Sensitive Detection of Hg$^{2+}$ with Switchable Electrochemiluminescence Luminophore and Disposable Bipolar Electrode

Hongxing Liu,[a] Xiaoming Zhou,*[a] Jinjin Shen,[a] and Da Xing*[a]

A novel paper-based bipolar electrode electrochemiluminescence (pBPE-ECL) switch system was used for the rapid, label-free, and sensitive detection of Hg$^{2+}$. In the proposed approach, Hg$^{2+}$ selectively mediates two thymine-enriched single-stranded DNA probes to form double-stranded DNA (dsDNA). In the presence of Hg$^{2+}$, the “light-switch” molecule [Ru(phen)$_2$dppz$^2$$^{2+}$ can freely intercalate into the base pairs of the dsDNA, resulting in intense ECL emission. The system has a limit of 0.1 nM for Hg$^{2+}$ detection. We also showed that the system can be used to distinguish Hg$^{2+}$ over other metal ions, including Pb$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Ba$^{2+}$, Co$^{2+}$, Cd$^{2+}$, Fe$^{3+}$, Mn$^{2+}$, and Li$^+$. Moreover, the proposed approach was rapid, low-cost, and label-free for effective sensing of Hg$^{2+}$ in drinking and lake water.

With the rapid development of the industry, a large amount of heavy metal ions are discharged into the environment, such as mercury ion. Mercury ion (Hg$^{2+}$) is a highly toxic heavy metal ion and the most stable form of inorganic mercury. Hg$^{2+}$ can accumulate in vital organs and cause cellular toxicity, which leads to variety of adverse health effects, such as DNA damaged, inhibited ligand–receptor interactions, disabled normal functions of the liver and kidney, disrupted immune system homeostasis, and even death. Mercury ions at very low concentrations can cause damage to the human nervous system and the digestive system, especially in the womb and the development of the baby. The World Health Organization (WHO) and the US Environmental Protection Agency (EPA) has set the maximum permissible levels of mercury in drinking water at 30 nM[6] and 10 nM,[5] respectively. Traditional techniques for Hg$^{2+}$ measurements include atomic emission spectrometry (AES),[6] atomic absorption spectrometry (AAS),[7] atomic fluorescence spectrometry (AFS)[8] and inductively coupled plasma mass spectrometry (ICPMS).[9] These techniques are reliable and sensitive for Hg$^{2+}$ detection. Nevertheless, these techniques usually require expensive instruments, well-trained operators, or sophisticated processes which limit their implement in routine monitoring of drinking water.[10] With the development of biological sensing technology, certain optical analytical methods utilizing colorimetric, fluorescence, electrochemistry and electrochemiluminescence (ECL) have been developed for qualitative and quantitative Hg$^{2+}$ detectionsince the property of thymine–Hg$^{2+}$–thymine (T–Hg$^{2+}$–T) coordination chemistry was proposed.[11] However, colorimetric was usually suffered from poor sensitivity and selectivity for Hg$^{2+}$ detection. The fluorometric methods for mercury still need a sophisticated instrumentation though it has a high sensitivity, since dye used in the assay usually require specific excitation light source to trigger the emission.[12] Electrochemical biosensor requires to immobilized the probe on the electrode surface resulting in a complicated pretreatment process.

We previously reported a wireless and label-free paper-based bipolar electrode electroluminescence (pBPE-ECL) switch biosensor by integrating the screen printing paper-based bipolar electrode into a highly sensitive ECL detection system.[13] The pBPE consists of a wax-screen-printed hydrophilic channel and a BPE as an electronic conductor plus another two carbon electrodes as the driving electrodes, which were fabricated through screen printing onto the hydrophilic channel.[14] The biosensor not only takes advantage of the wireless of the BPE with no need of a direct electrical connection required to activate the electrochemical reactions at its poles,[15] but also adopt the “light-switch” molecule [Ru(phen)$_2$dppz$^2$$^{2+}$ (phen = 1,10-phenanthroline; dppz = dipyrrolophenazine) as switchable electroluminescence luminophore for label-free detection.

