Analytica Chimica Acta xxx (2018) 1–7

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Highly sensitive impedimetric biosensor for Hg^{2+} detection based on manganese porphyrin-decorated DNA network for precipitation polymerization

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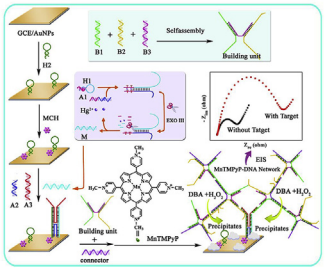
HIGHLIGHTS

- T-Hg^{2+}-T mismatches and Exo III are adopted for target converting and recycling.
- MnTMPyP-dsDNA peroxidase-like DNAzyme is used for precipitation polymerization.
- Biosensor shows good performances and is applied to Hg^{2+} detection in real samples.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 11 December 2017
Received in revised form
23 March 2018
Accepted 8 April 2018
Available online xxx

Keywords:
Impedimetric biosensor
Hg^{2+}
Target recycling
DNAzyme
Precipitation polymerization

ABSTRACT

In this work, a highly sensitive impedimetric biosensor was developed for mercuric ion (Hg^{2+}) detection. The biosensor design was based on Hg^{2+}-triggered exonuclease III (Exo III) cleavage for target recycling and DNAzyme-mediated catalytic for precipitation polymerization. Hg^{2+} induced thymine-thymine (T-T) mismatches were used to trigger the Exo III-catalyzed target recycling and produce free single-stranded DNA (defined as M). The outputted M then assisted the in formation of a DNA network on electrode surface to efficiently immobilize the porphyrin manganese (MnTmPyP). The formed MnTMPyP-double-stranded DNA (MnTmPyP-dsDNA) complex exhibited peroxidase-like activity capable of catalyzing a 3,3-diaminobenzidine (DAB) oxidation reaction, which produced an insoluble precipitate on the electrode surface. This reaction significantly enhanced the resistance signal for the quantitative determination of Hg^{2+}. Under optimal conditions, the impedimetric biosensor exhibited a wide dynamic working range of 0.005 nM−100 nM with a detection limit of 1.47 pM. This platform also demonstrated good reproducibility and selectivity, offering a promising avenue for the detection of other molecules.

1. Introduction

The mercuric ion (Hg^{2+}), one of the most highly toxic and hazardous known contaminants, poses a serious threat to human health and environment, even at low concentrations [1,2]. Thus, the routine detection of trace amounts of Hg^{2+} with high sensitivity and selectivity is of great importance in environmental protection and disease prevention [3]. Traditional detection methods, such as atomic absorption spectroscopy [4,5], atomic emission spectrometry [6,7] and inductively coupled plasma mass spectrometry (ICP-

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https://doi.org/10.1016/j.aca.2018.04.019
0003-2670/

MS) [8,9], have difficulty meeting the requirements for sensitivity, portable and simple detection of Hg^{2+}, due to the need for sophisticated instrumentation, long-term sample pretreatment processes and skilled personnel [10,11]. Since Ono and co-workers demonstrated that Hg^{2+} could specifically induce T-Hg^{2+}-T mismatches in DNA duplexes [12], different approaches including fluorescent [13,14], colorimetric [15,16], electrochemical [17,18], and electrochemiluminescent [19,20] methods, have been developed for highly sensitive detection of Hg^{2+}, promising a novel pathway for Hg^{2+} detection.

As a type of electrochemical detection technology, electrochemical impedance spectroscopy (EIS) has received considerable attention due to its powerful, nondestructive and informative virtues for the characterization of the electrical properties of biological interfaces [21]. In addition, EIS-based impedimetric biosensors with the advantage of label-free, simplicity, good sensitivity, and cost-efficiency, have been widely applied in research. In recent years, the use of enzymatic biocatalytic precipitation techniques have been a preferable approach for developing impedimetric assays. For example, Hou and co-workers [22] reported an impedimetric immunoassay protocol for the highly sensitive detection of carcinoembryonic antigen (CEA) using horseradish peroxidase (HRP) to catalyze the precipitation of 4-chloro-1-naphthol (4-CN) on the surface of the base electrode. Furthermore, Yang and co-workers [23] reported a sandwich-type electrochemical immunosensor based on a bienzyme system in which HRP and glucose oxidase biocatalyzed the precipitation for α-fetoprotein (AFP) detection. In addition, Li and co-workers [24] used the HRP-biocatalyzed oxidation of 3,3-diaminobenzidine (DAB) in the presence of hydrogen peroxide (H_2O_2) to produce insoluble precipitates to detect thrombin. These impedimetric biosensors showed extensive linear and low detection limits for target detection. However, the natural protein-assisted precipitates formation still suffered from high cost, easy loss of catalytic activity and fussy labeling progress, which restrict their further applications.

