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Electrocatalytic assay of mercury(II) ions using a bifunctional oligonucleotide signal probe

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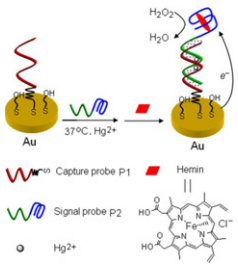
HIGHLIGHTS

- A bifunctional oligonucleotide was designed for Hg^{2+} electrochemical sensing.
- Electrocatalytic property of G4–hemin was investigated using cyclic voltammetry.
- Electrocatalytic reduction of H_2O_2 by G4–hemin provided amplified signal for Hg^{2+}.
- A simple and effective electrochemical Hg^{2+} biosensor was successfully developed.

GRAPHICAL ABSTRACT

The G-quadruplex–hemin (G4–hemin) complex exhibited good electrocatalytic activity toward the reduction of hydrogen peroxide when it was immobilized on a gold electrode through T–Hg^{2+}–T interaction mediated surface hybridization reaction, which enabled the design of a bifunctional oligonucleotide signal probe for label-free, electrocatalytic assay of Hg^{2+}.



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ABSTRACT

Engineered nucleic acid probes containing recognition and signaling functions find growing interest in biosensor design. In this paper, we developed a novel electrochemical biosensor for sensitive and selective detecting of Hg^{2+} based on a bifunctional oligonucleotide signal probe combining a mercury-specific sequence and a G-quadruplex (G4) sequence. For constructing the electrochemical Hg^{2+} biosensor, a thiolated, mercury-specific oligonucleotide capture probe was first immobilized on gold electrode surface. In the presence of Hg^{2+}, a bifunctional oligonucleotide signal probe was hybridized with the immobilized capture probe through thymine–mercury(II)–thymine interaction-mediated surface hybridization. The further interaction between G4 sequence of the signal probe and hemin generated a G4–hemin complex, which catalyzed the electrochemical reduction of hydrogen peroxide, producing amplified readout signals for Hg^{2+} interaction events. This electrochemical Hg^{2+} biosensor was highly sensitive and selective to Hg^{2+} in the concentration of 1.0 nM to 1 μM with a detection limit of 0.5 nM. The new design of bifunctional oligonucleotide signal probes also provides a potential alternative for developing simple and effective electrochemical biosensors capable of detecting other metal ions specific to natural or artificial bases.

1. Introduction

Natural pyrimidine bases or artificial bases in DNA duplexes can form metal-mediated base pairs with different metal ions. Thymine–thymine (T–T) base pair, for example, selectively captures mercury ion (Hg^{2+}) and forms T–Hg^{2+}–T base pair [1,2]. The impressive specificity and affinity of T–Hg^{2+}–T interaction have motivated growing interest on the development of high-performance sensors for Hg^{2+}, which is a serious environmental pollutant. These T–Hg^{2+}–T based Hg^{2+} sensors mainly included fluorescent [3–8], colorimetric [9–14] and electrochemical sensors [15–24]. While most of the fluorescent and colorimetric Hg^{2+}

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sensors had a common shortcoming of that the detection limit for Hg^{2+} was limited and could not reach the tolerated level of Hg^{2+} in drinkable water (10 nM, defined by the U.S. Environmental Protection Agency (EPA)), the electrochemical Hg^{2+} sensors recently developed have demonstrated significant advantages for Hg^{2+} detection attributing to the intrinsic high sensitivity of electrochemical signal transduction. Several signal transformation mechanics have been developed for converting the T–Hg^{2+}–T recognition events to sensitive electrochemical signals in the design of electrochemical Hg^{2+} sensors. For example, electrically contacted enzyme structure modulated by T–Hg^{2+}–T interaction was employed as a transducing element in Hg^{2+} recognition events [20]. Similarly, gold nanoparticles (AuNPs)-functioned reporter oligonucleotides were used as Hg^{2+} recognition probes and amplifying tags [16,18,19]. Also, Hg^{2+}-specific, redox-tagged oligonucleotide probe was designed to construct electrochemical structure-switching Hg^{2+} sensor [17]. With signal amplification of nanoparticle-labeled reporters, Hg^{2+} concentration as low as 1 nM has been reported to be detected by T–Hg^{2+}–T based electrochemical sensors. However, whereas these methods were sensitive and specific, they also suffered from a significant drawback: the fabrication of these electrochemical Hg^{2+} sensors relied on sophisticated chemical modification of the Hg^{2+}-specific oligonucleotide probes for labeling redox tags, enzyme units or nanoparticles, which was typically a multistep, time-consuming and costly process.

