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Electrochemistry

Sensitive pseudobienzyme electrocatalytic DNA biosensor for mercury(II) ion by using the autonomously assembled hemin/G-quadruplex DNAzyme nanowires for signal amplification

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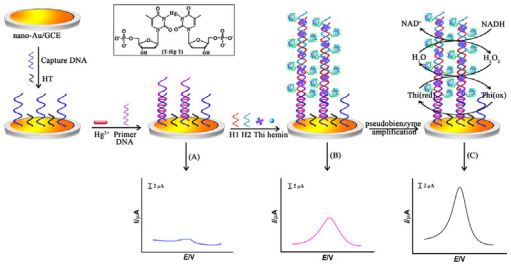
HIGHLIGHTS

- An ultrasensitive detection system for Hg^{2+} detection was presented.
- The autonomously assembled hemin/G-quadruplex DNAzyme nanowires were employed.
- The DNAzyme simultaneously served as an NADH oxidase and HRP-mimicking DNAzyme.
- The DNAzyme nanowires served as carrier for loading substantial redox probe Thi.

GRAPHICAL ABSTRACT



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ABSTRACT

Herein, a novel sensitive pseudobienzyme electrocatalytic DNA biosensor was proposed for mercury ion (Hg^{2+}) detection by using autonomously assembled hemin/G-quadruplex DNAzyme nanowires for signal amplification. Thiol functionalized capture DNA was firstly immobilized on a nano-Au modified glass carbon electrode (GCE). In presence of Hg^{2+}, the specific coordination between Hg^{2+} and T could result in the assembly of primer DNA on the electrode, which successfully triggered the HCR to form the hemin/G-quadruplex DNAzyme nanowires with substantial redox probe thionine (Thi). In the electrolyte of PBS containing NADH, the hemin/G-quadruplex nanowires firstly acted as an NADH oxidase to assist the concomitant formation of H_2O_2 in the presence of dissolved O_2. Then, with the redox probe Thi as electron mediator, the hemin/G-quadruplex nanowires acted as an HRP-mimicking DNAzyme that quickly bioelectrocatalyzed the reduction of produced H_2O_2, which finally led to a dramatically amplified electrochemical signal. This method has demonstrated a high sensitivity of Hg^{2+} detection with the dynamic concentration range spanning from 1.0 ng L^{-1} to 10 mg L^{-1} Hg^{2+} and a detection limit of 0.5 ng L^{-1} (2.5 pM) at the $3S_{blank}$ level, and it also demonstrated excellent selectivity against other interferential metal ions.

1. Introduction

Water-soluble mercury(II) ions (Hg^{2+}) are highly toxic environmental pollutants and harmful to human health. They can accumulate in the vital organs and tissues with combination to the sulfur-containing proteins and enzymes, which may result in brain damage, kidney failure, and various cognitive and motion disorders [1–5]. Up to now, substantial classical methods, such as atomic absorption spectroscopy [6], inductively coupled plasma optical emission spectrometry [7], and inductively coupled plasma mass spectrometry [8], have been widely used for Hg^{2+} detection. These methods, although sensitivity with low detection limits, are labor-intensive and require sophisticated instrumentation, skilled personnel as well as time-consuming sample pretreatment, which

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prohibit on-line and real-time monitoring [9]. Recently, DNA has attracted extremely attention as an emerging material for Hg^{2+} detection. It is reported that Hg^{2+} can be bind specifically with two DNA thymine bases (T) to form a stable T–Hg^{2+}–T complexes [10,11]. Therefore, substantial T–Hg^{2+}–T based detection systems, such as fluorescence [12,13], electrochemistry (EC) [14], electrochemiluminescence [15] and colorimetry [16–19] have been used for Hg^{2+} detection. Among them, EC-based biosensor has attracted particular attention owing to its high sensitivity, inherent simplicity, low cost, and excellent compatibility to miniaturization technology [20].

