RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Development of an Eco-friendly Immunochromatographic Test Strip and its application in detecting Hg^{2+} without chelators

Suyan Wang[1,a], Limin Wang[1,b,*], Hongfu Chen[b], Yulong Wang[b], Jia Cai[b], Mingming Yang[b], Fengquan Liu[a,*]

[a] Institute of Plant Protection, Jiangsu Academy of Agricultural Science; College of Plant Protection (Key Laboratory of Integrated Management of Crop Diseases and Pests), Nanjing Agricultural University, Nanjing, P.R.China

[b] College of Plant Protection (Key Laboratory of Integrated Management of Crop Diseases and Pests), Nanjing Agricultural University, Nanjing, P.R.China

* Corresponding Author. Email address: wlm@njau.edu.cn; fqliu20011@sina.com

[1] The first two authors contributed equally to this work.

Abstract

Here, a specific anti-Hg^{2+} monoclonal antibody (mAb) was generated and an Eco-friendly Immunochromatographic Test Strip (EFITS) based on a mAb-nanogold probe for rapid and specific detection of Hg^{2+} in water was developed. In the method, the conjugation of Hg-MNA (6-mercaptonicotinic acid) -BSA (bovine serum albumin) was synthesized as an immunogen, the conjugation of MNA-OVA (ovalbumin) was synthesized and selected as a coating antigen. The specific anti-Hg^{2+} mAb from BALB/C female mice was screened based on a competitive immunoassay. The coating antigen and goat anti-mouse IgG antibody were coated on a nitrocellulose membrane (NC membrane) as a test line and a control line, respectively. The anti-Hg^{2+} mAb-nanogold probe was applied to the conjugate pad. Hg^{2+} competes with OVA-MNA to the mAb-nanogold probe causing a color change on the test line corresponding to the Hg^{2+} contentThus, we can distinguish the subtle differences through a strip reader. The resulting EFITS is able to detect Hg^{2+} with LOD of 0.4 ng·mL^{-1} at 9 min by quantitative analysis. EFITS demonstrated here is eco-friendly (without Hg^{2+} on the strip), capable of rapid detection, and does not require chelators.

Key words: Hg^{2+}; Eco-friendly; MNA-OVA; mAb-nanogold; Immunochromatographic test strip

1. Introduction

With the rapid development of industrialization, mercury ion, which is highly toxic and can have adverse effect on human health, has been frequently detected in environment [1-2]. Researches have shown that dietary exposure to mercury in drinking water or sea food can seriously affect the

function of immune system, cardiovascular system, kidneys, lungs, bones and nervous tissues in mammals [3-6]. Minamata disease has alerted us the importance of monitoring mercury level in our surrounding. Therefore, sensitive and rapid analytical methods for detecting Hg^{2+} levels in water samples are crucial for monitoring water quality[7].

So far, traditional methods such as Atomic Fluorescence Spectroscopy [8], Atomic Absorption Spectroscopy [9-10], Inductively Coupled Plasma Spectroscopy-Mass Spectrometry [11-12], High Performance Liquid Chromatography (HPLC) and the HPLC-ICP-MS [13-14] coupling technique are sensitive and accurate. However, these methods called for high dependence on laboratory techniques requiring expensive and sophisticated instruments together with technical experts. All these restrict their extended application in the routine detection of heavy metals. In this context, lots of efforts had been done to develop novel, cheap, simple, portable and real time detection methods to trace mercury.

Immunochromatographic test strips had attracted much attention because of their rapid, low-cost and convenient character [15-18]. With the integration of colloidal gold and antigen-antibody reaction, we can detect the target analytes through a visible color reaction. Thus, the immunochromatographic test strips played a potential role in point of care assay to monitor our environment and human health.

To date, the developed immunochromatographic test strips for detecting heavy metals needed chelating agent to bind heavy metals and usually the strips contain heavy metals[2, 19-22]. In this research, a monoclonal antibody (mAb) was produced by using the Hg-MNA-BSA as immunogen, which can specifically recognize individual Hg^{2+} without any chelating agent. Based on the mAb, a high specific and sensitive immunochromatograpic strip was developed to detect the individual

Hg^{2+} from water. More importantly, the OVA-MNA (ovalbumin-6-mercaptonicotinic acid) was used as coating antigen, which without any heavy metal, resulting in realizing an eco-friendly and chelator-free strip for quantitative detection of Hg^{2+} in water samples. The developed Eco-friendly Immunochromatographic Test Strip enabeled a rapidand quantitative detection of Hg^{2+} in 10 min. The novelty of the developed EFITS is eco-friendly (without Hg^{2+} on strip), chelator-free and fast detection.

2. Materials and methods

2.1. Reagents and equipments

Gold nanoparticles (40 nm) were produced in the plant quarantine and applied immunology laboratory of Nanjing Agricultural University (Nanjing, China). 6-mercaptonicotinic acid (MNA), bovine serum albumin (BSA), ovalbumin (OVA), Tween-20, dimethyl sulfoxide (DMSO), 3,3´,5,5´-tetramethylbenzidine (TMB), dimethyl formamide (DMF), N-hydroxysuccinimide (NHS), N,N´-dicycohexylcarbodimide (DCC), Freund's complete/incomplete adjuvants and polyethylene glycol (PEG1500) were purchased from Sigma chemical Co. (St. Louis, USA). Ammonium carbonate, calcium chloride, manganese sulfate, lead sulfate, zinc sulfate, magnesium sulfate, ferric chloride, methylmercury chloride (CH_3HgCl), mercury (I) chloride (Hg_2Cl_2) and mercuric sulfate ($HgSO_4$) were purchased from Aladdin industrial corporation. Hypoxanthine aminopterin thymidine (HAT), hypoxanthine thymidine (HT) and culture media Dulbecco's Modified Eagle Medium (DMEM) were provided by Gibco (USA). Horseradish peroxidase labeled goat anti-mouse IgG conjugate (HRP-GaMIgG) was bought from Boster Biological Technology Co., Ltd (Wuhan, China). Fetal bovine serum (FBS) was provided by Hangzhou "Sijiqing" company (Hangzhou, China). Ultra-pure deionized water was produced with a

triple-distilled water system, and used to prepare all aqueous solutions. NC membranes, glass fibers and absorbent pads were purchased from Millipore Corp (Billerica, MA, USA). Bicinchoninic acid kit was purchased from sigma.