G-quadruplex–hemin complex (G4–hemin) is a class of horseradish peroxidase-mimicking DNAzyme formed by a special G-quadruplex structure with an intercalated hemin. It can catalyze the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonate disodium salt ($ABTS^{2-}$) by H_2O_2 to produce the colored radical ion ($ABTS^{\bullet-}$), which causes a detectable color change [25]. Substantial recent studies have focused on engineered nucleic acid probes employing G4–hemin as a optical signal producer for the colorimetric assay of DNA [26], proteins [27] and metal ions [27,14,28–34]. The simple probe design of integrating recognizing sequence with G4 unit facilitates nucleic acid probe fabrication without any chemical modification of the probe for labeling. On the other hand, it has been also reported that G4–hemin exhibits good electrocatalytic property toward the electrochemical reduction of hydrogen peroxide (H_2O_2) when it is immobilized on a pyrolytic graphite electrode by drop-coating or on a gold electrode through Au—S covalent binding, which enables the use of G4–hemin complex as a chemical modification-free electrocatalytic tag for amplified biosensing[35,36]. Here, we report the design of a bifunctional oligonucleotide signal probe combining a mercury-specific sequence with a G4 unit, and use of the electrocatalytic property of G4–hemin for electrochemical signal converting of Hg^{2+} recognizing event. We demonstrate that G4–hemin complex immobilized on a gold electrode through T–Hg^{2+}–T interaction mediated surface hybridization also shows good electrocatalytic activity toward the electrochemical reduction of H_2O_2. Based on this chemical modification-free bifunctional probe and its unique electrocatalytic property, a simple, sensitive and selective electrochemical Hg^{2+} biosensor was successfully developed.

2. Experimental

2.1. Reagents and apparatus

HPLC-purified oligonucleotides were obtained from sangon biotechnology Co. Ltd. (Shanghai, China). The sequences of the capture probe (P1) and the signal probe (P2) are as follows:

5′-HSC_6H_{12}-AAAGTCGCTTCGCTC-3′ (P1)

5′-GGGTTGGGCGGGATGGGAAAGAGCGTTGCGAC-3′ (P2)

Hemin, hexaammineruthenium (III) chloride ($[Ru(NH_3)_6]^{3+}$, RuHex) and 2-mercapto-ethanol (MCE) were purchased from Alfa Aesar. Triton X-100, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) were from Dingguo Biologic Products (Beijing, China). Hydrogen Peroxide (H_2O_2, 30%) and the used metal salts ($Hg(NO_3)_2$, $Zn(NO_3)_2$, $MgCl_2$, $Ca(CH_3COO)_2$, $Pb(NO_3)_2$, $CdCl_2$, $Mn(CH_3COO)_2$, $Cu(NO_3)_2$, $NiCl_2$, $FeCl_3$) were supplied by Sinopharm Group Chemical Reagent Co. Ltd. (Shanghai, China). All reagents were analytical grade and all solutions were prepared with 18.2 MΩ cm ultrapure water. The Tris buffer (10 mM Tris, 1 M NaCl, pH 7.4) was used for DNA immobilization. The HEPES buffer (10 mM HEPES, 50 mM KNO_3, pH 7.4) was used for hybridization, washing and electrochemical test solution. Electrochemical measurements were performed on an electrochemical workstation of CHI900B (CH Instructions, Austin, TX, USA) at room temperature. The used three-electrode system consisted of a gold working electrode (2 mm diameter), a saturated calomel reference electrode and a platinum foil auxiliary electrode.

