Development of ic-ELISA and an immunochromatographic strip assay for the detection of methylmercury

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ABSTRACT
In this study, we developed an indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) and an immunochromatographic strip assay based on a monoclonal antibody (mAb) against methylmercury (MeHg) to detect the presence of MeHg in tap water. Under optimum conditions (pH 8.0, 0.8% NaCl, and 0.1% Tween 20), the 50% half maximal inhibitory concentration (IC_{50}) and limit of detection (LOD) were 16.64 and 2.03 ng/mL, respectively. The anti-MeHg mAb was specific to mercury with no cross-reactivity with other metal ions. The cut-off value of the immunochromatographic strip assay was 500 ng/mL for semi-quantitative detection, and the LOD was 11.3 ng/mL for quantitative detection. The average recovery rates of the ic-ELISA and immunochromatographic strip assay were 98.13% and 107.87%, respectively, in tap water. Therefore, ic-ELISA and the immunochromatographic strip assay can be used to detect MeHg in tap water.

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Introduction
Mercury (Hg) is the only liquid metal at standard temperature and pressure conditions (Stwertka, 2002). Mercury, which exists in three forms (elemental mercury, inorganic mercury, and organic mercury), easily forms alloys with other metals, such as tin and gold, and is widely used in the industry. Natural and anthropogenic emissions are the main sources of mercury contamination in the environment. Natural emissions are from volcanoes, and anthropogenic emissions are from gold mining, burning coal, and the indiscriminate use of pesticides (Horowitz, Jacob, Amos, Streets, & Sunderland, 2014). Mercury is highly toxic, non-biodegradable, and has a long half-life in the biosphere like other heavy metals (Rajaganapathy, Xavier, Sreekumar, & Mandal, 2011; Singh, Sharma, Agrawal, & Marshall, 2010; Xing, Hao, Liu, Xu, & Kuang, 2013; Xing, Kuang, et al., 2014). Different forms of mercury have distinct toxicity properties (Alhibshi, 2012; Park & Zheng, 2012; Yang, Yang, Zhu, & Liu, 2009). Methylmercury (MeHg) has a higher toxicity than inorganic mercury. Specifically, MeHg contributes to central nervous system damage and fetal malformation (Korbas et al., 2010). Mercury through global transport has contributed to global contamination. Inorganic mercury is converted
into MeHg via oxidation and methylation reactions (Hsu-Kim, Kucharzyk, Zhang, & Deshusses, 2013). To control MeHg contamination, accurate and convenient detection methods are required.

A variety of methods have been developed for MeHg detection. Chen et al. (2013) analyzed mercury speciation in 11 fish samples via inductively coupled plasma mass spectrometry with anion exchange chromatography. Valdersnes, Fecher, Maage, and Julshamn, 2016 measured MeHg in seven marine products by gas chromatography with inductively coupled plasma isotope dilution mass spectrometry. Carrasco and Vassileva (2014) used gas chromatography coupled to pyrolysis-atomic fluorescence spectrometry for the detection of MeHg in marine biota samples with a limit of quantification of 0.85 pg. These methods are widely used in mercury speciation and MeHg detection and are precise; however, they require skilled personnel and are time-consuming for derivatization. In recent years, several techniques for the determination of mercury have been developed including biosensors based on nucleic acid functionalized gold nano-rods (Zhu et al., 2012), metal-specific DNAzymes (Hao, Xua, Xing, & Kuang, 2012; Kuang, Yin, Xing, & Xu, 2013), Raman label-encoded gold nanoparticles (Li et al., 2015; Ma et al., 2013), and antibodies (Xing, Liu, Zhang, Kuang, & Xu, 2014).

In this study, we developed an indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) and an immunochromatographic strip assay based on an anti-MeHg monoclonal antibody (mAb) for the determination of MeHg in tap water samples. To the best of our knowledge, this is the first time that the immunoassay is used to detect MeHg.