To obtain a high sensitivity, the EC-based biosensor is commonly assisted with signal amplification technique. The recently typical one is the hybridization chain reaction (HCR), which is a real protein-free isothermal amplification technique with a DNA fragment serves as an initiator and triggers a cascade of hybridization events with two ssDNA to simply in suit yield a long DNA nanowires [21,22]. In that case, a great deal of electrochemically active compound can be loaded *via* electrostatic adsorption or labeling with an amplified signal output. In order to further improve the sensitivity, this amplification technique is coupled with the enzyme amplification, which not only entails the enzymatic reaction taking place in the close vicinity of the biosensor but also allows its amplification according to the catalytic activity of the enzyme, providing the possibility to achieve high sensitivity [23]. However, the extra enzyme modification or conjugation is rather costly, time-consuming and sophisticated [24,25]. Therefore, it is necessary to explore a new method to solve this problem. The emerging of G-quadruplex DNAzymes (DNAzymes), which formed by the interlacing of hemin into a single-stranded guanine-rich nucleic acid and mimics the protein enzyme activity, may successfully resolve this issue [26]. If properly designed, the DNAzyme can *in situ* be propagated during the formation of HCR-induced long DNA nanowires with a simple operation and high protein enzyme-like activity.

It has been reported that the hemin/G-quadruplex DNAzyme can simultaneously serve as an NADH oxidase and HRP-mimicking DNAzyme [27]. Here, based on the above mentioned observation, we proposed a sensitive pseudobienzyme electrocatalytic DNA biosensor for Hg^{2+} detection by using the autonomously assembled hemin/G-quadruplex DNAzyme nanowires for signal amplification. The capture DNA was firstly immobilized on the surface of nano-Au modified glass carbon electrode (GCE). Owing to the coordination chemistry for the T–Hg^{2+}–T complexes, the primer DNA could be assembled on the electrode in the presence of target Hg^{2+}, which successfully triggered the HCR to form the hemin/G-quadruplex DNAzyme nanowires with substantial redox probe thionine (Thi). In the electrolyte of 0.1 M PBS (pH 7.0) containing NADH, the hemin/G-quadruplex nanowires firstly acted as an NADH oxidase to assist the concomitant formation of H_2O_2 in the presence of dissolved O_2. Then, with the redox probe Thi as electron mediator, the hemin/G-quadruplex nanowires acted as an HRP-mimicking DNAzyme that quickly bioelectrocatalyzed the reduction of the produced H_2O_2, which finally led to a dramatically amplified electrochemical signal. The results demonstrated that the developed pseudobienzyme electrocatalytic DNA biosensor could substantially improved the sensitivity for Hg^{2+} detection compared with the reported work.

2. Experimental

2.1. Chemicals and material

Nicotinamide adenine dinucleotide (NADH), thionine (Thi), hexanethiol (96%, HT), hemin and chloroauric acid ($HAuCl_4$) were obtained from Sigma-Aldrich (St. Louis, MO). Tris-hydroxymethylaminomethane hydrochloride (tris) was purchased from Roche (Switzerland). All the oligonucleotides used in the present study were synthesized and purified by HPLC by Shanghai Shenggong Biotechnology Co. (Shanghai, China) and used without further purification.

Capture DNA 5′-GGT TGG TGT GGT TGG-$(CH_2)_6$-SH-3′

Primer DNA 5′-GGT TGG TGT GGT TGG AGA AGA AGG TGT TTA AGT A-3′

Auxiliary single-strand (H_1) 5′-AGG GCG GGT GGG TGT TTA AGT TGG AGA ATT GTA CTT AAA CAC CTT CTT CTT GGG T-3′

Auxiliary single-strand (H_2) 5′-TGG GTC AAT TCT CCA ACT TAA ACT AGA AGA AGG TGT TTA AGT TGG GTA GGG CGG G-3′

DNA stock solutions were obtained by dissolving oligonucleotides in 20 mM Tris–HCl buffer (pH 7.4) containing 140 mM NaCl, 5 mM KCl and 1 mM $MgCl_2$. A quantity of 0.10 M sodium phosphate buffer (PBS, pH 7.0) containing 3.0 mM NADH was employed to investigate the performance of electrodes. All other chemicals were of reagent grade and used as received. Double distilled water was used throughout this study.

