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A novel label-free and sensitive electrochemical biosensor for Hg^{2+} based on ligase-mediated formation of DNAzyme

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ABSTRACT

A novel label-free electrochemical biosensor for the detection of Hg^{2+} based on ligase mediated creation of G-quadruplex-hemin DNAzyme has been developed. Firstly, Cp probe was immobilized on the gold electrode surface through Au–SH bond. In the presence of Hg^{2+}, Cp and Ap probes were partly hybridized with the LJ probe respectively through the specific T–Hg^{2+}–T interaction. Then, the adjacent 3′-OH terminal of Cp will link with the 5′-PO_4 terminal of Ap to form a G-rich DNA at the function of T4-ligase. After interaction with hemin, the G-rich DNA can form a G-quadruplex–hemin HRP-mimicking DNAzyme. Through measuring the current change caused by DNAzyme-catalyzed oxidation of 3,3′, 5,5′-tetramethylbenzidine(TMB), sensitive detection of Hg^{2+} can be achieved. The proposed sensor is simple, sensitive and selective, without the need of complicated labeling process, thus holds great potential for routine analysis of Hg^{2+} in environmental and biological samples.

1. Introduction

Heavy metal pollution has caused much concern throughout the world due to its universality and perniciousness. Among these heavy metals, mercury is one of the most noxious metals. Mercury ion (Hg^{2+}) is a highly toxic and the most stable form in its different oxidation states, so that can induce mercury poisoning. Elevated levels of Hg^{2+} in a biological species can cause damage to the brain, kidney, central nervous system and the endocrine system [1–3]. The Environmental Protection Agency of United States (U.S. EPA) allowable level of Hg^{2+} in drinking water should not exceed 10 nM [4]. Therefore, this raises a need to develop highly sensitive and selective methods for Hg^{2+} detection in environmental and biological samples.

Many analytical methods have been applied for the detection of Hg^{2+}, including surface enhanced Raman scattering (SERS) [5], surface plasmon resonances (SPR) [6], atomic absorption spectrometry (AAS) [7], inductively coupled plasma mass spectrometry (ICP-MS) [8], gas chromatography (GC) [9], high performance liquid chromatography (HPLC) [10], fluorescence sensing [11,12], and colorimetric sensors [13]. Although the methods can monitor Hg^{2+} successfully and most of them can provide low detection limits, they usually suffer from complex operations, expensive instruments, highly skilled persons and are time-consuming. Thus, it is still necessary to establish simple, affordable, portable and timesaving methods for the detection of Hg^{2+}. Electrochemical biosensors, as a promising detection technique, have received increasing attention due to the inherent advantages of facile operation, superior portability, fast response, high sensitivity, low cost, and the ability to provide in situ information.

G-quadruplex is a special folded conformation DNA which is formed from guanine-rich oligonucleotide strands in the end of telomere or oncogene promoters [14–18]. Recently, the application of G-quadruplex has attracted a lot of chemists' attention. G-quadruplex can be used in therapeutics as anticancer targets [19,20] or applied as antiviral/anticancer agents [21–23]. It also can be used for the construction of conductive nanowires [24,25] and electronic switches [26] in DNA nanotechnology. When coupled with small molecules, G-quadruplex exhibits great potential in biocatalysts as an artificial enzyme. For example, G-quadruplex-hemin DNAzyme has been widely applied to biosensors due to its high stability and considerable peroxidase-mimicking activity that can produce electrochemical [27], photoelectrochemical [28], electrochemiluminescent [29], chemiluminescent [30], colorimetric [31] and fluorescent signals [32].

In this work, we developed a novel electrochemical biosensor based on target-mediated DNA hybridization and nuclease-assisted formation of DNAzyme for the sensitive detection of Hg^{2+}. The G-rich DNA was formed on electrode surface by the combination of two short DNA probe at the assistance of T4-ligase, which avoids the tedious and expensive labeling steps for signal readout. After interaction with hemin, the DNAzyme is activated and then catalyzes the oxidation of TMB by H_2O_2 to produce detectable electrochemical signal. The fabricated biosensor is simple,

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label free, and exhibits high discrimination ability against other metal ions.

