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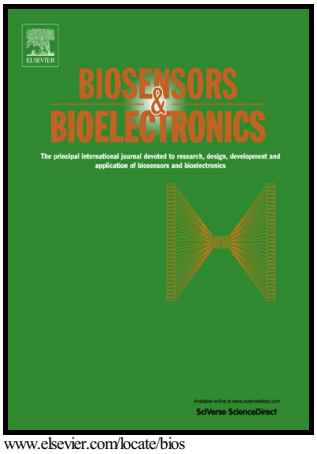

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Highly sensitive and selective electrochemical detection of Hg^{2+} through surface-initiated enzymatic polymerization

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Abstract:

A Hg^{2+} electrochemical biosensor is developed by integrating thymine-Hg^{2+}-thymine (T-Hg^{2+}-T) base pairs for the high selectivity with surface-initiated enzymatic polymerization (SIEP) for signal amplification. The fabrication begins with the covalent conjugation of capture DNA probe labeled with thiol at its 3'terminal onto the gold electrode. The presence of Hg^{2+} leads to DNA hybridization, in which complementary DNA was captured onto the biosensor surface, which subsequently catalyzed the addition of deoxynucleotides (dNTP) containing biotinlated 2'-deoxyadenosine 5'-triphosphate (biotin-dATP) by terminal deoxynucleotidyl transferase (TdT). The binding between biotin and strepavidin leads to the attachment of a large number of strepavidin functionalized silver nanoparticles (strepavidin-AgNPs), which could generate electrochemical stripping signal of silver to monitor the concentration of Hg^{2+} in KCl solution. Through utilizing the T-Hg^{2+}-T selectivity and SIEP amplification, this assay method can detect aqueous Hg^{2+} with a wide linear range from 0.05 nM to 100 nM and a detection limit of 0.024 nM. The application of this sensor in the analysis of drinking water demonstrates that the proposed method works well for real samples.

Keywords: Electrochemical sensor; Mercury; thymine-Hg^{2+}-thymine; Surface-initiated enzymatic polymerization; Signal amplification.

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1. Introduction

The emission of toxic heavy metal ions has posed a challenge to the global plan of maintaining a sustainable environment (Huang et al., 2014). Mercuric ion (Hg^{2+}) has been recognized as one of the worldwide issue for years due to its high toxicity and bioaccumulative effect (Onyido et al., 2004; Nolan and Lippard, 2008). Conventional analytical techniques such as inductively coupled plasma mass spectrometry (Bings et al., 2006), atomic absorption spectroscopy (Butler et al., 2012), and cold vapor atomic fluorescence spectroscopy (Fernández-Martínez et al., 2015) have been extensively used for the measurement of Hg^{2+} in water samples. Although these methods are very sensitive and accurate, they usually require sophisticated instruments and a large amount of sample, and involve time-consuming sample preparation steps (Huang et al., 2014). In contrast, electrochemical detection of Hg^{2+} has shown a number of attractive advantages such as simplicity, good portability, low cost and high sensitivity (Lost and Crespilho, 2012). Some Hg^{2+}-selective membrane electrodes based on Schiff crown ethers and mercapto compounds have been developed recently (Sanchez et al., 2010; Ebdelli et al., 2011). These electrodes take advantage of electrochemical changes upon the binding of Hg^{2+} due to the presence of N, O and S atoms in the structure compounds which coordinate with heavy metal ions. While the operation is simple and portable, the selectivity and sensitivity are not satisfactory for their broad applications.

In order to improve the selectivity, a new method based on the coordination between Hg^{2+} and bis-thymine has drawn considerable attention (Miyake, et al., 2006; Tanaka et al., 2007; Zhu et al., 2009), where Hg^{2+} is able to specifically bind two thymine bases together to form an Hg^{2+}-mediated complex (T-Hg^{2+}-T). Notably, other metal ions do not have this characteristic interaction with thymine. With the above strategy, several types of biosensors have been developed for the electrochemical determination of trace amounts

of Hg^{2+} (Han et al., 2009; Lai et al.,2011). As expected, these biosensors exhibit good selectivity. Unfortunately, some of their detection limits are above 10 nM, which may not be enough sensitive for certain applications.

