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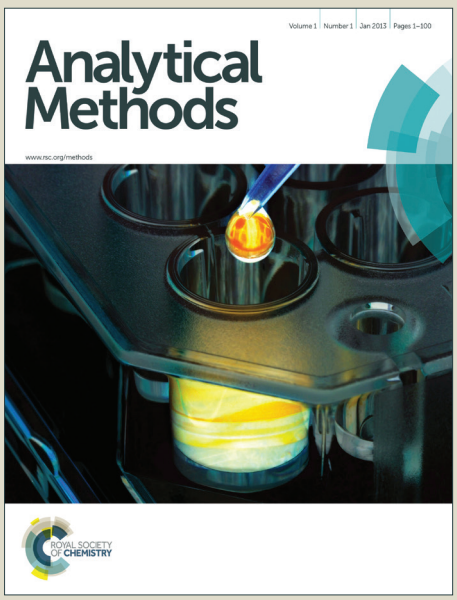

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Colorimetric detection of mercury based on a strip sensor

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Environmental pollution with mercury is a global problem. A fast and accurate detection method is urgently required as an alternative to instrument detection, whose cost is very high. Here we present a sensor for mercury (Hg(II)) detection which has promising analytical applications. The sample treatment process was integrated with the antibody–antigen reaction process. Adequate pre-treatment time before the test resulted in high sensitivity. The strip results shown an excellent linear relationship with the concentration of Hg(II) between 1 to 10 ng/mL, and the detection limit was 0.23 ng/mL. Other metals had a negligible effect on the detection of Hg(II). The accuracy of the method was evaluated by adding different concentrations of Hg(II) to tap water samples. The sample recoveries ranged from 103.2% to 108.7%. This immunoassay is simple and portable, which makes it very useful for rapid monitoring of Hg(II) contamination in field analysis.

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Introduction

Mercury pollution is one of the most severe global environmental problems.[1] The main sources of mercury include fossil fuel combustion, solid waste incineration, and chemical manufacturing.[2] Mercury is highly toxic and is extremely dangerous to all biological organisms as a result of its bioaccumulation and methylation. Exposure to mercury, even at low concentration, can damage the nervous and the digestive systems, especially the brain and kidney.[3] In the ecological chain, mercury bioaccumulation on plants and in waters is the major source of human exposure. In natural water, mercury is present as a divalent ionic form at trace concentration levels. The United States Environmental Protection Agency (EPA) has mandated the acceptable limit for Hg(II) in drinking water to be 2 ng/mL (10 nM).

Mercury contamination is ubiquitous in surface water and on agricultural crops, and monitoring of mercury frequently requires methods with high throughput. Conventional mercury analysis methods including atomic absorption spectrometry (AAS),[4] cold vapor atomic fluorescence spectrometry (CV-AFS),[5] and electrothermal vaporization–inductively coupled plasma mass spectrometry (ETV-ICP-MS).[6] Although these instrumental analyses are highly sensitive, stable and accurate, they require expensive instrumentation, skilled personnel, and complicated sample pre-treatment, which are time-consuming and unsuitable for point-of-use detection. To overcome these limitations, fast and simple methods have been developed for detection of Hg(II) in an aqueous solution; these methods involve simplified sample preparation and portable instrumentation. The gold nanoparticles(GNP)-based sensors modified with different recognition molecules for mercury have been widely used,[7] including T–T mismatches,[8-11] allosteric DNAzyme catalytic beacons,[12] and functional molecules.[13-15] As shown in Table 1, the detailed performance of the

different types of state-of-art Hg(II) sensors have been listed. Based on large instruments, some methods shown ultra-sensitivity for Hg(II) detection with the detection limit in the low ppt range. The performance of the GNP-based colorimetric sensors for detection of Hg(II) have been reviewed in detail by the Du et.al. However, most of colorimetric detection of Hg(II) sensors have low sensitivity and poor specificity and can be interfered with in quantitative measurements other metals that are closely related chemically.

