An electrochemiluminescence biosensor for mercury ion detection based on gamma-polyglutamic acid-graphene-luminol composite and oligonucleotides

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\section*{ABSTRACT}
An ultrasensitive electrochemiluminescence (ECL) biosensor based on gamma-polyglutamic acid-graphene-luminol (\(\gamma\)-PGA-G-luminol) composite and oligonucleotides for the detection of mercury ion (Hg\textsuperscript{2+}) was developed. \(\gamma\)-PGA-G-luminol composite coated on the glassy carbon electrode could produce a strong and stable ECL signal by luminol, and provide carboxyl groups to cross-link with amino-modified DNA1 enriching in thymine (T) by \(\gamma\)-PGA. Upon addition of Hg\textsuperscript{2+} and biotin-modified DNA2 enriching in T, T–Hg\textsuperscript{2+}–T interaction occurred and mediated the coordination between DNA1 and DNA2. Afterwards, streptavidin was connected to the end of DNA2 through specific binding of streptavidin to biotin, which obviously reduced the ECL intensity due to the inert protein layer with poor electron transfer ability. Based on the specific T–Hg\textsuperscript{2+}–T coordination chemistry and the specific binding of streptavidin-biotin system, ultrahigh sensitivity and selectivity for Hg\textsuperscript{2+} detection were obtained. Results revealed that the ECL intensity was logarithmically linear with the concentration of Hg\textsuperscript{2+} in a wide range from 0.01 to 100 nmol/L (about 0.002–20 ppb). Moreover, the biosensor also exhibited excellent selectivity for Hg\textsuperscript{2+} ions without significant interference from commonly co-existing metal ions in the sample matrix. Excellent sensitivity and selectivity make the developed biosensor a potential and simple tool for the detection of Hg\textsuperscript{2+}.

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\section*{1. Introduction}
Mercury is considered as one of the most toxic metallic pollutants in the environment [1]. Because of its high affinity for thiol group in proteins and enzymes, low level exposure to mercury can lead to serious bioaccumulation in organisms and cause damage to the nervous system, immune system, reproductive system, and cardiovascular system [2–4]. With an estimated annual release of 4400–7500 metric tons of mercury into the environment [5], mercury pollution has been considered as a common health concern. Considering its serious health hazard, different regulatory agencies have set maximum limits for mercury. The World Health Organization and the United States Environmental Protection Agency imposed a maximum mercury level of 6 ppb and 2 ppb, respectively, for drinking water [6,7], while the European Commission set the maximum limit of 1 ppb as early as 1998 [8]. These low maximum limits of mercury in drinking water necessitate sensitive analytical methods.

The most commonly used techniques for mercury detection mainly include inductively coupled plasma atomic emission spectrometry [9], inductively coupled plasma mass spectrometry [10], cold vapor generation-atomic absorption spectrometry [11], and cold vapor generation-atomic fluorescence spectrometry [12]. These methods are sensitive, selective, and have a low detection limit, but they are instrument-intensive, expensive, and require trained technicians. Furthermore, sample preparation may be tedious, and require a long time in some cases; thus hampering the on-site application. Currently, other assays such as electrochemical techniques [13], fluorescence methods [14], colorimetric methods [15], and test strips [16], have also been developed to allow miniaturization and field applications. However, these methods usually have practical limitations due to the poor aqueous solubility, sophistication needed to synthesize probe materials, and matrix interference or cross-sensitivity toward other metal ions. Thus, a
sensitive, selective and inexpensive method that requires minimal sample handling and permits rapid detection remains highly desirable.

Electrochemiluminescence (ECL) is a well-known detection method with acknowledged advantages such as simplified optical setup, strong signal response, low background values, wide dynamic range, and inherent sensitivity [17,18]. In the fields of biomedical and chemical analysis, ECL has been widely used as an important analytical method for the determination of extremely low concentrations of nucleic acids [19,20], proteins [21–23], cells [24,25], and small molecules [26–28]. It has been reported that Hg²⁺ could specifically bind two DNA thymine (T) bases to form strong and stable T-Hg²⁺-T complexes [29], which was directly confirmed using NMR spectroscopy [30]. According to this principle, biosensors that combine the advantages of ECL and the specific binding of T-Hg²⁺-T have been recently employed for the sensitive and selective detection of Hg²⁺ [31–38].