Recently, highly sensitive methods for Hg^{2+} detection have been developed with the assistance of DNA amplification techniques. Strategies using DNA superstructures (Yang et al., 2014), hybridization chain reaction (HCR) (Huang et al., 2014), and exonuclease III (Xuan et al., 2013; Wang et al., 2014), for examples, have been reported yielding high sensitivity. However, DNA superstructures require multistep operation processes. HCR require to prepare two specific target sequence hairpin DNA probes, and the activity of the exonuclease III may be affect by Hg^{2+} freed up from the T-Hg^{2+}-T hybrid. Fortunately, surface-initiated enzymatic polymerization (SIEP) can also play a role of signal amplification without the shortcomings of the above mentioned methods (Tjong et al., 2011; Wan et al., 2013; Lin et al., 2015). To the best of our knowledge, SIEP has not been investigated for the detection of Hg^{2+} ions. This approach uses terminal deoxynucleotidyl transferase (TdT), which is a template-free DNA polymerase that catalyzes the sequential addition of deoxynucleotides (dNTPs) at the 3'-OH group of an oligonucleotide primer and incorporates biotinlated 2'-deoxyadenosine5'-triphosphate (biotin-dATP) into the long single-stranded DNA (ssDNA) chain. Through the binding between biotin and strepavidin, a large number of signal tags could be assembled for the ultrasensitive detection of Hg^{2+}.

Herein, inspired by these DNA amplification techniques, this work designed a Hg^{2+} electrochemical biosensor through coupling T-Hg^{2+}-T base pairs with SIEP (Scheme 1). This biosensor involves covalent conjugation of capture DNA probe labeled with thiol at its 3'terminal onto a gold electrode. After the T-T mismatched DNA hybridization in the presence of Hg^{2+}, complementary DNA was captured on the biosensor surface, which

then catalyzed the addition of dNTP containing biotin-dATP by TdT. The above process results in the formation of long single-stranded DNAs labeled with numerous biotins. Though the binding between biotin and strepavidin, a large number of strepavidin-AgNPs were attached. Linear sweep stripping voltammetric detection of AgNPs was performed in a KCl solution, which showed remarkable amplification efficiency, high selectivity, low nonspecific adsorption and low background signal. This study therefore presents a very promising approach for highly selective and sensitive Hg^{2+} detection.

2. Material and methods

2.1.Reagents and apparatus

Terminal deoxynucleotidyl transferase (TdT) and DNA oligonucleotides were purchased from Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The capture DNA is 5'-thiol-$(CH_2)_6$-ACT GTC ATG ATC GT-3'; and complementary DNA is 5'-ACG TTC TTG TCT GT. There are some T-T mismatches between the capture and complimentary DNA. Bio-dNTP (a mixture of 0.25 mM biotin-dATP and 1 mM other dNTPs) was purchased from MBI Fermentas. Streptavidin was purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (China). Mercaptohexanol (MCH), trisodium citrate, sodium borohydride, silver nitrate and Hg^{2+} standard solution were purchased from Aladdin Industrial Corporation (Shanghai, China). Ultrapure water was obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore) and was used in all assays. All other reagents were of analytical grade and used as received.

All electrochemical assay measurements were performed on a CHI 660D

electrochemical workstation (Chenhua, Shanghai, China).

2.2. Preparation of streptavidin functionalized AgNP

Ag nanoparticles were prepared through the reduction of $AgNO_3$ by $NaBH_4$ and were stabilized using trisodium citrate according to literature (Liu and Li, 2011). The obtained solution was centrifuged at 15,000 rpm and the precipitate was redispersed in water. Streptavidin functionalized AgNP was prepared according to literature (Lin et al., 2015). 50.0 μL 1.0 mg/mL streptavidin was added to 1.0 mL AgNP colloidal solution, that was previously adjusted to pH 6.7 with 50 mM PBS. The mixture was stirred at room temperature for 2h and then centrifuged at 12,000 rpm for 15min. Afterwards the sediment was washed three times with pH 7.4 Tris-HNO_3 solution to remove the excessive streptavidin and finally dispersed in 1.0 mL pH 7.4 Tris-HNO_3 solution to obtain the streptavidin-AgNP strace tag.

2.3.Fabrication of biosensor

The overall process is illustrated in Scheme 1. Before modification, a gold electrode (GE) with 3 mm diameter was polished to a mirror using 1.0, 0.3, and 0.05 μm alumina slurry, followed by rinsing thoroughly with deionized water. Then, 5.0 μL capture DNA (1.0 μM) was dropped onto the GE for 2 h to covalently immobilize the 3'-SH modified capture DNA at room temperature in dark. After rinsing with deionized water, the modified GE was incubated with the blocking buffer solution of MCH for 30 min at room temperature to block the active site. The biosensor was obtained after washing with 10.0 mM Tri-HNO_3 buffer solution.

2.4. Electrochemical measurements

To carry out the hybridization and SIEP amplification, the above prepared biosensor was firstly incubated with 5 μL of Hg^{2+} standard solution or real water samples and T-T mismatched complementary DNA for 30 min at 37 °C to induce DNA hybridization. After washing with washing buffer and pH 7.4 PBS, mixed solutions containing bio-dATP/dNTP mixture, 1U TdT in reaction buffer were added to each biosensor for SIEP amplification. The TdT-mediated extension reaction was performed for 60 min at 37 °C in an incubator with constant temperature and humidity. After being washed, biosensors were treated with streptavidin-AgNPs. After 20 min incubation at room temperature, biosensors were washed with 10.0 mM pH 7.4 Tri-HNO_3 buffer prior to carrying out the linear sweep stripping analysis from 0.1 to 0.35 V at a scan rate of 50 mVs^{-1} in a 1.0 M KCl solution.