An XYZ3060 dispensing platform and CM4000 Guillotine Cutter (BioDot, Irvine, CA) were used to prepare test strips. Samples were validated using an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA). A membrane strip reader (TSR5000) was purchased from Jiening Biotech Co. Ltd (Shanghai, China).SP2/0 cells were stored in the plant quarantine and applied immunology laboratory of Nanjing Agricultural University (Nanjing, China), and BALB/c mice were purchased from the Center of Comparative Medicine of Yangzhou University (Yangzhou, China). All animals used in this study and animal experiments were approved by the Department of Science and Technology of Jiangsu Province. The license number was SYXK (SU) 2010-0005.

2.2 Synthesis of MNA-protein conjugation

The MNA was conjugated to BSA or OVA by the DCC/NHS ester method according to the previous literature with a slight modification [23, 24]. Briefly, MNA (0.014 g), NHS (0.011 g), and DCC (0.071 g) were dissolved in DMF (900 μL) and the reaction was stirred overnight at room temperature. After centrifugation of the solution at 13,400 rpm for 15 min, the supernatant was dropwise added to 7 mL of 0.13 $mol \cdot L^{-1}$ $NaHCO_3$ solution containing 117 mg BSA or OVA and kept stirring for 4 h at room temperature. Then, the resulting solution was centrifuged and the supernatant was dialyzed in 0.01 $mol \cdot L^{-1}$ PBS at 4 ºC for 2 days with five-times change of buffer. The protein concentrations of the MNA-protein (BSA or OVA) conjugations were determined by BCA kit.

The MNA-OVA solution was used as one of a potential coating antigen, and both the

111 MNA-BSA and MNA-OVA were used to preparation of Hg-MNA-protein conjugation.

112 **2.3. Preparation of Hg-MNA-Protein conjugation**

113 CH_3HgCl (0.07 mmol) was dissolved in 540 μL of methanol containing 10 % of 1 $mol \cdot L^{-1}$

114 NaOH (v/v). The solution of CH_3HgCl was added dropwise to MNA-Protein (BSA or OVA) while

115 stirring and the reaction was incubated overnight at room temperature. The solution was dialyzed

116 in 0.01 M $(NH_4)_2CO_3$ for 2 days with five-times change of buffer. The protein concentrations of

117 the Hg-MNA-protein (BSA or OVA) conjugations were determined by a Nanodrop 1000 UV-VIA.

118 The Hg-MNA-BSA was used as immunogen, Hg-MNA-OVA was used as the second potential

119 coating antigen.

120 **2.4. Production of monoclonal antibody**

121 Five BALB/C female mice of about 7 weeks old were immunized with the Hg-MNA-BSA

122 conjugation. The first dose consisted of 100 μg of conjugation intraperitoneally injected as an

123 emulsion of PBS and Freund's complete adjuvant. The subsequent injections were emulsified in

124 Freund's incomplete adjuvant. The second booster immunization was given to each mouse at

125 3-week intervals after the initial immunization, the third immunization was given at 4-week

126 intervals and the following immunization was given at 8-week intervals. One week after the last

127 injection, the antisera were obtained from the tail vein of each mouse. The sera were tested for

128 antibody titers and for analyte (Hg^{2+}) recognition by indirect incompetitive/competitive ELISA.

129 The process of ELISA was performed as described previously[24].

130 The mouse showing the highest serum immuno-reactivity was given a peritoneal cavity

131 injection of 100 μg Hg-MNA-BSA in PBS at 1 week intervals. Three to four days after the last

132 injection, the donor mouse was sacrificed. SP2/0 murine myeloma cells were cultured in DMEM

supplemented with 20% FBS. Splenocytes of selected mice were harvested aseptically. Cell fusion and hybridoma selection procedures were performed essentially as described previously[25]. The fusion cells were incubated at 37 ºC with 5% CO_2, and after 7 days, the supernatants were screened by an indirect ELISA using Hg-MNA-OVA and OVA-MNA as coating antigen. The supernatants which can recognize both coating antigens were detected for further indirect competitive ELISA using Hg^{2+} as competitor. The hybridomas whose supernatants recognized the two coating antigens and could be inhibited by Hg^{2+} were subcloned for three times using the limiting dilution method[25]. Four stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen. Abundant antibodies were collected and subjected to purification by ammonium sulfate precipitation. The unpurified mAb was stored at -20 ºC in the presence of 50% glycerol. The purified mAb was stored at -20 ºC.

2.5. Preparation of nanogold-mAb probe

The nanogold-mAb probe was prepared according to the method [26] with a little change. In brief, the pH value of the colloidal solution was adjusted to 8.2 with 0.2 M K_2CO_3. 66 μL of 2.5 mg•mL^{-1} solution were added into 10 mL adjusted colloidal solution with quick stir for 5 min and kept for 1 h at room temperature. The amount of the mAb was 10% more than the optimal amount which was determined referring to the method[27,34]. Afterwards, 1% BSA (final concentration) was added to stabilize the mixture with quick stir for 5 min. After one-hour standing at room temperature, the solution was centrifuged (10,000 rpm) at 4 ºC for 25 min, and the supernatant was carefully sucked up. The bottom sediment was resuspended with 2 mL 2% BSA containing 0.01 M sodium borate and centrifuged (10,000 rpm) at 4 ºC for 25 min to remove the redundant regent. The washing procedure was repeated twice, followed by a last washing using a solution

containing 2% BSA, 3% sucrose, 0.01 M sodium borate and 0.05% sodium azide. Finally, the sediment was resuspended with 1 mL of the last washing solution and the prepared gold-mAb solution was stored at 4 ºC for future study in one month.