The manganese porphyrin-double strand DNA (MnTMPyP-dsDNA) complex was reported as an excellent peroxidase-mimicking enzyme that exhibits high catalytic activity [25]. On the one hand, MnTMPyP can intercalate into the grooves of negatively charged dsDNA without any labeling steps [26]. On the other hand, the dsDNA scaffold can also efficiently maintain the catalytic activity of the MnTMPyP center and even improve its stability [27]. This special property make the DNA nanostructures with much dsDNA could be used as the ideal scaffolds for MnTMPyP binding. Consequently, in this work, self-assembled DNA networks were employed as efficient nanocarriers to achieve high a loading efficiency of MnTMPyP. Thereafter, the MnTMPyP decorated DNA networks were served as peroxidase-like artificial enzyme mimic that produced precipitates on the electrode surface for the development of an impedimetric biosensor. To achieve ultra-trace detection of Hg^{2+}, target recycling amplification was widely applied in biosensor fabrication. Exonuclease III (Exo III), an enzyme capable of digesting dsDNA beginning at the 3′-hydroxyl terminus in the case of a substrate with a blunt or recessed 3′ terminus [28], was adopted to achieve target recycling amplification.

Herein, a label-free impedimetric biosensor for Hg^{2+} detection was proposed by coupling MnTMPyP decorated DNA network for precipitation polymerization and Exo III-assisted target recycling for signal amplification. As displayed in Scheme 1, the assay protocol involved three primary steps (I-III): (I) In the presence of Hg^{2+}, the T-Hg^{2+}-T base pairs could trigger a toehold-mediated strand displacement reaction between DNA A1 and H1. In this case, the Exo III could be employed for target recycling and conversion of the input target Hg^{2+} into output DNA M. (II) With the help of the output DNA M, DNA A2 and A3 could open the hairpin structure of H1 and form "H"-type DNA nanostructures which contained two single-stranded overhangs at both ends of strand A3 on the electrode surface. Upon additional introduction of the building unit (the DNA structure self-assembled using B1, B2 and B3 that contained two DNA duplexes and four single-stranded sticky ends) and the connector (DNA cross-linker), the DNA network formed on the electrode surface *via* DNA self-assembly process. Simultaneously, MnTMPyP intercalated into the DNA network, resulting in a large number of MnTMPyP-dsDNA catalytic units. (III) The MnTMPyP-dsDNA catalytic units could catalyze DAB in the presence of H_2O_2 to produce an insoluble precipitate on the electrode surface, subsequently resulting in a greatly enhanced impedance value for quantification of the target.

2. Experimental section

2.1. Reagents and apparatus

A standard solution of Hg^{2+} (GSB04-1729-2004, 1000 μg/mL) was purchased from the National Center of Analysis and Testing for Nonferrous Metals and Electronic Materials (Beijing, China). Gold chloride ($HAuCl_4$), 6-mercapto-1-hexanol (MCH), 3,3-Diaminobenzidine (DAB), Mn(III)meso-Tetra(N-methyi-4-pyridyl) porphine (MnTMPyP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Exo III was obtained from New England Biolabs, Inc. (Beverly, MA, USA). All synthetic DNA sequences shown in Table S1 (see supplementary material) were provided by Sangon Inc. (Shanghai, China). Ultrapure water (18.2 MΩ/cm) was used throughout the experiment. All other chemicals were of analytical grade and used as received.

The following buffers were employed in this work: 20 mM Tris-acetate (Tris-Ac buffer, pH 7.4) containing 50 mM NaAc and 10 mM $Mg(Ac)_2$; 25 mM HEPES buffer (pH 7.4) containing 50 mM NaCl and 20 mM KCl solution; 0.1 M PBS buffer (pH 7.4) containing 10 mM KCl, 2 mM $MgCl_2$ and 5 mM $Fe(CN)_6]^{3-/4-}$.