The impedance spectra were recorded within the frequency range 0.1 Hz ± 50 kHz at the bias potential, 0.20 V; amplitude of alternating voltage, 5 mV, using in 0.1 M KCl solution containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$.

3. Results and discussion

3.1. Characterization of streptavidin-AgNPs and biosensor

Through interactions between the mercapto and amino groups of streptavidin and AgNPs, streptavidin could be easily functionalized onto the AgNPs to form streptavidin-AgNPs. The characterization of AgNPs and streptavidin-AgNPs have been reported in our earlier research (Lin et al., 2015). Electrochemical impedance spectroscopy (EIS) is an effective method to monitor the changes of interfacial properties, allowing the understanding of chemical transformation processes. As shown in Fig. 1, the bare GE demonstrated a larger semicircle, showing resistance towards the electron

transfer (ET) process on the GE, R_{et}, of 206 Ω (curve a). After SH-DNA was attached to the GE surface (DNA/GE), the R_{et} was increased to 540 Ω (curve b). This increase in R_{et} value was likely due to the immobilized DNA molecules serves as a blocking layer that increase in the thickness of interface and reduce effective area (Rafiee-Pour et al., 2016). In the presence of Hg^{2+}, the T-T mismatched complementary DNA were hybridized to the capture DNA. After incubating with reaction buffer solution containing bio-dATP/dNTP mixture and 1U TdT for 60 min at 37 °C, the R_{et} was further increased to 902 Ω (curve c). The increased R_{et} value attributed to the hybridization and hybridization and SIEP amplification, suggesting the successful extension of DNA chain by TdT.

3.2. Working mechanism

The detection mechanism of the proposed biosensor was illustrated in Scheme. 1. The capture DNA labeled with thiols at its 3' terminals and MCH are co-immobilized on gold electrodes via gold-thiol chemistry to form SH-DNA/MCH self-assembly monolayer. In the presence of Hg^{2+}, the T-T mismatched complementary DNA was hybridized to the capture DNA by T-Hg^{2+}-T coordination chemistry. Then, the T-T mismatched complementary DNA was catalyzed at its 3'-OH addition of dNTP containing biotin-dATP by TdT. This process results in the formation of long single-stranded DNA labeled with numerous biotins. Though the binding between biotin and strepavidin, a large number of strepavidin-AgNPs could be attached. Linear sweep stripping voltammetric detection of AgNPs was then performed in KCl solution.

To test the feasibility of the proposed strategy, linear sweep stripping voltammetric current responses of different samples were studied. As illustrated in Fig. 2, in the absence of Hg^{2+}, T-T mismatched DNA was not captured and SIEP did not occur, in which only relatively small background response was observed due to the nonspecific adsorption of AgNPs (curve a). On the other hand, in the presence of Hg^{2+}, T-T

mismatched DNA was captured. Still, without the SIEP amplification only a relatively small response was observed due to the nonspecific adsorption of AgNPs (curve b). On the contrary, a sharp stripping peak with the rapid increase of stripping current was obtained after SIEP amplification (curve c). It is significantly higher than that obtained without SIEP signal amplification. The above results provide solid support that T-Hg^{2+}-T base pair and SIEP signal amplification protocol could be integrated for highly sensitive detection of Hg^{2+}.

3.3. Optimization of detection conditions

The DNA hybridization efficiency is a key factor in improving the sensitivity of biosensor. With the increasing concentration of the capture DNA probe, the stripping peak current for Hg^{2+} increased initially and then started decreasing once the concentration of capture DNA probe was increased to 1.0 μM (Fig. 3A). It is likely due to the immobilized DNA molecules serves as a blocking layer that increase in the thickness of interface and reduce effective area (Wan et al., 2013; Rafiee-Pour et al., 2016.). Thus, 1.0 μM capture DNA probe was selected as the optimum concentration for the fabrication of biosensor in this study.

The DNA hybridization time is another important parameter affecting the analytical performance of the Hg^{2+} biosensor. With the increasing hybridization time, the stripping peak current for Hg^{2+} detection increased and then approached to a plateau once the hybridization time was extended to 30 min (Fig. 3B). Thus, 30 min was selected as the optimal time for the DNA hybridization time.

