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Flow injection chemiluminescence immunoassay based on resin beads, enzymatic amplification and a novel monoclonal antibody for determination of Hg^{2+}

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Abstract:

In the present work, a simple and sensitive flow injection chemiluminescent competitive immunoassay was developed for the determination of mercury (II) ion (Hg^{2+}) based on the carboxylic resin beads, a novel specific monoclonal antibody (McAb) and HRP enzyme-amplification. Resin beads with carboxyl group were creatively employed as supports for immobilizing more coating antigen through acylamide bond. With a competitive-type assay mode, the Hg^{2+} in solution competed with the immobilized coating antigen for the limited McAb. Then, the second antibody labeled with HRP was introduced, and an effectively increased CL was obtained, which was ascribed to the catalytic activity of HRP for the luminol-PIP-H_2O_2 reaction. With increasing concentration of Hg^{2+}, the CL of this system decreases because less HRP is present in the CL reaction. At optimal conditions, the CL signal displayed a good liner relation toward Hg^{2+} in range of 0.05-200 ng/mL with a detection limit (3σ) of 0.015 ng/mL. The immunosensor possessed high specificity, acceptable accuracy and reproducibility, and was examined in real samples with favorable results. This immunoassay will be of intriguing application prospect for the determination of other heavy metal ions and environmental contaminants.

***Keywords**:* Competitive immunoassay; Enzymatic amplification; Flow injection chemiluminescence; Hg^{2+}; Monoclonal antibody.

1. Introduction

Mercury, as a naturally occurring heavy metal element, is a kind of environmental pollutants. With the development of industry, a large amount of mercury has seeped into the environment via mercury mining and smelting, combustion of waste.[1,2] Due to its hazardous biotoxicity, this issue has aroused people's wide concern.[3,4] According to the literatures, mercury can result in immune system disorders and central nervous system damage of human body.[5,6] In view of this, the World Health Organization and Environmental Protection Law of China clearly stipulate that the content of mercury in drinking water shall not exceed 6 and 1 ppb, respectively.[7,8] Therefore, it is essential to develop a sensitive and selective method to monitor mercury (II) ion (Hg^{2+}) to ensure the public health. At present, there are some traditional methods for determination of Hg^{2+}, including atomic emission spectrometry (AES),[9] cold vapour atomic absorption spectrometry (CV-AAS),[10,11] high performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS),[12,13] electrochemical methods[14,15] and enzyme-linked immunosorbent assays (ELISA).[16] Although these methods are sensitive and selective, the inherent weaknesses limit their practical application to some extent, involving expensive and highly specialized apparatus, complex and inconvenient operation.[17] Accordingly, there is a great demand for establishing a simple, cost-effective and rapid method for field monitoring of mercury.

Flow injection chemiluminescence (FI-CL) has attracted increasing attention in many fields for determining trace species due to its various analytical merits. Such as high sensitivity and sample throughput, simple instrumentation, rapid in situ and real-time monitoring.[18-21] However, its low selectivity was an obstacle that hindered further development.[19,22] Fortunately, flow injection chemiluminescence

immunoassay (FI-CLIA), which combines FI-CL with immunoassay, will overcome this drawback and become a new promising method for application. [23,24]

To achieve high selectivity for Hg^{2+}, a monoclonal antibody (McAb) that binds specifically to Hg^{2+} was applied in immunoassay. 6-Mercaptonicotinic acid (MNA), containing two functional groups of carboxyl and thiol, was creatively employed as a bridge to link mercury and carrier protein to obtain mercury-MNA-protein conjugates. These conjugates were served as coating antigen and immune antigen, and further used to prepare McAb. Based on the previous report,[25] the McAb displayed a strong affinity recognition and high specificity for Hg^{2+}.

In this study, we designed a simple flow injection chemiluminescence competitive immunoassay for the quantitative detection of Hg^{2+} using carboxylic resin beads, specific monoclonal antibody and HRP enzyme-amplification. Carboxylic resin beads were used as solid phase for loading Hg^{2+} coating antigen. Due to the large surface-to-volume ratio and good biocompatibility, they are capable of immobilizing more antigens and improving the sensitivity of FI-CLIA. Monoclonal antibody with ultrahigh specificity was applied in competitive immunoassay, in which Hg^{2+} in solution will compete with the modified coating antigen for the limited McAb. Then, HRP labeled goat-anti-mouse second antibody was introduced. Owing to the excellent catalysis effect of HRP for the reaction of luminol-PIP-H_2O_2, the CL intensity observably increased. Because of the synergistic effect of the resin beads, McAb and HRP, the immunosensing strategy showed high sensitivity and specificity, and firstly applied in Hg^{2+} detection with excellent results. This approach provided an effective tool for the determination of Hg^{2+}, and will be suitable for the novel and interesting application in environmental monitoring.

