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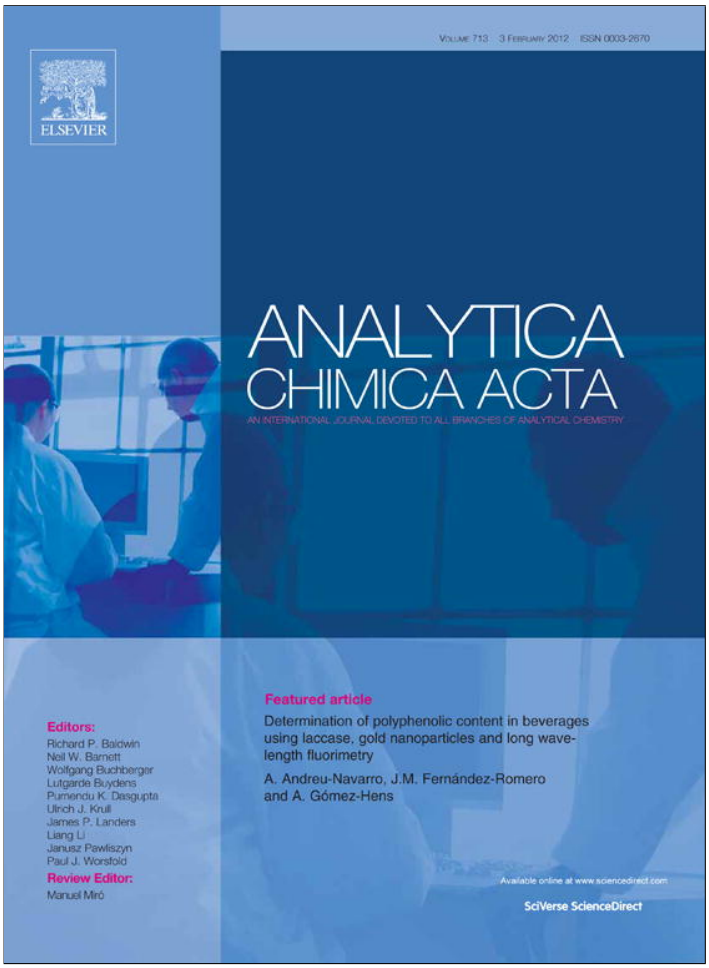

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Analytica Chimica Acta 713 (2012) 45–49

Contents lists available at SciVerse ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

An electrochemiluminescent assay for high sensitive detection of mercury (II) based on isothermal rolling circular amplification

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ARTICLE INFO

Article history:
Received 29 August 2011
Received in revised form 26 October 2011
Accepted 2 November 2011
Available online 25 November 2011

Keywords:
Eectrochemiluminescence
Rolling circular amplification
Biomimetic structure
Hg^{2+} detection

ABSTRACT

In this study, we firstly demonstrated that Bst DNA polymerase shows specific recognition and function on the T–Hg^{2+}–T biomimetic structure. Based on this, a novel available electrochemiluminescence (ECL) sensor for Hg^{2+} has been developed. In this strategy, magnet beads tagged primer was designed to complementary to the region of the circular padlock probe but with two T–T mismatches at the 3′ end. The mismatched primers cannot be extended by Bst DNA polymerase in the absence of Hg^{2+}. Stable T–Hg^{2+}–T can be formed in the presence of Hg^{2+}, thus induces the elongation and amplification reaction by DNA polymerase with a rolling circular amplification (RCA) mechanism. Subsequently, the resulted RCA products are hybridized with the tris (bipyridine) ruthenium (TBR)-tagged probes and detected by ECL platform. Current method shows a sub-nanomolar sensitivity and excellent selectivity over a spectrum of interference metal ions.

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1. Introduction

Mercury is a widespread and severe environmental pollutant, and it comes mainly from coal-burning power plants, oceanic and volcanic emissions, gold mining, and combustion of solid waste and fuels [1]. It is estimated that an annual release of 4400–7500 metric tons of mercury released into the environment [2]. The most usual and stable inorganic forms of mercury pollution, water-soluble divalent mercuric ion (Hg^{2+}), lead to wide variety of adverse health effects. Specifically, microbial biomethylation of solvated Hg^{2+} in aquatic sediments can generate methyl mercury that accumulates in the body through the food chain, and is known as a potent neurotoxin to cause permanent damage to the brain with acute and chronic cellular toxicity [3]. Therefore, it is highly desirable to develop practical mercury detection methods that can provide highly sensitive and selective routine determination of levels of Hg^{2+}. Indeed, there have been numerous reports on sensors for Hg^{2+} by using organic fluorophores [4–8] or chromophores [9–15]. Although the organic-molecule-based sensors show the high sensitivity, most of these sensors require the involvement of organic solvent, show quenched emissions, and suffer from poor selectivity in aqueous media.

