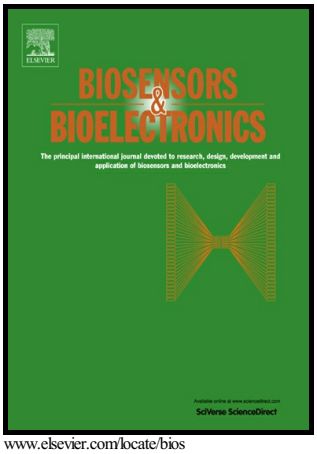

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PII: S0956-5663(18)30322-1
DOI: https://doi.org/10.1016/j.bios.2018.04.054
Reference: BIOS10450

To appear in: *Biosensors and Bioelectronic*

Received date: 5 March 2018
Revised date: 25 April 2018
Accepted date: 25 April 2018

Cite this article as: LeLe Wang, Yanli Wen, Lanying Li, Xue Yang, Nengqin Jia, Wen Li, Jiaoran Meng, Manlei Duan, Xiaoguang Sun and Gang Liu, Sensitive and Label-free Electrochemical Lead Ion Biosensor based on a DNAzyme triggered G-quadruplex/hemin conformation, *Biosensors and Bioelectronic,* https://doi.org/10.1016/j.bios.2018.04.054

Sensitive and Label-free Electrochemical Lead Ion Biosensor based on a DNAzyme triggered G-quadruplex/hemin conformation

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Abstract

Lead ion (Pb^{2+}) is a common environmental contaminant, which causes serious bioaccumulation and toxicity in human body. In this work, we developed a novel Pb^{2+} electrochemical biosensor using the specific DNAzyme on a DNA tetrahedron probe, in the presence of Pb^{2+}, the substrate strand was cleaved into two parts and released a "G-rich" oligo which subsequently formed a G-quadruplex/hemin complex, generating a detectable catalysis current signal with the assistant of H_2O_2. The 3-D

DNA tetrahedron regulated the density and orientation of the probe and thus improved the DNAzyme reaction, and facilitated the complex DNA conformational change in the confined space of the interface on the electrode surface, Finally, the LOD of our biosensor was proved to be 0.008 nM (3σ), which is 9000 times lower than the safety limit of EPA (15 μg/L or 72 nM), and 6000 times lower than IARC (10 μg/L or 48.26 nM), and more importantly, the specificity and reproducibility of the proposed biosensor was well demonstrated.

Keywords

Lead ion; DNA tetrahedron; Electrochemical biosensor; DNAzyme; G-quadruplex/hemin.

1. Introduction

Heavy metals ions, are ubiquitous environmental contaminants(Wang et al. 2016b) which can pass through shin, digestive system and respiratory organs into blood. As is well-known, lead ion (Pb^{2+}), due to its potential bioaccumulation and toxicity, causes neurological, reproductive, cardiovascular and developmental disorders, especially in children(Dolati et al. 2017; Zhou et al. 2016a). The safety limit of Pb^{2+} in drinking water established by the U.S. Environmental Protection Agency (EPA) was 15 μg/L (72 nM), while the International Agency for Research on Cancer (IARC) set a lower threshold of 10 μg/L (48.26 nM) in food and water(Guo et al. 2015). Traditional analytical techniques have been researched for sensitive Pb^{2+} analysis, including classical atomic absorption spectrometry (AAS)(Barbosa et al.

2016; Behbahani et al. 2015), inductively coupled plasma mass spectrometry (ICP-MS)(Liang et al. 2000), inductively coupled plasma atomic emission spectrometry (ICP-AES)(Ochsenkühn-Petropoulou and Ochsenkühn 2001) and anodic stripping voltammetry (ASV)(Sun et al. 2017; Zhao et al. 2015). Although these methods are sensitive and accurate, they often require complicated sample pretreatment, multiple analysis steps and sophisticated equipment, hindering their application in on-site or real-time analysis, and thus, it is urgently desired to develop sensitive, selective, and economical Pb^{2+} on-site detection strategies.