2.2. Sensing interface preparation

To prepare the sensing interface, gold electrodes were polished with 0.05 μm alumina slurries, ultrasonically washed in ultrapure water and electrochemically cleaned (a series of oxidation and reduction cycling in 1 M H_2SO_4 from −0.4 V to 1.2 V (*vs.* Hg/Hg_2SO_4)) before being modified with the thiolated probe P1. The cleaned gold surface was first interacted with the Tris buffer containing probe P1 for 2 h at room temperature, and then passivated with 1 mM MCE in ultrapure water for 1 h to eliminate the nonspecific-bonded P1. After being thoroughly rinsed with the washing buffer, the P1 covered gold electrode was stored at 4 °C in HEPES buffer for further use.

2.3. Quantification of surface density of capture probe P1

The surface density of capture probe P1 on gold electrode was evaluated using the chronocoulometric method developed by Tarlov and co-workers [37]. Briefly, the association of RuHex with the capture probes was monitored *via* chronocoulometry over a range of 0 V to −0.5 V (*vs.* SCE) at a period of 500 ms. Chronocoulometric measurements were first performed in 10 mM Tris buffer (pH 7.4) and then compared with those taken in 10 mM Tris buffer (pH 7.4) containing 500 μM RuHex to obtain the integrated charge of surface-confined RuHex. The surface density of P1 can be easily calculated from the equation as follows:

$$\Gamma_{DNA} = \left(\frac{QN_A}{nFA}\right)\left(\frac{z}{m}\right)$$

where Γ_{DNA} is the probe surface density, Q the integrated charge of surface-confined RuHex obtained by calculating the chronocoulometric intercept at $t = 0$, N_A Avogadro's number, n the number of electrons per molecule for RuHex reduction, F the Faraday constant, A the area of the working electrode, z the charge of RuHex, and m is the number of nucleotides in the probe P1.

2.4. Electrocatalytic detection of Hg^{2+}

For Hg^{2+} detecting in HEPES buffer, the prepared sensing interface was incubated in HEPES buffer containing 1 μM P2 and Hg^{2+} of different concentrations at 37 °C for 1 h and then interacted with 2 μM hemin in HEPES buffer (10 mM HEPES, 50 mM KNO_3, 0.05% Triton X-100, 2% DMSO, pH 7.4) at 25 °C for 20 min. After being rinsed with the washing buffer, the modified electrode was

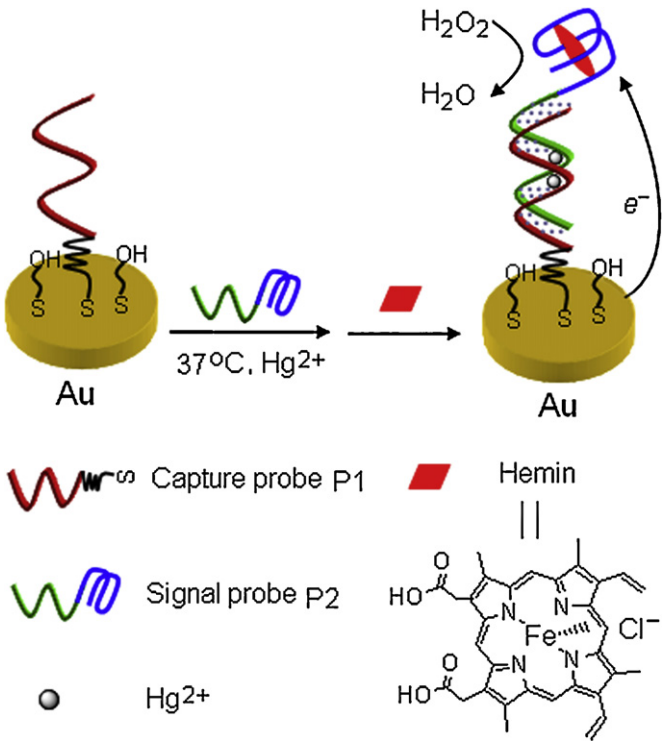

Fig. 1. Schematic representation of the electrochemical Hg^{2+} biosensor.

immersed in deoxidized test solution containing 1 mM H_2O_2 and the amperometric *i–t* curve measurement was performed.