**Materials and methods**

**Reagents, chemicals, and samples**

Methylmercury chloride (CH$_3$HgCl) was obtained from Dr Ehrenstorfer (Augsburg, Germany); 3,3′,5,5′-tetramethyl-benzidine (TMB) and TWEEN-20 were purchased from J&K Scientific (Shanghai, China). Bovine serum albumin (BSA), ovalbumin (OVA), 6-mercaptopenicotinic acid (MNA), incomplete Freund’s adjuvant (FIA), complete Freund’s adjuvant (FCA), 2-methylpropyl carbonochloridate, N-hydroxysuccinimide (NHS), N, N-dicyclohexyl-carbodiimide (DCC), gelatin, polyethylene glycol (PEG2000), ethylenediamine-$N,N,N',N'$-tetraacetic acid (EDTA), and chloroauric acid (HAuCl$_4$·4H$_2$O) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP)-goat anti-mouse IgG was purchased from Kang-cheng Bioengineering Co. (Shanghai, China). Cu(II), Hg(II), Cd(II), Pb(II), Mn(II), Zn(II), Al(III), Mg(II), Ni(II), and Ca(II) (1 mg/mL in 1 M HNO$_3$) were acquired from the National Institute of Metrology (Beijing, China). Cell culture media including polyethylene glycol solution, HAT supplement, HT supplement, and RPMI 1640 cell culture medium were obtained from Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA). Mouse SP2/0 myeloma cells were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). JieYi Biotechnology Co. Ltd. (Shanghai, China) provided the backing material (polyvinylchloride, PVC), sample pad (glass fiber membrane, GL-b 01), and absorbance pad (H5079). The nitrocellulose (NC) membrane was purchased from Merck-Millipore (Darmstadt, Germany).
Coating buffer (0.05 M CB), blocking buffer (0.2% gelatin in CB), washing buffer (phosphate buffer saline; 0.05% Tween-20, v/v in 0.01 M PBS), and stop solution (2 M sulfuric acid) were prepared in our laboratory. The substrate buffer consisted of solutions A (citric acid, H₂O₂, and Na₂HPO₄) and B (0.06% v/v TMB in glycol) at a 5 to 1 (v/v) ratio.

**Synthesis of immunogen and coating antigen**

MNA and proteins were conjugated using the DCC/NHS ester method (Suryoprabowo, Liu, Peng, Kuang, & Xu, 2014). Firstly, 5 mg MNA, 30 mg DCC, and 17 mg NHS were dissolved in 400 μL dimethylformamide and stirred overnight at room temperature. Secondly, the solution was centrifuged at 9600 × g for 10 min. The resulting supernatant was added dropwise to 10 mg BSA, previously dissolved in 2 mL of 0.1 M Carbonate buffer saline, under constant stirring for 8 h at room temperature. Following centrifugation at 9600 × g for 10 min, the supernatant was dialyzed against 0.01 M PBS for 3 d to remove unbound MNA. Thirdly, 2 mg CH₃HgCl dissolved in 200 μL methanol containing 10% of 1 M NaOH (v/v) was added dropwise to the MNA–BSA solution and incubated overnight at room temperature under constant stirring. Subsequently, the supernatant was dialyzed against 0.01 M PBS for 3 d. Finally, CH₃HgCl–MNA–BSA (immunogen) was stored at −20°C. CH₃HgCl–MNA–OVA (coating antigen) was prepared by the same method (Wang et al., 2012).

**Production of anti-MeHg mAb**

Six- to eight-week old mice were subcutaneously injected with 100 μg CH₃HgCl–MNA–BSA per mouse with an equal volume of FCA. The booster immunization was performed four weeks later with 50 μg CH₃HgCl–MNA–BSA per mouse with an equal volume FIA. One week following the fourth immunization, sera from the tail of mice were analyzed by ic-ELISA. The splenocytes of the mouse with the highest titer and lowest half maximal inhibitory concentration (IC₅₀) were fused with Sp2/0 myeloma cells to produce hybridomas. Hybridomas were screened by CH₃HgCl and Hg(II) as competitors. Following three sub-clones, the cell line 2F8 with the highest titer and lowest IC₅₀ was selected for the production of antibody. Antibody was purified by the ammonium-saturated ammonium sulfate precipitation method and stored at −20°C (Wang et al., 2016).