Graphene (G), as a one-atom-thick sheet of sp²-bonded carbon atoms, has gained much attention in recent years due to its distinctive properties of electrical conductivity, large surface area, high mechanical strength, and good biocompatibility [39,40]. Owing to these properties, G has been applied extensively in the fabrication of ECL biosensors [41]. The most commonly used ECL reagent, luminol, is an excellent luminesophore with good electrochemical stability and high ECL efficiency [42]. Gamma-polyglutamic acid (γ-PGA) has been reported to be the major component of the viscous sticky mucilage in natto produced by Bacillus subtilis and has been used in a wide variety of application due to its nontoxic nature [43].

In this work, an ultrasensitive ECL biosensor using gamma-polyglutamic acid-graphene-luminol (γ-PGA-G-luminol) composite and T-rich oligonucleotides for the determination of Hg²⁺ was developed. In the fabrication process, γ-PGA was used as the biopolymer to immobilize G-luminol on the glassy carbon electrode surface and to provide an aqueous microenvironment. Moreover, amino-modified DNA1 enriching in T can be cross-linked to γ-PGA through carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). To the best of our knowledge, use of γ-PGA in building a stable composite film to develop an ECL biosensor has not been reported. Upon addition of Hg²⁺ and biotin-modified DNA2 enriching in T, T-Hg²⁺-T interaction occurred directing a coordination between DNA1 and DNA2. Streptavidin, a 66 kDa protein purified from the bacterium Streptomyces avidinii, has been widely used as an intermediate link in immunoassay due to its high specificity and strong affinity for biotin [44,45]. Based on the highly selective recognition of streptavidin to biotin, streptavidin can be efficiently immobilized at the end of DNA2. Herein, streptavidin was first used as an inert protein to inhibit the electron transfer on the electrode interface, resulting in an obvious reduction on ECL intensity. The reduction in ECL intensity in presence of Hg²⁺ enabled realizing sensitive and selective determination of Hg²⁺.

Compared with other analytical methods based on ECL and T-Hg²⁺-T coordination chemistry [31–35], this proposed method using γ-PGA-G-luminol composite film as supporting matrix has a higher sensitivity. Because luminol was directly doped into G and γ-PGA without labeling procedures, a large number of luminophore can take part in the ECL reaction that will greatly enhance the sensitivity. Benefited from the specific binding of streptavidin to biotin, streptavidin-biotin system was first used to inhibit the ECL reaction and block the ECL signal more effectively, which also improved the sensitivity significantly. Furthermore, it also possesses other advantages such as good stability, high specificity, simple instrumentation and short assay time. Therefore, this novel biosensor demonstrated a balance between ultra-high sensitivity, high selectivity and easy operation.

2. Experimental

2.1. Apparatus

ECL measurements were performed with a custom-built ECL detection system which mainly contains a BPCL ultra-weak chemiluminescence analyzer (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China) and a CHI 1100A electrochemical workstation (Chenhua Instrument Company, Shanghai, China). A conventional three-electrode system including a bare or modified glassy carbon electrode (GCE, Φ = 3 mm) as the working electrode, a platinum wire electrode as the counter electrode, and a Ag/AgCl/3 mol/L KCl electrode as the reference electrode was used throughout the experiment. Electrochemical impedance spectroscopy (EIS) analysis was performed with a CHI 660E electrochemical workstation (Chenhua Instrument Company, Shanghai, China). The concentrations of Hg²⁺ in spiked water samples were validated using Agilent 7500cx ICP-MS (Agilent Technologies Inc., Santa Clara, USA).