To determine Hg^{2+} spiked in tap water, the water matrix was pretreated by acidic digestion. A 50 mL of tap water was filtered through a 0.22 μm membrane, added with 1 mL of nitric acid (69%) and heated to boiling. Then 2 mL of hydrogen peroxide (30%) was added and the mixture was left to digest for 30 min. After cooling to room temperature, the mixture was adjusted to about pH 7.4 using sodium hydroxide solution. Hg^{2+}-spiked tap water sample was obtained by diluting the Hg^{2+}-HEPES stock solution with equal volume of the digested tap water.

3. Results and discussion

3.1. Mercury sensor design

Fig. 1 shows the design of the electrocatalytic Hg^{2+} biosensor. The sensor recognition elements consist of a thiolated capture probe (P1: HSC_6H_{12}-5′-AAAGTCGCTTCGCTC-3′) and a bifunctional signal probe (P2: 3′-CAGCGTTGCGAG AAAGGGTAGGGCGGGTTGGG-5′). The probe P2 contains a 12-mer Hg^{2+}-specific sequence (underlined with solid line) in its 3′-end and a 17-mer G4 sequence (underlined with wavy line) in its 5′-end. The 12-mer Hg^{2+}-specific sequence is partly complementary to the 12-mer sequence of the capture probe P1 (underlined with solid line, including ten complementary base pairs and two mismatched T–T base pairs). To construct the sensor system, the gold electrode was first assembled with the thiolated capture probe P1 and then passivated by 1 mM MCE. In the presence of Hg^{2+}, the P1-covered electrode surface was incubated in the hybridization buffer containing probe P2, capturing the signal probe P2 on the electrode surface *via* T–Hg^{2+}–T interaction-mediated hybridization. The modified gold electrode was then immersed into a HEPES buffer containing hemin, forming a G4–hemin complex monolayer on the electrode surface. In the resulting bicomponent nucleic acid structure, the G4–hemin complex unit acted as an electrocatalyst for H_2O_2 electrochemical reduction, generating an amplifying electrochemical signal. In the absence of Hg^{2+}, the signal probe P2 could not hybridized with the capture probe P1 as the duplex containing two mismatched T–T base pairs was instable at the controlling hybridization temperature of 37 °C (the melting temperature of the duplex between P1 and P2 was 33 °C as evaluated by the DNA Melt Web Serve [38] under the current experimental conditions). Accordingly, the G4–hemin complex monolayer could not be formed on the sensor interface, resulting in a weak electrochemical reduction of H_2O_2. As the amount of G4–hemin complexes generated on the modified gold electrode was controlled by the concentration of Hg^{2+}, the resulting electrocatalytic reduction current of H_2O_2 could provide a quantitative measure for Hg^{2+}.

The T–Hg^{2+}–T interaction mediated surface hybridization of the signal probe P2 with the immobilized capture probe P1, and the electrocatalysis of G4–hemin complex toward the reduction of H_2O_2 were demonstrated by cyclic voltammetry. Fig. 2A shows that the P1–P2–hemin modified electrode (obtained by incubating the P1 and MCE-covered electrode with 1 μM P2 in the presence of 1 μM Hg^{2+} for 60 min and then interacting with 2 μM hemin for 20 min) reveals a well-defined, quasi-reversible redox wave in the deoxidized HEPES buffer (curve a). Control experiments indicated that other modified electrodes, prepared using the same procedures but in the absence of Hg^{2+} (curve b) or without hemin interaction (data not shown), did not show any significant voltammetric waves. Fig. 2B shows the electrocatalytic behaviors of different modified electrodes toward the electrochemical reduction of H_2O_2. Upon addition of 1 mM H_2O_2 in the test solution, the voltammogram of the P1–P2–hemin modified electrode (dash line, the same as curve a in Fig. 2A) was significantly changed, the cathodic current increased greatly and the anodic current almost disappeared (curve a), exhibiting a remarkable electrocatalytic effect on the reduction of H_2O_2. In contrast, the controlling electrodes, modified in the absence of Hg^{2+} (curve b) or without Hemin interaction (curve c), showed only small current responses to H_2O_2. These results implied that the G4–hemin complex was essential to activate the electrocatalytic reduction of H_2O_2, and that it was formed only by the T–Hg^{2+}–T interaction. Therefore, the reduction current increase of H_2O_2 between the P1–P2–hemin modified electrode and the Hg^{2+}-blank modified electrode could provide a readout signal for quantitative assay of Hg^{2+}. In the present study, the reduction current increase of 1 mM H_2O_2 occurred at −0.4 V (Δi in Fig. 2C) was adopted as the measurement signal. As shown in Fig. 2C, the Hg^{2+}-blank modified electrode showed only a small current response to the addition of 1 mM H_2O_2 into the test solution. The P1–P2–hemin modified electrode fabricated in the presence of 1 μM Hg^{2+}, however, exhibited remarkably increased current response toward the electrochemical reduction of H_2O_2.