2.2. Apparatus

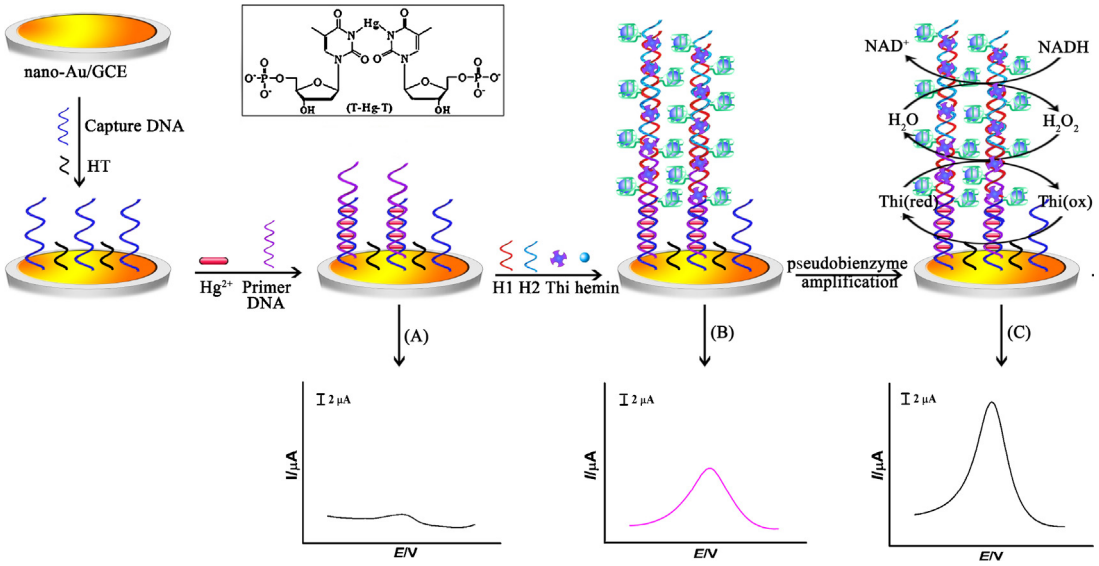
Electrochemical experiments, including electrochemical impedance spectroscopy (EIS) and differential pulse voltammograms (DPV), were carried out with a CHI 660D electrochemistry workstation (Shanghai CH Instruments, China) by using a bare or modified glassy carbon electrode as working electrode (GCE, Φ = 4 mm), a platinum wire as auxiliary electrode, and a saturated calomel electrode (SCE) as reference electrode. The value of pH was come out by pH meter (MP 230, Mettler-Toledo, Switzerland).

2.3. Preparation of DNA biosensor

Initially, the bare GCE was polished carefully with 0.3 and 0.05 μm alumina slurries and washed ultrasonically in double distilled water and ethanol for a few minutes. Then, in order to assemble the capture DNA, the cleaned GCE was electrodeposited a gold nanoparticles (nano-Au) layer from an aqueous solution of 1% $AuCl_4^-$ (ω%) at a constant potential of −0.2 V for 30 s. Then, the nano-Au/GCE was immediately followed by a 16 h incubation at 25 °C with 20 μL of 1.0 μM capture DNA. After thoroughly washing with buffer solution, 20 μL of 1.0 mM HT solution was dropped on the capture DNA/nano-Au/GCE surface and incubated for 40 min to eliminate nonspecific binding effects and obtain an optimal nucleic acid orientation. All resulting electrodes were rinsed with the washing buffer solution and stored at 4 °C until use. The schematic illustration of the DNA biosensor was dipalyed in Scheme 1.

2.4. Detection of Hg^{2+} by electrochemical measurement

Stock solutions of H_1 and H_2 were, respectively, heated to 95 °C for 5 min and then allowed to cool to room temperature (25 °C) to form hairpin structure at least for 2 h. The annealed hairpins were then stored in the refrigerator (4 °C) before use. For the analysis of Hg^{2+}, 30 μL mixture containing 1 μM primer DNA and varying concentrations of Hg^{2+} were dropped onto the prepared HT/capture DNA/nano-Au/GCE electrode and incubated for 30 min. In order to form the hemin/G-quadruplex DNAzyme nanowires with abundant redox probe Thi, the 30 μL mixture containing 1 μM H_1, 1 μM H_2, 0.25 mM redox probe Thi and 0.15 mM hemin was then coated on the obtained primer DNA/HT/capture DNA/nano-Au/GCE electrode and incubated for 2 h at 25 °C. Then, the electrochemical determinations were performed at room temperature in 1 mL PBS (pH 7.0) containing 3.0 mM NADH. The DPV measurement with the



Scheme 1. Schematic illustration of the sensitive pseudobienzyme electrocatalytic DNA biosensor for Hg^{2+} detection by using the autonomously assembled hemin/G-quadruplex DNAzyme nanowires for signal amplification.

potential range from −0.5 to 0.1 V, modulation amplitude 0.05 V, pulse width 0.06 s, and sample width 0.02 s was taken.

