzyme-substrate complex modified on the surface of electrode would be inconvenient to the Pb^{2+} recognition. As a result, the optimum reaction time was considered as 15 min.

Under optimal sensing conditions, the CV electrocatalytic current response of this sensor was investigated by varying the Pb^{2+} concentration with the Au/FcTH/GR-5/HRP-based sensor. The electrocatalytic current response of the sensor decreased with the increasing concentration of Pb^{2+} (Fig. 3C). The blank-subtracted electrocatalytic current changes of H_2O_2 were linearly proportional to the logarithm of the Pb^{2+} concentration. As shown in Fig. 3D, a wide dynamic linear range from 0.1 nM to 1000 nM was achieved with the fitting equation as follows: $\triangle I = 105.87+10.10X$ (R^2=0.992), where X was logarithm of Pb^{2+}concentrations, R was the regression coefficient. The detection limit of Pb^{2+} was determined to be 0.03 nM (S/N=3), which is lower than previously reported Pb^{2+} sensors (Table 1). The enhanced sensitivity originates from the amplification of the FcTH-mediated biocatalytic system.

Table 1. Comparison of several electrochemical biosensors for Pb^{2+} ions

Electrochemical Biosensors	Detection range	Sensitivity	References
MB-labeled 8-17 DNAzyme	0.5~10 μM	0.3 μM	30
8-17 DNAzyme/DNA-AuNP-biobar Codes	5 nM~0.1μM	1 nM	36
8-17 DNAzyme/TdTase/ALP amplification	0.05~500 nM	0.047 nM	34
8-17 DNAzyme/HCR amplification	0.1~75 nM	0.037 nM	37
GR-5 DNAzyme/$(Fe-P)_n$-MOF	0.05~200 nM	0.034 nM	46
Au/FcHT/GR-5/HRP	0.1~1000 nM	0.03 nM	This work

3.4 Selectivity, stability and reproducibility of electrochemical biosensor

To assess the selectivity of the sensor, the CV responses of the electrocatalytic reduction of H_2O_2 were measured for other metal ions including Fe^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, Hg^{2+}, Mn^{2+}, and Cd^{2+}. Fig. 4 showed the changes of catalytic current at -0.25V measured with the proposed sensor toward a 10 μM concentration of other metal ions. A negligible response change was observed when the sensor was exposed to these interference ions even at 10-fold higher concentration than Pb^{2+}, suggesting that these metal ions did not interfere the detection of Pb^{2+}. Only Pb^{2+} activated the GR-5 DNAzyme and resulted in the significant change of catalytic current. Therefore, the sensor is demonstrated to have an excellent selectivity for the Pb^{2+} detection and a potential application for analysis of real samples.

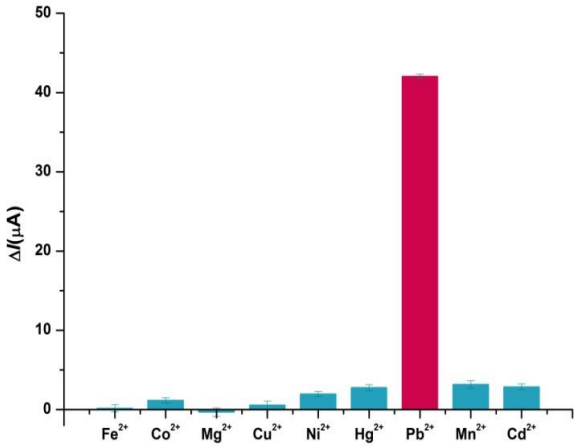


Figure 4. Selectivity in the analysis of Pb^{2+} ions by Au/FcHT/GR-5/HRP. The concentration of Pb^{2+} ion was 1μM. The concentration of other metal ions was 10 μM. The incubation time for all ions was 15 min. Error bars are standard deviations of three repetitive experiments.

For further application in the water samples, the stability of this sensor was tested by incubating with 50 mM Tris-acetate buffer (500 mM NaCl, pH 8.2) containing 1μM Pb^{2+} for 15 min.

After being washed with Tris-acetate buffer, the sensor was stored in darkness conditions at 4 ℃, and its current response was monitored every day. It was found that the initial current response did not significantly change during 3 days. However, after 3 days, the sensor response gradually decreased due to the deactivation of HRP. To check the reproducibility, six different Au/FcHT/GR-5/HRP electrodes were prepared and used for comparing their CV responses with 1μM Pb^{2+}. The relative standard deviation (*RSD*) of catalytic current changes obtained at 6 electrodes was calculated to be 4.7%, revealing the high reproducibility of the present sensor.

3.4 Real water samples analysis

To evaluate the analytical reliability and the practical utility of the proposed sensor in environmental analysis, it was applied in Pb^{2+}-spiked tap water samples for the detection of Pb^{2+}. The standard addition method was used to determine the Pb^{2+} concentration. The analytical results were summarized in Table 1. The recoveries of 95.2%~99.0% indicated good accuracy of the proposed sensor for the detection of Pb^{2+} in environment samples. Conventional ICP-MS method was used to detect the same spiked samples, and detailed results were presented in Table 1. The obtained Pb^{2+} concentrations determined by our sensor showed a good agreement with those obtained by ICP-MS. Therefore, the potential applicability of the sensor for the detection of Pb^{2+} in real samples was further confirmed.

Table 2. Determination of Pb^{2+} in tap water samples using the proposed electrochemical sensor and ICP-MS. The mean and standard deviation values are based on three repetitive experiments.

The proposed electrochemical sensor			ICP-MS (mean ± SD)
Added concentration (Pb^{2+})	Measured (mean SD)	Recovery (%)	
5 nM	4.95±1.2	99.0	5.2±0.9
50 nM	47.6±3.4	95.2	50.4±1.8
500 nM	487±12.7	97.4	496±9.8

4. CONCLUSIONS

In summary, we developed an ultrasensitive electrochemical biosensor based on the specific recognition of GR-5 with Pb^{2+}, together with FcHT-mediated signal amplification for the Pb^{2+} detection. FcTH was simultaneously modified with GR-5-substrate complex on one electrode to provide an electron mediator to accelerate the electron transfer reaction of HRP-electrocatalyzed H_2O_2 reduction, thereby enhancing the sensitivity of the proposed sensor. The linear dynamic range of the suggested sensor covered a 5-order wide concentration range in which Pb^{2+} detection is performed without dilution. In addition, the detection limit of the sensor was much lower than that of the

conventional electrochemical sensor. Importantly, the present sensor has successfully been applied for the reliable detection of Pb^{2+} in water samples and thus could be a valuable tool for Pb^{2+} sensing in environmental applications.

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