It is reported that the highly selective oligonucleotide-based sensor Hg^{2+} in aqueous has attracted significant interest. In detail, Hg^{2+} can specifically bind in thymine–thymine (T–T) base pairs in DNA duplexes [16]. The binding of mercury by T–Hg^{2+}–T pairs is strong and highly selective, based on which various Hg^{2+} sensors have been developed [17–23]. For example, the fluorescence based methods allowed the simple detection of Hg^{2+} with high selectivity [17]. In addition, DNA-modified gold nanoparticles (AuNPs) were employed as a colorimetric sensor for Hg^{2+}, which relied on the Hg^{2+}-induced aggregation of Au NPs and resulting red to blue color change [18]. Furthermore, Hg^{2+}-modulated G-quadruplex-based DNAzymes have been utilized for the colorimetric Hg^{2+} sensor, which can be easily read out with the naked eye [22].

Nevertheless, most of them are limited with respect to the insufficient sensitivity (limit of detection (LOD) > 40 nM). According to the US Environmental Protection Agency (EPA) standard, the maximum allowable level (MAL) for Hg^{2+} in drinking water is 10 nM (2.0 parts per billion (ppb)). However, few reported Hg^{2+} sensors can reach such sensitivity in aqueous solutions [22,24,25]. Thus, the development of a highly sensitive and selective Hg^{2+} sensor remains a challenge.

Recent developments in biotechnologies offer a wide variety of signal-amplification tools, among which rolling circle amplification (RCA) is a representative amplification technology due to its powerful amplification under isotherm condition [26–28]. By using a DNA primer complementary to a single-strand circular DNA, it was possible to generate long single-stranded DNA molecules containing thousands of repeated segments complementary to the original circle template by rolling circle DNA replication. So far, RCA has found numerous applications in the highly sensitive detection of DNA, RNA, and protein [29–33].

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doi:10.1016/j.aca.2011.11.003

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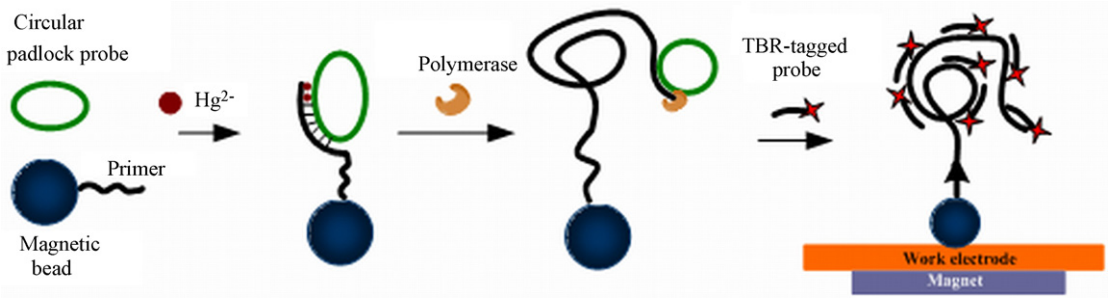

Fig. 1. Schematic description of the magnetic beads based Hg^{2+}-mediated rolling circle amplification-electrochemiluminescence sensor for the detection of Hg^{2+} in aqueous solution.