Herein, we fabricated a rapid, simple, disposable, wireless, label-free and sensitive pBPE-ECL switch system for Hg$^{2+}$ detection for the first time. Hg$^{2+}$ recognition relies on the T–T mismatch of two thymine enriched single-stranded DNA (ssDNA) probes. In the presence of Hg$^{2+}$, the two thymine enriched ssDNA probes can form T-Hg$^{2+}$-T double-stranded DNA (dsDNA) via mercury-mediated base pairs (T–Hg$^{2+}$–T), and then the “light-switch” molecule [Ru(phen)$_2$dppz$^2$$^{2+}$ can intercalate into the base pairs of the dsDNA, resulting in intense ECL emission. A low detection limit of 0.1 nM is achieved. Moreover, this approach shows high selectivity towards Hg$^{2+}$ over other metal ions, including Pb$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Ba$^{2+}$, Co$^{2+}$, Cd$^{2+}$, Fe$^{3+}$, Mn$^{2+}$, and Li$^+$. Additionally, it performs well in extended applications for Hg$^{2+}$ assays in drinking and lake water, which makes it great potential for future biosensor applications.

The proposed detection principle of this method is illustrated in Figure 1. It is well known that Hg$^{2+}$ can selectively

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mediate thymine mismatch to form a T-Hg$^{2+}$-T complex that is more thermally stable than the Watson–Crick A–T pair. To construct a label-free and sensitive system for the Hg$^{2+}$ detection, we designed two T–T mismatch thymine enriched ssDNA as the detection probes. In the absence of Hg$^{2+}$, the two T–T mismatch thymine enriched ssDNA are not capable of hybridization at room temperature because Tm is lower than the operating temperature, and even if mixed with [Ru(phen)$_2$dppz]$^{2+}$ and TPA, weak ECL signals are detected due to the fact that the ECL of [Ru(phen)$_2$dppz]$^{2+}$ is quenched by the protonation of the phenazine N atoms in the excited state in aqueous solution which only contains ssDNA. However, if Hg$^{2+}$ was presented in a solution, it would mediate the two thymine enriched ssDNA linked together by T–T base mismatch to form T–Hg$^{2+}$–T dsDNA. After mixed the dsDNA with [Ru(phen)$_2$dppz]$^{2+}$ and TPA, an ECL signal can be measured at the anode pole of the pBPE, because when [Ru(phen)$_2$dppz]$^{2+}$ binds to dsDNA, the N atoms are protected by the planar phenazine ligand interacted with the major groove of dsDNA, which produce a change in microenvironment that favored the population of a luminescent state, resulting in intense ECL emission. Once T–Hg$^{2+}$–T dsDNA formed, the emission intensity of the ECL is proportional to the Hg$^{2+}$ concentration in the presence of excess TPA, thereby enabling quantitative detection of Hg$^{2+}$.

As a proof of concept, we fabricated the pBPE as the procedure shown in Figure 2A. Briefly, the bipolar electrode and a couple of driving electrodes were screen printed on filter paper, firstly. Then, wax-screen printing was employed to form hydrophilic channels. The Table top microscope TM3030 (Hitachi High Technologies, America, Inc.) was used to get the images of the prepared hydrophilic channel, hydrophobic region and carbon electrodes to verify if they were formed well on the paper. Figure 2B shows that the hydrophilic channel of the pBPE possessed porous structures and high surface area microfibers which could offer an excellent hydrophilic microenvironment for the assay. It can be seen from Figure 2C that the wax had been well penetrated into the paper to form the hydrophobic barrier owing to the porous structure of the paper. As shown in Figure 2D, a continuous and dense conductive carbon paste layer on the surface of the paper was observed, facilitating the electrons transfer between the driving electrodes and the bipolar electrode.