DNAzymes are a kind of functional single-strand DNA or RNA, which have been successfully applied for the detection of metal ions(Huang et al. 2018; Li et al. 2018; Wang et al. 2016a; Yang et al. 2017; Yun et al. 2016; Zhang et al. 2017; Zhang et al. 2016; Zhou et al. 2016b), with their unique advantages such as chemical stability, low cost, selectivity and the capability of real-time analysis. As a classical model, the Pb^{2+}-specific DNAzyme is a double-strand DNA, that comprises of an enzyme strand and a substrate strand, and the substrate strand can be cleaved into two pieces in the presence of Pb^{2+} at the cleavage site that contains one ribonucleotide(Dalavoy et al. 2008; Liang et al. 2017; Zhu et al. 2014). In recent years, considerable research effort has been devoted to the development of DNAzyme-based Pb^{2+} biosensors, based on various detection techniques including fluorescence(Fu et al. 2016; Qian et al. 2015; Wen et al. 2016; Wen et al. 2011b), colorimetry(Kuo et al. 2015; Liu and Lu 2003; Yun et al. 2016), surface-enhanced Raman scattering (SERS)(Frost et al. 2015; Zhao et al. 2016), field effect transistor (FET)(Wang et al. 2016a) and electrochemical methods(Buica et al. 2017; Cui et al. 2015; Lin et al. 2011; Pelossof et al. 2012; Shen et al. 2008; Xiao et al. 2007; Yang et al. 2010).

Among all those DNAzyme-based biosensor platforms, electrochemical biosensors(Cui et al. 2015; Ge et al. 2016; Xiao et al. 2007) has been widely recognized with great potential in Pb^{2+} on-site biosensors, for its high sensitivity(Liu et al. 2008; Wang et al. 2017; Wen et al. 2013), low-cost(Hou et al. 2015), and most importantly, small-sized analysis equipment/chips(Liu et al. 2011; Liu et al. 2012; Liu et al. 2010; Wan et al. 2009). However, when utilized to electrochemical biosensor, the 3 dimension (3-D) conformation switch of the complex DNAzyme is usually hindered in the confined interface space on the traditional electrode surface(Lao et al. 2005). In order to construct an improved platform for the Pb^{2+} triggered DNAzyme reaction, an optimized interfacial engineer was essential, with regulable probe density, direction, and stability.

To improve the detection capability of Pb^{2+} biosensors, there is urgent necessity to develop better interfacial engineer. As proved by our previous work, a 3-D DNA tetrahedral nanostructure(Pei et al. 2010) showed remarkable capability of regulating the molecular distance and direction and improving the accessibility of the DNA probes, which played critical roles in many successful biosensors(Bu et al. 2011; Wen et al. 2012; Wen et al. 2011a).

In this work, we designed a 3-D DNA tetrahedral DNAzyme probe by adding a pendant substrate strand on a DNA tetrahedral nanostructure, and the substrate strand contained a G-rich fragment. During the analysis, a 2-step consequent conformational switch was performed: in the presence of Pb^{2+}, DNAzyme cleaved the substrate strand and released the G-rich part which subsequently combined with hemin and formed a G-quadruplex/hemin structure for an electrochemical catalysis signal. By using the G-quadruplex/hemin electrocatalytic reaction, we designed a promising Pb^{2+}

biosensor without any covalent labeled probe, which we believe is critical for the development of the Pb^{2+} electrochemical biosensors.