2.6. Preparation of the Immunochromatographic Test Strip

As shown in Figure 1, the Immunochromatographic Test Strip is made up of four parts including a sample pad, a conjugation pad, a nitrocellulose membrane, and an absorbent pad [26, 28]. The nanogold-mAb probe was dispensed by XYZ 3060 onto the dried, 2% BSA, 0.01 M sodium borate blocked glass fiber pad and vacuumized at 37 ºC for 30 min. The potential coating antigens were diluted with 0.09% NaCl and dispensed at test (T) line, while 0.15 $mg \cdot mL^{-1}$ goat anti-mouse IgG was dispensed at control (C) line when the NC membrane was dried at 37 ºC for 2 h. The distance between the T and C line was about 5 mm. The NC membrane, conjugate pad, sample pad and absorbent pad were pasted onto the PVC plate correctly which was then cut into 4-mm-wide strips using the programmable strip cutter CM4000. All strips were sealed in a plastic bag with a pack of desiccant gel and stored at 4 ºC.

2.7. Evaluation of developed Immunochromatographic Test Strip

2.7.1. Cross-reactivity of Immunochromatographic Test Strip

The specificity of the developed EFITS was determined as below, Fe (III), Ca (II), Mg (II), Zn (II), Pb (II), Mn (II) Cd (II), Hg (I), Hg(II) MNA, MNA-Hg, CH_3Hg^+.The concentration of all material was set as 1000 $ng \cdot mL^{-1}$.

2.7.2. Accuracy of Immunochromatographic Test Strip

Water samples for validation of the strip include mineral water from local supermarket, tap water from our lab, river water from Nanjing Agriculture University and lake water from Xuanwu

177 Lake (Nanjing, China). All water samples were centrifuged at 6000 rpm for 15 min and filtered
178 with a 0.22 μm filter membrane.

179 **2.7.3. Stability of Immunochromatographic Test Strip**

180 The stability of the strips was evaluated by running the same batch strips at 1, 2, 4 and 8
181 weeks intervals. The strips were stored at 4 $^{\circ}$C in sealed lucifugal bags. 0 and 1000 ng·mL^{-1}
182 concentration of mercury ions were tested. The ratio of color indensity of test line and control line
183 (T/C) was tested.

184 ## 3. Results and discussion

185 **3.1. Principle of the Immunochromatographic Test Strip**

186 The principle of the Immunochromatographic Test Strip was based on competitive
187 immunoreaction (Figure 1). The nanogold-mAb probe, which can bind to the coating antigen on
188 the test line, was used as the labeling material. During the lateral chromatography, the coating
189 antigen (immobilized on test line) acted as a competitive analogue of Hg^{2+}, binding to the
190 nanogold-mAb. When a sample solution is applied to the sample pad, the liquid sample can flow
191 to the other end of the strip according to the capillary action. The nanogold-mAb immobilized on
192 conjugation zone flow together with sample fluid to reach the test line and control line. For
193 positive experiment, signal intensity of the test line (red line from nanogold) showed an inverse
194 proportional corresponding relationship with Hg^{2+}content. The excess analyte and probe contained
195 in fluid fraction and continued flowing onto the control line and the absorbent pad. The probe
196 interacts with goat anti-mouse IgG immobilized on control line to form a red line. For negative
197 experiment, the probe can be fully binded to the coating antigen on test line and goat anti-mouse
198 IgG on control line with the same color intensity. The ratio of color intensity of the test line and

199 control line (T/C) was quantified at 9 min by using a test strip reader.

200 **3.2. The affinity analysis of mAb to MNA-OVA and individual Hg^{2+}**

201 2 potential coating antigens, MNA-OVA and Hg-MNA-OVA, were synthesized for
202 development of the Immunochromatographic Test Strip. The affinity analysis of mAb to coating
203 antigens and individual Hg^{2+} was performed on the Immunochromatographic Test Strip (Figure 2).
204 As illustrated in Figure 2, when the concentration of individual Hg^{2+} was 0 $ng \cdot mL^{-1}$, the intensity
205 demonstrated the affinity of nanogold-mAb to MNA-OVA and Hg-MNA-OVA. With the increase
206 of Hg^{2+}, the intensity decreased because individual Hg^{2+} is inhibiting the binding site of
207 nanogold-mAb so that the intensity of test line is decreasing.

208 From Figure 2, without Hg^{2+}, there was no significant difference in the affinity of
209 nanogold-mAb toward MNA-OVA and Hg-MNA-OVA. With the increasing of Hg^{2+}, individual
210 Hg^{2+} showed stronger competition towards nanogold-mAb when competing with MNA-OVA than
211 that with Hg-MNA-OVA. That means mAb has stronger affinity toward Hg-MNA-OVA than
212 MNA-OVA, in the meantime, it can be competed by individual Hg^{2+} without any chelator. More
213 importantly, the coating antigen MNA-OVA (in absence of Hg^{2+}) can be used as coating antigen
214 on the Immunochromatographic Test Strip, so the developed test strip for detection of Hg^{2+} was
215 called Eco-friendly Immunochromatographic Test Strip (EFITS).

216 **3.3. Optimization of analytical parameters for the EFITS**

217 Ionic strength (0-1.0 M), tween-20 (0.05%-0.4%) and pH values (5.5-9.0) were optimized to
218 obtain the best sensitivity. The results were judged by naked eye. Ionic strength (0 M, 0.05 M,
219 0.15 M, 0.5 M and 1.0 M NaCl additionally added into 0.01M PBST, pH 7.4) was optimized to
220 obtain the best sensitivity. As shown in Table 1, with the ionic strength in working solution

increased, the sensitivity of the strip improved. However, along with the ionic strength reached 0.15 M, the sensitivity diminished as the ionic strength increased. So, ionic strength of 0.15 M was chosen for future optimization. Extreme pH values may induce antibody structure changes thus destroying paratope of antibody[29~~27~~] and obstructing the binding of antibody with antigen. In this work, as we can see from Table 2, the sensitivity of the one-step strip improved with the pH value of working solution increased. But it reached the highest point at pH value 7.0 and 8.0. However color of T line was a little lighter in pH 8.0 than that of 7.0 while they maintained the same sensitivity. Consulting convenience of detecting mercury, we chose pH 7.0 for further study. As surfactant, moderate Tween-20 can block active group on NC membrane and nonspecific sites of our coating antigen, lowering nonspecific adsorption on NC membrane and enhancing color of T line. We found from Table 3 that with the increase of Tween-20 content, T line signal decreased. That is to say proper increase of Tween-20 content would improve sensitivity. Yet, too much Tween-20 weakened sensitivity. Thus, 0.2% Tween-20 was selected for working solution.