Meanwhile, electrochemiluminescence (ECL) is a general term used to describe a reaction or mechanism that produces light in the vicinity of the electrode. Because of the high sensitivity and selectivity, simple instrumentation, and low cost, ECL has recently become an important and a powerful analytical tool in analytical and clinical application [34–43]. Especially, recently two Hg^{2+} assays based on ECL techniques and T–Hg^{2+}–T construction have been developed [44,45]. Both two assays allowed very sensitive and specific detection of Hg^{2+}. In the present study, we describe a novel ECL sensor for Hg^{2+} detection by using magnetic beads based Hg^{2+}-mediated rolling circular amplification. The design of the proposed sensor is shown in Fig. 1. The principle for operation of this system relies on specific interactions between Hg^{2+} and the respective mismatched base pairs (T–T). The magnet beads tagged primer was designed to complementary to the region of the circular padlock probe but with two T–T mismatches at the 3′ end. The mismatched primers cannot be extended in the absence of the respective Hg^{2+}. In the presence of Hg^{2+}, the 3′ end of the primer can form a stable T–Hg^{2+}–T biomimetic structure, thus induces the elongation and amplification reaction by Bst DNA polymerase in the presence of dNTPs. The constantly replication of the circular padlock probe leads to the generation of long single-stranded DNA sequence with tandem repeats on the magnetic beads surfaces. Subsequently, these RCA products are hybridized with the TBR tagged probes that are complementary with a region of repeating unit. And then the excess signal probes can be easily washed off with the assist of magnetic separator. After that, the resulting magnetic beads–polymerization products–TBR complexes are detected in the custom-built ECL detection system.

2. Experimental

2.1. Materials

$Hg(ClO_4)_2$ was purchased from Alfa Aesar 97 (Royston, England). Carboxylic acid-modified magnetic beads (2.8 μM in diameter) were products of Dynal Biotech (Lake Success, NY, USA). All oligonucleotides used in this work are synthesized and purified by HPLC at Invitrogen, Guangzhou, China. Their sequences are listed in Table 1. The padlock probes are chemically 5′-phosphorylated, capture DNA was amine-functionalized with a $(CH_2)_6$ spacer at the 5′ end, and the detection probes are tagged with the $Ru(bpy)_3^{2+}$. TPA and the chemicals to synthesize the $Ru(bpy)_3^{2+}$ N-hydroxysuccinimide ester (TBR–NHS ester) are purchased from Sigma (Louis, MO, USA). The T4 DNA ligase, the exonuclease I, exonuclease III, and 100 bp DNA ladder markers and 6000 bp markers are from Takara Bio (Shiga, Japan). Bst DNA polymerase and the mixture of deoxyribonucleotides (dNTPs) are purchased from New England Biolabs. The SYBR Green I dye is offered from Invitrogen. Other chemicals employed were of analytical reagent grade and were used as received. The high-purity deionized water (resistance >18 MΩ cm) is used in all instances.

2.2. Experimental setup

The ECL system was developed in our laboratory [46,47]. The heart of the system is the electrochemical reaction cell, containing a working electrode, a counter electrode and an Ag/AgCl reference electrode. The working electrode (disk) and the counter electrode (ring) are made of platinum. A magnet under working electrode is used for capturing the magnetic beads based products. The voltage applied to the electrodes was controlled with a potentiostat (HDV-7C, Sanming, Fujian, China). Photon emission from the ECL reaction is collected by an optical fiber-bundle then detected by a single photon multiplier tube (PMT, MP-962, PerkinElmer, Wiesbaden, Germany). The signal from the PMT is amplified and discriminated. The output Transistor–Transistor Logic (TTL) pulses are converted with a multi-function acquisition card (PCL- 836, Advantech, Taiwan) and analyzed with Labview software.

2.3. Label of primer and detection probe

Magnetic bead-capture primers were prepared as follows: briefly, 100 μL of carboxyl-modified magnetic beads were washed 3 times with 500 μL of 100 mM imidazole buffer (pH 7.0), and then activated in 1000 μL of 100 mM imidazole buffer containing 30 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) with gently shaking for 30 min. 5 μL (10 μM) of primer was added into the mixture and incubated for 2 h at 37 °C with gentle mixing. Magnetic bead-capture primer were washed 3 times with 500 μL wash buffer (10 mM Tris–HCl, pH 8.0, 170 mM NaCl, 0.05% Tween20) and resuspended in 250 μL deionized water before use.

ECL detection probe was prepared according to our previously paper with minor modifications [43]. Briefly, 5′ amino-modified

Table 1
DNA sequences of and probes for discrimination of Hg^{2+} by RCA.