2. Experimental Section

2.1 Materials

All oligonucleotides were synthesised and purified by TaKaRa Biotech. Co., Ltd. (Dalian, China), and the DNA sequences are shown in Table 1. Tetra-A was the Pb^{2+}-specific substrate of DNAzyme, rA represents an adenosine ribonucleotide. $Pb(NO_3)_2$, $Cu(NO_3)_2$, $Zn(NO_3)_2$, $Cd(NO_3)_2$, $Co(NO_3)_2$, $Mn(NO_3)_2$, $Ni(NO_3)_2$, $Mg(NO_3)_2$, $Hg(NO3)2$, $Ca(NO3)2$, $BaCl_2$ were provided by Shanghai Institute of Measurement and Testing Technology (SIMT, Shanghai, China, 1 g/L). Hemin was purchased from Frontier Scientific, Inc. (Logan, UT, USA). HEPES (4-(2–Hydroxyethyl)-1-piperazineethane sulfonic acid) was from Aldrich-Sigma (U.S.A.). All solutions were prepared with Milli-Q water (18 MΩcm resistivity). Environmental water samples were taken from the pools and tap water in SIMT and treated with a 0.45 μm membrane filter to remove the insoluble impurities.

Table 1, DNA sequence information

Probe Name	**Sequence (5′-3′)**
Tetra-A	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAA GAGCCGCCATAGTATTTGGGTTGGGCGGGATGGGTTTCACT rAGGCACTTTGGTC
Tetra-B	SH-C_6-TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAAT AGATGCGAGGGTCCAATAC
Tetra-C	SH-C_6-TCAACTGCCTGGTGATAAAACGACACTACGTGGGAA

	TCTACTATGGCGGCTCTTC
Tetra-D	SH-C_6-TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTT TGTATTGGACCCTCGCAT
Pb-DNAzyme	CCAAAGTGCTCCGAGCCGGTCGAAGTGAAACC

2.2 Construction of the Pb^{2+}-specific tetrahedral nanostructure-based gold electrode surfaces

Gold electrodes were cleaned following a reported work(Zhang et al. 2007), and then, the Pb^{2+}-specific tetrahedral nanostructure-based substrate (sub-TSP) were produced as reported in our previous works(Bu et al. 2011; Wen et al. 2012; Wen et al. 2011a). DNA sequences for the construction of the tetrahedron were listed in table 1 (Tetra-A, Tetra-B, Tetra-C and Tetra-D), and for the probe assembling, 3 μL of sub-TSP probes (1 μM) were pipetted to a cleaned gold electrode and incubated overnight under room temperature.

2.3 Pb^{2+} DNAzyme reaction

Firstly, certain concentration of Pb^{2+} solution was mixed with 1 μM Pb-DNAzyme (table 1) in 10 mM HEPES buffer containing 1 M $NaNO_3$ and 20 mM $MgCl_2$ (pH 7.0). Secondly, the sub-TSP modified gold electrode (section 2.2) was immersed into 100 μL of above mixture in a 2 mL microtube (Axygen), and then heated to 60 °C for 5 min, and then cooled down to room temperature and incubated for 2 h, for the DNAzyme cleavage reaction. Finally, the gold electrode was rinsed

with 10 mM HEPES buffer and dried lightly with N_2, and then subjected to electrochemical measurement.

2.4 Electrochemical Measurement

Electrochemical measurement was performed in 10 mM HEPES buffer (50 mM $NaNO_3$, 100 mM KCl, pH 7.0) containing 1 mM H_2O_2 and 1 μM hemin. A CHI 630 electrochemical workstation (CH Instruments Inc., USA) and a conventional three-electrode configuration was employed all through the experiment, that involved a platinum wire auxiliary electrode, a gold working electrode, and an Ag/AgCl (3 M KCl) reference electrode. Cyclic voltammetry (CV) was carried out with scan rate of 100 mV/s.

3. Results and Discussions

3.1 The Principle of the 2-step label-free Pb^{2+} biosensor

We here report a sensitive Pb^{2+} electrochemical biosensor by using a novel Pb^{2+} specific DNAzyme (Pb-DNAzyme) on a DNA tetrahedral nanostructure, the novel Pb-DNAzyme consisted of two parts: a DNAzyme strand and a substrate strand on top of a DNA tetrahedral nanostructure (sub-TSP). The substrate strand contained a G-rich sequence which could form a G-quadruplex structure and generate a catalysis current when combined with a hemin. As shown in figure 1, when the DNAzyme strand hybridized to the substrate, the G-rich fragment was locked and thus inhibited the formation of the G-quadruplex. structure. In the presence of Pb^{2+} ions, the sub-TSP was cleaved at the rA site, and thus released the G-rich strand to combine with a hemin forming a G-quadruplex structure, which could then generate an

enhanced electrochemical current signal through the catalytically reduction of H_2O_2.