2.2. Reagents

Two types of oligonucleotides were synthesized and HPLC-purified by TaKaRa Biotechnology Co. Ltd. (Dalian, China), and the sequences are: 5′-NH₂–GTGTTCTCTCCCGTATC-3′ (DNA1) and 5′-biotin–GATTCCGTGATGACTCA-3′ (DNA2). Tris(2-Hydroxymethyl)aminomethane (Tris) was purchased from Acros Organics (Geel, Belgium). Gamma-polyglutamic acid (γ-PGA), bovine serum albumin (BSA, 98–99%), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 3-aminoephathalhydrazide (luminol) and streptavidin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Graphite powder (8000 mesh, 99.95%) and hydrazine monohydrate were purchased from Aladdin (Shanghai, China). Mercury nitrate and other metal nitrate salts were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Other chemicals were of analytical reagent grade or higher, and used without further purification or treatment. Tris–HCl buffer solution (pH 8.6) containing 0.05 mol/L Tris was used as the working solution for the ECL measurement. Ultrapure water (18 MΩ cm) obtained from a Deionized Power Purification System (Shanghai, China) was used throughout the experiment.

2.3. Preparation of gamma-polyglutamic acid-graphene-luminol (γ-PGA-G-luminol) composite

Graphene (G) was synthesized according to reported routes involving the steps of graphite oxidation, exfoliation and chemical reduction. Firstly, graphene oxide (GO) was synthesized from graphite powder using a method as presented by Kovtukhova et al. [46]. Then, exfoliation process was carried out by dissolving GO in deionized water using sonication, and the small amount of unexfoliated GO was removed by centrifugation. Finally, G was prepared from GO as described by Li et al. [47].

The γ-PGA-G-luminol composite was prepared as follows: 30 μL of 2 mg/mL G solution and 30 μL of 1 mmol/L luminol solution were mixed and vortexed for 5 min to obtain a homogeneous solution. Then, the mixed solution was left for 1 h for G and luminol to bond with each other adequately due to the π–π interaction between them. Next, 300 μL of 2 wt% γ-PGA was added and vortexed for 5 min to obtain the γ-PGA-G-luminol composite, which could produce a stable and strong ECL intensity after coated on GCE surface.
2.4. Fabrication of the ECL biosensor

Scheme 1A illustrated the fabrication steps of the ECL biosensor. Prior to fabrication, the bare GCE was polished to a mirror-like surface with 0.3 and 0.05 μm Al₂O₃ slurries successively, and then cleaned ultrasonically in 50% (v/v) ethanol–water solution and pure water, and dried using N₂ in order to remove any adsorbed substances on the GCE surface. Firstly 10 μL of γ-PGA-G-luminol composite was dropped onto the GCE surface and dried at room temperature. Then the electrode was immersed into a mixed solution containing 20 mg/mL EDC and 10 mg/mL NHS for 1 h to activate the carboxyl groups of γ-PGA on the electrode surface. After that, 5 μL of 1 μmol/L amino-modified DNA1 (dissolved in 0.05 mol/L PBS buffer solution, pH 7.4) was dropped onto the electrode surface and incubated for 1 h, facilitating the formation of covalent amide bond between the carboxyl group on γ-PGA and the amino group of amino-modified DNA1. Then, the electrode was immersed in 2 wt% BSA solution and incubated for 1 h to block non-specific binding sites on the electrode surface. Finally, the electrode was washed gently with pure water to remove any unbound substances and the ECL biosensor for Hg²⁺ assay was obtained.

2.5. Hybridization of DNA2 sequence

Mercury nitrate was dissolved in 0.1 mol/L nitric acid solution to prepare a 0.01 mol/L Hg²⁺ stock solution and stored in a cleaned brown glass bottle at 4 °C lightproof. Then, a series of Hg²⁺ working solutions were prepared from the stock solution by stepwise dilution with pure water. Next, the hybridization reaction was performed by dropping 5 μL of 1 μmol/L biotin-modified DNA2 solution (dissolved in 0.05 mol/L PBS buffer solution, pH 7.4) containing different concentrations of Hg²⁺ (100, 50, 10, 1, 0.1, 0.05, or 0.01 nmol/L) on the surface of electrode to react for 15 min. Finally, the electrode was rinsed with pure water to remove the non-hybridized DNA2 sequence.