To sum up, the working solution of the developed method contained 0.15 M ionic strength and 0.2% Tween-20 in 0.01 M PBS with pH 7.0.

3.4. The sensitivity of the developed EFITS

For this one-step strip assay, a series of mercury concentrations were dissolved in optimized working solution. Each solution was added on sample pad (100 μL per sample) and waited for 9 min. Water samples containing mercury standard concentrations ranging from 0 to 1000 $ng \cdot mL^{-1}$ were assayed by our developed strips. As shown in Figure 3, with mercury concentration increased, the intensity of red color on T line reduced. With mercury concentration of 5 $ng \cdot mL^{-1}$, the intensity of red color on T line was greatly weaker than that at zero concentration.

For quantification of Hg^{2+} on the test strip, mercury standard concentrations ranging from 0 ng·mL^{-1} to 1000 ng·mL^{-1} dissolved in optimized working solution were assayed by strips and the results were scanned by a strip reader. The obtained detection curve is shown in Figure 4A. From Figure 4A, we can observe that in the range of 0.5-500 ng·mL^{-1}, the diagram between B/B_0 and logarithm of mercury concentration (ng·mL^{-1}) was linear (Figure 4B). The regression equation was obtained ($y=-0.2656x+0.85$, $R^2=0.9966$). The IC_{50} value was calculated as 20.8 ng·mL^{-1}, and the detection limit was 0.4 ng·mL^{-1}.

In this study, the detection limit was 0.4 ng·mL^{-1}, which is lower than 2 ng·mL^{-1} recommended by United States Environmental Protection agency [21] and 6 ng·mL^{-1} recommended by World Health Organization [30]. This research provides a new view for detecting heavy metals with monoclonal antibodies. On one hand, we can detect mercury without using any chelators attributing to good quality of mAb. It could be owning to MNA, which contains a cyclic pyridine ring and a sulfhydryl group. MNA bare the mercury ion outside which makes it more likely for antibodies to recognize mercury ions. There is a possibility that unmask heavy metals from carrier proteins would increase probability of generating antibodies toward specific heavy metals. On the other hand, our developed one-step strip can be totally heavy metal-free and won't generate heavy metal pollution to our surrounding. Still, we don't know the principle of how the mAb works without using chelators. But it gives us confidence of the possibility of detecting single heavy metals without using chelators.

3.5. Evaluation of the developed EFITS

3.5.1. Cross-reactivity of the test strip

The cross-reactivity of the one-step strip is an important parameter when assessing accuracy

of detecting Hg^{2+}.The effects of common metal ions which may exist in water samples such as Mg^{2+}, Ca^{2+}, Zn^{2+}, Pb^{2+}, Fe^{3+}, Cd^{2+} were tested on the developed one-step strip. Also CH_3Hg^+, Hg^+, MNA, MNA-Hg were tested on the strip to get more information about cross-reactivity. As shown in Table. 5, Hg^{2+}, MNA and MNA-Hg showed cross-reactivity with Hg^{2+} while other compounds didn't show obvious cross-reactivity. The results demonstrated that the developed one-step strip was highly selective to Hg^{2+} ion.

3.5.2. Analysis of the spiked water samples

The analysis of spiked water samples was another parameter used to confirm the accuracy of the strip. The mineral water, tap water, lake water and river water were spiked with Hg^{2+} and detected in optimized working solution. As shown in Table 4, the average recoveries of spiked water samples ranged from 82.0% to 105.8%. The outcome showed that the strip could determine Hg^{2+} accurately.

3.5.3. Stability of the test strip

The test strips obtained from the same batch were stored in vacuum bag and preserved at 4 °C for 1, 2, 4 and 8 weeks. 1000 $ng \cdot mL^{-1}$ Hg^{2+} dissolved in optimized working solution and negative control (100 μL) were added on sample pad. Figure 6 showed no significant signal loss in T line, which is to say the strips were stable for at least 8 weeks. The stability assay ensures the possibility of practical application.

4. Conclusion

In this work, an Eco-Friendly Immunochromatographic Test Strip (EFITS) and a chelator-free method to detect mercury ion were developed. The quantitative detection limit was 0.4 $ng \cdot mL^{-1}$ measured by a test strip reader. The developed method showed a good specificity and

accuracy in application of detecting water samples containing mercury ion. Like most immunochromatographic test strips, the developed one-step strip enabled the detection with 9 min, and the quantified results could be obtained through a membrane strip reader.

Acknowledgements

The authors thank Dr. Pedro Laborda and Dr. Louis Conway for their comments.

This work was supported by the National Natural Science Foundation of China (31401771), the National High Technology Research and Development Program of China (2012AA101401-1, 2013AA065601) and the Natural Science Foundation of Jiangsu Province (BK20140685).