3.2. Optimization of experimental conditions

Three main parameters that may affect the amount of the G4–hemin electrocatalytic tags immobilized on the modified electrode surface and then the electrocatalytic current response to Hg^{2+} concentration were tested: the surface density of capture probe P1, the interaction time of G4 with Hemin for forming the G4–hemin complex and the hybridization time for P1–P2.

The surface density of capture probe P1 immobilized on gold electrode was optimized to let the maximum amount of signal probe P2 loading on the electrode surface through T–Hg^{2+}–T mediated hybridization. With a fixed immobilization time of 2 h, various P1 surface densities ranging from 1.7×10^{12} to 4.3×10^{12} molecules cm^{-2} were achieved by controlling the concentration of probe P1 varying from 0.3 μM to 3.0 μM in the immobilization buffer during the self-assembly process (curve a in

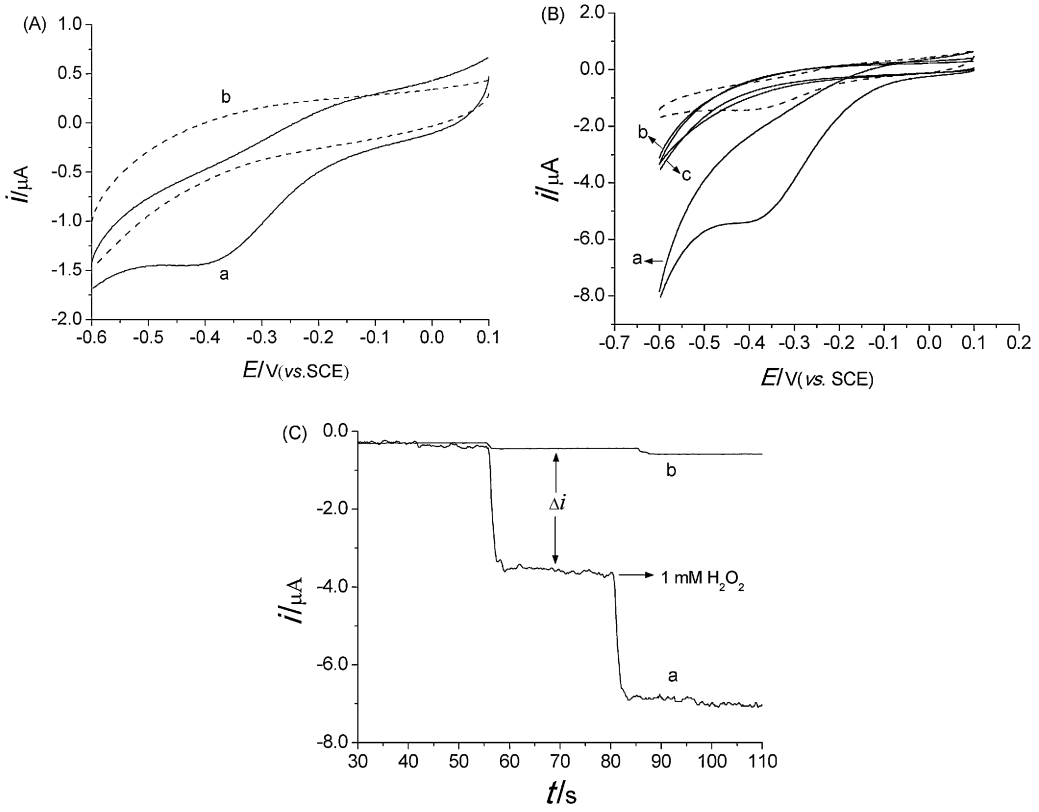

Fig. 2. Cyclic voltammograms for electrodes modified with probe P1 and MCE in the deoxidized HEPES buffer containing no H_2O_2 (A) and 1 mM H_2O_2 (B) (Scan rate: 100 mV s^{-1}). (a) the electrode was incubated