2. Experimental Section

2.1. Reagents

Monopotassium phosphate (KH_2PO_4, 99%), disodium hydrogen phosphatedodecahydrate ($Na_2HPO_4 \cdot 12H_2O$, 99%), potassiumchloride (KCl, 99.5%), sodium chloride (NaCl), Tris(hydroxymethyl)aminomethane (Tris, 99%), hydrogen peroxide (H_2O_2, 30%), *p*-iodophenol (PIP, 96%) and dimethyl sulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Ethyl-3-(dimethyl aminopropyl) carbodiimide (EDC, 98%), *N*-hydroxysuccinimide (NHS, 97%) and bovine serum albumin (BSA, 98%) were supplied by Fluka. Luminol (98%) and ovalbumin (OVA, 99%) were purchased from Sigma-Aldrich Co., Ltd. (USA). Methylmercury chloride (CH_3HgCl), 6-mercaptonicotinic acid (MNA) and mercuric chloride ($HgCl_2$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (HRP-GaMIgG) was purchased from Zhongshan Gold Bridge Biotechnology Co., Ltd. (Beijing, China). Carboxylic resin beads (diameter: 150 μm; sphericity: >99%) were obtained from Nanjing Microspheres Hi-Efficiency Isolation Carrier Co., Ltd. (Nanjing, China). All other reagents and chemicals were of analytical reagent grade. All aqueous solutions were prepared with sub-boiling distilled deionized water.

A stock solution of luminol was 0.01 M containing 1.0 M NaOH. The stock solution of 0.01 M PIP was obtained by dissolving 110 mg PIP in 5 mL DMSO and diluted with water to a final volume of 50 mL. These stock solutions were stored in the dark. Prior to use, luminol, PIP and H_2O_2 solutions were diluted with 0.1 M Tris-HCl buffer solution (pH 8.5) to the desired concentrations. Phosphate buffered saline (PBS) prepared using $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4, KCl and NaCl was used throughout the whole work.

2.2. Apparatus

Flow injection chemiluminescence measurements were carried out with an IFFM-E luminescence analyzer (Xi'an Remex Analytical Instruments Co., Ltd., China) equipped with an IFFS-A multifunction chemiluminescence detector (Xi'an Remex Analytical Instruments Co., Ltd., China). Polytetrafluoroethylene (PTFE) tubes with an inner diameter of 0.8 mm were used to connect all components in the flow system. Two peristaltic pumps were applied to convey all solutions, and an injection valve with a 100 μL loop was used to introduce the CL substrates. The chemiluminescence (CL) signals were measured with a photomultiplier (PMT) operating at –600V, and the corresponding CL intensity was used for the quantitative determination. The Scanning Electron Microscope (SEM) images were captured by a Hitachi S-4800 scanning electron microscope (Japan).

2.3 Preparation of the CH_3Hg-MNA coating antigen and monoclonal antibody

The preparation of CH_3Hg-MNA coating antigen and monoclonal antibody were referred to our previous works.[25,26] MNA acted as a linker to bond CH_3HgCl and carrier protein (BSA or OVA) due to its bifunctional groups. The obtained CH_3Hg-MNA-BSA and CH_3Hg-MNA-OVA were respectively used as immunogen and coating antigen. By using CH_3Hg-MNA-BSA to immune mice, and combining with hybridoma technique, the McAb was obtained. As reported in the literature,[25]the McAb displayed strong binding affinity and high specificity to Hg^{2+}. The above animal experiments were accomplished in accord with the relevant laws and regulations of China, and the local institutions have permitted these experiments.