References:

1 P. Miretzky, A. F. Cirelli, ***J. Hazard. Mater.*** 2009, 167, 10-23

2 Y. Zhou, Y. S. Li, X. Y. Meng, Y. Y. Zhang, L. Yang, J. H. Zhang, X. R. Wang, S. Y. Lu, H. L. Ren, Z. S. Liu, ***Sensor. Actuat. B-Chem.*** 2013, 183, 303-309

3 Z. Guo, G. Q. Chen, G. M. Zeng, Z. W. Li, A. W. Chen, M. Yan, L. Z. Liu, D. Y. Huang, ***RSC Advances.*** 2014, 4, 59275-59283

4 Y. Liao, Q. Li, N. Wang, S. J. Shao, ***Sensor. Actuat. B-Chem.*** 2015, 215, 592-597

5 X. J. Zhan, T. Xi, P. Zhou, ***Environ. Forensics.*** 2013, 14, 103-108

6 M. Y. Zhu, Y. Wang, Y. Deng, L. Yao, S. B. Adeloju, D. D. Pan, F. Xue, Y. C. Wu, L. Zheng, W. Chen, ***Biosens. Bioelectron.*** 2014, 61, 14-20

7 Y. H. Luo, L. L. Xu, A. H. Liang, A. P. Deng, Z. L. Jiang, ***RSC Advances***, 2014, 4, 19234-19237

8 K. Leopold, M. Foulkes, P. J. Worsfold, ***Anal. Chem.*** 2009, 81, 3421-3428

9 C. Burrini, A. Cagnini, ***Talanta.*** 1997, 44, 1219-1223

10 L. P. Yu, ***J. Agr. Food Chem.*** 2005, 53, 9656-9662

11 B. M. W. Fong, T. S. Siu, J. S. K. Lee, S. Tam, ***J. Anal. Toxicol.*** 2007, 31, 281-287

12 J. L. Gómez-Ariza, F. Lorenzo, T. García-Barrera, ***Anal. Bioanal. Chem.*** 2005, 382, 485-492

13 A. Dago, O. Gonzalez-Garcia, C. Arino, J. M. Diaz-Cruz, M. Esteban, ***J. Chromatogr. A.*** 2009, 1216, 6752–6757

14 J. L. Gomez-Ariza, D. Sanchez-Rodas, I. Giraldez, E. Morales, ***Analyst.*** 2000, 125, 401–407

15 Y. S. Li, Y. Zhou, S. Y. Lu, D. J. Guo, H. L. Ren, X. M. Meng, B. H. Zhi, C. Lin, Z. Wang, X. B. Li, Z. S. Liu ***Food Control.*** 2012, 24, 72-77

16 S. H. Huang, ***Sensor. Actuat. B-Chem.*** 2007, 127, 335-340

17 Q. K. Fang, L. M. Wang, Q. Cheng, J. Cai, Y. L. Wang, M. M. Yang, X. D. Hua, F. Q. Liu, ***Anal. Chim. Acta.*** 2015, 881, 82-89

18 C. R. Xing, L. Q. Liu, S. S. Song, M. Feng, H. Kuang, C. L. Xu, ***Biosens. Bioelectron.*** 2015, 66, 445-453

19 K. Abe, K. Nakamura, T. Arao, Y. Sakurai, A. Nakano, C. Suginuma, K. Tawarada, K. Sasaki, ***J. Sci. Food Agri.*** 2011, 91, 1392-1397

20 A. M. Lopez_Marzo, J. Pons, D. A. Blake, A. ***Biosens. Bioelectron.*** 2013, 47 190-198

21 Y. Zhou, X. L. Tian, Y. S. Li, Y. Y. Zhang, L. Yang, J. H. Zhang, X. R. Wang, S. Y. Lu, H. L. Ren, Z. S. Liu, ***Biosens. Bioelectron.*** 2011, 30, 310-314

22 Y. Zhou, Y. Y. Zhang, F. G. Pan, Y. S. Li, S. Y. Lu, H. L. Ren, Q. F. Shen. Z. H. Li, J. H. Zhang, Q. J. Chen, Z. S. Liu. ***Biosens. Bioelectron.***, 2010, 25, 2534-2538

23 F. D. Cai, Q. Zhu, K. Zhao, A. P. Deng, J. G. Li, ***Environ. Sci. Technol.*** 2015, 49, 5013-5020

24 Y. Z. Wang, H. Yang, M. Pschenitza, R. Niessner, Y. Li, D. Knopp, A. P. Deng. ***Anal. Bioanal. Chem.*** 2012, 43, 2519-2528

25 J. C. Howard, G. W. Butcher, G. Galfre, C. Milstein. Springer Berlin Heidelberg, 1979: 54-60

26 L. M. Wang, J. Cai, Y. L. Wang, Q. K. Fang, S. Y. Wang, Q. Cheng, D. Du, Y. H. Lin, F. Q. Liu, ***Microchim. Acta.*** 2014, 181, 1565-1572

27 X. D. Hua, G. L. Qian, J. F. Yang, B. S. Hu, J. Q. Fan, N. Qin, G. Li, Y. Y. Wang, F. Q. Liu. *Biosens. Bioelectron*., 2010, 26, 189-194

28 X. Liu, J. J. Xiang, Y. Tang , X. L. Zhang, Q. Q. Fu, J. H. Zou, Y. H. Lin, ***Anal. Chim. Acta.*** 2012, 745, 99-105

29 R. Reverberi, L. Reverberi, ***Blood. Transfusion***. 2007, 5 (4): 227

30 H. He, F. Wu, M. J. Xu, S. G. Yang, C. Sun, Y. H. Yang, ***Anal. Methods.*** 2011, 3, 1859-1864

Figure Legends:

Figure 1: The schematic diagram of an eco-friendly immunochromatographic test strip (EFITS) and a competitive immunoreaction on the EFITS

Figure 2: The affinity of nanogold-mAb to potential coating antigen (MNA-OVA and Hg-MNA-OVA) and Hg^{2+} on the test strip. When the concentration of Hg^{2+} is 0 ng·mL-1, the intensity means the affinity of mAb to coating antigens. While the increase of concentration of Hg^{2+}, the intensity is decrease because of the competitive reaction of Hg^{2+} with coating antigen for the limited binding sites of mAb (n=3).

Figure 3: Determination of Hg^{2+} on the eco-friendly immunochromatographic test strip. The concentrations of Hg^{2+} were 1000, 500, 250, 100, 50, 25, 10, 5, 2, 0 ng·mL^{-1}, respectively. CL: control line; TL: test line.

Figure. 4. A: Standard inhibition curve of Hg^{2+} in developed EFITS. The curve was obtained using the relationship between the values of signal intensity of the test line and the concentration of Hg^{2+}. B: The calibration curve from "A". B/B_0 is binding ratio of antibody/antigen on the test strip.