DNA	Sequence (5′-3′)
Padlock probes	CTGCCATCTTAACAAACCCGTACATCATCAGATTCTTGCTACTTCTGAATAGACTAAGACATGCGA TTACCGGGCT
Target oligonucleotides	GCCCATTGTTAAGATGGCAGAGCCCGGTAATCGCA ACAGACCA
Primer	AAAAAAAAAA TCAGAAGTAGCAAGTT
Detection probes	ACCCGTACATCATCAGA

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detection probe (ACCCGTACATCATCAGA) were dissolved in 100 mM sodium bicarbonate buffer (pH 8.5), then TBR–NHS ester was added to the solution at 20-fold concentration with respect to the DNA. This mixture was left to react in the dark and was gently shaken during 10 h. Labeled DNA was precipitated by addition of cold absolute ethanol. The mixture was kept 30 min at −20 °C and then centrifuged 20 min at 12,000 rpm. The supernatant was removed and the pellet was rinsed twice with cold 80% ethanol. The pellet was allowed to dry in vacuum during 10 min, then was dissolved in pure water and stored at −20 °C until use.

2.4. RCA template preparation

In experiments where DNA circles were formed in a target-specific padlock probe ligation reaction, DNA circles were prepared by ligation of 1 μM padlock probes in 100 μL of ligation system [66 mM Tris–HCl (pH 7.5), 0.1 mM ATP, 10 mM DTT, 6.6 mM $MgCl_2$], with 300 U of T4 DNA ligase and 2 μL of 10× BSA templated by 1 μM synthetic target oligonucleotides at 30 °C for 60 min. After ligation, 5 U exonuclease I and 100 U exonuclease III were added to reaction system, and the samples were incubated at 25 °C for 10 h, followed by inactivation at 95 °C for 10 min. The ligated probes were then purified by DNAmate assisted ethanol precipitation. The concentration of the purified products were measured and stored at −20 °C for further use.

2.5. Analysis of real water samples

Tap water samples were collected from South China Normal University (SCNU), and the lake water samples were from the central lake of SCNU. Before the addition of Hg^{2+} for recovery test, the water samples were all passed through 0.22-μM filters.

2.6. RCA reaction

RCA experiments were performed in 40 μL reaction system (20 mM Tris–HCl, 10 mM KCl, 10 mM $(NH4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton-100, pH 8.8) that contained 1 μM magnetic beads linked primers and 100 nM DNA circle probe. 2 μL Hg^{2+} with different concentrations (or the spiked real water samples) were added to the mixture, and was then denatured at 70 °C for 5 min and cooled down to 37 °C. Then, 10 U Bst DNA polymerase and 500 μM dNTPs were added to the mixture, and incubated at 55 °C for optimal times. The reaction was terminated by heating the mixture at 90 °C for 5 min.

2.7. ECL detection

For analyzing the samples, the 50 μL total reaction volume containing 20 μL RCA products, 5 μL detection probes (5 μM), and 25 μL hybridization buffer (20 mM TE, 600 mM NaCl, pH 7.4) was heated to 95 °C for 5 min and then incubated for 60 min at 40 °C. After the hybridization, the reaction mixture was separated by using magnetic racks (Dynal, mpc-s) and washed twice with bind buffer to remove the unbound ECL probes. The remained target analytes were resuspended in 100 μL ECL assay buffer (200 mM phosphate, 50 μM NaCl, 7 mM NaN_3, 0.8 μM Tirton X-100, 0.4 mM Tween 20, 100 mM TPA, pH 8.0). Then, the mixture was intermixed and transferred into the reaction cell, where the magnetic beads–polymerization products–TBR complexes were captured and temporarily immobilized on the working electrode by a magnet under it. A voltage of 1.25 V was applied across the electrode and the photon signal was measured.

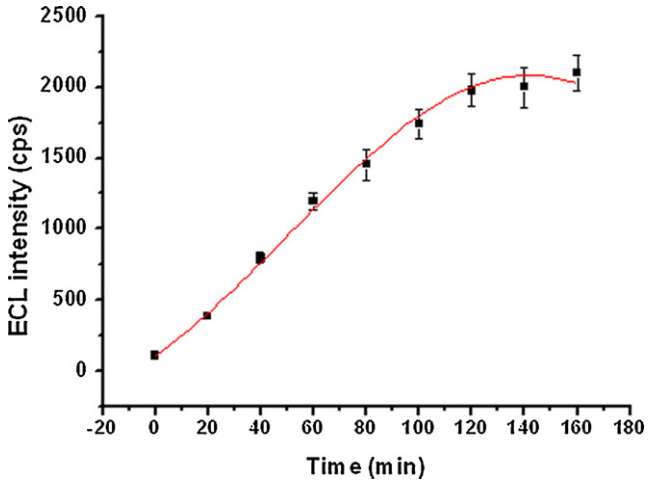

Fig. 2. ECL intensity-amplification time profile of the Hg^{2+}.