**Ic-ELISA**

A microtiter 96-well plate was coated with 100 μL/well of 1 μg/mL CH₃HgCl–MNA–OVA diluted in coating buffer and incubated at 37°C for 2 h. The plate was washed three times with washing buffer. Blocking buffer (200 μL/well) was added and incubated at 37°C for 2 h. Subsequently, the plate was washed and dried at 37°C for 30 min. Different concentrations of CH₃HgCl standard solution (50 μL) dissolved in 0.01 M PBS and 50 μL diluted mAb (2 μg/mL) were successively added to each well, and the plate was incubated at 37°C for 30 min and washed. Goat anti-mouse IgG labeled with HRP (100 μL) was added, and the plate was incubated at 37°C for 30 min. After three washes, 100 μL TMB substrate solution was added to each well, and the plate was incubated at 37°C for 15 min. Finally, following the addition of 10% sulfuric acid (50 μL/well), absorbance was measured at 450 nm in a microplate reader (Liu, Xing, Yan, Kuang, & Xu, 2014).
**Cross-reactivity**

To assess cross-reactivity (CR) in ic-ELISA, Hg(II), Cd(II), Pb(II), Cr(III), Mn(II), Ni(II), Al(III), Zn(II), Mg(II), and Ca(II) were used. CR was calculated using the following equation, CR (%) = \((\text{IC}_{50}\text{ of MeHg}/\text{IC}_{50}\text{ of the other heavy metals}) \times 100\%\) (Kong, Liu, Song, Kuang, & Xu, 2016).

**Preparation of antibody–colloidal gold conjugate**

Gold nanoparticles (20 nm in diameter) were synthesized by the sodium citrate reduction method (Kuang, Xing, et al., 2013). Briefly, 4 μL of 0.1 M K₂CO₃ and 10 μg mAb were added to 1 mL of the colloidal gold solution and mixed at room temperature for 30 min. Subsequently 50 μL of 10% BSA was added to the gold nanoparticle–mAb solution and mixed at room temperature for 2 h to block any unbound sites on the antibodies. Finally, the solution was centrifuged at 6200 × g for 45 min, re-suspended in 1 mL resuspension buffer (0.02 M PBS, 5% sucrose, 2% sorbitol, 1% mannitol, 0.1% PEG, 0.1% Tween 20, and 0.04% NaN₃), and stored at 4°C.

**Assembly of the immunochromatographic strip**

The NC membrane was pasted onto the center of the PVC plate. Subsequently, 1 mg/mL of antigen (CH₃HgCl–MNA–OVA) and 0.5 mg/mL of goat anti-mouse IgG were sprayed at 1 µL/cm onto the NC membrane using a dispenser as the test (T) and control (C) lines, respectively, and dried at 37°C for 2 h. Finally, the sample pad and absorbent pad were laminated with the NC membrane on the PVC plate, and the PVC plate was cut into 2.8-mm wide strips (Xu et al., 2016).

**Principle of the immunochromatographic strip assay**

The principle of immunochromatographic strip assay is shown in Figure 1 (Xing et al., 2015). Gold nanoparticle–mAb solution (50 μL) was allowed to react with 150 μL of sample solution for 5 min. The sorbent pad of the strip was inserted into the solution.

![Figure 1. Principle illustration of the immunochromatographic strip assay for MeHg detection.](image)
After 5 min, the strip was removed, blotted, and observed. MeHg in the sample solution competes with the antigen embedded in the T line for the anti-MeHg mAb. Therefore, the higher MeHg concentration present in the sample, the less antibody is bound to the antigen, thereby resulting in a weak color on the T line.