Cyclic voltammetry (CV) and EIS were performed on a CHI 660E electrochemistry workstation (CHI Instruments of Shanghai, China). A platinum wire was used as the counter electrode, a saturated calomel was used as the reference electrode, and a modified glassy carbon electrode (GCE, $\Phi = 4$ mm) was used as the working electrode. pH measurements were obtained with a pH meter (PHS 3C, Shanghai Leici Instrument Company, Ltd., China).

2.2. Exo III-catalyzed target recycling and conversion progress

Prior to use, the hairpin DNA H1 and H2 were heated at 95 °C for 5 min and then cooled slowly to room temperature to form the stem-loop structure. In a typical operation process, 2 μM hairpin DNA H1, 1 μM ssDNA A1 and Hg^{2+} (different concentrations) were incubated together at 37 °C for 30 min. Subsequently, 5 units of Exo III was added into the above solution, and the mixture was incubated at 37 °C for another 30 min. The total volume of the mixed sample solution prepared for further use was 25 μL.

2.3. Preparation of the impedimetric biosensor

First, three different single strands were assembled into a building unit *via* hybridization. A detailed description of this process is as follows: an equal ratio of three single strands (B1, B2 and B3) was diluted into the Tris-Ac buffer, resulting in a final concentration of 2 μM for each strand. Subsequently, the mixture was heated to 95 °C for 5 min and then gradually cooled to room temperature to obtain the building unit.

After a thorough polish and cleaning with Al_2O_3 powder and water, respectively, a thin layer of AuNPs in 1% $HAuCl_4$ solution was

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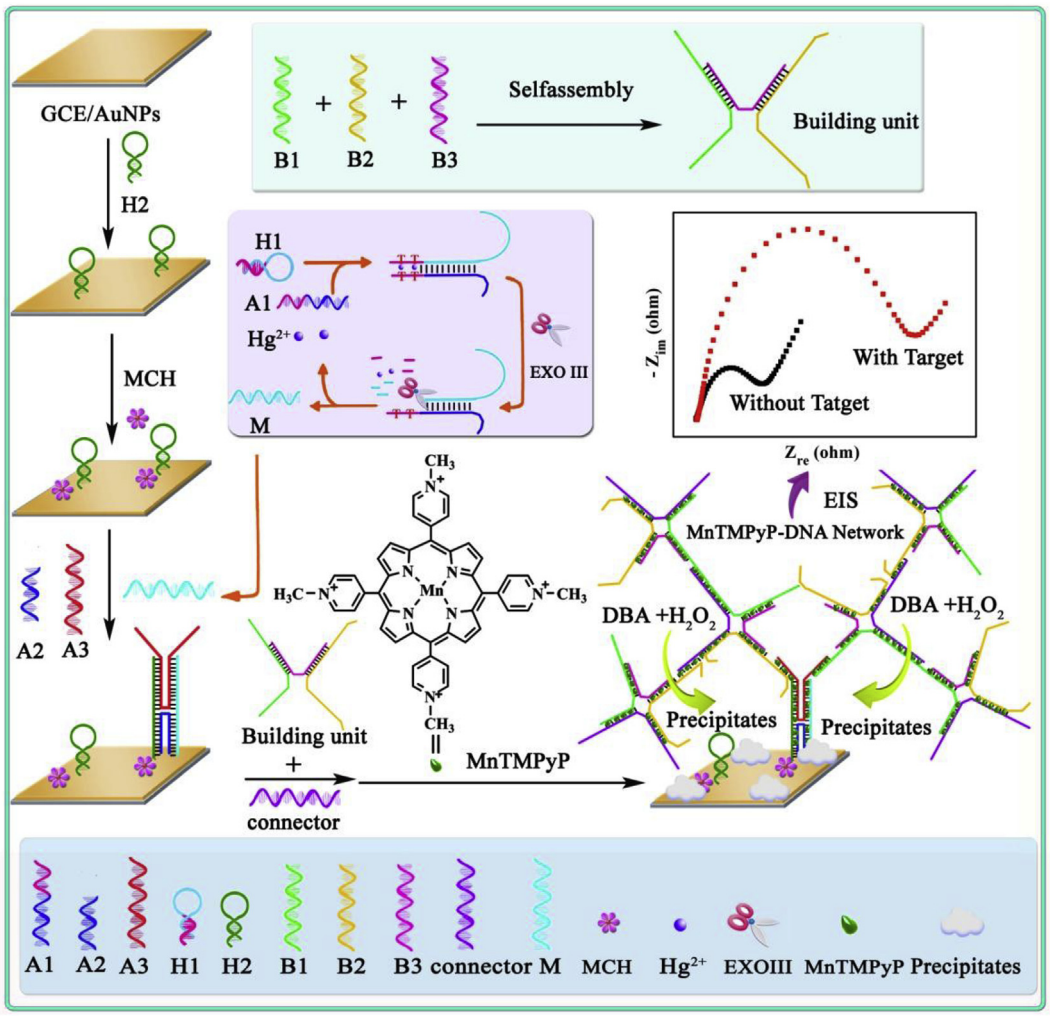