3. Results and discussion

3.1. The EIS characterization of the stepwise modified electrodes

The fabricating process of the proposed Hg^{2+} DNA biosensor was confirmed by EIS and the analysis was performed in 0.1 M PBS containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ (pH 7.0) (Fig. 1). The impedance spectrum of the bare GCE displayed a very small semicircle domain (curve *a*). After the electrodeposition of the nano-Au, a much smaller semicircle could be observed owing to the superior conduction of nano-Au (curve *b*). However, the assembly of capture DNA formed a negatively charged interface, which may electrostatically repel the negatively charged redox species $[Fe(CN)_6]^{3-/4-}$ with an obviously increased diameter of the semicircle (curve *c*). After the employment of inert HT to block nonspecific sites, a further increase of diameter of the semicircle was acquired (curve *d*). It has been reported that Hg^{2+} can stabilize the DNA duplex containing mismatched T–T base pair *via* the formation of a T-Hg^{2+}-T complex, therefore, there was an increased diameter of semicircle after the HT/capture DNA/nano-Au/GCE electrode incubated with

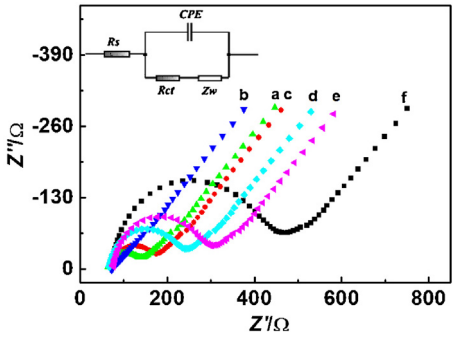


Fig. 1. Nyquist plots of bare GCE (a), nano-Au modified GCE (b), capture DNA/nano-Au/GCE (c), HT/capture DNA/nano-Au/GCE (d), HT/capture DNA/nano-Au/GCE after incubated with 1 μM primer DNA and 100 μg L^{-1} Hg^{2+} (e), and the obtained electrode after treated with HCR (f). EIS experiments were conducted in 0.1 M PBS solution containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$.

100 μg L^{-1} Hg^{2+} and primer DNA (curve *e*). Finally, there was also an increased diameter of semicircle after the assembly of hemin/G-quadruplex DNAzyme nanowires *via* hybridization (curve *f*).

To investigate the kinetic parameters of the modified electrode, CVs of HT/capture DNA/nano-Au/GCE electrode at different scan rates were recorded and the results were shown in Fig. 2A. As illustrated, a well-defined redox couple was observed corresponding to redox probe $[Fe(CN)_6]^{3-/4-}$. The peak currents of the redox system were directly proportional to the square root of scan rate for sweep rates below 150 mV s^{-1} (Fig. 2B), indicating a diffusion controlled redox process.