2. Experimental

2.1. Materials

High-performance liquid chromatography (HPLC) purified DNA oligonucleotides were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). The sequences of oligonucleotides used in this study were as follows:

Cp: 5′-SH-AAAAGGGTGGGTG-3′; Ap: 5′-PO_4-GGTGGGT-3′; LJ: 5′-CCCTCCCTCCCT-3′. T4 DNA Ligase and 10 × T4-Ligase buffer were purchased from New England Biolabs, Inc. (Beijing, China) and used as received. 6-Mercapto-1-hexanol (MCH), Hemin, H_2O_2 (30%), Tris-(2-carboxyethyl)-phosphine (TCEP), 3,3′,5,5′-Tetramethylbenzidine (TMB), glacial acetic acid and dimethylsulfoxide (DMSO) were obtained from Aladdin Chemistry Co. Ltd. (Shanghai, China). A stock solution of hemin (5 mM) was prepared in DMSO and stored in the dark at −20 °C. Before use, the hemin solution was diluted to required concentration with dilution buffer (25 mM Tris–HCl, pH 8.0, containing 20 mM KCl, 200 mM NaCl and 1% DMSO). The ultrapure water supplied with a Milli-Q system (18.5 MΩ Millipore, USA) was used throughout the experiment.

2.2. Instruments

Electrochemical experiments were conducted on a CHI 660E electrochemical workstation purchased from Shanghai Chenhua instruments co. Ltd. (Shanghai, China). A three-electrode system consisting of a bare or modified gold electrode, an Ag/AgCl reference electrode and a platinum wire electrode, was employed in all electrochemical measurements. Polyacrylamide gel electrophoresis (PAGE) was performed in a DYY-6C electrophoresis apparatus (Beijing, China). The concentrations of Hg^{2+} in water samples were determined by an X SERIES II inductively coupled plasma mass spectroscopy (ICP-MS) purchased from Thermo Electron Corporation (Waltham, MA, USA).

2.3. Pretreatment of the gold electrode

The gold electrode was immersed in a freshly prepared piranha solution (a 7:3 v/v mixture of concentrated H_2SO_4 and 30% H_2O_2) for 10 min, followed by a thorough rinse with deionized water. Then, the electrode was polished with 0.05 μm alumina slurry to obtain a mirror surface, followed by sonication in ethanol and deionized water for 5 min respectively to remove residual alumina powder. The electrode was then electrochemically cleaned over the potential range −0.2 and +1.5 V in H_2SO_4 (0.5 M) at a scan rate of 100 mV s^{-1} and 50 mV s^{-1} for 20 cycles repeatedly, and then a stable cyclic voltammogram was obtained. The gold electrode was rinsed with abundant deionized water before it was blow-dried with nitrogen.

2.4. Fabrication of the electrochemical biosensor for Hg^{2+} detection

Firstly, the capture probe (Cp) was treated with TCEP (2.0 mM, 0.1 M PBS) at 37 °C for 30 min to reduce the possible existence of a disulfide bond. Then, 5 μL pretreated Cp (1.5 μM) was applied to the cleaned gold electrode and left overnight at −4 °C. This was followed by immersion in 1 mM MCH for 1 h to block the nonspecific adsorption sites. Following this, 5 μL mixed solution, contains 1 μL Ap (10 μM), 1 μL LJ (10 μM), 1 μL Hg^{2+} (a series of different concentrations from 10 nM to 1 μM), 1.5 μL T4-ligase (1 U μL^{-1}) and 0.5 μL 10 × T4-Ligase buffer, was added to the electrode surface and incubated for 1.5 h at 22 °C. After that, the electrode was thoroughly rinsed with deionized water to produce the free DNAzyme sequence. 5 μL hemin (0.5 μM) was added to the electrode and incubated for 40 min at 25 °C. During this time, the DNAzyme was activated which led to its folding into special G-quadruplex conformation. Unless otherwise specified, the electrode was rinsed with washing buffer (10 mM PBS, pH 7.4, containing 0.1 M NaCl) completely after every step.