Scheme 1. Schematic diagram of the impedimetric biosensor stepwise assembly procedure and electrocatalysis detection principle.

deposited at −0.2 V for 30 s on the GCE electrode to obtain the GCE/AuNPs electrode. Afterward, 20 μL H2 (2 μM) was dropped onto the GCE/AuNPs electrode over the course of 12 h to complete the assembly of H2 on the electrode surface. Subsequently, the modified electrode was incubated with 20 μL of MCH (1.0 mM) for 30 min to block the unspecific sites. And then, 10 μL of the above Hg^{2+} conversion solution, 10 μL of a mixture of A2 and A3 (2 μM of each DNA) were simultaneously dropped onto the MCH blocked electrode and incubated for 60 min at room temperature to form H-type DNA nanostructures. After washing the electrode, the electrode was incubated with 20 μL of a mixture containing building units (2 μM), connectors (2 μM) and MnTMPyP (3 μM) at 37 °C for 90 min. After a thorough rinse with ultrapure water, the modified electrode was dipped into a 1.0 mM DAB solution containing 0.1 mM H_2O_2 and then incubated for 15 min to complete the enzymatic biocatalytic precipitation reaction. Finally, the impedimetric biosensor was prepared for EIS analysis.

2.4. Polyacrylamide gel electrophoresis

The different DNA strands and nanostructures were subjected to polyacrylamide gel electrophoresis (PAGE) using a freshly prepared 16% non-denaturing gel at a 100 V constant voltage for 150 min. Subsequently, the gels were stained with ethidium bromide and photographed with a digital camera under UV light illumination.

3. Results and discussion

3.1. Characterization of the impedimetric biosensor

Each preparation step of the impedimetric biosensor was confirmed with CV and EIS using a 5.0 mM $[Fe(CN)_6]^{3-/4-}$ solution. As shown in Fig. 1A, the bare GCE displayed a good reversible redox of $[Fe(CN)_6]^{3-/4-}$ (curve a). Upon the deposition of AuNPs, the peak current showed an obvious increase (curve b) due to the excellent conductivity of the AuNPs. After the H2 assembled onto the GCE/AuNPs electrode, the peak current decreased (curve c) due to the electron transfer hindrance of the DNA chains. After employing MCH to block nonspecific binding sites, the current decreased further due to the non-conductivity of MCH (curve d). After incubation with the Hg^{2+} conversion solution, A2 and A3, H-type DNA nanostructures formed, resulting in a current that continuously decreased (curve e) because the negative charge on the surface of the electrode continued to increase. After the assembly of the MnTMPyP-DNA network *via* incubation with the building units, connectors and MnTMPyP, an increasing peak current trend was observed (curve f) despite formation of the DNA network. This phenomenon might have contributed to the desired electron transfer characteristics from the massive loading of MnTMPyP, which has a large π-conjugated system. After the modified electrode was incubated with the precipitation solution (curve g), a