By using the DNA tetrahedral nanostructure as a base of the DNA probe, a well-regulated surface interfacial was achieved: the rigid TSP strongly supported the probe in an up-right orientation, which is important for the 2-step DNA conformational switch, what is more important, the side length of the TSP (D_{TSP}) was about 5.8 nm which is larger than the dimension of the G-quadruplex/hemin (D_{G4} less than 2 nm), in this situation, adequate molecular distance was provided for the conformation of G-quadruplex/hemin structure.

3.2 Characterization of the G-quadruplex/hemin complex based Pb^{2+} biosensor

Firstly, the construction of our biosensor was verified by CV in 10 mM HEPES buffer (50 mM $NaNO_3$, 100 mM KCl, pH 7.0) with 1 μM hemin. As shown in figure 2A, dramatically enhanced electrocatalytic reduction peak of H_2O_2 was found in the presence of Pb^{2+} (100 nM) and H_2O_2 (1 mM) (Fig 2A d), indicating the DNAzyme cleavage of the probe and the formation of the G-quadruplex/hemin structure, on the contrary, only negligible current signal was observed in the absence of Pb^{2+} (Fig 2A a and b), which demonstrated the specificity of the DNAzyme reaction. Interestingly, the reduction peak decreased distinctively without H_2O_2 (Fig 2A c) under exactly the same condition, which further proved a fact that the reduction peak at -200 mV was from the reduction of H_2O_2.

3.3 Optimization of the detection conditions

Several crucial experiment parameters that would impact the specific interaction

between DNAzyme and Pb^{2+} were investigated, including the cleavage time, the reaction temperature and the hemin concentration. As figure 2B shows, when 100 nM Pb^{2+} was analyzed, the peak current signal increased when the reaction time was increased, until reached a steady state at 2 hours, thus 2-hour was chosen as the optimum cleavage time in the following experiment.

Then, the DNAzyme cleavage was performed under 3 different temperatures: 20 °C, 25 °C and 37 °C, and the signal increase (ΔI, figure 2) from 100 nM Pb^{2+} was compared, as figure 2C shows, the ΔI at 25 °C was about 4 times higher than 20 °C and 2 times higher than 37 °C, demonstrating that 25 °C is the best reaction temperature for an optimized signal-to-noise ratio.

Another key condition is the amount of hemin, which was designed to insert into the G-quadruplex structure and show electrochemical catalyzing capability. As shown in figure 2D, when we compared the current signal while increasing the hemin concentration from 0.1 μM to 1 μM, ΔI increased accordingly until reached a plateau at 1 μM, which indicated the saturation concentration of hemin. Thus, we chose 1 μM as the optimal concentration in all our following analysis.

3.4 Sensitivity of the Pb^{2+} biosensor

Under optimal conditions, we investigated the sensitivity of the Pb^{2+} biosensor based on DNA nanostructure and G-quadruplex/hemin complex, by analyzing a series of Pb^{2+} samples with concentration ranged between 0.01 nM to 1000 nM.

As anticipated, when the concentration of Pb^{2+} was increased, the reduction peak located at -0.2 V (vs. Ag/AgCl) apparently increased (Figure 3A), demonstrating a typical electrocatalysis reduction of H_2O_2. As a result, the detection signal for 0.01 nM Pb^{2+} could be distinguished from the background (Figure 3B), and the electrocatalytic cathodic currents showed a linear relation with the logarithm concentration of Pb^{2+} from 0.01 nM to 1000 nM: $I=297\times\log[Pb^{2+}]+873$, where $[Pb^{2+}]$ is the concentration of Pb^{2+}. Finally, the limit of detection (LOD) of our Pb^{2+} biosensor is calculated to be 0.008 nM (3σ), which is quite competitive in reported electrochemical Pb^{2+} sensors based on DNAzyme (Table 2).