2.5. Electrochemical measurements

The i-t curves were performed in the time window from 0 s to 100 s in 0.1 M PBS buffer (pH 6.4), containing 0.15 M NaCl, 1 mM TMB and 1 mM H_2O_2. The modified electrode was immersed into the PBS buffer where the subsequent enzymatic reaction steps took place. The i-t curves were conducted after 1 min of catalytic reaction.

2.6. Gel electrophoresis experiments

Native 15% PAGE gel was prepared by mixing 5.43 mL ultrapure water, 2.0 mL 5 × TBE buffer (0.45 M Tris–boric acid–EDTA, pH 8.0), 2.5 mL 30% bis-acrylamide, 84 μL 10% ammonium persulfate and 12 μL N,N,N′,N′-tetramethylethylenediamine (TEMED) together. Before electrophoresis analysis, the samples were prepared in a 10 μL reaction solution which was made of 5 μM DNA samples and 1 × T4 buffer (50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ and 1 mM ATP). After the DNA hybridization at 22 °C for 1.5 h, the resulting DNA samples were mixed with 600-fold diluted super green and 1 × loading buffer for 5 min, then the mixture were applied to the gel. Electrophoresis was carried out in a 1 × TBE buffer (0.09 M Tris–boric acid–EDTA, pH 8.0) at 70 V for 2 h. The resultant gel was photographed with a Gel Image System.

3. Results and discussion

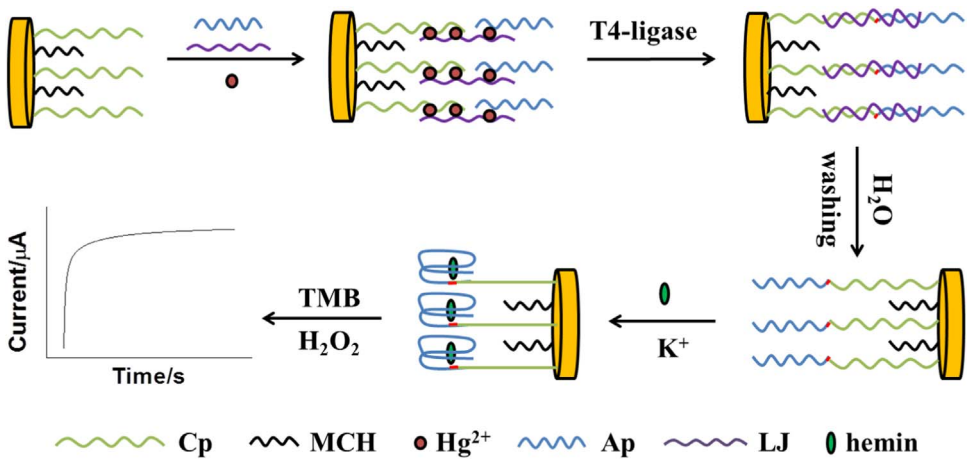
3.1. Sensing principle of the fabricated electrochemical biosensor

The schematic diagram of the proposed biosensor is shown in Scheme 1. Firstly, the capture probe (Cp) with a sulfhydryl group at the 5′-terminal was covalently immobilized on a gold electrode through Au–SH bond. Then, the modified electrode was blocked by MCH to inhibit nonspecific adsorption and promote the DNA hybridization. In the presence of Hg^{2+}, Cp and Ap probe would hybridize with the LJ probe through T–Hg^{2+}–T interaction. With the help of T4-ligase, the 5′-terminus phosphate radical of Ap would link with the 3′-terminus hydroxyl group of Cp, which resulted in the formation of hybridized G-rich DNA sequence. After washing with double-distilled water, the double-strand DNA would unwind and released the LJ probe from electrode surface due to the lack of ionic strength. When incubated with hemin, the free G-rich DNA would form G-quadruplex-hemin compounds, which can catalyze the oxidation process of TMB by H_2O_2 and result in amplified electrochemical signal. As a result, sensitive and selective detection of Hg^{2+} was achieved.