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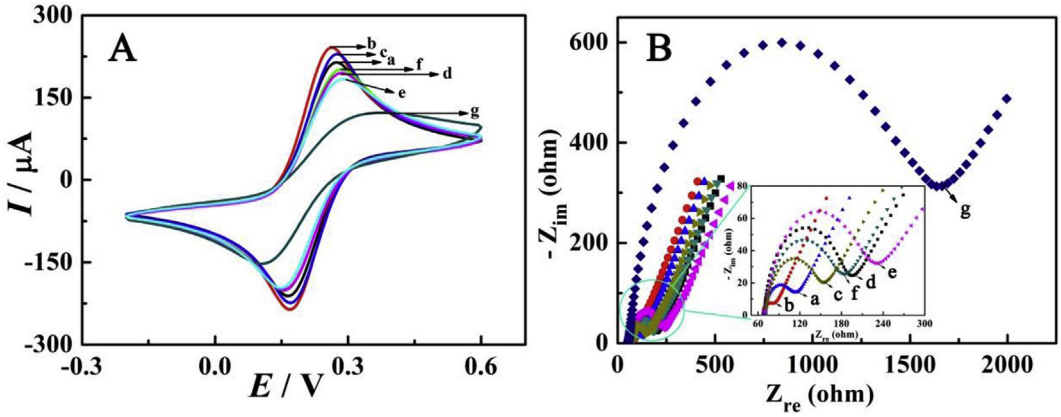

Fig. 1. CV (A) and EIS (B) results of the impedimetric biosensor at each preparation step: bare GCE (a), GCE/AuNPs (b), GCE/AuNPs/H2 (c), GCE/AuNPs/H2/MCH (d), GCE/AuNPs/H1/MCH/H2/H DNA nanostructures (e), GCE/AuNPs/H1/MCH/H2/H DNA nanostructures/MnTMPyP-DNA network (f) and GCE/AuNPs/H1/MCH/H2/H DNA nanostructures/MnTMPyP-DNA network/precipitation (g). Detection buffer: 2 mL 5 mM $Fe(CN)_6]^{3-/4-}$ (pH 7.4).

significantly decreased current response was observed, demonstrating DAB had been oxidized and formed part of the precipitate via the biocatalysis of the MnTMPyP-DNA network.

EIS was also employed to verify the assembly process of the impedimetric biosensor. As displayed in Fig. 1B, the bare GCE showed a very small impedance values (curve a). After the AuNPs had been deposited on the bare GCE, the GCE semicircle diameter decreased (curve b) due to the conductivity of AuNPs. However, the semicircle diameter increased consecutively upon the sequential incubation of negatively charged H2 (curve c) and non-conductive MCH (curve d). Resistance increased further after the electrode was incubated with the mixture of the Hg^{2+} conversion solution, A2 and A3 (curve e). The resulting formation of H-type DNA nanostructures further hindered electron transfer on the electrode surface. In addition, after the assembly of the MnTMPyP-DNA network, the resistance decreased (curve f) due to the excellent electron conductivity of the immobilized MnTMPyP. Finally, the semicircle diameter increased significantly after deposition of the insoluble precipitate on the electrode surface (curve g). The results displayed in Fig. 1B were in good agreement with those of CV (Fig. 1A), suggesting the fabrication of the impedimetric biosensor was successful and followed Scheme 1.

3.2. Investigation of the DNA network and its signal amplification capability

The reaction pathways of the proposed DNA network were characterized *via* PAGE. As depicted in Fig. 2A, without the Hg^{2+} conversion solutions containing the output DNA M, two individual bright bands with low molecular weight were observed in lane 1 for the mixture of hairpin DNA H2, ssDNA A2 and A3. The same band was observed for both H2 and A2 due to their similar molecular weights. Upon the introduction of the Hg^{2+} conversion solution into the H2, A2 and A3 mixture, the four DNA strands self-assembled into H-type DNA nanostructures. Corresponding bright bands at a higher position were observed in lane 2, demonstrating that both the target transformation and DNA assembly had occurred. After ssDNA B1, B2 and B3 self-assembled into a tripartite building unit *via* annealing, a bright band was observed in lane 3. As expected, after treating the whole system comprising H2, A2, A3, building units and connector DNA with the Hg^{2+} conversion solution, a band with a very large molecular weight was observed in lane 4, indicating the successful formation of the DNA network. The above results verified the feasibility of the proposed DNA network.