To investigate the sensitivity of our newly developed pBPE-ECL molecular switch based Hg$^{2+}$ detection system, different concentrations of Hg$^{2+}$ (0, 0.05, 0.1, 0.3, 0.5, 0.8, 1, 5, 10, 50 nM) were mixed with 10 μM two thymine enriched ssDNA to form T–Hg$^{2+}$–T dsDNA, respectively. The T–Hg$^{2+}$–T dsDNA was then incubated with 0.5 mM [Ru(phen)$_2$dppz]$^{2+}$ and 50 mM TPA to evaluate the sensitivity of the pBPE-ECL molecular switch based Hg$^{2+}$ detection system. As shown in Figure 3A, the ECL intensities increase obviously with increasing Hg$^{2+}$ concentration from 0.05 to 50 nM. The luminescence observed in the experiments was not constant and actually decayed over time. This behavior was likely caused by the irreversible decomposition of the luminophore. So, the maximum luminescence signal observed within 8 s was recorded as the detection signal.

The mechanism of the [Ru(phen)$_2$dppz]$^{2+}$-DNA complex reaction with TPA at the anode of the pBPE is depicted in the Equations (1)–(4):

$$\text{[Ru(phen)$_2$dppz]}^{2+}-\text{dsDNA} + e^- \rightarrow \text{[Ru(phen)$_2$dppz]}^{3+}-\text{dsDNA}$$  

$$\text{TPA} - e^- \rightarrow \text{TPA}^+ + \text{H}^+$$  

$$\text{[Ru(phen)$_2$dppz]}^{3+}-\text{dsDNA} + \text{TPA}^+ \rightarrow \text{[Ru(phen)$_2$dppz]}^{2+}-\text{dsDNA} + \text{products}$$  

$$\text{[Ru(phen)$_2$dppz]}^{2+}-\text{dsDNA} \rightarrow \text{[Ru(phen)$_2$dppz]}^{2+}-\text{dsDNA} + \text{hv}$$

The maximum luminescence signals corresponding to each concentration of Hg$^{2+}$ and the negative control are presented.
Suggests that this system is capable of reliably performing Hg\(^{2+}\) detection. The results depicted in Figure 4 showed that weak ECL intensity was much lower than the maximum permissible level of Hg\(^{2+}\), as calculated by three times signal-to-noise ratio, which demonstrates the ECL detection against other metal ions at a concentration of 50 nM. All of the experiments were carried out under the same reaction conditions to facilitate comparison of the results with different metal ions.

To further verify the selectivity of the pBPE-ECL molecular switch based Hg\(^{2+}\) detection system, 50 nM Hg\(^{2+}\), Pb\(^{2+}\), Cu\(^{2+}\), Pb\(^{2+}\), Cd\(^{2+}\), Fe\(^{3+}\), Mn\(^{2+}\) and Li\(^+\) were subjected to the two thymine enriched ssDNA, respectively. The analysis solution contained metal ions was then incubated with 0.5 mM [Ru(phen)\(_2\)dppz]\(^{2+}\) and 50 mM TPA to evaluate the selectivity of the pBPE-ECL molecular switch based Hg\(^{2+}\) detection system. The results depicted in Figure 4 showed that weak ECL intensity occurred for Pb\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\), Ba\(^{2+}\), Cd\(^{2+}\), Cd\(^{2+}\), Fe\(^{3+}\), Mn\(^{2+}\) and Li\(^+\) or the control, which indicate that interfering ions have little effect on the detection of Hg\(^{2+}\). However, only the samples containing Hg\(^{2+}\) formed T-Hg\(^{2+}\)-T dsDNA coupled with [Ru(phen)\(_2\)dppz]\(^{2+}\) exhibited significant ECL intensity. These results confirmed that the designed pBPE-ECL molecular switch based Hg\(^{2+}\) detection system shows excellent selectivity towards Hg\(^{2+}\) over other metal ions, including Pb\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\), Ba\(^{2+}\), Cd\(^{2+}\), Cd\(^{2+}\), Fe\(^{3+}\), Mn\(^{2+}\) and Li\(^+\).

It is usually difficult to quantitatively the concentration of Hg\(^{2+}\) in the environment as the level is always quite low. To verify the sensitivity of the proposed approach is sufficient high for theoretical utility, we applied our pBPE-ECL molecular switch based Hg\(^{2+}\) detection system to the analysis of real samples, including drinking and lake water. The levels and recoveries of spiked Hg\(^{2+}\) are listed in Table 2. The results demonstrate the accuracy of this method for the detection of Hg\(^{2+}\) with low levels in real samples.