3. Results and discussions

We first optimized the amplification time and the quantity of TBR-tagged probes for the RCA based ECL detection. The RCA reaction, and ECL detection are carried out as described in the experimental section (see supporting information). Fig. 2 depicts the effect of RCA reaction time on the ECL readout. It is shown that the 100 min was selected as the optimum amplification time in the following experiments. To test the Hg^{2+} mediated RCA, the resulting products of RCA were electrophoresed in a 1% agarose gel stained with SYBR Green I dye and observed under the image analysis software (Quantity One™, Bio-RAD, CA, USA). As shown in Fig. 3a, there is an obvious strap appeared in the presence of Hg^{2+} (1 μM and 10 μM Hg^{2+}), but the strap is not found in the blank control, suggesting that Hg^{2+} can specifically initiates the RCA reaction. Subsequently, the resulting products of RCA, hybridizing with TBR probes, were detected in the custom-built ECL detection system. The Fig. 3b shows that the average ECL value obtained from analysis of 1 μM Hg^{2+} was 2539 ± 71 counts per second (cps), which was significantly higher than the value of control (152 ± 18 cps). These results are consistent with the results of gel electrophoresis, and demonstrated that the proposed method could be used to discriminate Hg^{2+} markedly.

To evaluate the sensitivity of the assay, different concentration of Hg^{2+} from stock solution were tested. The ECL value increased as the concentration of Hg^{2+} increased as shown in Fig. 4. The results show an ultrahigh sensitivity obtained from the proposed method, even when the target concentration decreased to 100 pM (20 ppt). At this concentration, the ECL value obtained from analysis of the Hg^{2+} and control was 298 ± 41 cps and 156 ± 24 cps, respectively. To define if a sample is Hg^{2+}-positive, a threshold value was calculated based on the mean of blank control plus three times the standard deviation (S.D.). According to the formula, the threshold value was set as 228 cps, indicating the Hg^{2+} could be discriminated fairly at the concentration of 100 pM Hg^{2+}. The high sensitivity can be attributed to the following factors. For instance, the Bst DNA polymerase has shown a strong and reliable ability to displace newly synthesized DNA strands under isothermal conditions, so the long single-stranded DNA sequences are produced with tandem repeats. And a large number of signal probes can be bound to the single-stranded sequences for ECL detection. In addition, the magnetic beads–polymerization products–TBR complexes can readily be collected on the electrode surface by using a magnet under the electrode. Then the ECL detection of magnetic beads enriched RCA products is executed *in situ* at the surface of a platinum electrode, leading to a construction of a highly condensed $Ru(bpy)_3^{2+}$ domain.

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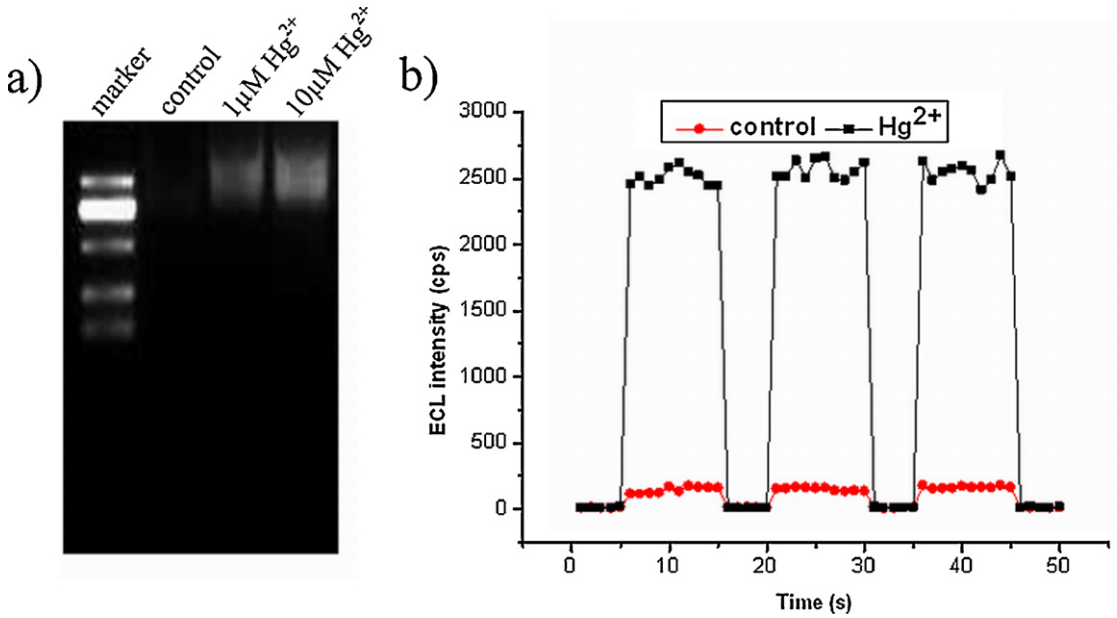