The DNA network played an essential role in the signal amplification strategy. To evaluate the signal amplification capability of the DNA network, a comparison of the experimental results for biocatalytic precipitation both with and without a DNA network is presented in Fig. 2B. The assay performed solely with H-type DNA nanostructures for the loading of MnTMPyP and subsequent precipitation (curves a, $R_{et} \approx 776\,\Omega$) resulted in a distinct increase in resistance after the H-type DNA nanostructures had assembled into a DNA network (curve b, $R_{et} \approx 1660\,\Omega$). In sum, the presence of the DNA network caused a dramatic signal increase of 114% (curve b *vs.* curve a in Fig. 2B). The results revealed that the DNA network functioned as a nanocarrier could cause significant amplification of the impedimetric signal.

3.3. Optimization of experimental conditions

As discussed previously, the DNA network played an essential role in signal amplification. A suitable assembly time was important in ensuring sufficient hybridization and massive MnTMPyP loading. Thus, the assembly time of the DNA network was investigated to achieve optimal assay performance. As shown in Fig. 3, as the assembly time increased, the resistance increased gradually at first and then plateaued to a constant value at an assembly time of 90 min. This trend is indicative of a complete hybridization of the DNA network. Therefore, a duration of 90 min was employed for DNA network assembly and MnTMPyP loading.

3.4. Analytical performance of the sensing platform

Under optimal conditions, the sensitivity of the described strategy toward Hg^{2+} was examined. Fig. 4A shows the resistance value response toward the precipitation polymerization for different Hg^{2+} concentrations. The resistance value increased as the Hg^{2+} concentration increased in the range of 0.005 nM−100 nM with a detection limit of 1.47 pM and a signal-to-noise ratio of 3σ (in which σ represents the standard deviation of a blank sample, n = 11). The plot of the resistance *vs.* the logarithm of the Hg^{2+} concentration showed a linear relationship with a regression coefficient (r) of 0.9913 (Fig. 4B) and a regression equation of $\Delta R_{et} = 1561.01 + 509.90 \lg c$ (nM). To further evaluate the advantages of the developed assay, its analytical properties were compared with other reported models (Table S2, see supplementary material). The proposed biosensor exhibited excellent analytical performance, which could be attributed to its highly effective target conversion and precipitation polymerization strategies.

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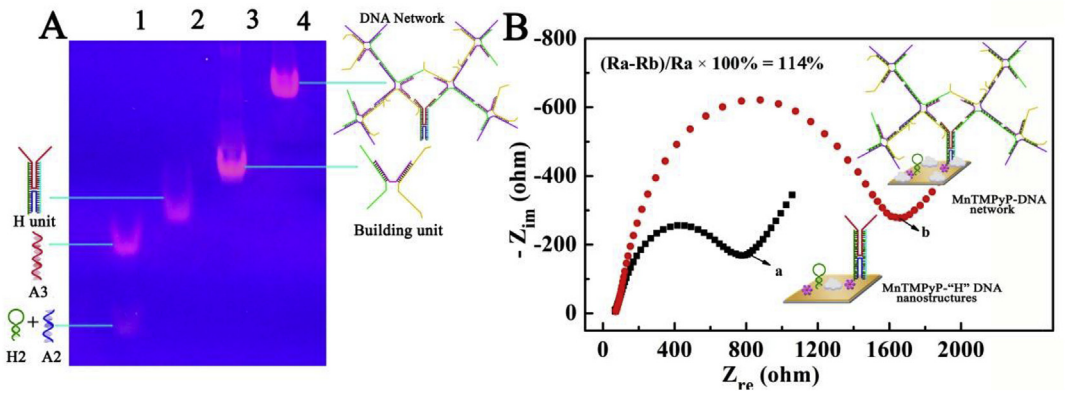

Fig. 2. (A): PAGE characterization of the reaction pathways of the DNA network. Lane 1: H2 + A2 + A3; lane 2: H2 + A2 + A3 + output DNA M; lane 3: H-type DNA nanostructures formed using B1, B2 and B3; and lane 4: connection of H-type DNA nanostructures with building units to form a DNA network using connector DNA as a cross-linker. (B) H-type DNA nanostructure (a) and the DNA network (b) used for loading MnTMPyP for the precipitation reaction.

