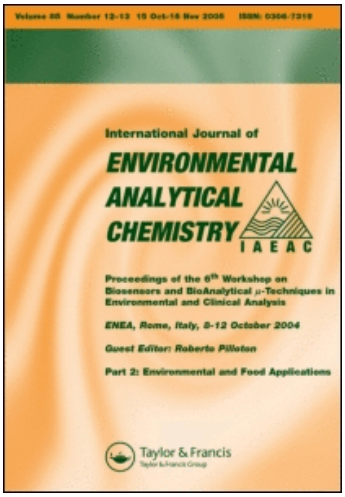

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Chenlin Hu[ab]; Nanqin Gan[a]; Zhike He[c]; Lirong Song[a]
[a] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Wuhan 430072, PR China [b] Graduate School of the Chinese Academy of Sciences, Beijing, PR China [c] College of Chemistry and Molecular Sciences, University of Wuhan, Wuhan 430072, PR China

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A novel chemiluminescent immunoassay for microcystin (MC) detection based on gold nanoparticles label and its application to MC analysis in aquatic environmental samples

CHENLIN HU†‡, NANQIN GAN†, ZHIKE HE§ and LIRONG SONG*†

†State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, CAS, Donghu Nanlu No. 7, Wuchang, Wuhan 430072, PR China
‡Graduate School of the Chinese Academy of Sciences, Beijing, PR China
§College of Chemistry and Molecular Sciences, University of Wuhan, Wuhan 430072, PR China

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A novel chemiluminescent immunoassay method based on gold nanoparticles was developed for the detection of microcystins (MCs). The immunoassay included three main steps: indirect competitive immunoreaction, oxidative dissolution of gold nanoparticles, and indirect determination for MCs with Au^{3+}-catalysed luminol chemiluminesent system. The method has a wide working range (0.05–10 $\mu g\,L^{-1}$, $r^2 = 0.9914$), the limit of detection was determined to be 0.024 $\mu g\,L^{-1}$, which is much lower than the World Health Organization's proposed guidelines (1 $\mu g\,L^{-1}$) for drinking-water. The proposed method was applied to MC analysis in natural water and fish tissue samples, and most results in the proposed method were in agreement with the conventional indirect competitive enzyme-linked immunosorbent assay method, which indicated that the new chemiluminescent immunoassay was sensitive, reliable, and suitable for MC analysis in natural water and fish tissue samples.

Keywords: Microcystins; Gold nanoparticles; Chemiluminescence; ELISA

1. Introduction

Microcystins (MCs) are a large family of heptotoxic cyclic peptides primarily produced by some freshwater cyanobacteria genera (blue-green algae) *Microcystis*, *Anabaena*, *Oscillatoria* (*Planktotrix*), and so far 80 variants of MCs have been identified [1–3]. Structurally, Microcystins (MCs) include two variable amino acids and an unusual aromatic amino acid (3-amino-9-methoxy-2, 6, 8-trimethyl-10-penyl-deca-4, 6-dienoic acid) usually called Adda [2]. As a matter of fact, it is Adda that plays an important role in the toxicity of MCs compounds [4].

MCs have caused many fatalities in wild and domestic animals, and freshwater mussel and farmed fish [5]. Meanwhile, the presence of MCs in freshwater body can lead to human illness, sometimes even death when humans are frequently exposed to

*Corresponding author. Fax: +86–27–68780806. Email: lrsong@ihb.ac.cn

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MCs through the consumption of microcystin-contaminated water and food or recreational activities such as swimming [6, 7]. For these reasons, the World Health Organization (WHO) has established safe guidelines with the recommended limitation of $1\,\mu g\,L^{-1}$ of MC-LR in drinking-water which is suitable for human daily consumption [8].