As an alternative, simple, fast, and cost-effective immunoassays,[16-20] based on suitable antibodies and protocols, have been used for the detection of different heavy-metal species. Given that heavy metals are too small to elicit an immune response, different organic compounds, including 1-(4-isothiocyanobenzyl) ethylenediamine-N,N,N',N'-tetraacetic acid (ITCBE),[17, 21-23], 1-(4-aminobenzyl) ethylenediamine-N,N,',N'-tetraacetic acid (aminobenzyl-EDTA),[24] glutathione,[25] and 6-mercaptonicotinic acid,[26] have been used to link the Hg(II) and the protein. This technique has been used successfully to produce monoclonal antibodies specific for mercury or mercury conjugates. However, these monoclonal antibodies either have high cross-reactivity with cadmium[18, 21] or show poor detectability.[22]

Many immunoassays, especially the indirect enzyme-linked immunosorbent assay (ELISA), have long detection times of between 1 to 2 hours because they require a competitive reaction, color development, and washing steps. Antibody-based immunochromatography has been used successfully to circumvent this disadvantage and has been applied in many fields, including medical services,[27, 28] agricultural pollution,[29, 30] environmental monitoring,[31, 32] and food safety.[33]

Immunochromatography is a powerful tool in the bioassays field. This platform reduces the incubation and washing times because of the use of paper chromatography

has high throughput, and needs only small sample volumes, giving short analysis time and high sensitivity with the use of some enhancement technology.[32, 34, 35] The use of immunochromatography for the rapid determination of heavy metals in food and water samples has also been researched.[36-43] The strip sensor used has a short detection time, within 10–15 minutes, and is applicable to rapid on-site detection. Conventionally, however, compared with the ELISA, the efficacy of the antibody is sacrificed in an immunosensor and the limit of detection is higher. This is mainly caused by the lack of incubation time provided with the antibody, the analyte and the competitor. We have identified antibodies specific for Hg(II)–EDTA and developed an immunochromatographic method for the detection of trace levels of Hg(II) in environmental aqueous samples. There is no loss in sensitivity compared with the ELISA, as a result of the inclusion of an incubation process.

Experimental methods

Reagents and instruments

The 1-(4-isothiocyanobenzyl)ethylenediamine-N,N,N',N'-tetraacetic acid (ITCBE) was purchased from Dojindo Laboratories (Shanghai, China). All metal ions were atomic absorption standards. The Hg(II), Cu(II), Cd(II), Pb(II), Cr(III), Mn(II), Co(II), Fe(III), Zn(II), Al(III), Mg(II), and Ca(II) (1000 μg/mL in 1% HNO_3 or 5% HCl) were purchased from the National Institute of Metrology, P.R. China (Beijing, China). Keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) (fatty acid ultrafree), Tween-20, 3,3',5,5'-tetramethylbenzidine (TMB), Freund's complete and incomplete adjuvants, horseradish peroxidase labeled goat anti-mouse IgG, hypoxanthine aminopterin thymidine (HAT), hypoxanthine thymidine (HT), polyethylene glycol (PEG 4000), and chloroauric acid ($HAuCl_4 \cdot 4H_2O$) were

purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse SP2/0 myeloma cells were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Anti-Hg(II)-EDTA monoclonal antibody was produced in our laboratory. All plasticware was soaked overnight in 3 M HCl and glassware was mixed-acid washed and rinsed liberally with purified water before use. All water used was purified to 18.2 MΩ.cm (Millipore).

The backing material (polyvinylchloride), the sample pad (glass fiber membrane, GL-b01), the absorbance pad (H5079), and the conjugate pad (Ahlstrom 8964) were purchased from JieYi Biotechnology Co., Ltd. (Shanghai, China). The nitrocellulose (NC) membrane was supplied by the Millipore Corporation (Bedford, MA, USA).

The CM4000 Guillotine Cutting Module (BioDot Inc., Irvine, CA, USA) and the Dispensing Platform (BioJet Quanti3000 dispenser) were used to manufacture the test strips. The BioDot TSR3000 Membrane Strip Reader was used to test the color intensity of colloidal gold on the test line. The pH was adjusted with an acidometer (Tianda Apparatus Co. Ltd., Shanghai, China). Inductively coupled plasma mass spectrometry (Thermo Fisher Scientific, Waltham, MA, USA) was used to confirm the detection results.