2.6. ECL detection

A 5 μL of 0.1 mg/mL streptavidin solution was dropped onto the above hybridized electrode and incubated for 5 min to connect large size of streptavidin through specific binding of streptavidin-biotin system, followed by washing thoroughly with pure water to remove unbound streptavidin. After that, the electrode was scanned using chronamperometry in 0.05 mol/L Tris–HCl buffer solution (pH 8.6) with the potential stepped from −0.4 V to +0.6 V, pulse width of 0.3 s, time interval between two pulses of 30 s. In addition, the voltage of the photomultiplier was set at 800 V, and the ECL signals were measured at the same time.

2.7. Detection of Hg²⁺ in real water samples

To evaluate the application of the proposed method in environmental water samples, real water samples were collected from Yong River near the Ningbo University. The samples were filtered through 0.22 μm membrane and spiked with standard Hg²⁺ solution. Then, the spiked water samples and original water samples were analyzed separately using ICP-MS and the proposed method.

3. Results and discussion

3.1. Mechanism of the ECL biosensor

The fabrication and detection process of the ECL biosensor are depicted in Scheme 1. γ-PGA-G-luminol composite was first coated on the GCE surface to form a robust film. When compared with the bare GCE (Fig. 1A, curve a) and the GCE coated with γ-PGA-G compound (Fig. 1A, curve b), this composite produced a strong and stable ECL signal (Fig. 1A, curve c). γ-PGA not only is an ideal biopolymer with great adhesivity to immobilize special substances on solid substrates but also contains a lot of carboxyl groups. Thus, amino-modified DNA1 could be cross-linked to the surface of γ-PGA-G-luminol composite through EDC/NHS. In order to block non-specific binding sites on the composite surface, BSA was immobilized to finally construct the ECL biosensor. Meanwhile, the ECL intensity decreased obviously due to the immobilization of DNA1 and BSA (Fig. 1A, curve d and curve e, respectively), because they hindered the electron transfer on the electrode interface that inhibited the ECL reaction and blocked the electrode surface from emitting ECL signal simultaneously.

The principle for Hg²⁺ detection can be described as follows (Scheme 1B): In the absence of Hg²⁺, T–Hg²⁺–T interaction between DNA1 and DNA2 did not occur so that streptavidin could not be connected to the surface of GCE. Therefore, a higher ECL intensity was obtained. However, in the presence of Hg²⁺, biotin-modified DNA2 hybridized with DNA1 through T–Hg²⁺–T coordination chemistry and formed a stable double-stranded DNA causing a reduction in
EIS (Fig. 582, indicating successive transfer of Hg\textsuperscript{2+}, T–Hg\textsuperscript{2+}–T interaction, due to the good conductor of electricity. However, after G was added to build γ-PGA-G-luminol composite, the \( R_{ad} \) decreased distinctly due to the good conductivity of G (curve c). During the successive modification on the electrode surface with DNA1 and BSA, \( R_{ad} \) increased greatly due to its hindrance to the interfacial electron transfer (curve d), which confirmed the immobilization of these substances. In the presence of Hg\textsuperscript{2+}, DNA1 hybridized with DNA2 resulting in further increase of the semicircle (curve e), indicating that DNA2 has been successfully immobilized through T–Hg\textsuperscript{2+}–T mismatches. Similarly, immobilization of streptavidin also resulted in an increased impedance of the biosensor due to the inert protein layer and showed a larger \( R_{ad} \) (curve f).