Table 2, Comparison of similar Pb^{2+} sensors

Detection Methods	Label style	Detection Limt	Reference
DNAzyme-based electrochemical sensor	MB	0.3 μM	(Xiao et al. 2007)
DNA functionalized porphyrinic metal−organic framework based electrochemical sensor	iron−porphyrinic metal−organic framework	0.034 nM	(Cui et al. 2015)
DNAzyme based fluorescence biosensor	FAM	0.53 nM	(Guo et al. 2015)
Fluorescent sensing based on DNAzyme and ThT/G-quadruplex	Label free	0.06 nM	(Wen et al. 2016)
Colorimetric sensor using molecular beacon and DNAzyme	Label free	0.02 nM	(Yun et al. 2016)
Colorimetric biosensor using DNAzyme-directed assembly of	gold nanoparticles	100 nM	(Liu and Lu 2003)

gold nanoparticles			
Single strand DNAzyme and G-quadruplex/hemin based electrochemical sensor	Label free	0.1 nM	(Pelossof et al. 2012)
DNA nanostructure DNAzyme and G-quadruplex/hemin based electrochemical sensor	Label free	0.008 nM	This work

3.5 The specificity and reproducibility of the proposed biosensor

To challenge the specificity of our as-prepared Pb^{2+} biosensor, several environmentally relevant divalent metal ions including Hg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Ba^{2+}, Cu^{2+}, Cd^{2+}, Mg^{2+}, Zn^{2+}, Hg^{2+} and Ca^{2+} were analyzed under the same analysis condition. As shown in Figure 4, ΔI for 100 nM Pb^{2+} was about 1210 nA, while the unspecific ions produced much lower ΔI, which demonstrated the selectivity of our Pb^{2+} biosensor.

3.6 Practicability of the Pb^{2+} biosensor

Finally, the practicability of the Pb^{2+} biosensor was studied. First, we investigated the reproducibility of the as-prepared Pb^{2+} biosensor, by performing the same analysis experiment on 6 different gold electrodes independently. The results indicated that the relative standard deviation (RSD) was 7.1% at 100 pM and 3.8% at 100 nM. Then, the stability of our Pb^{2+} biosensor was challenged by storing the DNA

probe assembled electrodes for 7 days under 4 °C before being subject to the specific DNAzyme reaction and electrochemical analysis.

In order to demonstrate the applicability and reliability of the proposed biosensor when facing real samples, tap water and pool water were analyzed. Water samples were collected from the tap-water and a pool, and then filtered with a 0.45 μm membrane filter to remove the insoluble impurities, and then, recovery test was carried out by analyzing the real samples with the addition of certain amount of Pb^{2+}. High resolution inductively coupled plasma mass spectrometer (HR-ICP-MS) was applied at the same time as a verification for each sample. As shown in table 2, before the addition of Pb^{2+}, the analysis results were 0.63 nM and 0.03 nM respectively in tap water and pool water, and after the addition of 1 nM Pb^{2+}, the analysis results increased to 1.56 nM and 0.95 nM, which were quite consistent with the results from HR-ICP-MS, and the recovery in tap water and pool water were calculated to be 93% and 92%, strongly demonstrated the practicability of our Pb^{2+} biosensor when applied in environment water analysis.

4. Conclusions:

In conclusion, we have developed a novel analysis strategy using specific DNAzyme reaction for the sensing of Pb^{2+}, in the presence of Pb^{2+}, the substrate strand was cleavaged into two parts and thus released a “G-rich” oligo which subsequently formed a G-quadruplex/hemin complex, generating a detectable catalysis current signal with the assistant of H_2O_2. A key challenge of this work is to perform a complex “two-step” DNA conformational change in the confined space on