A long SIEP reaction time is expected to lengthen DNA chain for the signal amplification, which is clearly an important parameter affecting this analytical performance of the biosensor. The effect of SIEP reaction time on the stripping peak current was shown in Fig. 3C, where the peak current increased rapidly with the

increasing SIEP reaction time and then trended to a constant value when the time became longer than 60 min. The result indicate the saturation of SIEP product due to the exhaustion of substrates or inactivation of the TdT. Thus, 60 min was selected as the optimum time for the SIEP reaction.

The reaction time of biotin with the streptavidin functionalized AgNPs also affects the analytical performance. With the increasing streptavidin-biotin reaction time, the stripping peak current increased and then trended to a constant value after 20 min (Fig. 3D), which arose from saturated binding between the streptavidin functionalized AgNPs and the biotin. As such, 20 min was selected as the optimal time for the biotin-streptavidin reaction.

3.4. Analytical performance

Under the optimum conditions selected above, the stripping peak current of AgNPs showed gradually increase with increasing concentration of Hg^{2+} in the sample (incubation) solution (Fig. 4A). The calibration plot showed a good linear relationship between the stripping peak current and the logarithm of the analyte concentration within the range between 0.05 nM and 100 nM, which the linear regression equations I (μA) = 2.41×lg[Hg^{2+}] (nM) + 8.55 with a correlation coefficient of 0.9996 (Fig.4B). The limit of detection was estimated about 0.024 nM, which was the value of 3σ/S (where σ is the standard deviation of the background signal, and S is the slope of the current-target concentration line). Comparison of the performance of this biosensor and other biosensors reported in literature is summarized in Table 1S. The present SIEP amplification strategy reveals a superior sensitivity as compared to those reported previously using T-Hg^{2+}-T without amplification process (Han et al., 2009; Niu et al., 2011; Tortolini et al., 2015). it is even lower than most of the other existing Hg^{2+} sensor based HCR, Exo III or gold nanoparticle amplification. The high sensitivity and low limit

of detection could improve the detection precision and make it suitable for detecting low concentration Hg^{2+} in water samples.

The relative standard deviation (RSD) for five parallel measurements with one DNA/GE (intra-assay) incubated with 1.0 nM Hg^{2+} was 5.6%, indicating a good precision. The detection of 1.0 nM Hg^{2+} with five DNA/GE fabricated independently (inter-assay) showed a RSD of 8.2%, giving an acceptable fabrication reproducibility of the biosensors. In addition, when the biosensor was stored in dry at 4 ^{o}C, over 95% of the initial response was retained after a storage period of 15 days. These results indicate that this Hg^{2+} biosensor has acceptable reliability and stability, and is suitable for the detection of Hg^{2+} in water samples.

3.5. Detection selectivity

The selectivity of the above biosensor was evaluated by monitoring the response current when the sensing system was interfered with other metal ions, such as Mg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, Mn^{2+}. 1.0 mM other metal ions versus 5.0 nM Hg^{2+} were added into the hybridization solution, respectively. As shown in Fig. 5, these metal ions, although presented at a much higher concentration, only showed a little effect on the detection of Hg^{2+}. The high selectivity of this biosensor is due to the fact that T-T mismatch shows high affinity for Hg^{2+} against other metal ions and the binding force for T- Hg^{2+}-T is even stronger than that of the A-T base pair (Zhu et al., 2009).

3.6. Practical application

The analytical reliability and application potential of the proposed method was evaluated by recovery experiments with drinking water containing different amounts of Hg^{2+}. The drinking water samples were obtained from the tap in our laboratory (Wenzhou University). The electrochemical signal generated from the drinking water samples was

about 5.82 μA , implying that Hg^{2+} concentrations was far below 10.0 nM. Recovery is between 96% and 106% (Table S2), which certifies that this sensor can be used in the environmental monitoring of Hg^{2+}.

4. Conclusions

A novel electrochemical strategy for the highly sensitive and selective detection of Hg^{2+} in aqueous solution was developed. It integrates the selectivity of T-Hg^{2+}-T pair with SIEP amplification. Here, T-T mismatched complementary DNA is mediated by Hg^{2+} to hybridize, which subsequently catalyzes the formation of long ssDNA. The SIEP and T-Hg^{2+}-T coordination chemistry warrants respectively high sensitivity and selectivity of the biosensor. The proposed method shows a wide detection range and an ultralow detection limit. Considering the high sensitivity and selectivity of this assay, with the simple, convenient, and portable features of electrochemical technique, this strategy will be a promising candidate for Hg^{2+} detection in environmental water samples. Moreover, it can be further rationalized to detect trace amounts of other metal ions by utilizing other metal ion-interacting bases.