3.2. The amplification properties of the hemin/G-quadruplex DNAzyme nanowires and pseudobienzyme electrocatalytic system

In our work, the signal amplification was derived from the hemin/G-quadruplex DNAzyme nanowires and pseudobienzyme electrocatalytic system with the hemin/G-quadruplex simultaneously acting as an NADH oxidase and HRP-mimicking DNAzyme. To demonstrate the amplifying action, a comparison was made by constructing various biosensors obtained in different working buffers. Fig. 3A displayed the DPV response of the HT/capture DNA/nano-Au/GCE electrode incubated with 30 μL mixture containing 1 μM primer DNA, 0.25 mM redox probe Thi and 100 μg L^{-1} Hg^{2+}. As can been seen that a low current peak was obtained in PBS (pH 7.0), which could ascribe to the electrostatic adsorption of redox probe Thi onto the DNA duplex. After the primer DNA triggered the HCR to form the hemin/G-quadruplex DNAzyme nanowires, an obviously increase of peak current could be seen, indicating that the hemin/G-quadruplex DNAzyme nanowires can successfully load abundant redox probe Thi (Fig. 3B). Additionally, the HT/capture DNA/nano-Au/GCE electrode treated with primer DNA and HCR was also investigated in PBS (pH 7.0) containing 3.0 mM NADH. The peak current increased largely (Fig. 3C) compared with the electrode investigated in PBS only (Fig. 3B), suggesting the feasibility of proposed pseudobienzyme amplification strategy as expected. On the basis of these results, we might make the conclusion that the hemin/G-quadruplex DNAzyme nanowires and pseudobienzyme electrocatalytic system could be utilized for amplifying the electrochemical signal.

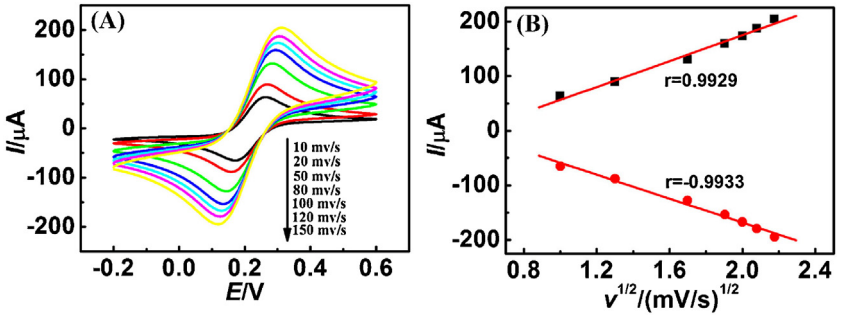


Fig. 2. (A) CV responses of HT/capture DNA/nano-Au/GCE in 0.1 M PBS solution containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$ at scan rates (from inner to outer) 10, 20, 50, 80, 100, 120 and 150 mV s^{-1}. (B) The corresponding plot of peak current *vs.* square root of scan rate.

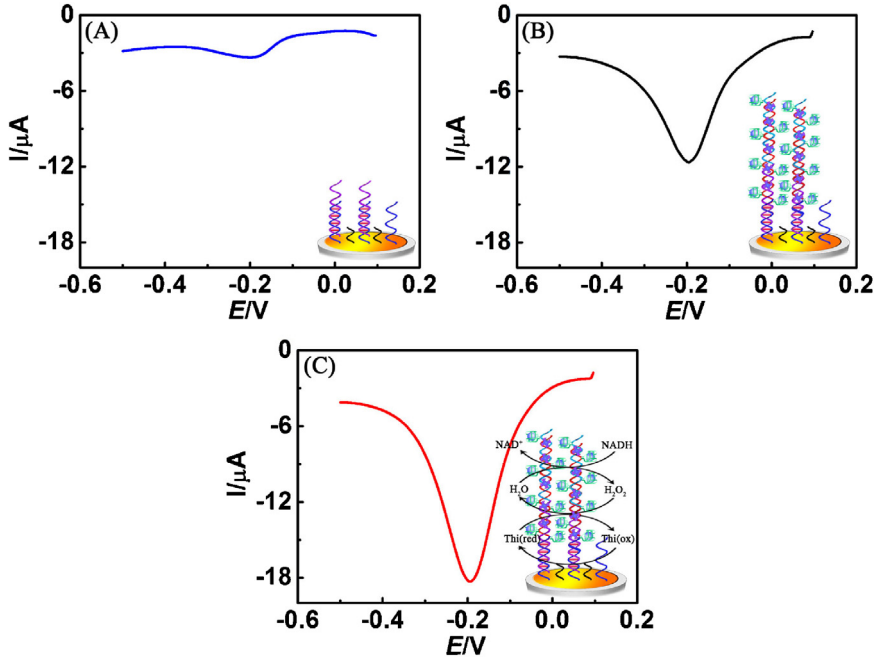


Fig. 3. The DPVs of various electrodes obtained in different working buffer (A) was the HT/capture DNA/nano-Au/GCE after treated with 1 μM primer DNA, 0.25 mM redox probe Thi and 100 μg L^{-1} Hg^{2+}, and investigated in 0.1 M PBS (pH 7.0); (B) and (C) were the HT/capture DNA/nano-Au/GCE after treated with HCR, 0.25 mM redox probe Thi and 100 μg L^{-1} Hg^{2+}, and investigated in 0.1 M PBS without and with 3.0 mM NADH, respectively.