the gold electrode surface, thus, we designed a 3-D DNA tetrahedral base for the DNAzyme probe, which better controlled the density and orientation of the probe to improve the DNAzyme reaction. Several critical experiment conditions were systematically optimized, such as cleavage time, reaction temperature and the hemin concentration, and the practicability of our Pb^{2+} biosensor was investigated by analyzing tap water and pool water. Finally, the LOD of our biosensor was demonstrated to be 8 pM (3σ), which is at least 9000 times lower than the safety limit of EPA (15 μg/L or 72 nM), and nearly 6000 times lower than that of IARC (10 μg/L or 48.26 nM). The DNAzyme triggered G-quadruplex/hemin biosensor was proved to be simple, rapid and convenient for Pb^{2+} detection, and the sensitivity (0.008 nM) exceeded most of the previously reported Pb^{2+} sensors by several orders of magnitude (Table S1).

Acknowledgments:

This work was financially supported by National Natural Science Foundation of China (No. 21775104), the National Quality Infrastructure Program of China (2017YFF0204605), and Shanghai Rising-Star Program (16QB1403100).

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Figure 1, Illustration of the label-free Pb^{2+} electrochemical biosensor based on the Pb-DNAzyme triggered G-quadruplex/hemin on a DNA tetrahedran. D_{G4} represents the side length of hemin/G-quadruplex structure, and the D_{TDP} represents the dimension of TSP.

Figure 2, (A) CV results of our biosensor in the presence of (a) 0 M Pb^{2+} with 1 mM H_2O_2 and (b) 0 M Pb^{2+} without H_2O_2, and (c) 100 nM Pb^{2+} without H_2O_2 and (d) 100 nM Pb^{2+} with 1 mM H_2O_2. The analysis was performed in 10 mM HEPES buffer (50 mM $NaNO_3$, 100 mM KCl, pH 7.0), and the optimization results of (B) incubation time for DNAzyme reaction, (C) incubation temperature, and (D) concentration of hemin. $\Delta I=I-I_0$, and I_0 was the blank current, while I was the current signal achieved by 100 nM target Pb^{2+} under different conditions.

Figure 3. (A) CV results of Pb^{2+} analysis with the concentrations as following: 0, 0.01 nM, 0.1nM, 1 nM, 10 nM, 100 nM, 1000 nM from top to bottom. (B) Logarithmic plot of electrocatalytic cathodic currents to the concentrations of Pb^{2+}, the error bars represent standard deviations for measurements taken from at least three independent experiments. The peak current was taken for quantification at -0.2 V (vs Ag/AgCl).

Figure 4. Specificity of the Pb^{2+} biosensor based on 3-D DNAzyme and G-quadruplex/hemin complex. The concentration of all the ions was 100 nM.