To avoid health risks posed by MCs, it is essential to develop a sensitive, reliable, and conventional method for their determination. Fortunately, numerous methods have been developed to determine MCs in drinking-water and food, including biochemical screening methods such as mouse bioassays, enzyme-linked immunosorbent assay (ELISA), and protein phosphatase inhibition assay (PPiA); physicochemical methods such as high-performance liquid chromatography (HPLC), liquid chromatography with mass spectrometry (LC/MS), liquid chromatography with electrospray mass spectrometry (LC/ESI/MS), capillary electrophoresis (CE), nuclear magnetic resonance (NMR), gas chromatography (GC) and GC with MS detection (GC/MS). Those methods mentioned above for MC detection have been reviewed by Msagti *et al*. [9]. However, every method reported has its advantages and disadvantages. Indeed, as a common tool for MC analysis, ELISA has a relatively narrow working range [10–12]. HPLC requires complex sample pre-treatment and standards which are not always available, though it has excellent accuracy and reproducibility [13, 14]; other methods such as CE and HPLC/MS are relatively expensive and require skilled personnel.

Until recently, all kinds of the immunoassays were developed with the purpose of improving the sensitivity of immunoassays. A time-resolved fluorescence immunoassay (TRFIA) for MC detection was developed, based on the europium chelate of MC-LR by Mehto *et al* [15]. A similar study was also developed based on secondary antibody labelled with europium in our laboratory [10], and a more sensitive range ($0.01–10\,\mu g\,L^{-1}$) was obtained previously in that work. Recently, a series of analytical methods based on gold nanoparticle labels have been continuously proposed for the analyte detections [16–18]. As a biological label, gold nanoparticles involve easier and less expensive preparation, excellent compatibility with biomolecules [19–22], and flexible measurements such as chemiluminescene (CL) [23], anodic stripping voltammetry (ASV) [24], and induced coupled plasma mass spectrometry (ICPMS) [25].

Based on these facts, gold nanoparticles and the chemiluminescent detection method were first introduced into this work for the detection of MCs in an attempt to improve the sensitivity of the immunoassay. The reaction format of indirect competitive ELISA (icELISA) was employed in the new method, and the determination of MCs was indirectly performed by measuring the amounts of gold with CL system. Under the optimized conditions for the assay, the recovery for MCs was studied. Moreover, the new method was applied to MC analysis in natural water and tissue samples, and was compared with the conventional icELISA.

2. Experimental

2.1 *Chemicals*

Microcystin-LR ($>95\%$, HPLC-grade purity) was purified from cultured *Microcystis* in our laboratory. $HAuCl_4$, gelatin, and luminol were purchased from Sigma

(St. Louis, MO). Bovine serum albumin (BSA) and Tris base were obtained from Roche (Basel, Switzerland). Goat-anti-mouse IgG (Affinipure, H + L) and Goat-anti-mouse IgG (Affinipure, H + L) conjugated HRP (horseradish peroxidase) were purchased from Pierce (Rockford, IL). 3,3′,5,5′-Tetramethylbenzidine (TMB) was obtained from Cayman Chemical Company (Ann Arbor, MI). All other chemicals used in the experiment were of analytical grade. All the solutions were prepared by the deionzed water (18.2 MΩ cm), which was obtained from a MilliQ System (Millipore, Bedford, MA).

2.2 *Instrumentation*

UV-visible spectra of colloidal gold were collected on an Ultrospec 3000 UV-Visible spectrometer (Amersham Pharmacia Biotech, Uppsala, Sweden). The diameters of gold nanoparticles were confirmed using a JEM-1230 transmission electron microscope (TEM) (JEOL, Tokyo). Chemiluminescence's intensity was obtained on a BioOrbit 1251 luminometery system (LKB, Uppsala, Sweden). The optical density of the ELISA assay was measured using microplate reader (Model 550 microplate reader BIO-RAD Laboratories, Hercules, CA).

2.3 *Sample preparation*

2.3.1 Environmental water samples. Three different water samples (sample I, II, and III) were collected from Dianchi Lake (Yunnan, China) and Guanqiao (Wuhan, China) for MC analysis. Sample I was collected from Fu Baowan of Dianchi Lake on 26 April 2005, sample II was collected from Fu Baowan of Dianchi Lake on 23 April 2006, and sample III was collected from Guanqiao. All raw water samples were immediately filtered through 0.45-μm filters (GF/C, Whatman) after they were collected from the surface of the pond and lake between 0 and −0.5 m, and a portion (1 mL) of the filtered sample was stored at −20°C for MC analysis.

2.3.2 Fish tissues. The experimental fishes were exposed to MCs for 20 days through the diet after several weeks of acclimatization, and then slaughtered. Liver and muscle parts were selected, dried, and stored at −20°C for further extraction.