Figure 5: Stability of the EFITS by running the same batch strips after the storage of 1 (a), 2 (b), 4 (c), 8 (d) weeks at 4 □ at 0 and 1000 ng·mL^{-1} of Hg^{2+}.

Table Legends:

Table 1 Effect of ionic strength of working solution on the EFITS assay (n=3)

Table 2 Effect of pH values of working solution on the EFITS assay (n=3)

Table 3 Effect of Tween-20 of working solution on the EFITS assay (n=3)

Table 4 Recovery studies of real water sample spiked with Hg^{2+} by the developed EFITS (n=4)

Table 5 Cross-reactivity of the monoclonal antibody with metal ions and related compounds

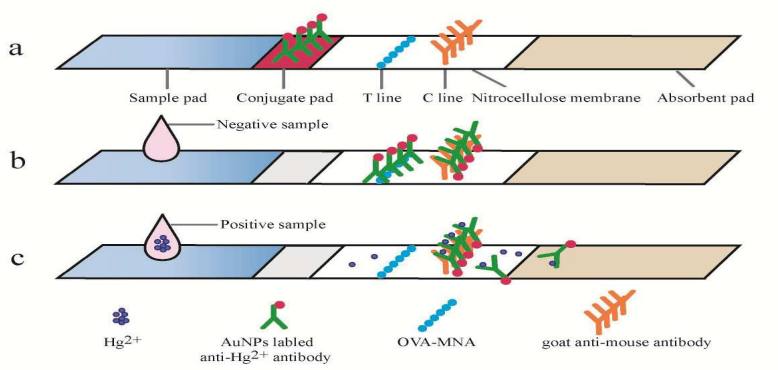
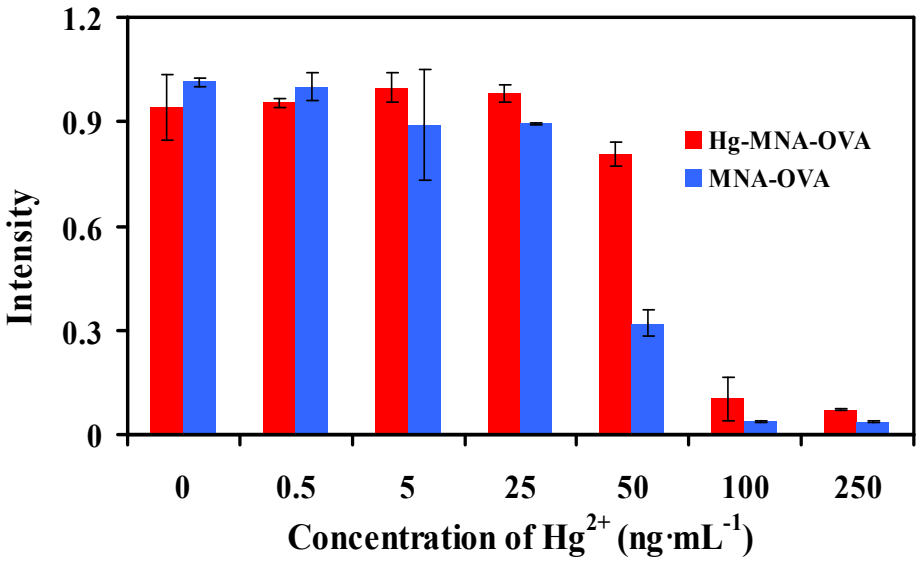


Figure 1: The schematic diagram of an eco-friendly immunochromatographic test strip (EFITS) and a competitive immunoreaction on the EFITS



397

398 **Figure 2: The affinity of nanogold-mAb to potential coating antigen (MNA-OVA and Hg-MNA-OVA)**

399 **and Hg^{2+} on the test strip. When the concentration of Hg^{2+} is 0 ng·mL^{-1}, the intensity means the affinity of**

400 **mAb to coating antigens. While the increase of concentration of Hg^{2+}, the intensity is decrease because of the**

401 **competitive reaction of Hg^{2+} with coating antigen for the limited binding sites of mAb (n=3).**