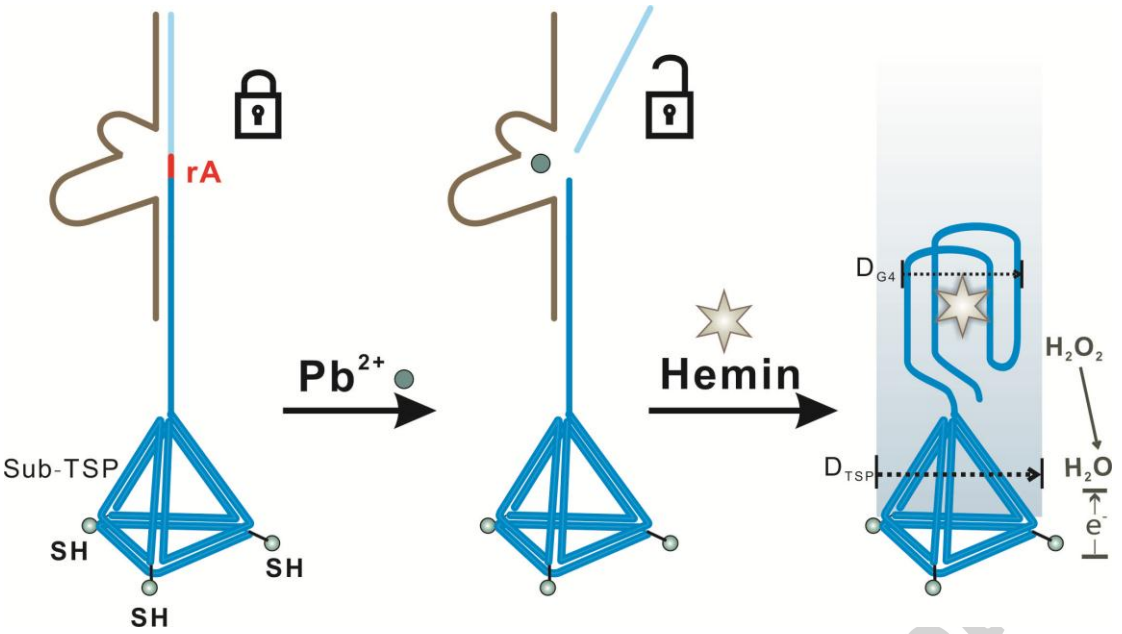

Fig. 1.

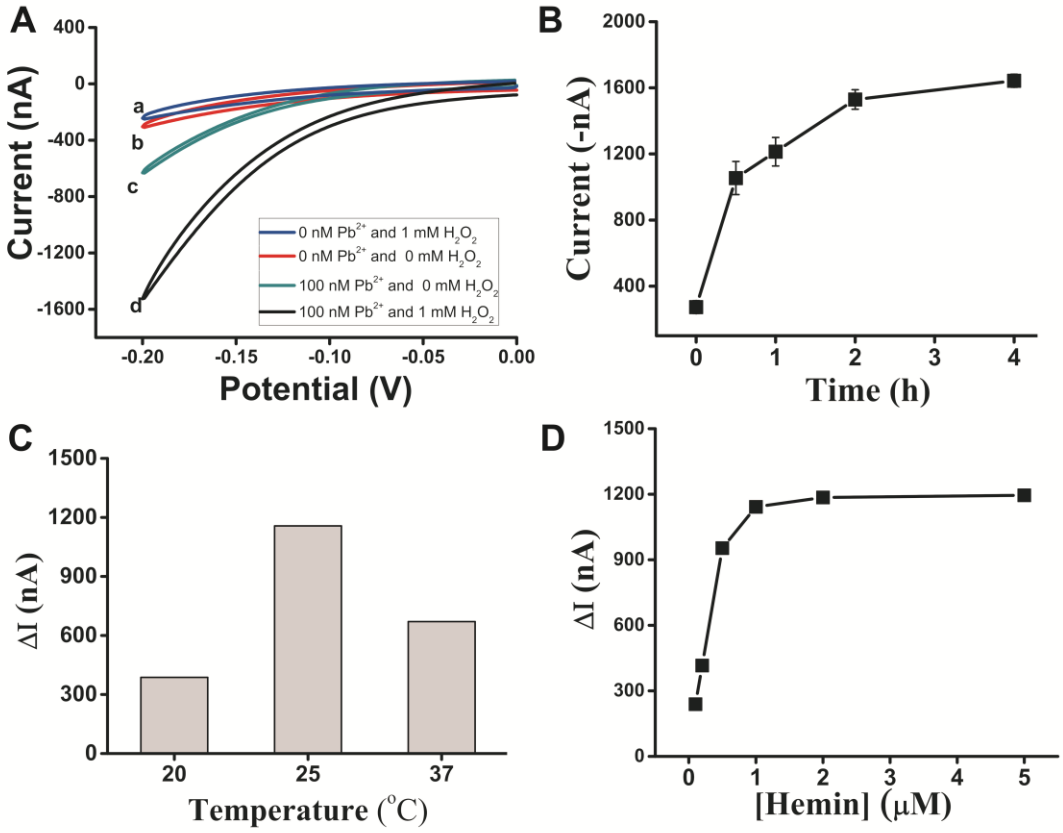

Fig. 2.