The procedure of MC extraction from fish tissue samples was as follows. Dried fish tissues after lyophilization (about 500 mg) were extracted first with 5% acetic acid (10 mL) for 20 min, then with 80% methanol aqueous solution (20 mL) for 20 min twice. The three extracts were combined, diluted, and passed through a pre-conditioned Sep-Pak cartridges (Waters, Milford, MA). MCs were eluted with 10 mL of 100% methanol. After evaporation at 35°C to dryness in a rotary evaporator, the residue containing microcystins was dissolved in 1 mL of ultrapure water; the samples obtained were also stored at −20°C for the MCs analysis.

2.4 *Production of monoclonal antibody*

The immunogen (MC-LR)-BSA was synthesized according to the method developed by Chu *et al*. [26]. The production of the monoclonal antibody (mAb) was the same as our previous report [10]. In brief, female BALB/c mice were immunized with (MC-LR)-BSA. Hybridomas were produced by fusing the spleen cells from the immunized mouse with myeloma cell SP2/0 in polyethylene glycol, and then selected with hypoxanthine aminopterin thymidine (HAT) medium. After two weeks of fusion, the hybridomas were screened for the production of anti-MC-LR antibodies by icELISA. Hybridomas were estimated as positive for the generation of specific antibodies when they were positive for (MC-LR)-BSA, (MC-LR)-PLL and negative for EDPC-treated proteins in the ELISA. The positive hybridomas were recloned several times by a limiting dilution method. The mAbs were purified by protein G column (Amersham Biosciences, Sweden) according to the manufacturer's instructions. The cross-reactivity of the antibody with three different microcystins (MC-LR, MC-RR, and MC-YR) was also tested in our previous work [10]. The results of the test indicated that the monoclonal antibody had a similar affinity for MC-LR and MC-RR, but less affinity for MC-YR. Since MC-LR and MC-RR account for more than 80% of hepatic toxin congeners produced by freshwater cyanobacteria [27], more toxic MCs in the samples can be determined in the novel immunoassay based on gold nanoparticles and anti-(MC-LR)-BSA monoclonal antibody.

2.5 *Synthesis of the colloidal gold*

Colloidal gold was prepared according to Frens' method with a minor modification [28], and all the glassware was cleaned with a solution-aqua regia (HCl-HNO_3) (3 : 1 v/v) and rinsed with ultrapure water before used. Briefly, 100 mL of 0.01% $HAuCl_4$ (by weight) solution was added into the flask equipment with magnetic stirring. After boiling for 5 min, 2 mL of 1% sodium citrate (by weight) was added with vigorous stirring for 5 min, and then cooled. The solution was stirred for an additional 15 min until room temperature was reached and subsequently filtered through a 0.45 μm Millipore membrane. Colloidal gold freshly made was stored in the fridge at 4°C. The diameter of the gold nanoparticles was ~13 nm (figure 1) as given by TEM (the accelerating voltage of the electron beam was 100 kV), and the peak of the absorbance was 519 nm.

2.6 *Preparation of gold-coated goat-anti-mouse IgG*

Preparation of gold-coated goat-anti-mouse IgG was performed according to the methods reported by Verkleij *et al*. [29], Ni *et al*. [30], and Lyon *et al*. [31]. The goat-anti-mouse IgG (10% more than the minimum amount, which was determined using a flocculation test) was added to 12.5 mL of pH-adjusted colloidal gold suspension (pH 8.2 adjusted with 0.01 M K_2CO_3), and then 4 mL of 5% BSA solution was added after vigorous stirring for about 10 min. The solution was incubated for 40 min at room temperature followed by centrifugation at 11 000 rpm for 1 h at 4°C, and then the centrifugation was performed again after the soft sediment from the first centrifugation was resuspended in 0.02 M TBS (pH 8.2, Tris-HCl containing 1%