Acknowledgments

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Figure captions:

Scheme 1. Schematic presentation of the biosensor fabrication and the SIEP signal amplification strategy for Hg^{2+}. The capture DNA labeled with thiols at its 3' terminals and MCH are co-immobilized on gold electrodes. The presence of Hg^{2+} leads to DNA hybridization, in which complementary DNA was captured onto the biosensor surface, which subsequently catalyzed the addition of dNTP containing biotin-dATP by TdT. A large number of strepavidin-AgNPs is attached through between biotin and strepavidin, which could generate electrochemical stripping signal of silver to monitor the concentration of Hg^{2+} in KCl solution.

Fig.1. EIS of (a) bare GE, (b) DNA/GE, and (c) biosensor after hybridization with 0.5 nM Hg^{2+} and SIEP amplification conducted in 0.1 M KCl solution containing 5.0 mM $[Fe(CN)_6]^{3-/4-}$. Inset: equivalent circuit used to model impedance data in the presence of redox couples; Rs, electrolyte solution resistance; Ret interfacial electron transfer resistance; CPE, constant phase element; Zw, Warburg impedance resulting from the diffusion of ions.

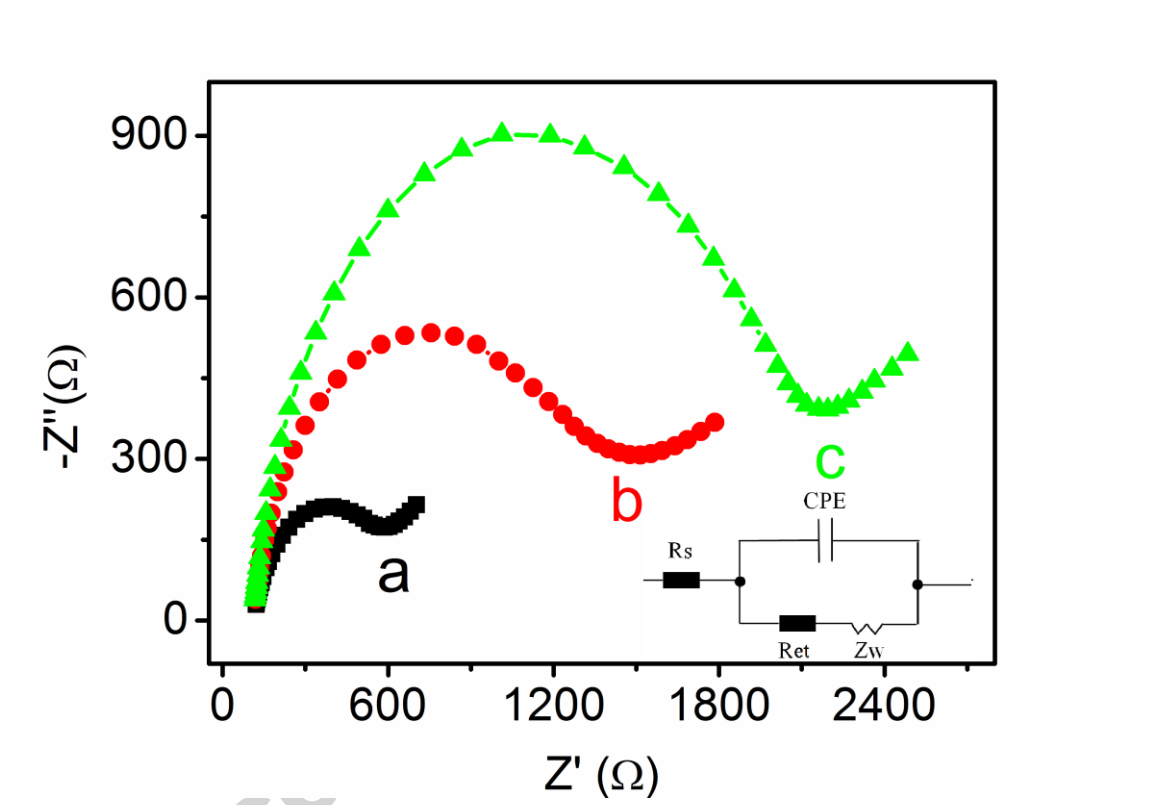
Fig.2. Linear sweep stripping voltammogram of the biosensor in 1.0 M KCl solution after incubation with 0 (a), or 0.5 nM Hg^{2+} (b, c) and T-T mismatch DNA probes, without (b) and with SIEP amplification (a, c).