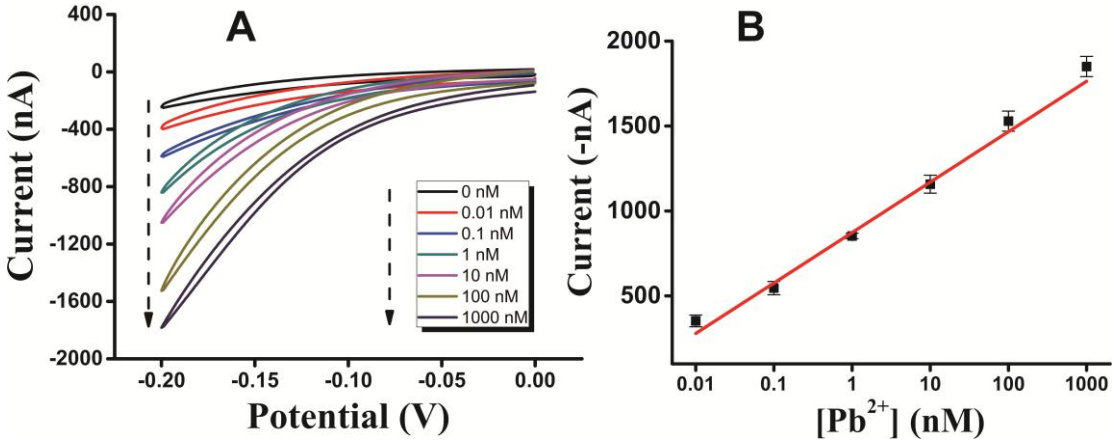

Fig. 3.

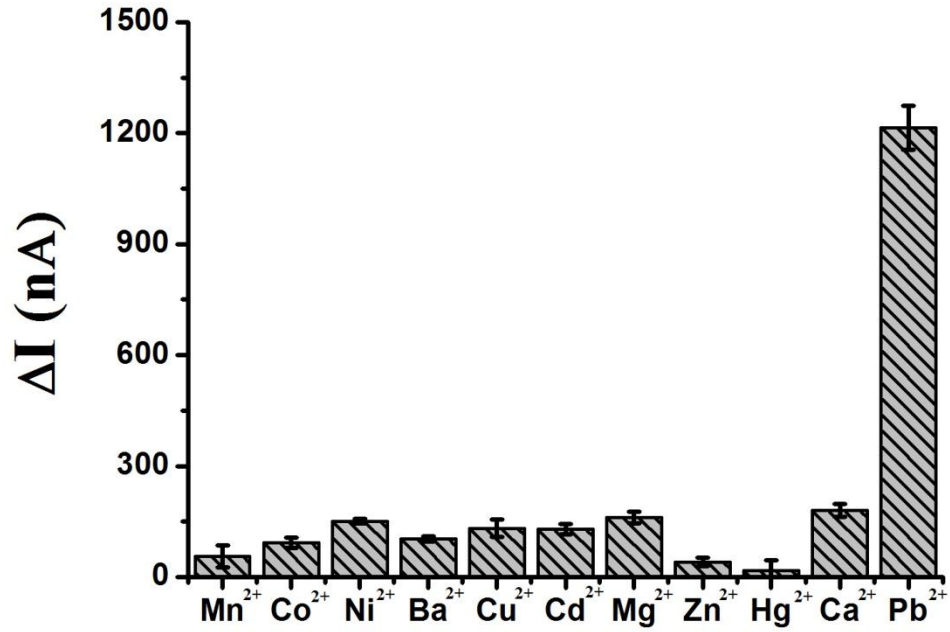

Fig. 4.

Highlights:

1 We developed a novel Pb^{2+} electrochemical biosensor using the specific DNAzyme on a DNA tetrahedron probe;

2 Pb^{2+} triggered the DNAzyme reaction and realized the G-rich fragment to form a G-quadruplex/hemin structure.

3 DNA tetrahedron regulated the density and orientation of the probe and thus improved the DNA conformation switch;

4 the LOD of our biosensor was proved to be 0.008 nM