Production of monoclonal antibody

The monoclonal antibody 5E3 was prepared as previously described;[41] it recognizes Hg(II)–EDTA complexes. In detail, the Hg(II) was conjugated to carrier proteins (BSA and KLH) via ITCBE. Subsequently, the Hg(II)–ITCBE–KLH was used as the immunogenic antigen, and 100 μg antigen was mixed with Freund's complete adjuvant for the first immunization. For the next four booster immunizations, 50 μg of immunogen emulsified with incomplete Freund's adjuvant was given at 3-week

intervals after the initial immunization. Five injections later, the serum collected from the tails of the mice was identified using the indirect ELISA. The mouse that shown the highest titer of antibody against Hg(II)–EDTA that could not be inhibited by the high concentration of EDTA was selected for cell fusion. The Hg(II)–EDTA and EDTA were both used for cell screening. After several subcloning procedures, a cell line that produced antibody against the Hg(II)–EDTA complex was selected and expanded for ascites production. The BALB/c mice were primed with incomplete Freund's adjuvant 7 days before injection of the cells. The collected ascitic fluid was purified by the caprylic acid–ammonium method. The sensitivity and cross-reactivity were tested using the ELISA. The affinity constant between the antibody and Hg(II)–EDTA was calculated using a non-competitive enzyme immunoassay.

Preparation of GNP–antibody conjugates

To prepare the GNP-labeled antibodies, the pH of GNP solution was adjusted using 0.1 M K_2CO_3. Subsequently, 0.5 mL of antibody(0.2 mg/mL) in 2 mM borate buffer solution (pH 8.2) was mixed gently with 10 mL of the monodispersed GNP (30 nm in size) solution. To block the GNP surface, bovine serum albumin (BSA) solution was added, to a concentration of 0.5%. The conjugate was centrifuged at 9000 rpm for 15 min, and the sediment (1 mL from 10 mL original solution) was collected in PBS containing 2% (w/v) BSA, 2% (w/v) sucrose, and 0.02% (w/v) sodium azide. This procedure was repeated twice.

Preparation of the immunochromatographic assay

The immunochromatographic assay was fabricated as previously described. As shown in Figure 1, the coating antigen (1 mg/mL Hg(II)–EDTA–BSA) and goat anti-mouse

IgG (0.5 mg/mL) were used to produce the test line (T line) and control line (C line) on the membrane using the dispenser at 1 μL/cm. Detection of Hg(II)–EDTA was based on a competitive format. The Hg(II) solution was first treated with the EDTA solution to form Hg(II)–EDTA. Subsequently, 80 μL of each solution was added to the pre-dried, GNP-labeled antibody, and the solution was vortexed for 5 s. Under these conditions, the Hg(II)–EDTA complex is formed and this binds simultaneously to the anti-Hg(II)–EDTA antibody to form an antigen–antibody complex. The amount of this complex is proportional to the amount of Hg(II) in the original sample. This complex, in solution, is added to the strip sample pad and migrates into the NC membrane. The Hg(II)–ITCBE–BSA and the goat anti-mouse IgG was sprayed on the NC membrane and formed the test line and control line.

If there is no Hg(II) in the sample solution, the GNP-labeled antibody binds to the immobile Hg(II)–EDTA–BSA antigen (on the T line) and the goat anti-mouse IgG (on the C line). This result is considered negative. If a certain amount of Hg(II) exist in the sample solution, free antigen (in the sample solution) combines with the antibody. Less GNP–antibody combines with the immobile Hg(II)–EDTA–BSA antigen and the intensity of the T line will decrease. If the Hg(II) concentration is high enough, the T line will disappear. Excess reagents that cross the NC membrane become entrapped in an absorbent pad.