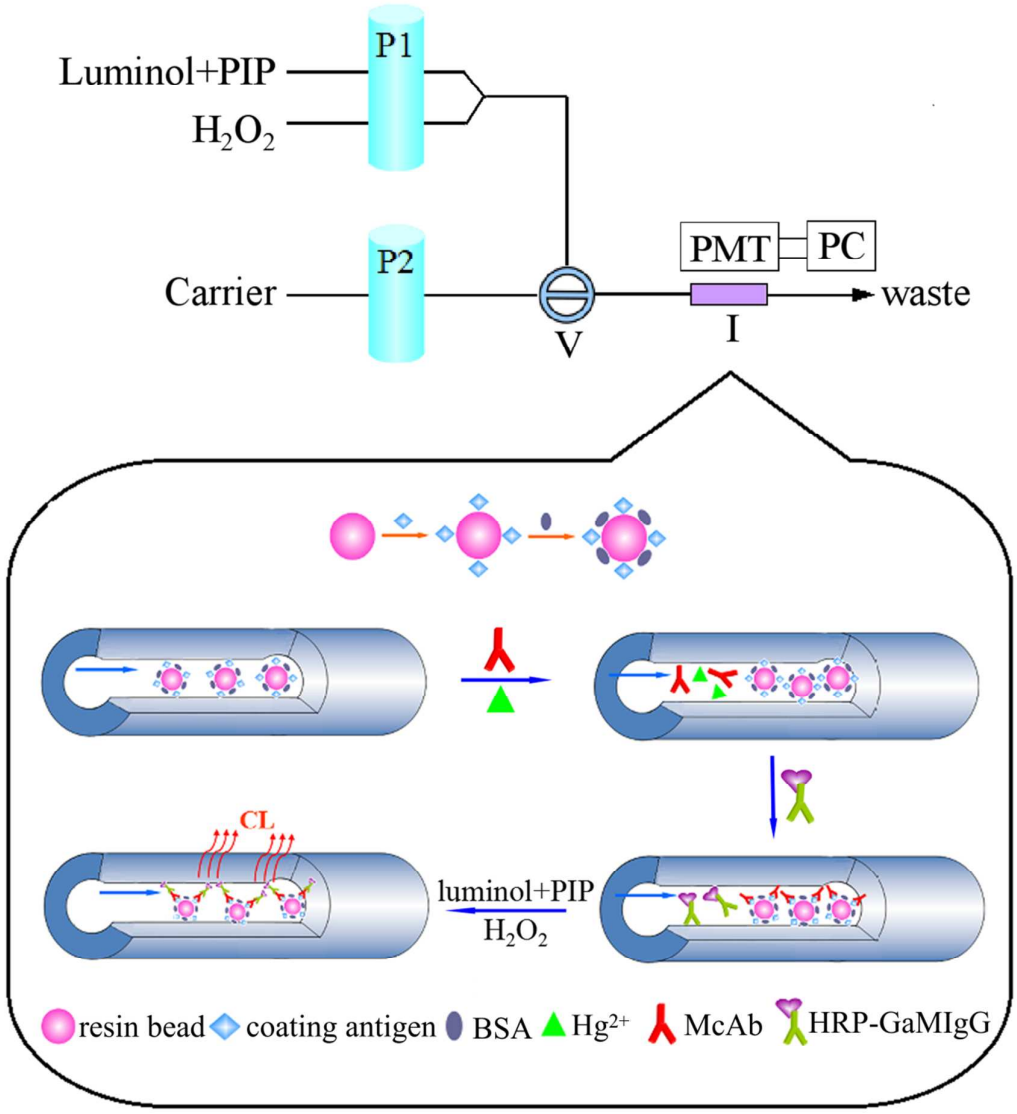
2.4 Preparation of CL immunosensor

Carboxylic resin beads were firstly swollen with 20% ethanol, then washed with 0.01 M 2-morpholinoethanesulfonic acid (pH 5.5) three times, and resuspended in 1 mL PBS buffer. After that, EDC/NHS was added to activate the carboxyl groups for 2

h with constant oscillation at room temperature (RT). After being washed thrice with PBS buffer, 10 μL of 15 μg/mL Hg^{2+} coating antigen was added into the suspension overnight in a refrigerator at 4 °C. Subsequently, the resulting coating antigen immobilized resin beads were rinsed with PBS and treated with 10 μL of 5% BSA to block the residual reactive sites. Finally, they were washed with PBS again and loaded into a glass tube for later use (Scheme 1).

2.5 Detection of chemiluminescence

Scheme 1 outlines the strategy of the determination of Hg^{2+} by this immunosensor. To obtain the incubation solution, 5 μL of different concentrations of standard Hg^{2+} solutions were mixed with 5 μL of 6 μg/mL McAb, and pre-incubated 23 min at 37 °C. Then, the incubation solution was diluted with PBS to a final volume of 8 mL, and conveyed by syringe pump through the immunosensor with a flow rate of 0.5 mL/min. There was a competitive immunoassy between Hg^{2+} standard solution and coating antigen modified on the resin beads for reacting with the limited binding sites of McAb to form the immunocomplex. Afterwards, the immunocomplex was washed with streams of PBS, and 10 μL of 5μg/mL HRP-GaMIgG was diluted to 8 mL with PBS and introduced into the immunosensor to react with McAb. To remove the uncombined immunoreagents, the glass tube was washed with PBS. Eventually, 100 μL CL substrate solutions composed by 0.6 mM luminol, 0.7 mM PIP and 4 mM H_2O_2 were delivered to the immunosensor by carrying liquid and the CL signal related to the Hg^{2+} concentration was measured. After that, the regeneration of coating antigen modified resin beads was performed by flowing 0.1 M glycine-HCl (pH 2.2) and washing with 0.01 M PBS solutions at flow rate of 2 mL/min for 1 min, respectively.



Scheme 1. Schematic diagram of FI-CLIA system: (P1) peristaltic pump, (P2) syringe pump, (V) injection valve, (I) immunosensor, (PMT) photomultiplier and (PC) personal computer.