**Establishment of the immunochromatographic strip assay**

A series of standard solutions of MeHg (0–500 ng/mL) were prepared. Standard solution (150 µL) was mixed with 50 µL of gold nanoparticle–mAb solution and incubated at room temperature for 5 min. The sample pad of the strip was inserted in the solution. After 5 min, the results were analyzed with the naked eye and a scanning reader.

**Sample analysis**

EDTA (1 mg) was added to 10 mL tap water sample to reduce the effect of Hg(II). Subsequently, the sample was spiked with different concentrations of MeHg and analyzed by ic-ELISA (5, 20, 50, 100, and 500 ng/mL) and the immunochromatographic strip assay (20, 50, 100, and 500 ng/mL).

**Results and discussion**

**Antigen characterization**

We characterized CH$_3$HgCl–MNA–BSA using ultraviolet (UV) spectroscopy. Figure 2 shows that the characteristic peaks of MNA and BSA were located at 296–342 nm and 280 nm, respectively, while the conjugate of MNA–BSA had the characteristic peaks of MNA (296 nm sharp peak and 342 nm shoulder peak) and BSA (~280 nm). Following conjugation with CH$_3$HgCl, the characteristic peaks shifted to blue (260 nm and 285 nm) and the shoulder peak at 342 nm disappeared. This result revealed that the

![Figure 2](image-url)

**Figure 2.** The UV spectrum of MNA, BSA, MNA–BSA and the complete antigen of CH$_3$HgCl–MNA–BSA.
synthesis of CH$_3$HgCl–MNA–BSA was successful. Similarly, we characterized CH$_3$HgCl–MNA–OVA.

**Sensitivity and specificity of mAb**

To determine the optimal conditions of ic-ELISA, we evaluated the effects of pH, ionic strength, and methanol concentration on mAb sensitivity. Different concentrations of NaCl (0.4%, 0.8%, 1.6%, and 3.2%) in 0.01 M PBS (Figure 3(a)) were tested. The maximum optical density (OD) values were not affected at 0.4% or 0.8% NaCl. However, with increasing NaCl concentration, OD values decreased. Figure 3(b) shows that when the concentration of Tween 20 was 0.1%, the $A_{\text{max}}$/IC$_{50}$ ratio ($A_{\text{max}} = 1.63$; IC$_{50} = 16.64$ ng/mL) was the highest. OD values increased with increasing pH up to 9 (Figure 3(c)). Therefore, the ic-ELISA optimum conditions consisted of 0.8% NaCl, 0.1% Tween 20, and pH 8.0 0.01 M PBS (Figure 3(d)). Under these optimum conditions, IC$_{50}$ was 16.64 ng/mL and the limit of detection (LOD) was 2.03 ng/mL.

CR with other heavy metals, that is, Hg(II), Cd(II), Pb(II), Cr(III), Mn(II), Ni(II), Al (III), Zn(II), Mg(II), and Ca(II), was tested to evaluate the specificity of mAb. The antibody was specific to MeHg and Hg; the CR with other metals was <0.2% (Table 1).

![Figure 3](image)

**Figure 3.** Optimization of assay buffer for ic-ELISA. (a) The influence of ionic strength; (b) the influence of Tween 20; (c) the influence of pH; (d) standard inhibition curve for the ic-ELISA analysis of MeHg under the optimal conditions.
Table 1. CR with other heavy metals of the antibody (2F8).

| Metal ions | IC$_{50}$ ng/mL | CR (%) |
|------------|-----------------|--------|
| MeHg       | 16.64           | 100    |
| Hg(II)     | 2.17            | 766.8  |
| Cd(II)     | >1000           | <0.2   |
| Cu(II)     | >1000           | <0.2   |
| Ca(II)     | >1000           | <0.2   |
| Zn(II)     | >1000           | <0.2   |
| Mn(II)     | >1000           | <0.2   |
| Mg(II)     | >1000           | <0.2   |
| Al(III)    | >1000           | <0.2   |
| Pb(II)     | >1000           | <0.2   |
| Ni(II)     | >1000           | <0.2   |