Detection of Hg ions

For evaluation of the linearity of this method, Hg(II) standard solutions were prepared in the concentration range of 1 to 100 ppb by dilution of an Hg(II) standard (1000 ppm in 2% nitric acid) in HBS containing EDTA (5 mM), at pH 7.4. Eighty microliters of Hg(II) standard solution was added to the pre-dried, GNP-labeled

antibody, and the solution was vortexed for 5 s. After 30 min incubation at 37°C, 80 μL of each Hg(II) solution was deposited onto the sample pad, and the results were determined by the naked eye after 10 min. For quantitative measurement, the intensity of each strip was recorded using a strip reader. The approach for visual LOD calculation was followed. As showed in the Fig.5, the calibration curve was plotted as

$$y = a + b \cdot x \quad (1)$$

When

$$y = C_{blank} - 3s.d. \quad (2)$$

The LOD was calculated as follows:

$$LOD = 10^{\frac{a-(C_{blank}-3s.d.)}{b}} \quad (3)$$

where C_{blank} is the color intensity of signal of blank sample (without Hg(II)).

The *s.d.* was calculated according to the well-known formula:

$$s.d. = \sqrt{\frac{1}{n_r-1} \cdot \sum_{i=1}^{n_r} \left(x_i - x_{avg}\right)^2} \quad (4)$$

where n_r is the total number of the samples. X_i is the *i*th sample of the series of measurements.

X_{avg} is the average value of the color intensity (or other) signals obtained for the specific series of identical samples repeated n_r times.

Sample analysis

In order to apply this method to environmental samples, tap water samples spiked with different concentrations of Hg(II) were tested by this method. First, the tap water samples were filtered using a 0.45 μm nylon filter and treated subsequently with 10-fold concentrated HEPES buffer solution (HBS, 100 mM HEPES, 1.37 M NaCl, 30 mM KCl, and 50 mM EDTA, pH 7.4) to adjust the pH and the ion concentration.

The spiked samples were detected using the strip three times. All detection results were confirmed by ICP-MS.

Results and discussion

Screening and characterization of the monoclonal antibody

The hybridomas were initially screened using the indirect ELISA to test their ability to bind to the Hg(II)–EDTA–BSA obtained after cell fusion. These clones were screened subsequently by competitive ELISA using the EDTA and the Hg(II)–EDTA chelate complex. The antibodies synthesized by the hybridoma bound to Hg(II)–EDTA–BSA; this binding could not be inhibited by 5 mM EDTA, but could be inhibited by 50 ng/mL Hg(II) in 5 mM EDTA. The hybridomas that synthesized such antibodies with specificity for the Hg(II)–EDTA was identified and subcloned by limiting dilution. The subclone of interest (5E3) was found to secrete an antibody of the IgG1 type. The sensitivity and specificity of the monoclonal antibody purified from the ascites fluid was tested by the competitive ELISA. A typical inhibition curve is shown in Figure 2. The half-maximal inhibitory concentration (IC 50) was 14.3 ng/mL and the dynamic range (IC20–IC80) was from 5.3 to 50 ng /mL. The K_{aff} value was 1.66×10^{9} L/mol, according to the calculation**.**

In order to ensure the specificity of the antibody, the cross-reactivity with other metal ions, including Cu(II), Cd(II), Pb(II), Cr(III), Mn(II), Fe(III), Al(III), Mg(II), and Ca(II), was examined using the ELISA. As shown in Figure 3, Cd(II) had a low level of cross-reactivity (less than 5.2%) and the other metals shown negligible cross-reactivity (less than 1.34%) with Hg(II). This verified the specificity and sensitivity of the antibody. Therefore, coexisting metals in the water samples will not interfere with the accuracy of the Hg(II) immunoassay.