3. Results and Discussion

3.1 Characterization of carboxylic resin beads

The different surface morphologies of the resin beads before and after modified with coating antigen are shown in Figure 1. The resin beads are polystyrene-divinyl benzene containing carboxyl groups. The carboxyl can be activated by EDC/NHS, and used to bond proteins through acylamide bonds between functional groups. Figure

1a and 1c showed the SEM images of naked resin bead in different scales. The porous structure of the swollen carboxylic resin bead provides a large surface-to-volume ratio, which is beneficial to link biomolecules. After coating antigen was bound to the carboxyl, it can be clearly observed that the surface of resin beads became smoother as shown in Figure 1d, which implied coating antigen had successfully modified on the resin beads.

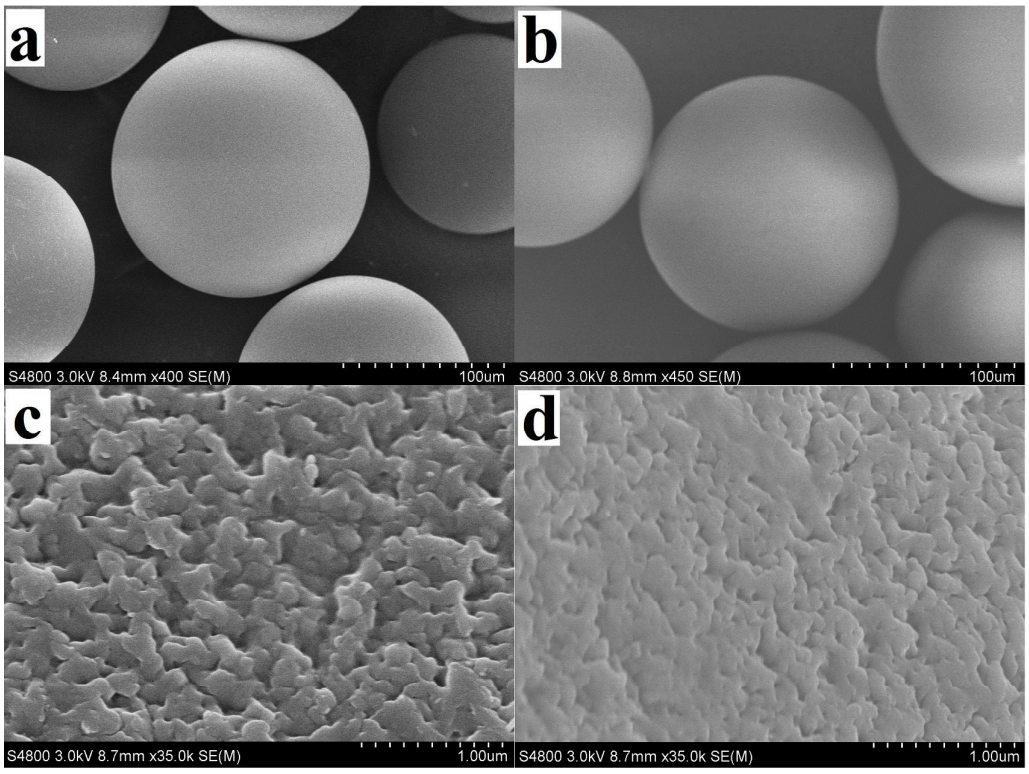


Figure 1. SEM images of naked carboxylic resin bead (a) and (c), and coating antigen immobilized resin bead (b) and (d).

3.2 Kinetic characteristic of the CL reaction

To prove the feasibility of the CL strategy relies on the catalytic effect of HRP for the luminol-PIP-H_2O_2 reaction, we investigated the kinetic characteristic of the CL system. Immunocomplex with HRP-GaMIgG, and immunocomplex with PBS were separately carried into the reactor, and the CL intensity-time curves were shown in Figure 2. When injecting the CL substrates into the immunosensor, a CL reaction was

initiated promptly and a maximum CL signal was obtained at 8 s, then it decayed to baseline after 18 s approximately. Through the comparison of curve a and b, we can observe an obvious enhancement of CL signal when HRP-GaMIgG was present, which was caused by the favorable enzymatic amplification effect of HRP for the CL reaction.