Fig. 3. The testification of the proposed method. (a) RCA products are electrophoresed in a 1% agarose gel stained with SYBR Green I dye. (b) ECL intensities corresponding to 1 μM Hg^{2+}, target-free control. On: potentiostat on. Off: potentiostat off. The DNA ladder is indicated in lane M. Lanes 1, line 2 and line 3 represent the RCA products derived from target-free control, 1 μM Hg^{2+} and 10 μM Hg^{2+}. The magnetic beads based RCA reactions are performed for 90 min at 55 °C.

The electrochemical reactions and ECL mechanisms of $Ru(bpy)_3^{2+}$ and TPA in solutions have mainly been investigated by Bard and Leland group [34,35]. This is well explained the high sensitivity obtained by current magnetic beads based ECL assay for Hg^{2+}.

The selectivity of this proposed method has was evaluated by testing the response of the assay to other environmentally relevant ions, including Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ag^{+}, Ni^{2+}, Pb^{2+} and Zn^{2+} at two concentrations (1 and 100 μM). As can be observed in Fig. 5, none of the metal ions gave ECL values higher than half of that produced by 1 nM Hg^{2+} ions, and the selectivity was determined to be at least 100 000-fold higher for Hg^{2+} over any other metal ions (1 nM Hg^{2+} versus 100 μM competing metal ions). In addition, the co-existence of other metal ions with Hg^{2+} in the sample also did not affect Hg^{2+} detection (Fig. 6). It is indicating that the assay has very high selectivity, which is attributed mainly to its ability to chelate T–T mismatches.

We calculated that precision (intra-assay variance) of the assay from 3 replicate determinations at Hg^{2+} concentrations of 1 nM and 10 nM. The CV values are 11.3% and 9.6%, respectively. To evaluate the potential practicability of current Hg^{2+} assay, we further assessed the utility of the assay by studying analytical recovery. 5 nM and 50 nM of Hg^{2} were added to tap water and lake water samples, then measured by ECL method, analytical recovery ranged from 86% to 96% (Table 2).

The assay takes advantage of the high amplification efficiency of RCA and the intrinsically high sensitivity of ECL, leading to a limit of detection of 100 pM (20 ppt), which satisfactorily meets the sensitivity requirement of EPA [2]. In addition, the RCA-based Hg^{2+} sensor exhibits excellent selectivity over a spectrum of interference metal ions. This method demonstrates several analytical advantages. First, the high sensitivity with a detection limit of 20 ppt can be three to four orders of magnitude more sensitive than many other sensing systems. Moreover, it is highly selective, which allows detection of Hg^{2+} in the presence of an excess (100 000-fold) of

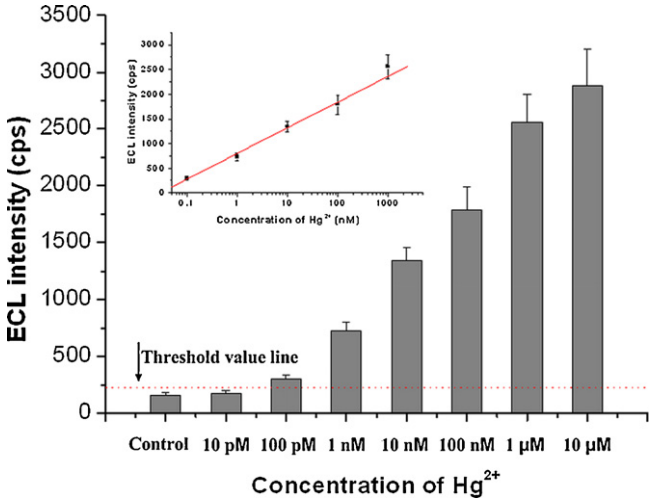

Fig. 4. Sensitivity of the Hg^{2+} sensor. The dashed line represents the threshold value that was calculated based on the mean of blank control plus three times the standard deviation (S.D.). The ECL value is increased with the increase of concentration of Hg^{2+}. Inset: the calibration curve from 0.1 to 1000 nM Hg^{2+}.