3.3. Analytical performance of electrochemical DNA biosensor

The sensitivity and dynamic range of the as-prepared DNA biosensor were evaluated with Hg^{2+} standards. The DPV measurement was carried out in 0.1 M PBS (pH 7.0) containing 3.0 mM NADH. As shown in Fig. 4A, the peak currents increased with the increasing Hg^{2+} concentration in the range of 1.0 ng L^{-1} to 10 mg L^{-1}. And a linear relationship between the DPV peak currents and the logarithm of Hg^{2+} concentrations was observed (Fig. 4B). The detection limit of 0.5 ng L^{-1} was then evaluated using a signal three-fold the background noise. Such a low detection limit was obviously lower than those of other T–Hg^{2+}–T based biosensors recently developed (Table 1), indicating that the present DNA biosensor with the amplification of hemin/G-quadruplex DNAzyme

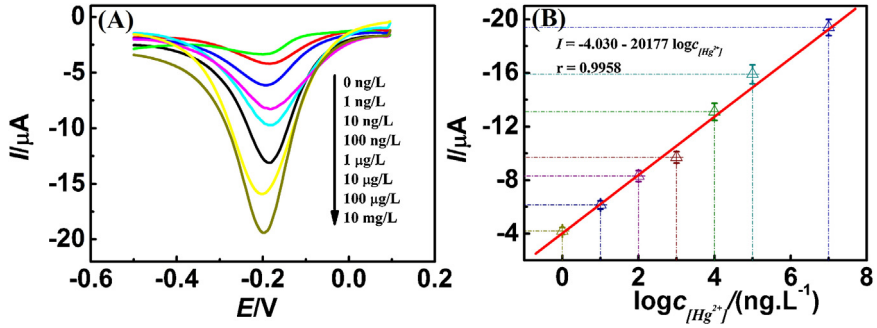


Fig. 4. (A) DPV responses and (B) the corresponding calibration curve acquired at the HT/capture DNA/nano-Au/GCE electrode treated with varying concentrations of Hg^{2+}, primer DNA and HCR. DPV experiments were conducted in 0.1 M PBS solution containing 3.0 mM NADH.

Table 1
Comparisons of proposed DNA biosensor with other reported methods for Hg^{2+} detection.

Analytical method	Detection limit	Linear range	Ref.
Fluorescence	0.92 nM	1–50 nM	[28]
EIS	100 pM	1 nM–1 mM	[29]
DPV	1 nM	8.0–100 nM	[9]
DPV	2.5 nM	5.0 nM–1.0 mM	[30]
Chronoamperometry	500 pM	1 nM–1 μM	[31]
Chemiluminescence	500 pM	1 nM–10 μM	[32]
Magnetic resonance imaging	300 pM	1.5–225.7 nM	[33]
DPV	0.5 ng L^{-1} (2.5 pM)	1 ng L^{-1}–10 mg L^{-1} (5 pM–50 μM)	Our work

nanowires and pseudobienzyme electrocatalytic system possessed a superior sensitivity and completely met the requirement of Hg^{2+} analysis.

3.4. Reproducibility, specificity, stability and regeneration of the DNA biosensor

The reproducibility of the electrochemical aptasensor was tested *via* the intra- and interassay coefficients of variation. We repeatedly assayed the Hg^{2+} with three different levels using the identical batch sensors. Experimental results revealed that the coefficients of variation of the intra-assay between 3 times were 6.1%, 5.9% and 8.2% for 1 ng L^{-1}, 10 μg L^{-1}, and 100 μg L^{-1} Hg^{2+}, respectively, whereas the coefficients of variation for the inter-assay with various batches were 8.9%, 6.9% and 7.6% toward the above mentioned analyte, showing that the proposed DNA biosensor had good reproducibility.