402

403

404

405

406

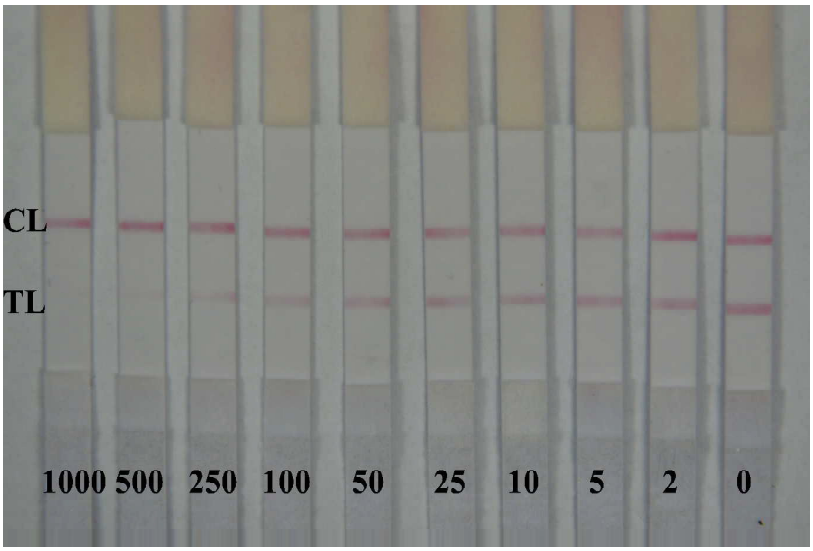
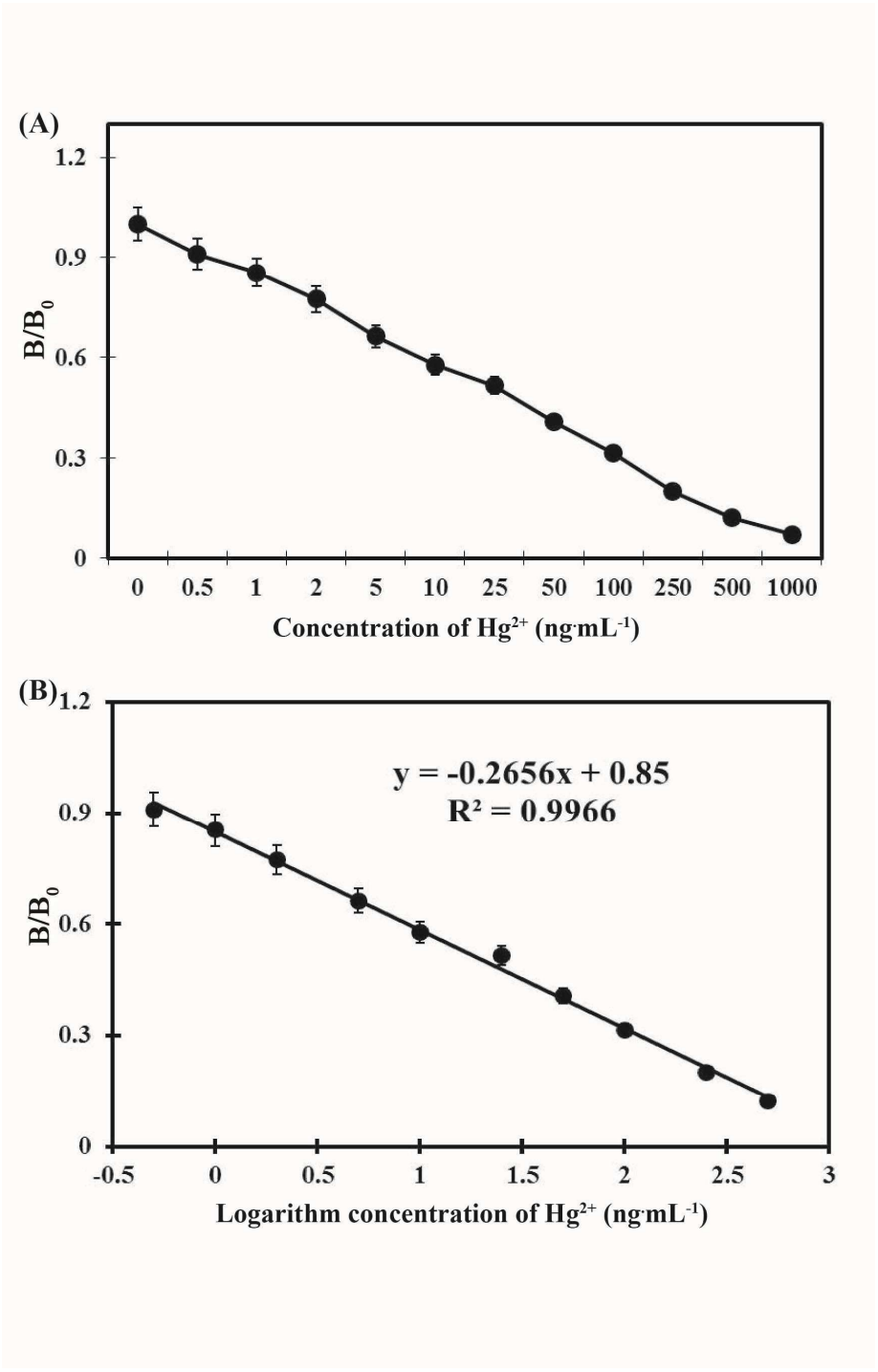


Figure 3: Determination of Hg^{2+} on the eco-friendly immunochromatographic test strip. The concentrations of Hg^{2+} were 1000, 500, 250, 100, 50, 25, 10, 5, 2, 0 $ng \cdot mL^{-1}$, respectively. CL: control line; TL: test line.



423

424 **Figure 4. A: Standard inhibition curve of Hg^{2+} in developed EFITS. The curve was obtained using the**

425 **relationship between the values of signal intensity of the test line and the concentration of Hg^{2+}. B: The**

426 **calibration curve from "A". B/B0 is binding ratio of $(T/C)_{mercury\ sample}/(T/C)_{no\ mercury}$ on the test strip.**

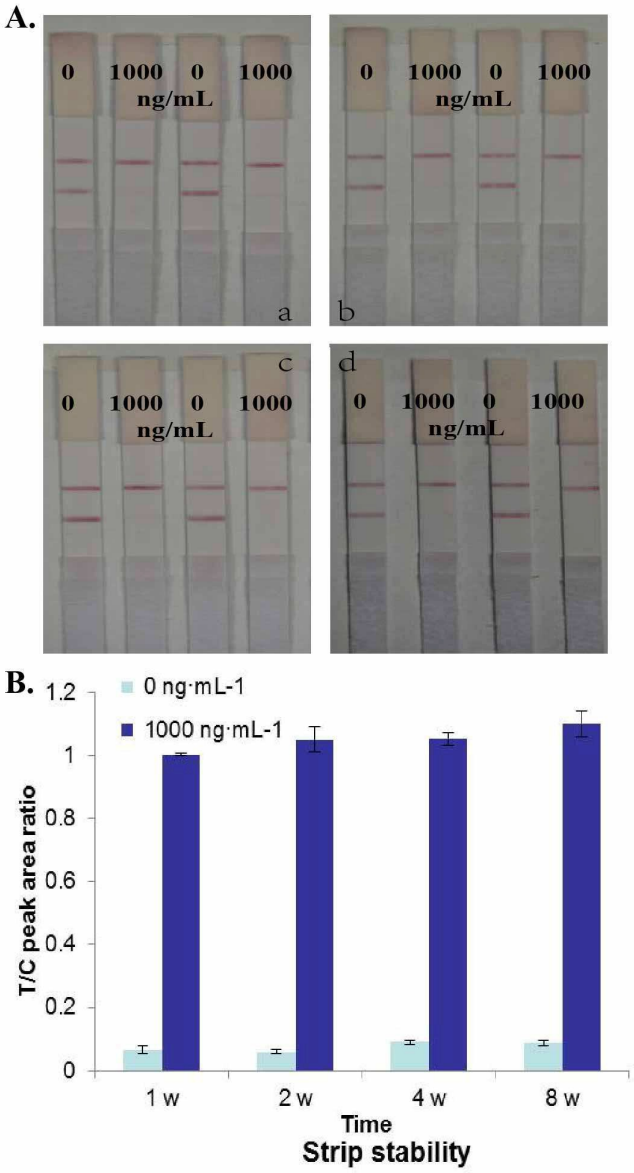


Figure 5: A. stability of the EFITS by running the same batch strips after the storage of 1 (a), 2 (b), 4 (c), 8 (d) weeks at 4 ℃ at 0 and 1000 ng·mL^{-1} of Hg^{2+}. B. stability test of the EFITS read by membrane strip reader.