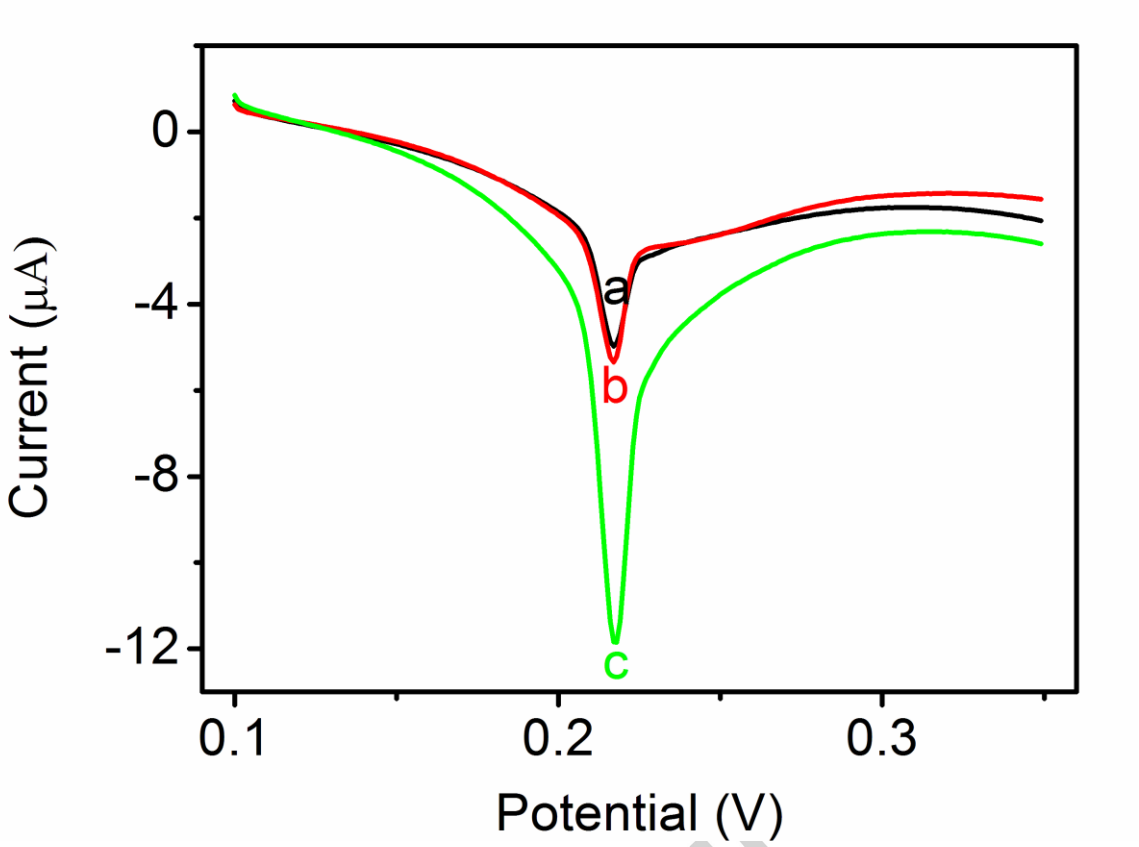
Fig.3. Effects of (A) different concentrations of capture probes, (B) reaction time of DNA hybridization, (C) SIEP reaction time and (D) streptavidin functionalized AgNPs-biotin reaction time on linear sweep stripping voltammetric responses. The concentration of Hg^{2+} was 5.0 nM.

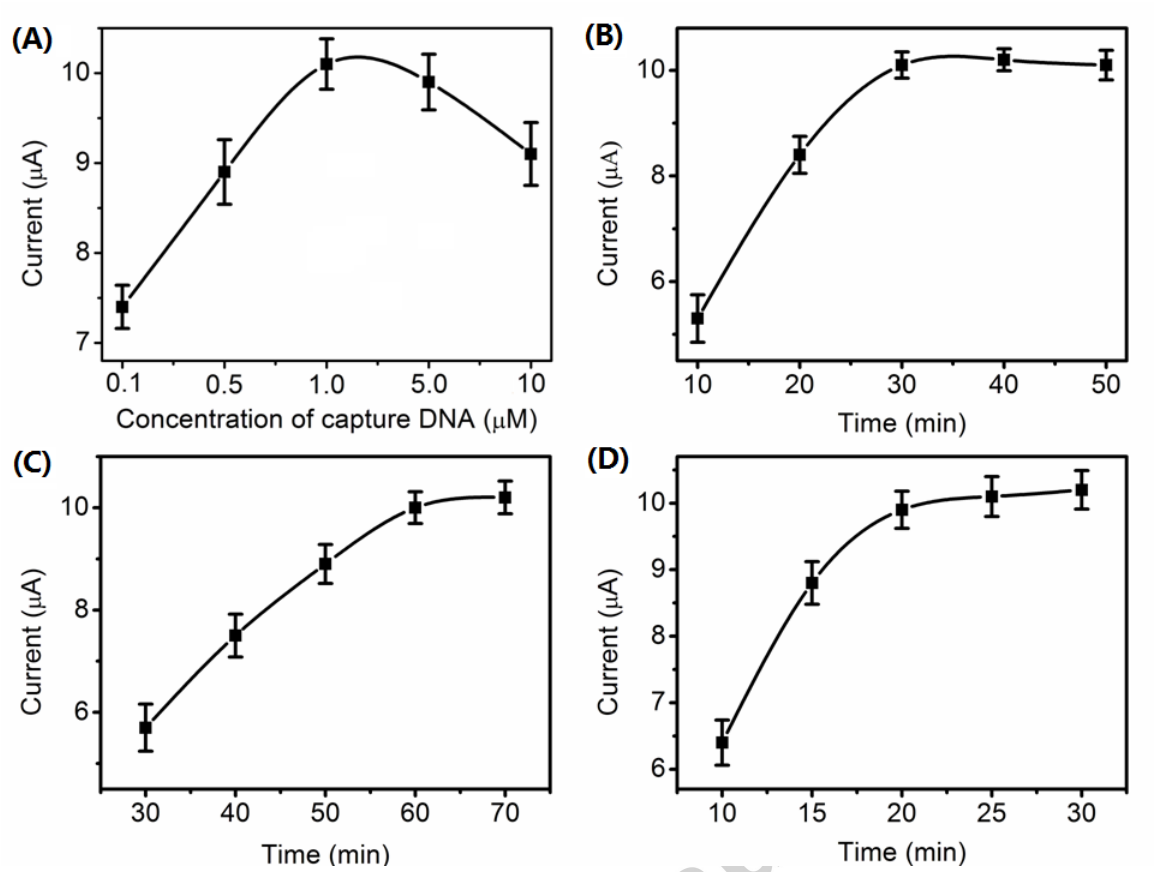
Fig.4. (A) LSV responses and (B) calibration curve of the proposed method for