3.2. Feasibility of the developed Hg^{2+} electrochemical biosensor

3.2.1. Characterization of the structure change of DNA

In order to verify the Hg^{2+} mediated hybridization of DNA in this experiment, the PAGE experiment was performed to monitor the structural changes of DNA probe. Samples including Cp probe, Ap probe and LJ probe before (1) and after (2) addition of Hg^{2+} were analyzed (Fig. 1A). As expected, in the presence of Hg^{2+} (lane 1), the DNA migrated slower than that without Hg^{2+} (lane 2). At



Scheme 1. The principle of the electrochemical biosensor for Hg^{2+} sensing based on ligase-mediated formation of DNAzyme.

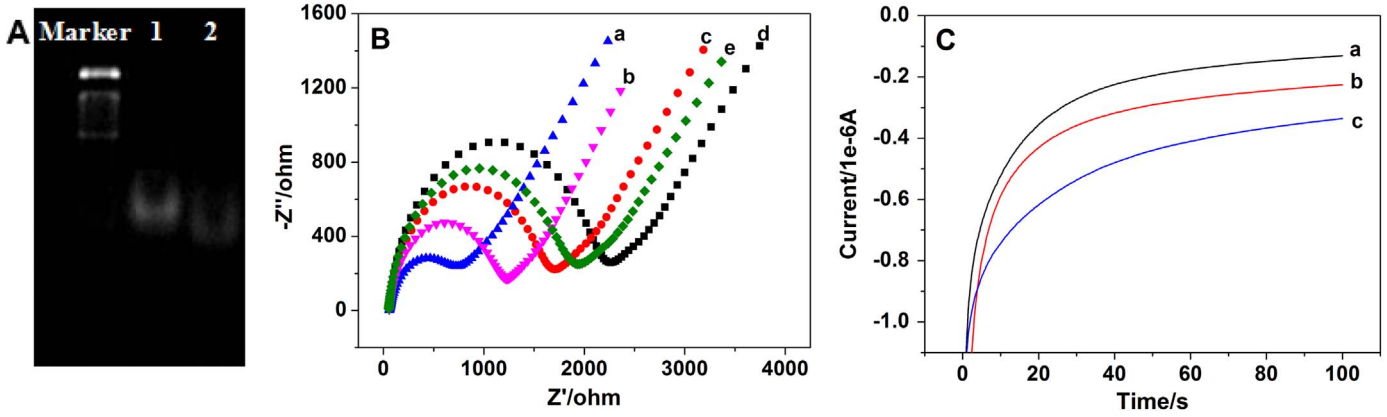


Fig. 1. (A) Gel electrophoresis graph of the sensing system. Cp, Ap, LJ, T4-ligase with (1) and without (2) addition of Hg^{2+}. (B) Nyquist plots of the different modified electrodes. (a) bare gold electrode, (b) Cp modified gold electrode, (c) after MCH blocked gold electrode, (d) dsDNA modified gold electrode, (e) dsDNA modified gold electrode after washing with deionized water. The EIS experiments were performed using 5 mM $[Fe(CN)_6]^{3-/4-}$ contains 0.5 M KCl. (C) i-t curves of the electrochemical biosensor at different concentrations of Hg^{2+} (a) 0, (b) 300 nM, (c) 800 nM.

the same time, the electrophoresis strip of lane 1 was brighter than that of lane 2 under ultraviolet irradiation. These results can be attributed to the following factors: firstly, the specific T–Hg^{2+}–T interaction can mediate the T bases contained DNA probes hybridization with each other into high molecular weight double-stranded DNA. Secondly, super green nucleic acid stain is more sensitive to double-stranded DNA than single-stranded DNA.