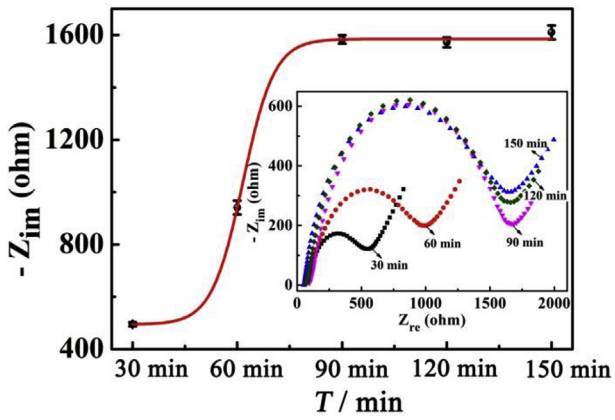

Fig. 3. Dependence of EIS responding to 1 nM target Hg^{2+} on the DNA network assembly time. The error bars represent the standard deviations for three different measurements.

3.5. Selectivity and reproducibility of the biosensor

To explore the selectivity of the proposed biosensor, several samples containing environmental relevant metal ions were tested, including Pb^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+}, at a concentration of 50 nM. The resistance to each type of interfering ion is reported in Fig. 5A. At concentrations 50-fold higher than that of Hg^{2+} (1 nM), the response from these environmental relevant metal ions showed no obvious changes in resistance in contrast to the blank test. However, significant resistance was observed in the presence of the Hg^{2+}. Moreover, the cross-sensitivity of the biosensor was examined by analyzing a mixture containing all ions. The resistance of the mixture was almost the same as that obtained from a solution containing only Hg^{2+}. The results confirmed the proposed biosensor demonstrated high selectivity. To investigate the reproducibility of the biosensor, we studied batch-to-batch precision and reproducibility by analyzing the same concentration of Hg^{2+} (1 nM) using an intra-assay and inter-assay. The relative standard deviation (RSD) of the intra-assay and inter-assay were 2.26% and 1.43%, respectively (Fig. 5B). The results demonstrated that the proposed biosensor exhibits acceptable reproducibility.

3.6. Analysis of Hg^{2+} present in samples

With excellent sensitivity and selectivity in buffer solution, the analytical applicability of the proposed impedimetric biosensor was evaluated by testing environmental samples, which included tap water, river water and landfill leachate. The content of free Hg^{2+} in the tap and river water samples exceeded the lower detection limit of the proposed method. However, obvious increases in the readout signal were observed after three environmental water samples were spiked with various concentrations of Hg^{2+}. The analytical results are summarized in Table 1. The acceptable recovery (94%–108%) and RSD (2.39%–5.14%) results indicate that the

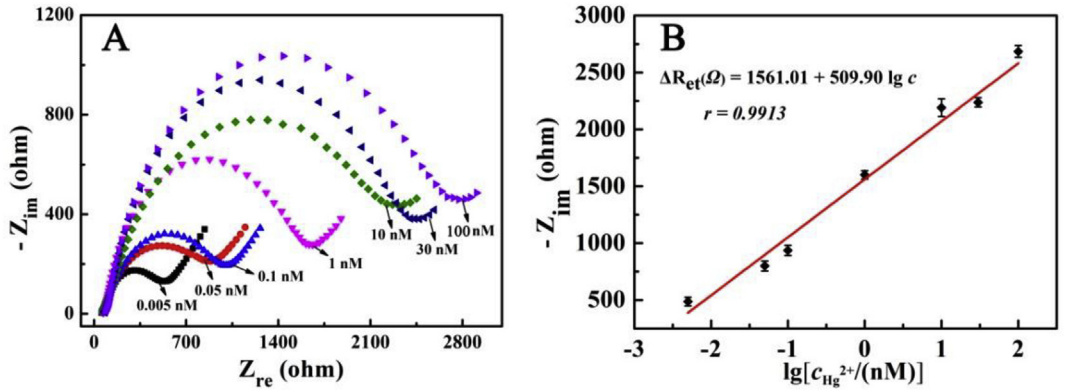

Fig. 4. (A) EIS response of the impedimetric platform toward standards containing different Hg^{2+} concentrations. (B) Calibration plot of resistance values *vs.* logarithm of the Hg^{2+} concentration.