Hg(II) immunoassay using the strip sensor

Several parameters were optimized, including the concentration of Hg(II)–ITCBE–BSA antigen on the T line in the nitrocellulose membrane and that of the GNP–antibody conjugate (results not shown). The color intensity of the T line is inversely related to the Hg(II) concentration in the sample. The sample solutions at different concentrations (0, 1, 2, 5, 10, 20, 50, 100 ng/mL) were prepared and tested by the assay. The result is shown in Figure 4A. The differences in color intensities of T lines among samples containing 0, 1, 2, and 5 ng/mL were able to be discriminated with the naked eye. The T line was disappeared completely with sample spiked at 10 ng/mL. Figure 5 shows the calibration curves obtained by measuring the intensity of color at the T line with the strip reader using the strip sensor (the X – axis scale was given in log 10 type in Fig. 5). Using the strip reader equipment, the intensity of color was detectable and the range linearity was from 1 to 10 ng/mL; the limit of detection (LOD) reached 0.23 ng/mL. The repeatability of this method at different concentrations was evaluated using the coefficient of variation (CV). The results are summarized in Table 2; the intra- and inter-assay CVs were 1.8–8.9% and 1.1–7.9%, respectively.

Detection of Hg(II) in water samples

Tap water samples spiked with Hg(II) at concentrations between 1 and 100 ng/mL were analyzed. In tap water, Ca(II), Mg(II), Cu(II), Zn(II), Al(III) and Fe(III) are the major potential metallic ions; they had a negligible effect on the accuracy of the assay. As shown in Figure 4B, the color intensity decreased as the spiked Hg(II) concentration increased; the cut-off value was 10 ng/mL. The result indicated that this

method is robust in the analysis of tap water samples. The recovery data were calculated by the following equation:

$$\text{Recovery (\%)} = \frac{\text{Calculated concentration of analyte in spiked sample - Original concentration of analyte}}{\text{Theoretical spiked concentration}}$$

As shown in Table 3, good recovery (103.2%–108.7%) was obtained. The results indicated that the strip method is sensitive and accurate, and able to detect Hg(II) in tap water samples at low Hg(II) concentrations.

Conclusion

We have developed a strip that performs Hg(II) detection with high sensitivity, which is greatly needed for on-site detection in field analysis. The chelation process integrated with antibody–antigen reaction in one step makes the strip highly sensitive in the detection of trace Hg(II). The Hg(II) in spiked tap water samples was detected by this method with good recovery, which indicates that this assay could be used as a potential alternative tool for on-site detection of Hg(II) pollution.

Acknowledgements

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Captions:

Fig. 1 Schematic of the antibody-based mercury immunoassay.

Fig. 2 The standard inhibition curve for the Hg(II) indirect competitive ELISA

Fig. 3 Metal ion specificity

Fig. 4 Sensitivity test of strip.

Fig. 5 Lineal range of the calibration curves obtained during Hg(II)-EDTA analysis by strip immunoassay.

Table 1 The performance of different types of state-of-art Hg(II) sensors

Table 2 The intra- and inter-assay of the assay for Hg(II) detection

Table 3 Recovery test of Hg(II) in Tap water samples

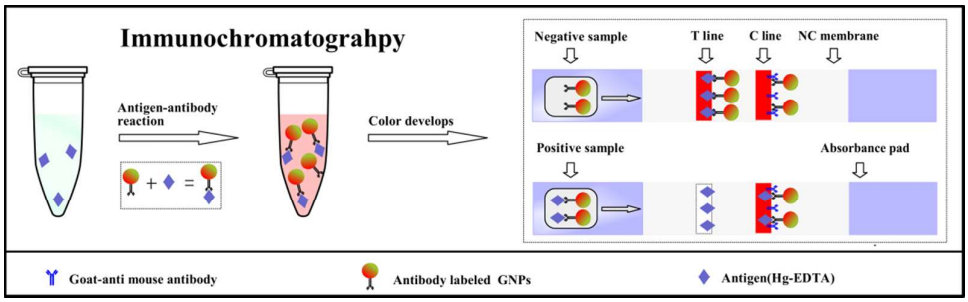

Fig. 1 Schematic of the antibody-based mercury immunoassay.