3.2.2. *Characterization of the modified electrodes*

To verify the assembly process, the interfacial properties of the modified electrodes were evaluated by electrochemical impedance spectroscopy (EIS). Fig. 1B shows the Nyquist plots at different conditions. There are two distinct parts of the Nyquist plots, one is a semicircle part in the high frequency region and the other is a linear line in the low frequency region. The diameter of the semicircle represents the charge transfer resistance (Rct) of the electrode surface. As we can see, bare gold electrode exhibited the smallest semicircle radius (curve a), indicating that the electron transfer rate was fast. The semicircle radius was increased after immobilization of the Cp probe (curve b), which is mainly due to the electrostatic repulsion between the negatively charged Cp probe and $[Fe(CN)_6]^{3-/4-}$, which inhibits the electron transfer process. When the electrode was further blocked by non-electrically conductive MCH, Rct value significantly increased as a result of the formation of an insulating layer on the electrode surface (curve c). In the presence of Hg^{2+} and T4 ligase, Cp probe and Ap probe can hybridize with the LJ probe to form double-stranded DNA, which resulted in an increase in the negative charge and the Rct value (curve d). It should be noted that, after washing with ultrapure water, the Rct value decreased significantly (curve e). This is due to the fact that without sufficient ionic strength to confer thermal stability, double-stranded DNA unwinding and LJ probe releasing occurred, resulting in the decrease of electrostatic repulsion. All these results indicated the successful fabrication of the electrochemical biosensor.

3.2.3. *Electrochemical measurement*

Some electrochemical experiments were conducted to verify the feasibility of the fabricated biosensor for Hg^{2+} detection. Fig. 1C shows the i-t curves with the addition of different concentrations of Hg^{2+}. It can be seen that the current was relatively low in the absence of Hg^{2+} (curve a). However, the current rose after addition of Hg^{2+}, and was positively correlated with Hg^{2+} concentration. These results showed that through Hg^{2+} mediated DNA hybridization and T4 ligase ligation, DNAzyme can be effectively formed to produce amplified electrochemical signals. Thus, the fabricated biosensor has great potential for sensitive detection of Hg^{2+}.

3.3. *Optimization of experimental conditions*

Since the amount of capture probe modified to electrode surface is a key factor to electrode performance, we investigated the concentration of Cp probe (0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 μM) firstly.