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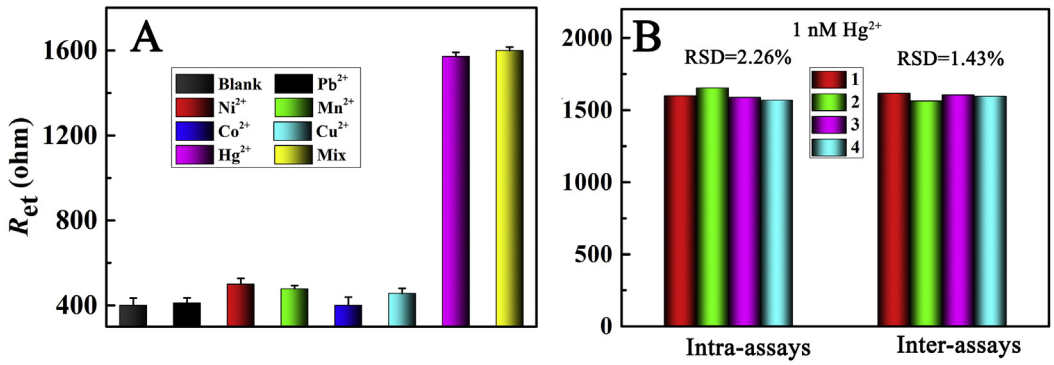

Fig. 5. (A) Specificity of the impedimetric biosensor toward the blank control, Pb^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+} and the mixture of Hg^{2+} with other interfering ions. (B) Reproducibility of the impedimetric biosensor.

Table 1
Hg^{2+} concentration in environmental sample determined by impedimetric biosensor.

Sample	Concentration found/nM	Concentration of added Hg^{2+}/nM	Concentration found after added Hg^{2+}/nM	Recovery/%	RSD /%
Tap water	none	–	–	–	–
	none	10	10.4	104	3.15
	none	30	29.5	98	2.39
River water	none	–	–	–	–
	none	10	10.8	108	4.73
	none	30	31.6	105	3.96
landfill leachate	10.5	–	9.9	94	4.47
	10.5	10	19.6	96	5.14
	10.5	30	41.7	103	3.46

impedimetric biosensor developed in this study shows potential in the analysis of complex environmental samples.

4. Conclusion

In summary, this work describes a highly sensitive impedimetric biosensor that couples a manganese porphyrin-decorated DNA network for precipitation polymerization and Exo III-assisted recycling for signal amplification. Two components of this biosensor are responsible for its significant improvement in detection performance. The first component is its *highly effective target converting and recycling strategy*. The rationally designed oligonucleotide probes help fully optimize the effect of T-Hg^{2+}-T coordination chemistry to ensure the high selectivity and sensitivity of the detection system. Furthermore, Exo III achieves highly effective target conversion and recycling. The second component is the *excellent catalytic ability of the MnTMPyP-DNA network DNAzyme*. The DNA network used in this work is self-assembled via assistance from the output DNA, which is associated with the Hg^{2+} concentration. The hybridized dsDNA configuration in the DNA network permits the 1000-fold loading of MnTMPyP without labeling or complex operations in the DNA nanostructure, resulting in a large number of MnTMPyP-dsDNA catalytic units with high catalytic activity and stability in precipitation polymerization. The excellent performance of the proposed impedimetric biosensor demonstrates its great promise for use in the environmental monitoring of Hg^{2+}. Future applications of this biosensor could include using other natural or synthetic bases that selectively bind different targets for alternative sensing devices.

Acknowledgements

This work was financially supported by the NNSF of China (21705013), the Chongqing Science and Technology Commission of China (cstc2017jcyjAX0282, cstc2015jcyjBX0126, cstc2016shmsZX20001), the Science and Technology Research Program of Chongqing Municipal Education Commission China (KJ1711265), the Foundation for High-level Talents of Chongqing University of Arts and Sciences, China (R2016CH08). Chongqing Yongchuan District Water Authority (2016–07).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.04.019.

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Please cite this article in press as: S. Xie, et al., Highly sensitive impedimetric biosensor for Hg^{2+} detection based on manganese porphyrin-decorated DNA network for precipitation polymerization, Analytica Chimica Acta (2018), https://doi.org/10.1016/j.aca.2018.04.019