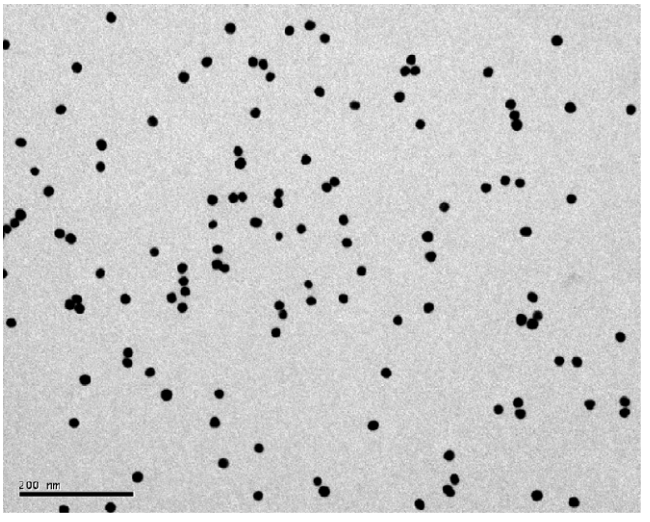

Figure 1. TEM photograph of gold nanoparticles.

BSA and 0.05% NaN_3). Then, following the removal of the supernatant, the red soft sediment was resuspended in 0.02 M TBS (pH 8.2, Tris-HCl containing 1% BSA and 0.05% NaN_3). The gold-coated goat-anti-mouse IgG was stored at 4°C for further use.

2.7 *Immunoassay protocol*

2.7.1 Immunoassay based on gold nanoparticles. Indirect competitive immunoreaction model was chosen for the determination of MCs in the new method. Each well was coated with 100 μL of (MC-LR)-BSA at 4°C overnight, the unbound antigen was washed away with 0.01 M PBST (PBS containing 0.05% Tween-20, pH 7.4), and the well was washed again with 0.01 M PBST followed by blocking with 0.01 M PBS (pH 7.4) containing 0.5% gelatin at 4°C overnight. Fifty microlitres of standards or samples was pipetted into the coated wells, and then 50 μL of mAb was added into each well. After incubation at 37°C for 90 min followed by washing three times with PBST, 100 μL of goat-anti-mouse IgG labelled with gold nanoparticles (1 : 200 dilution with PBS containing 0.5% gelatin) was added into each well and incubated at 37°C for 50 min followed by washing five times with PBST. The determination of MCs was performed using a chemiluminescent system.

2.7.2 Indirect competitive ELISA. To evaluate the reliability of the proposed method, MCs in the same sample were detected using both the icELISA and the proposed method. In the procedure of icELISA, the similar conditions were used, except that goat-anti-mouse-HRP was added to each well after the competitive immunoreation. One hundred microlitres of goat-anti-mouse-HRP (1 : 3000 dilution with PBS containing 0.5% gelatin) was added to each well and incubation followed by washing five times with PBST. Then, 100 μL of TMB substrate solution was added, the plate was

incubated for 10 min, the reaction was stopped by addition of 50 μL of 1 M H_2SO_4, and the absorbance was read at 450 nm in the automatic microplate reader.

2.8 *Chemiluminescence detection*

After the immunoreaction based on gold nanoparticles was completed, 100 μL of oxidative solution (0.5 M NaCl–0.01 M HCl–0.5 mM Br_2) was pipetted into the well and incubated for 25 min at room temperature for dissolving gold nanoparticles, and the well was then placed in the oven at 60°C for 20 min to remove the remaining Br_2 in the solution [23]. The final solution was transferred into a glass tube, and 100 μL of the solution (10 μM luminol in 1 M NaOH) was injected into the glass tube for the CL detection.

3. Results and discussion

The principle of the competitive CL immunoassay with a colloidal gold label was depicted in figure 2. This was applied to the analyte-MCs, and primary antigen (MC-LR)-BSA was adsorbed passively on the surface of the polystyrene microwell. The MCs and mAb were in turn added into the microwell. Because of the specific binding between MCs and mAb, the remaining mAb was captured by the primary antigen (MC-LR)-BSA, and then by the goat-anti-mouse IgG labelled with gold nanoparticles, after the removal of the unbound labelled antibody, the gold contained in the bound phase was thus dissolved in an oxidative solution, and the gold ions released into the solution were quantified by the Au^{3+}-catalysed luminol chemiluminescent reaction system. The CL intensity is inversely proportional to the content of MCs in the sample.