with 1 μM P2 in the presence of 1 μM Hg^{2+} for 60 min and then interacted with 2 μM hemin at 25 °C for 20 min prior to test; (b) the electrode was treated following the same procedure for (a) in the absence of Hg^{2+}; (c) the electrode was treated following the same procedure for (a) in the absence of Hemin. In (B), a CV curve (dash line) from (A) (curve a) is included for comparison. (C) Chronoamperometric curves of the different modified electrodes in the successive addition of 1 mM H_2O_2, amperometric measurements were performed by applying a working potential of −0.40 V (*vs.* SCE) under unceasing stirring.

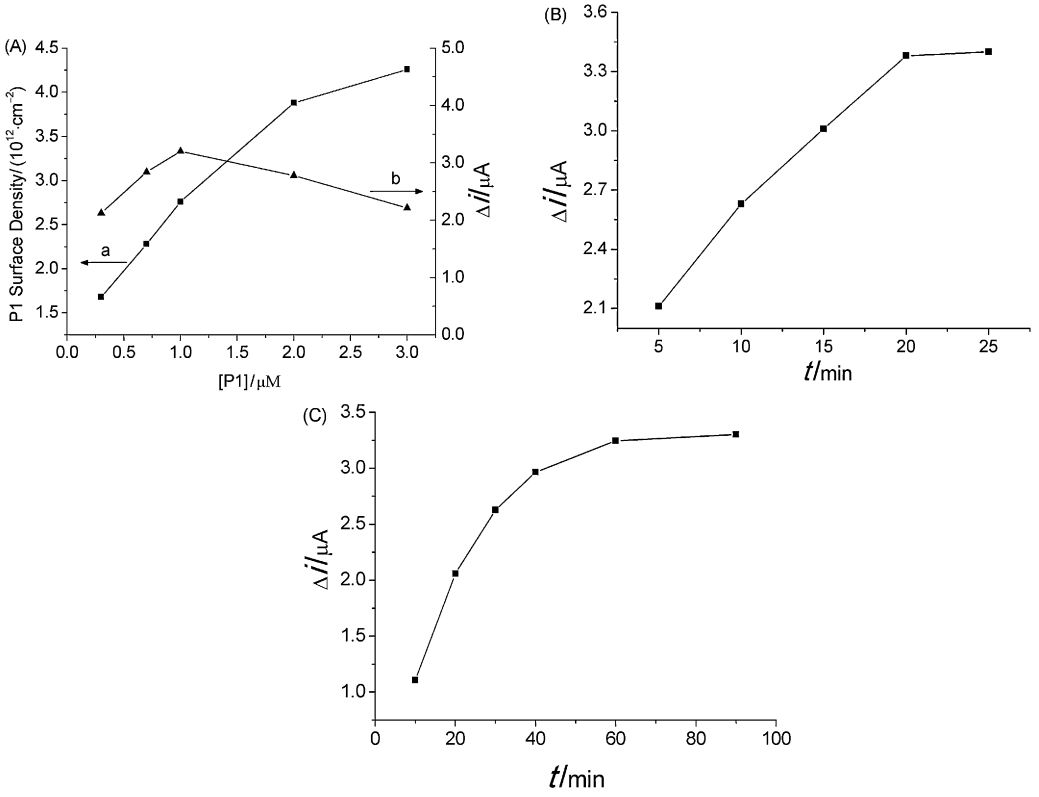

Fig. 3. (A) Effect of P1 surface density on the performance of the electrocatalytic Hg^{2+} biosensor. (a) Dependence of P1 surface density on the concentration of P1 in immobilization buffer. (b) Current responses to 1 μM Hg^{2+} on the modified electrodes prepared by different concentrations of P1 in immobilization buffer. (B) Effect of the interaction time of G4 with Hemin on the current response to 1 μM Hg^{2+}. (C) Effect of the hybridization time on the current response to 1 μM Hg^{2+}.