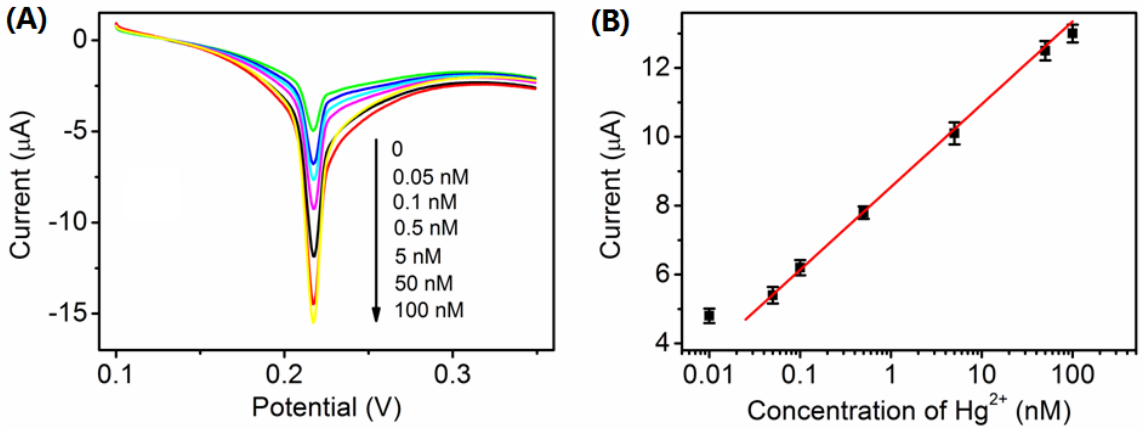
Hg^{2+}detectionin (0.05 nM, 0.1 nM, 0.5 nM, 5 nM, 50 nM, and 100 nM) in 1.0 M KCl solution (*n*=3 for relative standard deviation).

Fig. 5. Selectivity of the biosensor against other metal ions. Concentrations of Hg^{2+} and other metal ions were 5.0 nM and 1.0 mM, respectively.






(A)
Current (μA)
Concentration of capture DNA (μM)
(B)
Current (μA)
Time (min)
(C)
Current (μA)
Time (min)
(D)
Current (μA)
Time (min)