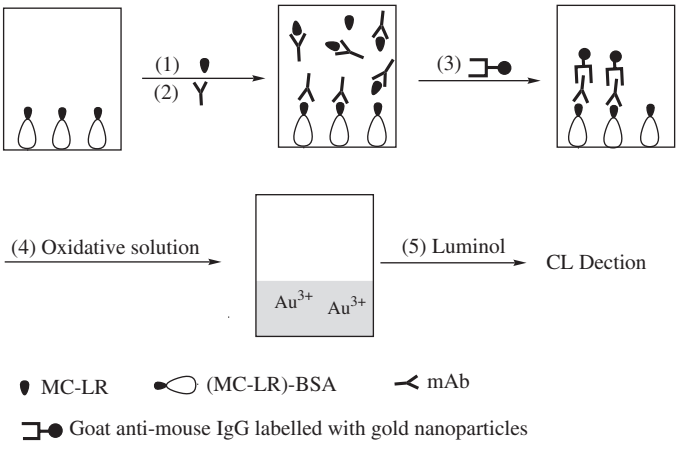

Figure 2. Schematic illustration of indirect competitive CL immunoassay based on anti-MC-LR monoclonal antibody and goat anti-mouse IgG labelled with gold nanoparticles.

3.1 *Optimization of the CL detection condition*

In the present assay, a sensitive chemiluminescent system was used to determine the amount of gold nanoparticles after immunoreaction. Gold nanoparticles must be transferred into the Au^{3+} form, which can catalyse the chemiluminescent reaction of luminol in alkaline medium. The chemiluminescent intensity of the catalytic reaction was proportional to the content of the complex $AuCl_4^-$. Therefore, it is essential to choose the optimum gold-dissolving solution. In the previous work, 0.1 M hydrochloric aicd solution containing 0.1 M NaBr (0.1 M HCl–NaBr) was used by Dequaire *et al.* to dissolve gold nanoparticles [24], and 0.1 M HBr containing 10^{-4} M Br_2 was also chosen in Authier's work to dissolve gold [16]. Recently, the oxidative solution (NaCl–HCl–Br_2) has been proved to be more efficient than a typical solution (HNO_3–HCl) in Fan's research [23]. Considering the successful use of the oxidative solution (NaCl–HCl–Br_2) in Fan' work [23], the oxidative solution (NaCl–HCl–Br_2) was also selected as the gold-dissolving solution in our experiment. Meanwhile, parameters such as the concentrations of NaCl, HCl, and Br_2 were investigated to establish the optimum gold-dissolving condition; our preliminary experimental results were consistent with those previously obtained by Fan *et al.* [23].

As the critical factor of chemiluminescent reaction, the concentration of luminol was also studied in the range of 0.1–10 μM. It was found that 10 μM luminol in 1 M NaOH was the optimum concentration using the mixture (0.5 M NaCl–0.01 M HCl–0.5mM Br_2) to dissolve gold nanoparticles in our experiment.

3.2 *Standard curves for the new method and icELISA*

Standard curves for both of the two methods were obtained from a series of standard MC-LR solution in the 0.0001–100 μg L^{-1} range (figure 3). The responses in both two

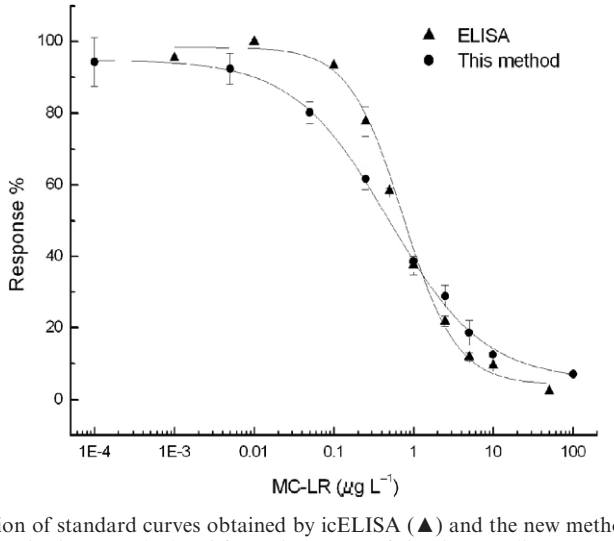

Figure 3. Comparsion of standard curves obtained by icELISA (▲) and the new method (●) with MC-LR. Error bars are the standard error calculated from the means of the three replicates.