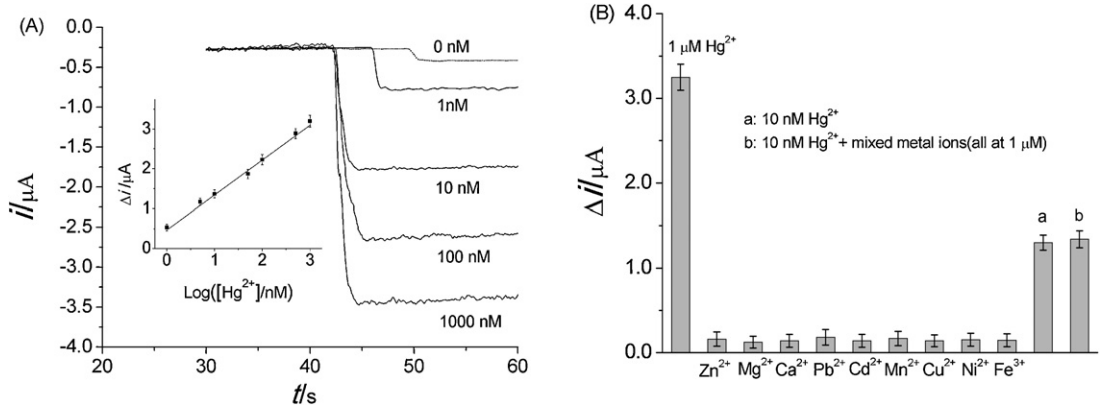

Fig. 4. (A) Representative current response curves for Hg^{2+} of different concentrations (inset: linear dependence of the reduction current increase of 1 mM H_2O_2 (Δi) on the logarithm of Hg^{2+} concentration). (B) Specificity and selectivity of the designed biosensor for Hg^{2+}. The concentration of each tested interferential metal ion was 1 μM.

Fig. 3A). As shown in Fig. 3A, the largest current response to 1 μM Hg^{2+} was observed at the modified electrode prepared with 1.0 μM probe P1 in the immobilization buffer, indicating that the surface density of 2.8×10^{12} molecules cm^{-2} was optimal to the surface hybridization between P1 and P2 and thus favored forming the maximum amount of G4–hemin electrocatalytic units on the electrode surface. This result is consistent with the previous report that capture probe densities in the range of $(1–3) \times 10^{12}$ molecules cm^{-2} are often of most interest for DNA biosensors due to the high surface density of hybridized target [39].

The interaction kinetics of G4 with Hemin and the hybridization kinetics of P1–P2 were also examined. The results indicated that a period of 20 min was fully enough for forming a complete G4–hemin complex monolayer on the modified electrode through the specific interaction between Hemin and G4 sequence of the surface-confined signal probe P2 (Fig. 3B). On the other hand, the electrocatalytic current response to 1 μM Hg^{2+} intensified as the time of T–Hg^{2+}–T mediated hybridization reaction increased, and they reached to a saturation value after about 60 min (Fig. 3C). Accordingly, all subsequent analyses of Hg^{2+} used the optimal conditions.

3.3. Analytical characteristics

The designed biosensor system was sensitive for Hg^{2+} detection. Fig. 4A shows that the electrocatalytic current response increased with the increasing Hg^{2+} concentration in the range of 1.0 nM to 1 μM. A linear relationship between the reduction current increase (Δi) and the logarithm of Hg^{2+} concentration was obtained ($\Delta i/\mu A = 0.50 \times \log([Hg^{2+}]/nM) + 0.88$, $R^2 = 0.9947$). The existence of a linear relationship between the signal and the logarithms of Hg^{2+} concentration is difficult to explain with our current knowledge, although similar logarithmic signal-*versus*-concentration relationships have been reported for other solid-state DNA sensors [40–42]. The detection limit was 0.5 nM evaluated using a signal three-fold the background noise, which exceeds that of a variety of previously reported colorimetric or fluorescent assays based on T–Hg^{2+}–T interaction. Such a low detection limit is much lower than the toxicity level of Hg^{2+} in drinkable water (10 nM) defined by the U.S. EPA. The specificity was also evaluated by substituting Hg^{2+} in the hybridization buffer with various metal ions such as Zn^{2+}, Mg^{2+}, Ca^{2+}, Pb^{2+}, Cd^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+} and Fe^{3+} at 1 μM concentration, respectively. As shown in Fig. 4B, the biosensor exhibits remarkable response to Hg^{2+}, but hardly responds to other metal ions. Likewise, the sensor's response to Hg^{2+} (10 nM, Fig. 4B-a) is not significantly affected by the presence of mixed interferential metal ions (all at 1 μM, Fig. 4B-b), which indicates a good selectivity for Hg^{2+} detection