3.3. Optimization of experimental conditions

To further improve the performance of the ECL biosensor, the effects of (a) concentration of γ-PGA, (b) pH of ECL detection solution, (c) reaction time for T–Hg\textsuperscript{2+}–T interaction, and (d) incubation time for streptavidin binding to biotin were investigated. As shown in Fig. 2A (curve a), the ECL intensity increased dramatically with the increasing concentration of γ-PGA during the initial stages, and the increase was very slow after the concentration of 1.75 wt%. Continuous increase of the PGA would reach its solubility and become difficult to obtain a homogeneous solution. Therefore, 2 wt% was chosen as the optimal concentration of PGA. The pH of ECL detection solution was an important factor affecting the ECL intensity. Therefore, the effect of pH on ECL intensity was investigated after the biosensor was constructed. As presented in Fig. 2A (curve b), the ECL intensity was low in the pH lower than 8.4 while rose to a higher value when the pH was increased to 8.6, due to the fact that the ECL behavior of luminol system had the optimal intensity in alkaline solution. Considering a higher pH may affect the stability of T–Hg\textsuperscript{2+}–T complexes [33, 34], pH 8.6 was chosen as the optimal value to produce a suitable ECL intensity. Since the reaction time for T–Hg\textsuperscript{2+}–T interaction and incubation time for streptavidin binding to biotin were also expected to influence the ECL intensity in detection process, they were studied using Hg\textsuperscript{2+} at the concentration of 1 mmol/L. As shown in Fig. 2B (curves c and d), the ECL intensities decreased during the initial stages, and reached a plateau and remained nearly unchanged thereafter. The longer time during the initial stages was due to the hybridization of DNA1 and DNA2 as well as the bonding of streptavidin and biotin. When DNA1 hybridized with DNA2 for more than 15 min and streptavidin bound to biotin for more than 5 min, the ECL intensities were barely changed because the reactions were almost effectively ended. All these results indicated that the optimal concentration of γ-PGA, pH of ECL detection solution, reaction time for T–Hg\textsuperscript{2+}–T interaction and incubation time for streptavidin binding to biotin were 2.5 wt%.

3.4. Application of ECL biosensor

The ECL biosensor was applied to the determination of Hg\textsuperscript{2+}. As shown in Fig. 3A (curve e), the ECL intensity increased with the concentration of Hg\textsuperscript{2+}, which indicates a high sensitivity of the ECL biosensor to the Hg\textsuperscript{2+} detection. The ECL biosensor also shows good selectivity and specificity to other metal ions, such as Cu\textsuperscript{2+}, Cd\textsuperscript{2+}, Pb\textsuperscript{2+}, and Zn\textsuperscript{2+}. The results show that the ECL biosensor has potential applications in the detection of Hg\textsuperscript{2+} in real samples.

Fig. 2. (A) Effect of (a) concentration of γ-PGA and (b) pH of ECL detection solution, and (C) reaction time for T–Hg\textsuperscript{2+}–T interaction and (D) incubation time for streptavidin binding to biotin on the ECL intensity.
for streptavidin binding to biotin were 2 wt%, 8.6, 15 min, and 5 min respectively.

### 3.4. Detection of Hg^{2+} using the ECL biosensor

Operational stability is one of the important factors affecting the application of ECL biosensor. G contains large π bond, while luminol contains small aromatic ring and is a weak electronic delocalization system, so there exists noncovalent π–π interaction and weak bonding force between G and luminol [35,48]. Due to the bonding force between G and luminol as well as the high viscosity of γ-PGA, GCE modified with γ-PGA–G–luminol composite gave a strong and stable ECL intensity. Fig. 3A showed the ECL emission of the biosensor under 18 cycles of continuous potential scan from −0.4 to +0.6 V for the detection of 10 nmol/L Hg^{2+}. Stable ECL signals indicated that the stability of the biosensor was excellent and suitable for ECL detection.

Under the optimal conditions, the ECL biosensor was used for the detection of Hg^{2+} at various concentrations. As presented in Fig. 3B, the ECL intensity decreased gradually with increasing concentrations of Hg^{2+}. Fig. 3C illustrates the calibration plots of the ECL intensity with the Hg^{2+} concentration in the range from 0.01 to 100 nmol/L (about 0.002–20 ppb), while the inset showed a good linear relationship between ECL intensity and the logarithm of Hg^{2+} concentration. The regression equation was $I_{ECL} = 2499.35 - 624.35 \times \log C (\text{Hg}^{2+})$ with a correlation coefficient of 0.994. According to the method previously reported for indirect detection involving the reduction or quenching of signal [49], the limit of detection (LOD) of this method is estimated to be 0.005 nmol/L (about 0.001 ppb), which was about 3 orders of magnitude lower than the Maximum Contaminant Level in drinking water set by the United States Environmental Protection Agency. Therefore, the proposed method can be used for the ultrasensitive determination of Hg^{2+}.