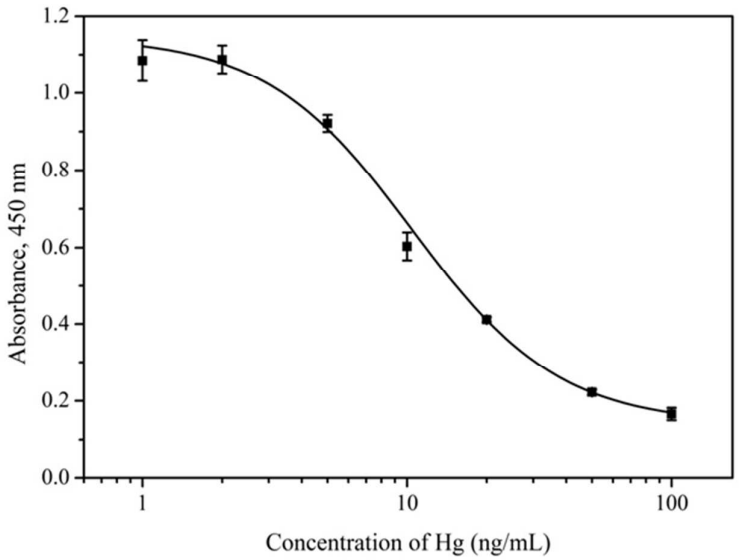

Fig. 2 The standard inhibition curve for the Hg(II) indirect competitive ELISA

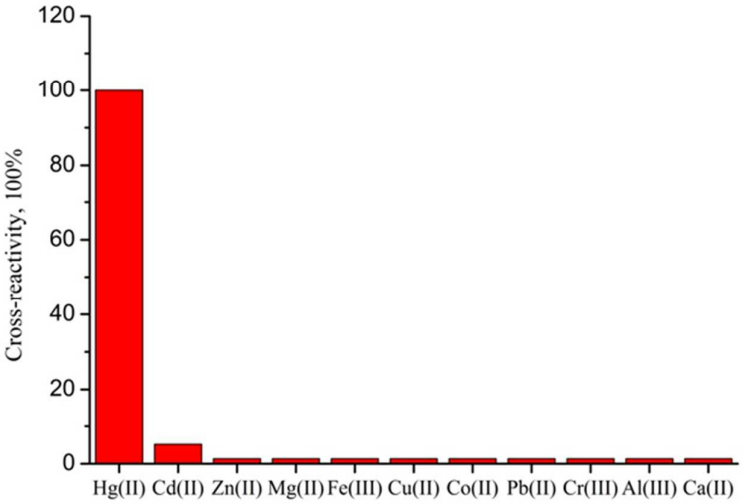

Fig. 3 Metal ion specificity

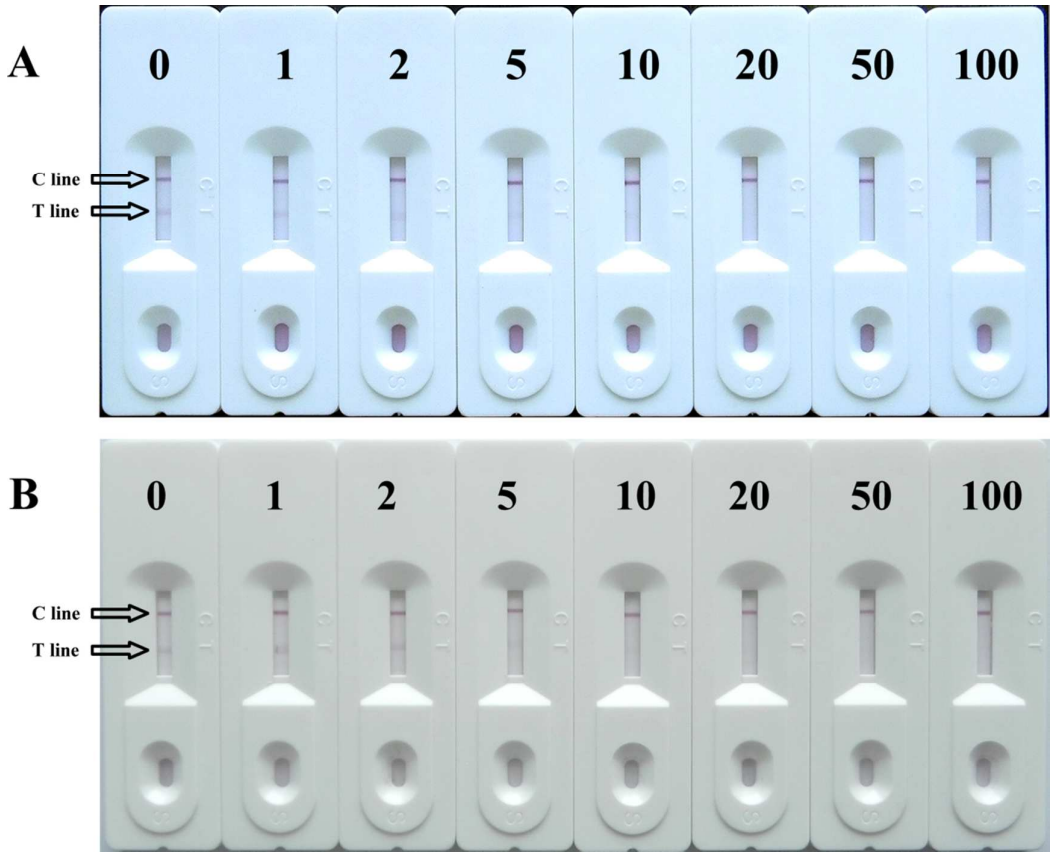

Fig. 4 Sensitivity test of strip. A. Typical photo image of detection Hg(II) by strip sensor in buffer solution. B. Typical photo image of detection Hg(II) by strip sensor in tap water.

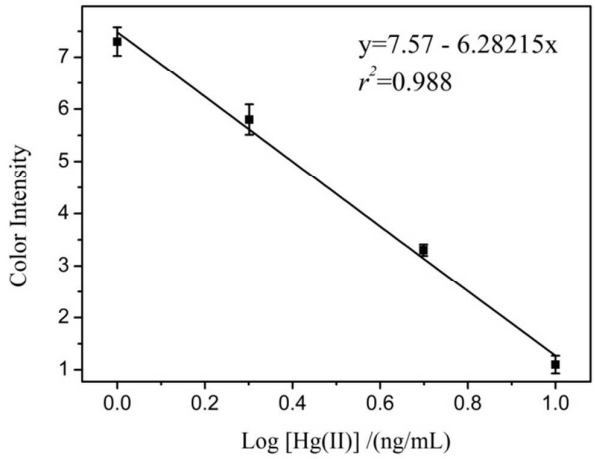

Fig. 5 Lineal range of the calibration curves obtained during Hg(II)-EDTA analysis by strip immunoassay.

Table 1 The performance of different types of state-of-art Hg(II) sensors

Probe Platform	Signal Transducer	LOD [ng/mL]	Reference
DNA	Surface-enhanced raman scattering	0.0008(3σ)	8
	Plasmon-enhanced infrared spectroscopy	0.037	9
	Visual Detection[a]	1.2	11
	Fluorescence Enhancement	0.48(3σ/slope)	12
	Visual Detection	0.2	13
	Visual Detection	0.6	38
	Visual Detection	0.02	42
Functional Molecule	UV/Vis Spectroscopy	11(3σ)	14
	Visual Detection	1	15
Antibody	Visual Detection	1.6	23
	Strip Reader	0.23(3σ)	Our method
	Visual Detection	1	Our method

[a] The visual detection limit was the concentration causing color changes that could be judged with the naked eyes.

Table 2 The intra- and inter-assay of the assay for Hg(II) detection

Concentration Hg(II) (ng/mL)	Intra-assay CV (%)	Inter-assay CV (%)
1	1.8	7.9
2	5.8	7.8
5	8.9	1.1

Table 3 Recovery test of Hg(II) in Tap water samples

Tap water samples	Original concentrations[a] (ng/mL)	Spiked concentration (ng/mL)	Recovery (%) (mean ± SD, n = 3)	CV(%)
1	0.01	1	108.7	9.2
2	0.01	2	104.2	7.5
3	0.01	5	103.2	7.3

[a]Original concentrations were detected by ICP-MS.