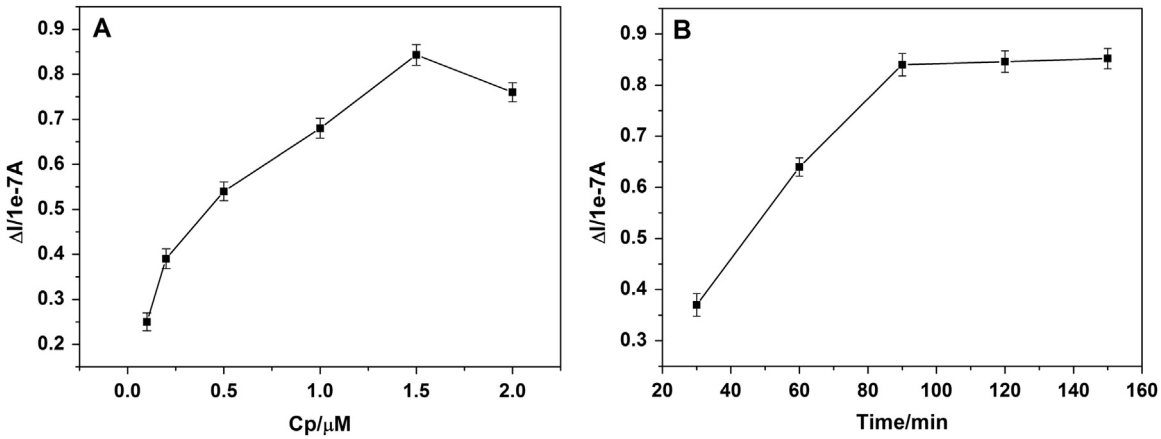


Fig. 2. The effect of Cp concentration (A) and DNA hybridization time (B) on the sensing performance (Hg^{2+}: 500 nM).

The result shown in Fig. 2A indicated the concentration of Cp probe at 1.5 μM gave the largest current response. With further increase of the Cp concentration, the density of Cp immobilized to the electrode surface was higher, and gave rise to a greater steric hindrance effect which restricted the DNA hybridization efficiency and effective assembling of DNAzymes. This may be responsible for the lower electrochemical response at higher concentration of Cp probe (2.0 μM). Thus, 1.5 μM was chosen as the optimal Cp concentration in the following experiments.

Considering that the hybridization efficiency is highly influenced by hybridization time, the DNA hybridization time was also investigated in the range of 30–150 min. As shown in Fig. 2B, the ΔI value increased accordingly with increase in incubation time until it reached a plateau at 90 min. The appearance of the plateau may be due to the hybridization of almost all the Cp probe on the electrode surface. Therefore, 90 min was used as the hybridization time in subsequent work.

3.4. Sensitivity and selectivity of the sensing system

The sensitivity of the biosensor was investigated by measuring the current changes during catalytic reaction. Different concentrations of Hg^{2+} were measured under the optimized reaction conditions. The currents increased along with the concentration of Hg^{2+}. The dependency relationship between the Hg^{2+} concentration and the currents confirmed the detection principle that, the more Hg^{2+} was added, the more DNAzymes were assembled on the electrode surface. We found that the currents showed a good linear correlation with the Hg^{2+} concentration ranging from 10 nM to 1 μM (Fig. 3A). According to the 3σ method, a detection limit of 5.8 nM was achieved, which is sensitive enough for the analysis of Hg^{2+} in drinking water. These results indicated that the fabricated biosensor can afford sensitive electrochemical detection of Hg^{2+}.

Selectivity is a very important parameter for evaluating the performance of a chemosensor which directly determines its use in the analysis of complex environmental and biological matrices. To investigate the selectivity of the electrochemical biosensor for Hg^{2+} detection, a blank sample (ultrapure water), Hg^{2+} and other metal ions were tested under the same conditions. The concentrations of other ions were 10-fold higher than Hg^{2+}. As shown in Fig. 3B, the ΔI value of other ions was obviously lower than Hg^{2+}, which was almost as low as that of the blank. These results indicated that the fabricated biosensor possessed excellent selectivity for Hg^{2+} detection. This high selectivity was mainly owing to the high affinity of T–Hg^{2+}–T coordination.

3.5. Recovery experiment

Herein, we also investigated the practical application of the developed electrochemical biosensor by determining Hg^{2+} in spiked water samples. The water samples were collected from laboratory tap water and waste water (a gift from Longyan Rare Earths LTD) from a point source suspected to discharge metal ions, respectively. After filtering through a 0.22 μm membrane to remove insoluble matter, these water samples were measured by