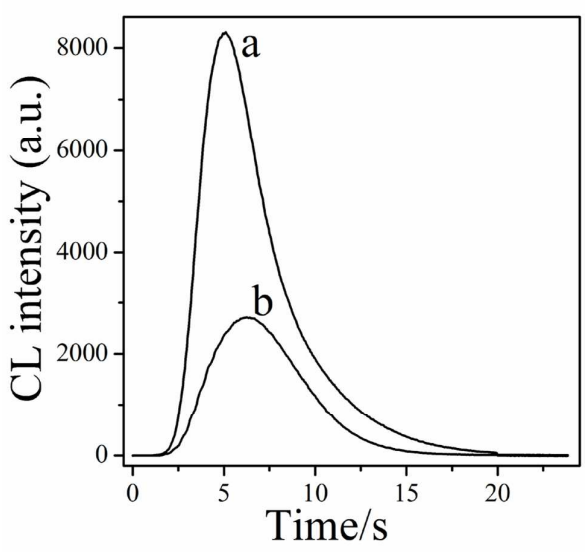


Figure 2. Kinetic curve of the luminol-PIP-H_2O_2 CL system: PBS (pH 7.4) in the presence (a) and absence of 10 μL of 5 μg/mL of HRP-GaMIgG (b), respectively. Conditions: 100 μL of Tris-HCl (pH 8.5) containing 0.6 mM luminol, 0.7 mM PIP and 4 mM H_2O_2 was injected.

3.3. Optimization of CL detection conditions

To build optimal conditions for CL reaction system, the acidity of CL system, the concentrations of CL substrate solutions and the flow rate of carrier liquid were investigated systematically. Comparing the CL emission in PBS, Na_2CO_3-$NaHCO_3$ and Tris-HCl buffer solutions, we found the CL was more stable in Tris-HCl. Therefore, all CL substrate solutions were diluted with Tris-HCl, and the corresponding optimized concentration of luminol, PIP and H_2O_2 was 0.6, 0.7 and 4 mM. The flow rates of carrier liquid would affect the CL intensity, and the higher flow

rate was helpful to collect the rapid CL emission signal. However, too high flow rate would reduce the contact time of CL substrates and HRP, which will result in the decrease of the CL signal. Based on the above analysis, we studied the effects of flow rates on CL response, and concluded that 2 mL/min of P1 and 0.5 mL/min of P2 was appropriate for the FI-CLIA system.

3.4 Optimization of the immunoassay conditions

The detection sensitivity of the immunosensor was affected by the concentrations of coating antigen, McAb and HRP-GaMIgG, and the pre-incubation time of antigen and McAb to form immunocomplex. Thus, a series of tests were conducted to pick out the optimal conditions. As shown in Figure 3a, the Δ CL intensity (the difference of the CL signal from the immunosensor for detection Hg^{2+} at 0 and 1000 ng/mL) increased with the increase of the concentration of coating antigen, and reached a relatively stable value at 15 μg/mL, indicating that the amount of coating antigen was enough for subsequent immunoassay. Hence, 15μg/mL was the optimum concentration of coating antigen. The effect of the concentration of McAb added in the incubation solution was also investigated. As shown in Figure 3b, when the concentration exceeded 6 μg/mL, the Δ CL intensity intended to be basically stable at a constant value. Thus 6 μg/mL of McAb was selected for competitive immunoassay. Due to the effective catalysis of HRP for the reaction of luminol-PIP-H_2O_2, the impact of the concentration of HRP-GaMIgG on the performance of this CL immunosensor was tested from 2-7 μg/mL (Figure 3c). The Δ CL intensity enhanced and began to flatten at 5 μg/mL, thus 5 μg/mL was the optimal concentration of HRP-GaMIgG.

In addition, the pre-incubation time also influenced the sensitivity of the immunosensor because a suitable reaction time was essential for McAb to capture Hg^{2+} containing solution. The result shown in Figure 3d indicated that 23 min of

pre-incubation time ensured the complete immunoreaction. Thus, 23 min was chosen for the further detection.