Table 2. Recovery of MeHg spiked in tap water samples with ic-ELISA.

| Tap water samples | Spiked concentration (ng/mL) | Detected concentration (mean ± SD, n = 5) (ng/mL) | Recovery (%) | CVs (%) |
|-------------------|------------------------------|----------------------------------------------------|--------------|---------|
| 1                 | 5                            | 4.24 ± 0.37                                        | 84.8         | 8.73    |
| 2                 | 20                           | 19.67 ± 1.14                                       | 98.35        | 5.8     |
| 3                 | 50                           | 50.75 ± 2.08                                       | 101.5        | 4.1     |
| 4                 | 100                          | 103.25 ± 5.16                                      | 103.3        | 5.00    |
| 5                 | 500                          | 513.49 ± 23.77                                     | 102.7        | 4.63    |

**Analysis of samples by ic-ELISA**

Tap water samples were spiked with different concentrations of MeHg (5, 20, 50, 100, and 500 ng/mL). Recovery rates from ic-ELISA ranged between 84.8% and 103.3% (Table 2).

![Figure 4](image-url)  
Figure 4. Optimization of 13 surfactants. 1 = suspension buffer, 2 = polyvinyl pyrrolidone, 3 = polyethylene glycol, 4 = polyvinyl alcohol, 5 = BSA, 6 = Casein, 7 = Sucrose, 8 = Trehalose, 9 = Sorbitol, 10 = Mannitol, 11 = Tween-20, 12 = Brij-35, and 13 = Triton X-100.
Optimization of the immunochromatographic strip assay

To allow the solution to spread on the surface of NC membrane, surfactants are usually added to the resuspension buffer. In this study, 13 surfactants were evaluated. The optimum surfactant was 5% BSA (Figure 4).

Qualitative and quantitative determination of MeHg

A series of MeHg standard solutions (0, 10, 20, 50, 100, 200, and 500 ng/mL) were measured under optimum conditions (Figure 5). At 500 ng/mL MeHg, the T line disappeared; therefore, the cut-off value of the strip was 500 ng/mL for semi-quantitative detection. The intensity of the signal of the T line was evaluated using a scanning reader. The LOD was 11.3 ng/mL based on the calibration curve (Figure 6).

Detection of samples by the immunochromatographic strip assay

Tap water samples were spiked with different MeHg concentrations (20, 50, 100, and 500 ng/mL) and analyzed by the immunochromatographic strip assay (Figure 7). Figure 7
**Figure 6.** The calibration curve of concentration of MeHg with color density.

**Figure 7.** Image of MeHg detection with immunochromatographic strip assay in tap water: 1 = 20 ng/mL, 2 = 50 ng/mL, 3 = 100 ng/mL, and 4 = 500 ng/mL.
shows that at 500 ng/mg MeHg, the T line disappeared, which was consistent with the results of the MeHg standard solution. The recovery rates (105.3–112.1%) were calculated from the calibration curve (Table 3).

| Tap water samples | Spiked concentration (ng/mL) | Detected concentration (mean ± SD, n = 6) (ng/mL) | Recovery (%) | CVs (%) |
|-------------------|-----------------------------|--------------------------------------------------|--------------|---------|
| 1                 | 20                          | 21.24 ± 1.23                                     | 106.2        | 5.8     |
| 2                 | 50                          | 52.67 ± 2.54                                     | 105.3        | 4.82    |
| 3                 | 100                         | 112.05 ± 5.08                                    | 112.1        | 4.53    |

**Conclusion**

In the study, we developed ic-ELISA and an immunochromatographic strip assay based on anti-MeHg mAb to detect the presence of MeHg in tap water. The average recovery rates obtained from ic-ELISA and the immunochromatographic strip assay were 98.13% and 107.87%, respectively. The advantage of immunoassays is that the sample does not require any derivatization. In summary, we developed two immunoassays for the potential detection of MeHg in foods.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**Table 3.** Recovery of MeHg with immunochromatographic strip assay spiked in tap water samples.
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