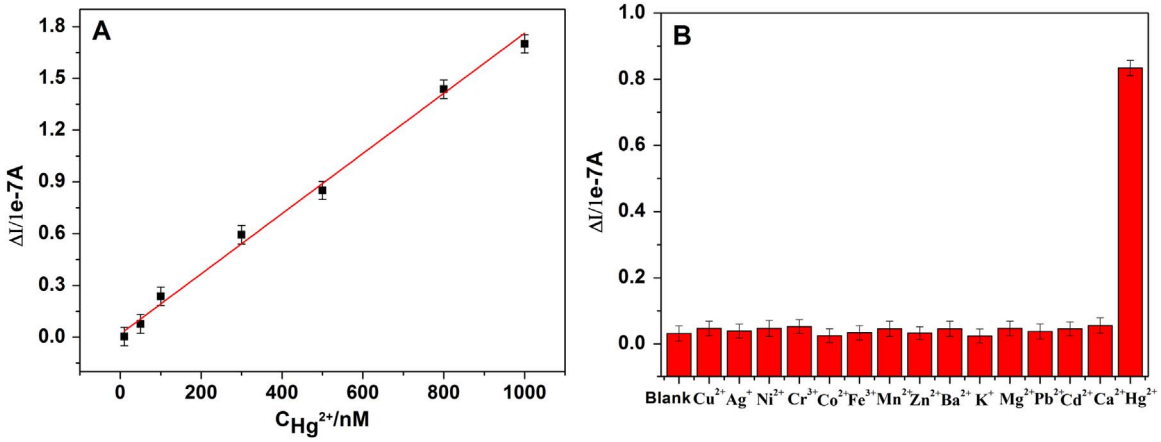


Fig. 3. (A) The calibration plot of ΔI values against the concentrations of Hg^{2+} in the range 10 nM–1 μM. (B) Selectivity investigation of the biosensor for Hg^{2+} detection by comparing the current intensity of 500 nM Hg^{2+} with that of other metal ions (The sample matrix was tap water).

Table 1
Hg^{2+} analysis in real water samples.

Sample	ICP-MS/nM	Add/nM	Found/nM	Recovery/%	RSD/%
Tap water	Not detected	50.0	53.0	106.0	5.87
		200.0	187.2	93.6	4.25
Waste water	4.8	800.0	834.4	104.3	6.02
		20.0	21.0	105.0	5.30
		100.0	95.7	95.7	4.51
		500.0	512.5	102.5	5.82

ICP-MS first. The original concentration of Hg^{2+} in the waste water was determined to be ~4.8 nM by ICP-MS, which is below the detection limit of the proposed assay. The concentrations of other metal ions, including Cr^{3+}, Co^{2+} and Cu^{2+} (detected by ICP-MS) in the waste water were ~100 fold of that of Hg^{2+}, and the concentrations of Ag^{+}, Cd^{2+}, Pb^{2+} etc. were comparable or lower than that of Hg^{2+}. Then, the water samples spiked with different concentrations of Hg^{2+} standard solutions were analyzed by the electrochemical method. Every sample was detected in triplicate. The results are shown in Table 1. Recovery values ranging from 93.6% to 106.0% and RSD between 4.25% and 6.02% were obtained, indicating that other metal ions in the complex water matrix have no significant effect on Hg^{2+} detection and the proposed electrochemical biosensor was well applicable for Hg^{2+} analysis in real water samples.

4. Conclusions

In summary, we have established a simple, label-free electrochemical biosensor for Hg^{2+} detection based on target-mediated DNA hybridization and nuclease-assisted DNAzyme formation. The specific recognition ability of Hg^{2+} to T-T mismatch was utilized to produce double-stranded DNA. T4-ligase was added to connect the two short DNA and then formed a long G-rich DNA which can fold up into G-quadruplex. In the presence of hemin, the formed G-quadruplex-hemin DNAzyme can catalyze the oxidation of TMB to realize amplified electrochemical signal and then improve the sensitivity of the biosensor. The proposed biosensor also exhibited excellent selectivity for Hg^{2+} against other metal ions. The electrochemical biosensor was successfully applied to the detection of Hg^{2+} in spiked water samples. Due to the advantage of easy operation, high sensitivity and selectivity, the proposed biosensor offers great potential for on-site analysis of Hg^{2+} in a wide range of samples.

Acknowledgments

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