(A)
Current (μA)
Potential (V)
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0.1 nM
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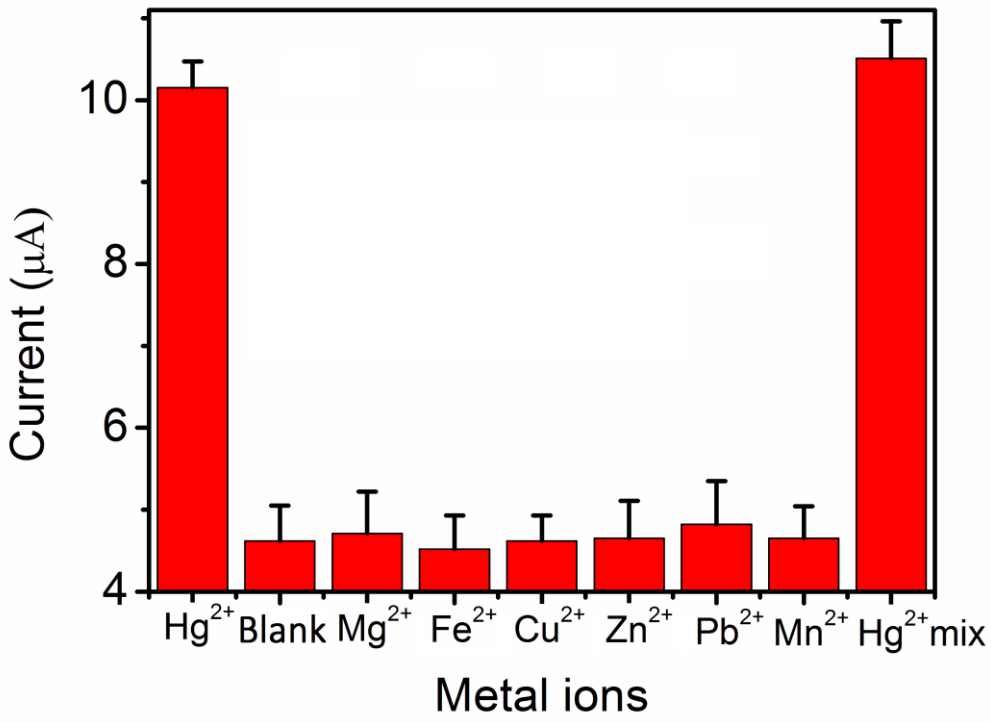
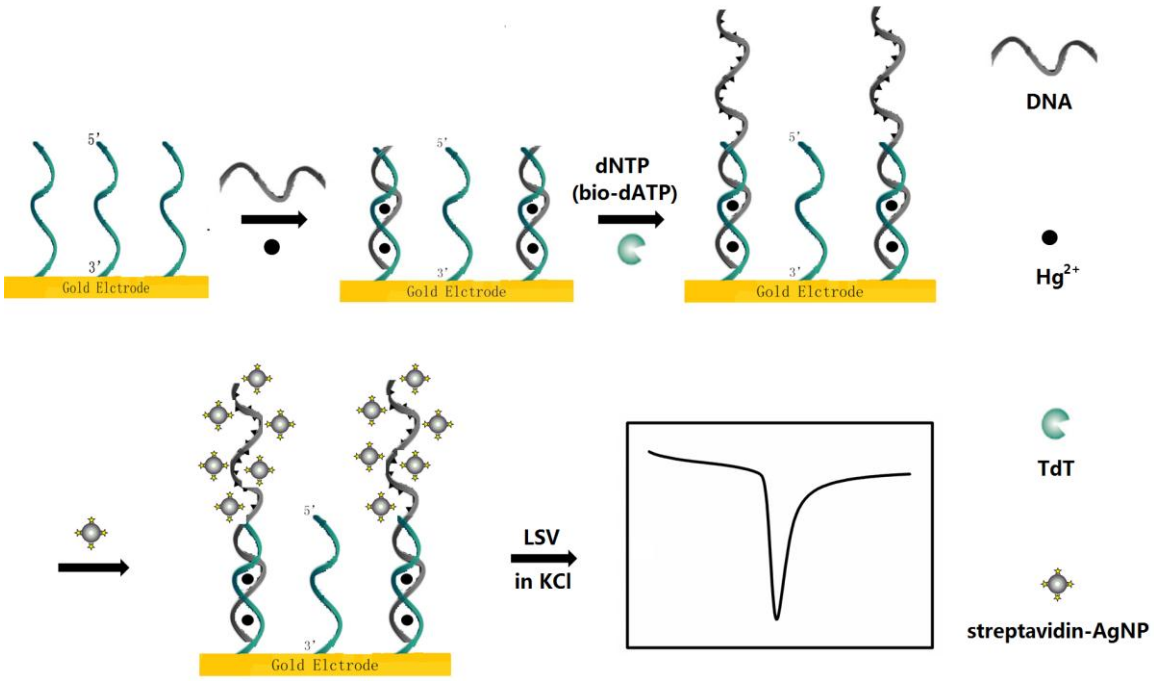
5 nM
50 nM
100 nM
(B)
Current (μA)
Concentration of Hg2+ (nM)



Scheme



Highlights

- A highly sensitive and selective platform for the Hg^{2+} detection was developed.
- This method can detect Hg^{2+} down to 0.024 nM for drinking water
- The method integrates the selectivity of thymine-Hg^{2+}-thymine base pair with DNA surface-initiated enzymatic polymerization amplification.
- This system can be further rationalized to detect trace amounts of other metal ions by utilizing other metal ion-interacting bases.