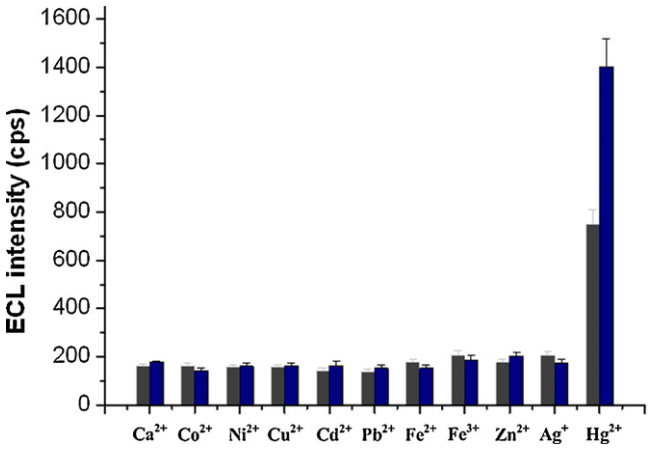

Fig. 5. Selectivity of the Hg^{2+} sensor. All competing metal ions were tested at 1 (gray) and 100 μM (blue). For comparison, sensor responses to 1 (gray) and 10 nM (blue) of Hg^{2+} were also presented. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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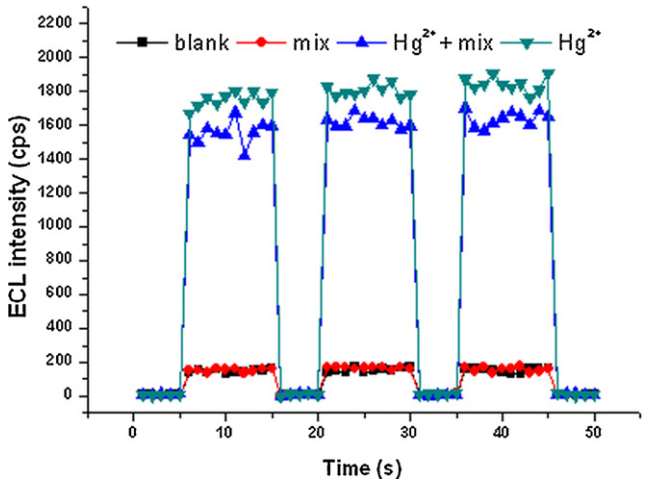

Fig. 6. Solutions containing a mixture of other metal ions (Mix; Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Ag^{+}, Ni^{2+}, Pb^{2+} and Zn^{2+} (each 100 nM)).

Table 2
Analytical recovery of Hg^{2+} added to real water samples.

	Added (nM)	Found (nM)	Recovery (%)
Tap water	0	0	–
	5	4.6	92
	50	47.7	95
Lake water	0	1.4	–
	5	5.7	86
	50	49.4	96

other metal ions in samples. Furthermore, combining with the magnetic beads based ECL technique, the proposed assay can be easily extended to a high-throughput and automatic screening format.

4. Conclusions

We have developed a highly sensitive and selective Hg^{2+} determination method using an ECL technique based on Hg^{2+}-mediated RCA. However, for developing a practical assay, more research needs to be done on improvement of the ECL intensity variation with the analysis of low concentrations of Hg^{2+} samples. It may also be possible to improve the ECL intensity by the scavenging of dithiothreitol from the storage buffer of Bst DNA polymerase. Because dithiothreitol might competitively bind with Hg^{2+} from T–Hg^{2+}–T structure, thus will affect the sensitivity. Optimized method for scavenging of dithiothreitol will need to be further evaluated. In summary, we expect this proposed method has enormous potential for the application of Hg^{2+} monitoring in environment, water, and food samples.

Acknowledgements

This research is supported by the National Basic Research Program of China (2010CB732602), the Key Program of NSFC-Guangdong Joint Funds of China (U0931005), and the Program for Changjiang Scholars and Innovative Research Team in University (IRT0829), and the National Natural Science Foundation of China (81101121).

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