Traditional miniaturized BPE-ECL devices for Hg\(^{2+}\) detection may require complicated chip-manufacturing techniques, or expensive peripheral equipment. The present pBPE-ECL system have advantages over: 1) The assay strategy expanded the application of Hg\(^{2+}\) specific oligonucleotides based ECL method for Hg\(^{2+}\) detection. As best as we known, it is the first

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<td>Our biosensor</td>
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![Figure 3](image3.png)

Figure 3. Sensitivity assay of the pBPE-ECL analysis system. A) ECL intensities obtained in the presence of different concentrations of Hg\(^{2+}\) (0, 0.05, 0.1, 0.3, 0.5, 0.8, 1, 5, 10, 50 nM). B) A quantitative correlation between the ECL intensity and the Hg\(^{2+}\) concentrations over a wide range from 0.05 to 50 nM. Inset is the linear relationship between the ECL intensity and the Hg\(^{2+}\) concentrations ranging from 0.1 to 50 nM. The error bars indicate the relative standard deviation of measurements performed in triplicate.

![Figure 4](image4.png)

Figure 4. Selectivity investigation of the proposed detection system for Hg\(^{2+}\) detection against other metal ions at a concentration of 50 nM. All of the experiments were carried out under the same reaction conditions to facilitate comparison of the results with different metal ions.
Methods. The device has a limit of detection of 0.1 nM of Hg$^{2+}$, which is less than most of the ECL based detection time required to run an assay from start to finish is about 31.5 min which is less than most of the ECL based detection method. 3) The paper-based bipolar electrode used in the proposed method not only eliminated fussy electrode pretreatment process processes, but also avoided the direct power wire connection to working electrode, which make the detection unit disposable and simple. It is also low-cost, disposability and biodegradability

However, further developments or improvements are still required before the pBPE-ECL device could be widely used both in the laboratories and in low-resource settings for Hg$^{2+}$ detection. Currently, the layout of the pBPE should be modified and further improving the detection sensitivity. Another drawback of the current device is that the ECL signal acquire and analysis system is still relatively expensive, bulky and complicated. Although this will require future evaluation and optimization, this detection strategy can be developed into a portable platform for point of care Hg$^{2+}$ detection if integrate a DC power supply or paper-based batteries and smartphone detection model in the future.

In summary, we have demonstrated a low-cost and disposable pBPE-ECL molecular switch system for the wireless, label-free and sensitive detection of Hg$^{2+}$ for the first time. The assay takes advantage of the inherent character of the “light-switch” molecule, which can intercalate into the base pairs of DNA for label-free detection, and the wireless pBPE-ECL platform to yield a low-cost, disposable and sensitive analysis. The time required to run an assay from start to finish is about 31.5 min which is less than most of the ECL based detection methods. The device has a limit of detection of 0.1 nM of Hg$^{2+}$. We also showed that the system can be used to distinguish Hg$^{2+}$ over other metal ions, including Pb$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Ba$^{2+}$, Co$^{2+}$, Cd$^{2+}$, Fe$^{3+}$, Mn$^{2+}$ and Li$^+$, attributed to the employment of specific T-Hg$^{2+}$-T coordination chemistry to form double-stranded. Moreover, the proposed approach was rapid, low-cost, and label-free for effective sensing of Hg$^{2+}$ in drinking and lake water.

<table>
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<th>Table 2. pBPE-ECL detection of Hg$^{2+}$ in drinking and lake water.</th>
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<td>Samples</td>
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<tr>
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Whatman chromatography paper (Φ = 125.0 mm, pure cellulose paper) was purchased from Hangzhou WoHua filter paper Co., Ltd. (Zhejiang, China) and used after adjustments in size. The conductive carbon paste (model number CNB-7, < 60 Ω square$^{-1}$), which was used as a fabrication material for the driving/working electrodes, was obtained from Xuzhou Bohui New Materials Technology Co., Ltd. (Xuzhou, China). Solid wax and smooth spoon-like metal utensils were obtained from a local department store.[Ru(phen),dpbz]2(IPF$_6$), was synthesized and characterized by Professor Caiping Tan from Sun Yat-Sen University.TPA (≥ 98%, article number: 102-69-2) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Deionized water was prepared with a water purification system ($>$ 18 MΩ) and used in all the experiments. All the chemical reagents used were of analytical reagent grade without any further purification.