The usefulness of the designed biosensor for the analysis of real samples was tested by determinizing the recovery of spiked Hg^{2+} in tap water. Tap water samples spiked with Hg^{2+} at three different concentrations of 2.0, 10.0 and 50.0 nM were tested. The results obtained *via* the above calibration curve were 2.06 ± 0.12, 10.22 ± 0.21 and 49.02 ± 0.36 nM, with the recovery values of 103.0%, 102.2% and 98.04% for the samples containing 2.0, 10.0 and 50.0 nM Hg^{2+}, respectively. The good recovery confirmed the reliability of the proposed for the analysis of low Hg^{2+} concentrations in real water samples.

These results above fully demonstrate that the developed biosensor was sensitive and selective for Hg^{2+} assay. Compared to other T–Hg^{2+}–T interaction-based electrochemical or optical Hg^{2+} sensors, the present sensor platform can provide many potential advantages (see comparison in Table 1). Firstly, the use of an integrated bifunctional oligonucleotide probe as a recognition

Table 1
Comparison of the performances of Hg^{2+} biosensors based on T–Hg^{2+}–T interaction.

Detection methods	Signal probe	Detection limit	Signal mode
Electrochemistry			
This work	Bifunctional oligonucleotide	0.5 nM	Signal on
Ref. [24]	Ferrocene-labeled oligonucleotide	100 nM	Signal on
Ref. [17]	Ferrocene-tagged oligonucleotide	0.5 nM	Signal off
Ref. [16]	AuNP-functionalized oligonucleotide	0.5 nM	Signal on
Fluorescence			
Ref. [8]	Dye-labeled oligonucleotide	14.5 nM	Signal on
Ref. [6]	Fluorophore-labeled oligonucleotide	20 nM	Signal off
Colorimetric			
Ref. [13]	AuNP-functionalized oligonucleotide	200 nM	Signal off
Ref. [14]	G-quadruplex-based DNAzyme	50 nM	Signal off

element and a signal label for Hg^{2+} sensing simplifies the sensor design. It does not require either the multiple self-assembly steps involved in AuNPs-based amplification assays or the sophisticated passivation and washing steps required by enzymatic amplification assays. Secondly, our electrocatalytic platform generates positive readout signals for the Hg^{2+} sensing events, in contrast to some previously reported electrochemical Hg^{2+} sensors that provided negative responses. And lastly, the electrocatalysis of the G4–hemin labels toward the electrochemical reduction of H_2O_2 provides amplified readout signals, which bring about remarkably increased sensitivity for Hg^{2+} sensing in comparison to the optical Hg^{2+} biosensors. The simplicity and effectivity make the present electrocatalytic system to be a potential alternative for Hg^{2+} detecting.

4. Conclusions

In summary, the present study has demonstrated that the G4–hemin complex assembled on a gold electrode through T–Hg^{2+}–T interaction-mediated surface hybridization exhibits good electrocatalytic activity toward the electrochemical reduction of H_2O_2. This feature enabled the use of G4–hemin complex as an effective electrocatalytic label for developing a simple, sensitive and selective electrochemical Hg^{2+} biosensor. As other natural or artificial base pairs also selectively bind other metal ions (*e.g.*, Ag^+ by cytosine–cytosine base pairs) [43,44], new electrocatalytic sensor systems capable of detecting various metal ions in aqueous solution can also be developed.

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