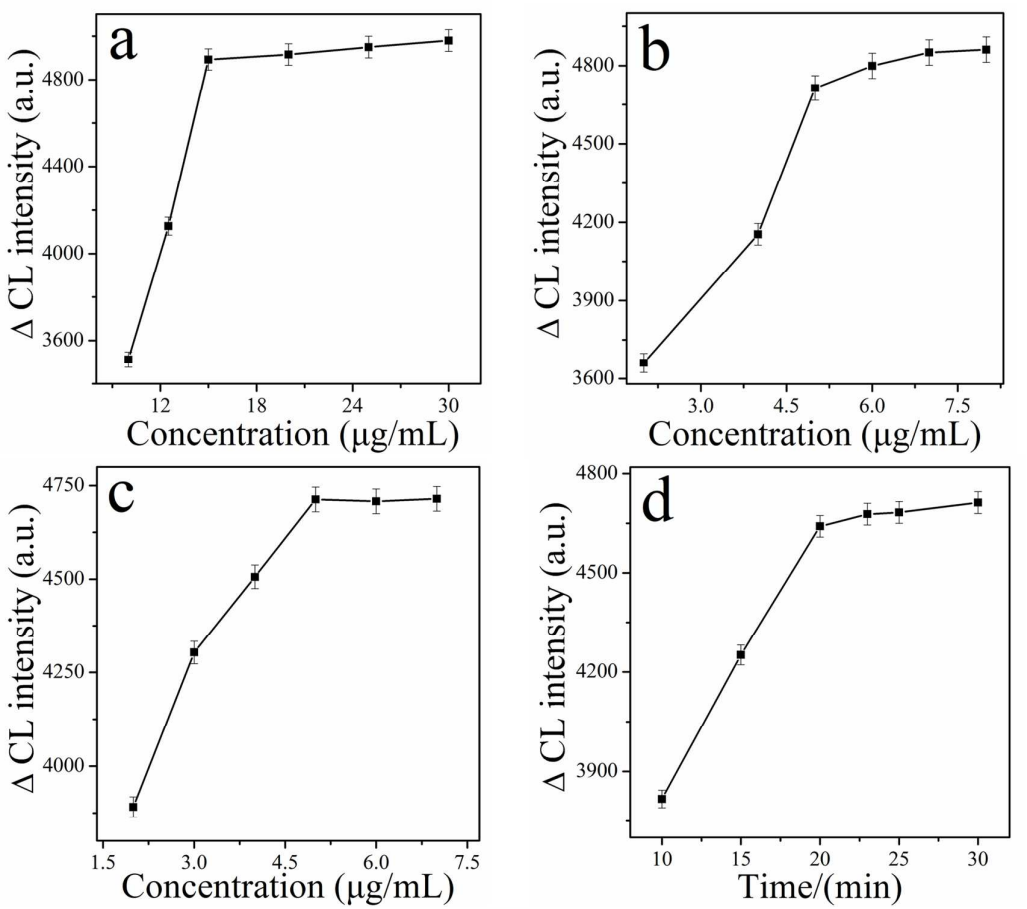


Figure 3. Optimization of concentrations of (a) coating antigen, (b) McAb, (c) HRP-GaMIgG and (d) pre-incubation time of Hg^{2+} and McAb for immunoreaction conditions.

3.5 FI-CL competitive immunoassay for Hg^{2+}

Under the optimized conditions, competitive immunoassay was applied to detect Hg^{2+}. Hg^{2+} standard solution and the coating antigen modified on the beads will compete for the primary McAb, which result in the reduction of the amount of secondary antibody/enzyme captured by McAb. Thereby, the CL signal was decreased as increasing the concentration of Hg^{2+} in incubation solution (shown in Figure 4a). This is because of the effective catalyst HRP, which would catalyze the

luminol-PIP-H_2O_2 reaction to produce enzyme-enhanced CL signal. Figure 4b showed the linear relation of the CL intensity with the Hg^{2+} concentration in the range of 0.05-200 ng/mL, and the regression equation was I_{CL} = 8100.0 - 27.3$C_{Hg^{2+}}$ (ng/mL) (R^2 = 0.9970), the limit of detection (LOD) was 0.015 ng/mL at S/N of 3. Compared with other methods for detecting Hg^{2+} (Table 1), this work shows a wider linear range and a lower LOD, implying that the proposed CL immunosensor possessed commendable analytical performance.

Table 1. Properties of comparable methods for determination of Hg^{2+}.

Methods	LODs	Analytical ranges	References
AES	0.75 ng/mL	1-100 ng/mL	9
CV-AAS	0.08 ng/mL	2.5-10 ng/mL	10
Fluorescence	0.18 nM	1-10 nM	27
HPLC	22.8 ng/mL	80-480 ng/mL	28
ELISA	0.042 ng/mL	0.087-790.4 ng/mL	29
Colorimetric Assay	8 nM	0.01-5μM	30
Electrochemistry	0.5 nM	0-100 nM	31
Electrochemi-luminescence	0.1 nM	1-100 nM	32
FI-CLIA	0.015 ng/mL (0.07 nM)	0.05-200 ng/mL	This work

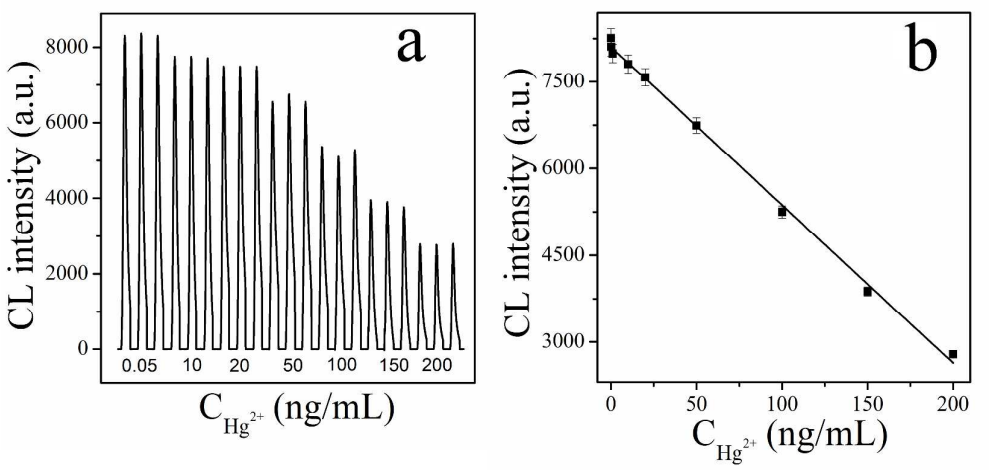


Figure 4. (a) The CL responses of the immunosensor to varying concentrations of Hg^{2+}. (b) Linear calibration curve for Hg^{2+} detection (n=3). All the tests were performed under the optimum conditions.