435 **Table 1 Effect of ionic strength of working solution on the EFITS assay (n=3)**

Ionic strength of working solution		Mercury(II) standard concentration ($ng \cdot mL^{-1}$)				
		0	50	250	500	1000
0 M NaCI	Test line	7	6	5	4	2
	Control line	7	7	7	7	7
0.05M NaCI	Test line	7	6	5	4	2
	Control line	7	7	7	7	7
0.15 M NaCI	Test line	7	5	3	2	1
	Control line	7	7	7	7	7
0.5 M NaCI	Test line	7	5	4	3	1
	Control line	7	7	7	7	7
1.0 M NaCI	Test line	7	6	5	3	1
	Control line	7	7	7	7	7

436 7+++: Red line appeared.

437 6++±: Red line appeared but was weaker than +++.

438 5++: Red line appeared but was weaker than ++±.

439 4+±: Red line appeared but was weaker than++.

440 3+: Red line appeared but was weaker than +±.

441 2±: Red line appeared but was weaker than +.

442 1-: Red line did not appear.

443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466

Table 2 Effect of pH values of working solution on the EFITS assay (n=3)

the pH values of working solution		Mercury(II) standard concentration($ng \cdot mL^{-1}$)				
		0	50	250	500	1000
pH 5.5	Test line	7	6	5	4	2
	Control line	7	7	7	7	7
pH 6.0	Test line	7	6	5	4	2
	Control line	7	7	7	7	7
pH 7.0	Test line	7	5	3	2	1
	Control line	7	7	7	7	7
pH 8.0	Test line	5	5	3	2	1
	Control line	5	5	5	5	5
pH 9.0	Test line	5	5	4	2	1
	Control line	5	5	5	5	5

7+++: Red line appeared.

6++±: Red line appeared but was weaker than +++.

5++: Red line appeared but was weaker than ++±.

4+±: Red line appeared but was weaker than++.

3+: Red line appeared but was weaker than +±.

2±: Red line appeared but was weaker than +.

1-: Red line did not appear.

Table 3 Effect of Tween-20 of working solution on the EFITS assay (n=3)

Tween-20 (v/v) of working solution		Mercury(II) standard concentration ($ng \cdot mL^{-1}$)				
		0	50	250	500	1000
0.05%	Test line	7	6	5	4	2
	Control line	7	7	7	7	7
0.1%	Test line	7	6	5	3	2
	Control line	7	7	7	7	7
0.2%	Test line	7	5	3	2	1
	Control line	7	7	7	7	7
0.4%	Test line	5	5	4	3	1
	Control line	5	5	5	5	5

7+++: Red line appeared.

6++±: Red line appeared but was weaker than +++.

5++: Red line appeared but was weaker than ++±.

4+±: Red line appeared but was weaker than++.

3+: Red line appeared but was weaker than +±.

2±: Red line appeared but was weaker than +.

1-: Red line did not appear.

533 **Table 4 Recovery studies of real water sample spiked with**
534 **Hg^{2+} by the developed EFITS (n=4)**

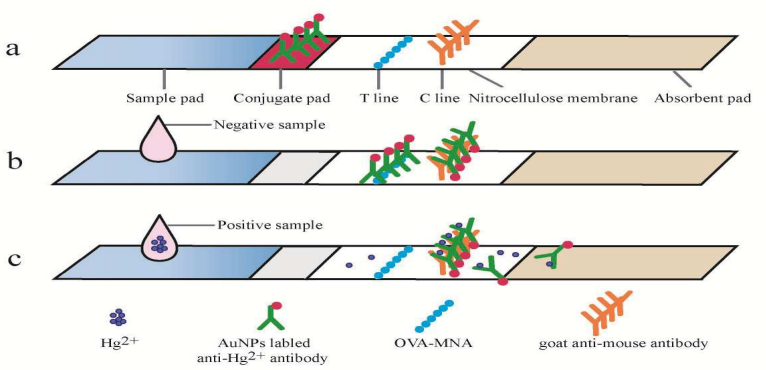
Samples	Theoretical ($ng \cdot mL^{-1}$)	Measure ($ng \cdot mL^{-1}$)	Mean recovery (%)± SD
Mineral water	400	400.0	100.0±10.3
	200	204.7	102.4±10.3
	100	104.2	104.2±8.6
Tap water	400	407.8	102.0±7.2
	200	202.1	101.0±4.6
	100	105.8	105.8±3.5
Lake water	200	203.6	101.8±4.29
	100	81.9	82.0±3.0
	50	44.3	88.7±1.7
River water	200	191.5	95.8±4.8
	100	96.2	96.2±7.2
	50	46.5	93.1±3.6

535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550

Table 5. Cross-reactivity of the monoclonal antibody with metal ions and related compounds

Metal ions and related compounds	IC_{50} (ng•mL^{-1})	CR (%)
Hg^{2+}	20.8	100
Hg^{+}	>1000	<0.1
CH_3Hg^{+}	>1000	<0.1
MNA- Hg	77	27
MNA	112	18.6
Ca^{2+}	>1000	<0.1
Cd^{2+}	>1000	<0.1
Zn^{2+}	>1000	<0.1
Mg^{2+}	>1000	<0.1
Pd^{2+}	>1000	<0.1
Fe^{2+}	>1000	<0.1
Mn^{2+}	>1000	<0.1

Table of contents



The schematic diagram of an eco-friendly immunochromatographic test strip (EFITS)

and a competitive immunoreaction on the EFITS