### 3.5. Selectivity

In addition to sensitivity, the selectivity of the ECL biosensor for the detection of Hg^{2+} was also investigated. The other non-specific metal ions including Pb^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, and Cd^{2+} at a concentration of 1 μmol/L was tested under the same conditions as in the case of Hg^{2+}. The experimental results were compared with blank sample and Hg^{2+} sample at a concentration of 1 nmol/L. As shown in Fig. 4, strong ECL intensity were obtained with non-specific metal ions and the intensities were almost similar to that of the blank sample, whereas Hg^{2+} at the concentration of 1 nmol/L has a weak ECL intensity though it has the concentration 1000 times lower than the non-specific metal ions. The obvious difference between Hg^{2+} and other non-specific metal ions on ECL intensity was due to the specific binding ability of Hg^{2+} to chelate T–T mismatches and formation of stable T–Hg^{2+}–T complexes, which contributed to the high selectivity.

### 3.6. Stability and reproducibility of the ECL biosensor

The biosensor was stored at 4 °C and lightproof in a refrigerator when not used and its stability was assessed by periodically checking its relative activity. The initial ECL intensity for 1 nmol/L Hg^{2+} detection was obtained using the biosensor constructed freshly. After 4 weeks of storage, the ECL intensity for the detection of 1 nmol/L Hg^{2+} was 90.3% of the intensity observed with freshly fabricated biosensor. Thus, the ECL biosensor has acceptable storage stability and comparable lifetime of various heavy metal ions sensors [50–52].

The reproducibility of the biosensor was evaluated through the analysis of Hg^{2+} at the same concentration using five biosensors fabricated under the same conditions. All biosensors exhibited similar levels of ECL intensity and the relative standard deviation (RSD) was 6.2%, demonstrating that the reproducibility of the proposed biosensor was excellent.

### 3.7. Application of the ECL biosensor for the detection of Hg^{2+} in river water samples

To investigate the applicability of the proposed method, the assay was validated by comparison with the analysis performed...
Table 1: Detection of Hg²⁺ in spiked river water samples using ICP-MS method and the proposed method (x = n = 5).

<table>
<thead>
<tr>
<th>Samples (nmol/L)</th>
<th>ICP-MS (nmol/L)</th>
<th>This assay (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.00</td>
<td>49.15 ± 1.42</td>
<td>51.33 ± 2.31</td>
</tr>
<tr>
<td>5.00</td>
<td>4.87 ± 0.17</td>
<td>4.84 ± 0.21</td>
</tr>
<tr>
<td>0.5</td>
<td>0.48 ± 0.02</td>
<td>0.47 ± 0.03</td>
</tr>
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using ICP-MS. Neither this biosensor nor the ICP-MS-based method detected the presence of Hg²⁺ in the original river water samples. Table 1 presents the analytical results of Hg²⁺ in spiked water samples, which demonstrated that there was no significant difference (P > 0.10) between these two methods as analyzed using group t-test. Therefore, this developed ECL biosensor was an efficient tool for ultrasensitive detection of Hg²⁺ in real water samples.

4. Conclusions

A novel ECL biosensor for the detection of Hg²⁺ based on γ-PGA-G-lumilin composite and oligonucleotide was developed. The hybridization of the two oligonucleotides through T-Hg²⁺-T coordination chemistry leads to the combination of streptavidin, which reduced the ECL intensity. The measured ECL intensity was logarithmically linear with the concentration of Hg²⁺ in the range from 0.01 to 100 nmol/L (about 0.002–20 ppb) with a linear correlation coefficient of 0.994. Moreover, the proposed method also possesses other advantages such as good selectivity even in the presence of high concentration of other common metal ions, time saving without labeling procedures and easy operation with simple instrument. Particularly, the proposed method was applied successfully to the determination of Hg²⁺ in real environmental water samples. Therefore, the developed method shows great potential for easy, rapid, low-cost and highly sensitive Hg²⁺ detection in environmental analysis.

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References


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