The influences of some other heavy metal ions including Zn^{2+}, Pb^{2+}, Mg^{2+}, Ni^{2+}, Fe^{3+}, Cd^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+} were tested under the same conditions as in the case of Hg^{2+}. As shown in Fig. 5, although the concentrations of Hg^{2+} were 50 times lower than other metal ions, respectively, the Hg^{2+} showed the highest peak current among the various metal ions studied. The satisfying result could ascribe to the specific recognition of T–T mismatch bases for Hg^{2+}.

Here, the stability of proposed DNA biosensor was investigated *via* the successive assays of 20 cycles using CV measurements in 0.10 M PBS (pH 7.0). As a result, the peak currents of the DNA biosensor treated with 100 μg L^{-1} Hg^{2+} and HCR decreased only 4.12% after the successive assays of 20 cycles, indicting acceptable stability of proposed DNA biosensor. Moreover, the storage stability of the proposed DNA biosensor was also investigated. After being stored in a refrigerator at 4 °C for 10 days, the proposed DNA biosensor retained 92.8% of its initial response, which also indicated the good stability of proposed DNA biosensor. Additionally, it reported that the ascorbic acid could reduce Hg^{2+} into Hg^{+}, which effectively reduced the coordination with T [34]. Therefore, the regeneration of the DNA biosensor could be obtained by washing in 100 mM ascorbic acid solution for 1 h. Then, the obtained DNA biosensor retreated with 100 μg L^{-1} Hg^{2+} and HCR. The results showed that less than ±5.2% change in current was observed from an DNA biosensor over six times regeneration.

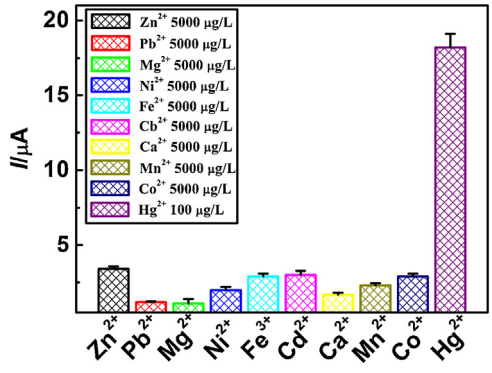


Fig. 5. Study of the possible interferences tested with the proposed DNA biosensor.

Table 2
Detection results of water samples spiked with Hg^{2+}.[a]

Samples	Added Hg^{2+} (ng L^{-1})	Found Hg^{2+} (ng L^{-1})[b]	RSD(%)	Recovery (%)
1	10	9.2	8.5	92
2	100	106	5.9	106
3	1000	982	6.2	98.2
4	10,000	10,320	7.3	103.2

RSD stands for Relative standard deviation.
[a] The DPV measurements were carried out within the range of −0.5–0.1 V in 0.1 M PBS (pH 7.0) containing 3.0 mM NADH.
[b] Calculated as a mean of three measurements.

3.5. Analysis of environmental water samples

The application of the DNA biosensor was evaluated *via* the determination of Hg^{2+} in river water (collected from Yangtze Gorges, China). And the standard addition method was used here. The sample was directly spiked with certain amounts of Hg^{2+} standard solution. As shown in Table 2, the proposed DNA biosensor displayed good consistencies between the actual and the measured Hg^{2+} concentrations, which confirmed that the proposed DNA biosensor was applicable for Hg^{2+} detection with enough precision and accuracy even in real environmental sample matrices.

4. Conclusions

In conclusion, we have designed a highly sensitive and selective pseudobienzyme electrocatalytic DNA biosensor for Hg^{2+} detection by using the autonomously assembled hemin/G-quadruplex DNAzyme nanowires for signal amplification. Owing to the specific coordination between Hg^{2+} and T, primer DNA thus could be assembled on the electrode, which successfully triggered the HCR to form the hemin/G-quadruplex DNAzyme nanowires with substantial redox probe Thi and further realized the pseudobienzyme electrocatalytic amplification. Our analytical strategy was simple, rapid, and cost-effective, and could easily detect pM level of Hg^{2+}. The limit of detection was far below the upper limit mandated by Environmental Protection Agency (EPA, 10 nM in drinking water). All of these observations demonstrated that the designed biosensor presented here could be extended toward the on-site monitoring of the other metal ions or trace pollutants in environmental matrices by using different DNA or aptamer probes.

Acknowledgments

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