3.6 Regeneration, reproducibility and specificity of the immunosensor

The regeneration of coating antigen modified resin beads can be realized because the affinity binding between antigen and McAb was non-covalent. In this work, 0.1 M glycine-HCl (pH 2.2) was applied as regenerative reagent due to its rapid and complete dissociation of the immunocomplex.[33] The regeneration efficiency was tested by using 50 ng/mL Hg^{2+} standard solution and calculated to be 93.3% (shown in Figure 5a), suggesting this immunosensor was renewable.

The reproducibility and stability of the immunosensor for Hg^{2+} were investigated with inter- and intra-assay precision. Inter-assay precision was evaluated by fabricating five immunosensors independently to detect 50 ng/mL Hg^{2+}, the relative standard deviation (RSD) of the homologous CL responses were 4.37%. The stability was estimated by performing five repetitive measurements of one immunosensor for detection of 20, 100 and 200 ng/mL Hg^{2+}, and the RSD was in range of 0.48 to 1.91% (Figure 5b). These results indicated the proposed immunoassay possessed satisfactory reproducibility and stability.

Specificity is an important parameter for assessing the immunosensor. We have investigated the response of other metal ions to the immunosensor. Pb^{2+}, Ni^{2+}, Mg^{2+}, Cu^{2+}, Cd^{2+}, Ca^{2+}, Ba^{2+}, Fe^{3+}, at the same concentration of 100ng/mL, used as interfering species added into the incubation solution instead of Hg^{2+}, and detected with this immunosensor. As shown in Figure 5c, the effects of other metal ions were insignificant. The results indicated this immunosensor has excellent specificity for Hg^{2+}, owing to the unique molecular structure of CH_3Hg-MNA which made the mercury atom exposed completely and recognized by immune system.[25]

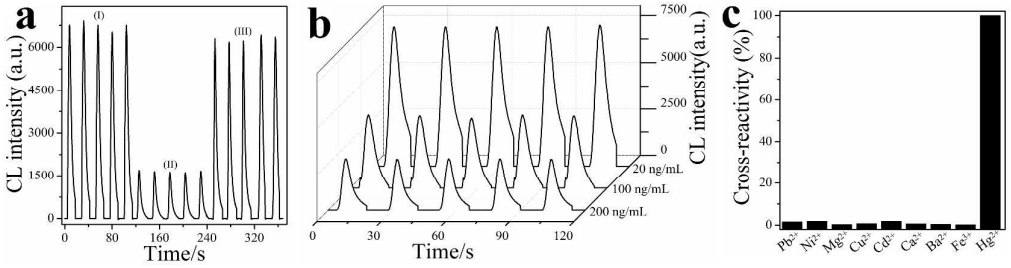


Figure 5. (a) CL intensity measured in a newly assembled immunosensor (I), the immunosensor washed with 0.1 M glycine-HCl (II) and the regenerated immunosensor (III), n=5, respectively. (b) CL emission from the immunosensor formed at 20, 50 and 200 ng/mL Hg^{2+} standard solution, n=5. (c) Detection specificity of the developed CL immunosensor. The concentrations of Hg^{2+} and other metal ions were 100 ng/mL, n=3. All the tests were under the optimum conditions.

3.7 Application of real samples analysis

To further probe the analytical practicability of the proposed method, several environmental water samples were collected and tested with this immunosensor. The tap and natural water samples were separately obtained from our laboratory, Jinji Lake and a local river in Suzhou, China. Firstly, the water samples were filtered with 0.45 μm membrane to remove insolubles. Then, they were detected with the proposed method, and there was no detectable Hg^{2+} residue in our collected samples. Thus, they could be regarded as blank samples and used to make the spike recovery tests. The

results were given in Table 2 with satisfactory recoveries ranged from 94.7 to 110.3%, which indicated that our immunosensor showed good accuracy for practical application.

Table 2. Recovery tests of Hg^{2+} spiked in the real samples by the proposed method (n=3).