**Instrumentation**

The DC power supply (Model LW-K60SD) was purchased from Lonewe Instrument Meter Co., Ltd. (Hong Kong, China). The voltage of the photomultiplier tube (PMT; MP-962, Perkin Elmer, Wiesbaden, Germany) was set to 850 V for detection. The signal was then amplified and discriminated with a transistor-transistor logic (TTL) and quantified using a multi-function acquisition card (PCI-1751, Advantech, Taiwan) controlled by a Labview-based software program that was configured in-house.

**Fabrication of the Mold for Screen Printing and Wax-Screen Printing**

The mold for screen printing and wax-screen printing were fabricated as previously report with slight modification.[11]Firstly, pave a 300 nylon mesh to a hollow frame with a stretcher machine to use as the under layer of the photosensitive material to coat onto. A photo mask is produced by printed the screen printing and wax-screen printing shapes designed by Adobe Illustrator on a silver halide photosensitive film. Subsequently, exposed the as-prepared nylon mesh to ultraviolet (UV) light. Finally, developed by wash out the unexposed photosensitive material, dried and properly mended to obtain a desired mold. The wax in the wax-screen printing process can melt into the paper through screen mesh interspace and form the hydrophobic barriers. While areas with a cross-linked photosensitive material blocked the melted wax and yielded the hydrophilic channel in the screen printing process.

**Fabrication of the pBPE**

The pBPE were fabricated as previously report,[12] with slight modification, as shown in Figure 2A. First, the qualitative filter paper was cut into a suitable size, and then the driving electrodes and the bipolar electrode were screen-printed onto the paper by the mold of screen printing with conductive carbon paste, simultaneously. The semi-finished pBPE were dried at room temperature for 1 h. Second, the semi-finished pBPE was immobilized under the mold of wax-screen printing, and solid wax was evenly coated on the screen, in order to allow the solid wax to contact with the paper through the screen, a smooth metal spoon was used to further rub the screen. After the paper was wax-screen printed, it was placed on a hot plate together with the screen and heated at 80 °C for approximately 8 s to melt the wax into the paper to form the hydrophobic barriers.

**Experimental Section**

**Materials and Chemicals**

Hg(ClO$_4$)$_2$ was purchased from Alfa Aesar (Royston, England). The two thymine enriched ssDNA probes was received from Shanghai Sangon (Shanghai, China).5'-TTCTTTTCTTTTCTTTCTT-3’ and 5’-TTGTTGTTTGTGTTGTTT-3’.
pBPE-ECL Based Hg$^{2+}$ Quantitative Assays

Once the pBPE chip fabrication was completed, it can be used for the pBPE assays. It is detailed described in Figure 1. The pBPE chip was immobilized on a substrate which was fabricated by 3D-printed. And the driving electrodes were connected with a 14 V DC power supply. Then, 30 μl of the assay containing 10 μM two thymine enriched ssDNA probe, 0.5 mM [Ru(phen)2dppz]2++, 50 mM TPA and varied concentrations of Hg$^{2+}$ being mixed and incubated for 30 min at room temperature. The assay solution was then add on the center of the pBPE chip. The substrate containing the pBPE chip was turned over to ensure ECL from the pBPE chip can be detected by PMT, and the paper chip was immobilized in a black box. Before the device was powered, a 30–60 s wait time was used to ensure that the entire channel was completely filled with the assay solution. Next, turn on the DC power supply and the ECL at the BPE anodic pole could be obtained. The ECL intensity collected by the PMT and recorded by a LabVIEW-based photon-counting computer program for further analysis.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: electrochemiluminescence · label-free detection · “light-switch” molecules · mercury ions · paper-based bipolar electrodes

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