Samples		Added (ng/mL)	Found (ng/mL)	RSD (%)	Recovery (%)
River water	No. 1	0	N. D.*		
	No. 2	10	9.55±0.076	0.8	95.5
	No. 3	20	19.76±0.26	1.3	98.8
	No. 4	50	48.37±0.77	1.6	96.7
Lake water	No. 1	0	N. D.		
	No. 2	10	9.62±0.16	1.7	96.2
	No. 3	20	20.20±0.040	0.2	101.0
	No. 4	50	48.19±0.34	0.7	96.4
Tap water	No. 1	0	N. D.		
	No. 2	10	10.54±0.074	0.7	105.4
	No. 3	20	22.06±0.42	1.9	110.3
	No. 4	50	47.33±0.14	0.3	94.7

* N. D. =Not detected.

4. Conclusions

In conclusion, a novel immunosensor based on carboxylic resin beads and specific McAb was designed, coupled with the excellent catalysis effect of HRP in luminol-PIP-H_2O_2 CL system to sensitively detect Hg^{2+} residue in water samples for the first time. Resin beads with activated carboxyl groups exhibited good

biocompatibility and were directly applied to attach more coating antigen, then Hg^{2+} antibody was simply conjugated. Subsequently, HRPtagged second antibody was brought in and catalyzed the CL reaction of luminol-PIP-H_2O_2 which resulted in the efficient enhancement of CL intensity. Compared with previous literature methods, the established competitive immunoassay for Hg^{2+} showed excellent performance with high sensitivity, wide detection range, acceptable stability, simple manipulation and low-cost, and displayed acceptable precision in real sample test. This method will open up a promising platform for detecting other metal ions and small molecules in biological and environmental monitoring.

Acknowledgments

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Table 1. Properties of comparable methods for determination of Hg^{2+}.

Methods	LODs	Analytical ranges	References
AES	0.75 ng/mL	1-100 ng/mL	9
CV-AAS	0.08 ng/mL	2.5-10 ng/mL	10
Fluorescence	0.18 nM	1-10 nM	27
HPLC	22.8 ng/mL	80-480 ng/mL	28
ELISA	0.042 ng/mL	0.087-790.4 ng/mL	29
Colorimetric Assay	8 nM	0.01-5μM	30
Electrochemistry	0.5 nM	0-100 nM	31
Electrochemi-luminescence	0.1 nM	1-100 nM	32
FI-CLIA	0.015 ng/mL (0.07 nM)	0.05-200 ng/mL	This work

Table 2. Recovery tests of Hg^{2+} spiked in the real samples by the proposed method (n=3).

Samples		Added (ng/mL)	Found (ng/mL)	RSD (%)	Recovery (%)
River water	No. 1	0	N. D.*		
	No. 2	10	9.55±0.076	0.8	95.5
	No. 3	20	19.76±0.26	1.3	98.8
	No. 4	50	48.37±0.77	1.6	96.7
Lake water	No. 1	0	N. D.		
	No. 2	10	9.62±0.16	1.7	96.2
	No. 3	20	20.20±0.040	0.2	101.0
	No. 4	50	48.19±0.34	0.7	96.4
Tap water	No. 1	0	N. D.		
	No. 2	10	10.54±0.074	0.7	105.4
	No. 3	20	22.06±0.42	1.9	110.3
	No. 4	50	47.33±0.14	0.3	94.7

* N. D. =Not detected.

Figure captions

Scheme 1. Schematic diagram of FI-CLIA system: (P1) peristaltic pump, (P2) syringe pump, (V) injection valve, (I) immunosensor, (PMT) photomultiplier and (PC) personal computer.

Figure 1. SEM images of naked carboxylic resin bead (a) and (c), and coating antigen immobilized resin bead (b) and (d).

Figure 2. Kinetic curve of the luminol-PIP-H_2O_2 CL system: PBS (pH 7.4) in the presence (a) and absence of 10 μL of 5 μg/mL of HRP-GaMIgG (b), respectively. Conditions: 100 μL of Tris-HCl (pH 8.5) containing 0.6 mM luminol, 0.7 mM PIP and 4 mM H_2O_2 was injected.

Figure 3. Optimization of concentrations of (a) coating antigen, (b) McAb, (c) HRP-GaMIgG and (d) pre-incubation time of Hg^{2+} and McAb for immunoreaction conditions.

Figure 4. (a) The CL responses of the immunosensor to varying concentrations of Hg^{2+}. (b) Linear calibration curve for Hg^{2+} detection (n=3). All the tests were performed under the optimum conditions.

Figure 5. (a) CL intensity measured in a newly assembled immunosensor (I), the immunosensor washed with 0.1 M glycine-HCl (II) and the regenerated immunosensor (III), n=5, respectively. (b) CL emission from the immunosensor formed at 20, 50 and 200 ng/mL Hg^{2+} standard solution, n=5. (c) Detection specificity of the developed CL immunosensor. The concentrations of Hg^{2+} and other metal ions were 100 ng/mL, n=3. All the tests were under the optimum conditions.