Table 1. Recovery analysis of MCs determined by the proposed method in water samples.

MC-LR added[a] ($\mu g\,L^{-1}$)	Recovery (%)	CV (%)
0.05	90	14.7
0.5	90	17.3
1	102	5.4
5	103	12.6

[a]Recovery analysis was performed with three independent measurements for water samples.

methods were plotted against the logarithm of the concentrations of the MCs. The response was calculated using the following equation:

$$\text{Response (\%)} = \frac{B - B_{\text{background}}}{B_0 - B_{\text{background}}} \times 100,$$

where B is the signal of samples, $B_{\text{background}}$ is the background signal, and B_0 is the maximal signal in the absence of MCs. Figure 3 showed that a linear range for icELISA was 0.1–5 $\mu g\,L^{-1}$ ($r^2 = 0.99$), and a linear range of 0.05–10 $\mu g\,L^{-1}$ ($r^2 = 0.9914$) obtained by the new method. The limit of detection (LOD), which was defined as three times the standard deviations of blank samples ($N = 21$), was 0.024 $\mu g\,L^{-1}$ for the new method. As can be seen, although the new method was less sensitive than the TRFIA method (0.01–10 $\mu g\,L^{-1}$) developed previously [10], both the sensitivity and linear range were still superior to that of icELISA as well as those by the other immunoassay [12, 32–34], which can be explained by the fact that the chemiluminscent analytical method was sensitive than classic colorimetric ELISA [35].

3.3 *Accuracy evaluation of the proposed method*

The accuracy of the proposed method was validated with recovery experiments. Considering that the proposed method may be applied as an immunoassay for the environmental monitoring of MCs, recovery analysis was performed using both water samples and fish tissues as experimental materials. For the recovery of MC-LR in the water samples, Blank freshwater was spiked with MC-LR at four different levels (0.05, 0.5, 1, and 5 $\mu g\,L^{-1}$). As for the recovery of MC-LR in fish tissues, non-infected fishes were chosen, and the dry tissue powder samples were spiked with two levels (1500 and 2000 $ng\,g^{-1}$) for liver samples, and two levels (3 and 5 $ng\,g^{-1}$) for muscle samples. These tissue samples were extracted, diluted in ultrapure water, and quantified with the proposed method. The recovery results for water and tissue samples were summarized in tables 1–3.

From tables 1–3, it is clear that the recovery results of fish tissue (58–76%, CV 7.97–16.75%) are relatively unsatisfactory in comparison with those of water samples (90–103% CV, 5.4–17.3%). It is likely that MC-LR in water samples without any extraction procedure was detected directly, whereas MCs in fish tissue samples must be extracted before being determined. The inevitable loss of MC-LR in the process of extraction resulted in a lower recovery. However, as for the poor recovery analysis for the muscle of fish, this is not an unusual phenomenon, because similar results were also reported in the MC recovery analysis of fish muscle tissue [36]. In the Bogialli's

Table 2. Recovery analysis of MCs determined by the proposed method in liver tissue of fishes.

MC-LR added[a] ($\mu g\,L^{-1}$)	Recovery (%)	CV (%)
1500	76	16.75
2500	71	7.97

[a]MCs in spiked samples were extracted using SPE method as described in section 2. Recovery analysis was performed with two independent measurements for muscle samples.

Table 3. Recovery analysis of MCs determined by the proposed method in muscle tissue of fish.

MC-LR added[a] ($\mu g\,L^{-1}$)	Recovery (%)	CV (%)
3	58	2.4
5	58	18.3

[a]MCs in spiked samples were extracted using the SPE method as described in section 2. Recovery analysis was performed with two independent measurements for liver samples.

work, it was found that the recovery of MCs was not substantially affected by factors including extractant volume, MC concentrations, and type of fish. Meanwhile, Williams *et al*. showed that MCs in fish tissues partially bound to PP-1 and PP-2A enzymes [37, 38], which affected the extraction efficiency. Therefore, the specific solution to dissociate the covalent bound between MCs and PP-1or PP-2A [36], the use of immunoaffinity cartridges (IAC) [39], and the appropriate method for MCs analysis should be taken into consideration for better recovery in fish tissues. Although the new method was more sensitive than the colorimetric assay, problems associated with a low reproducibility at random were also observed during the recovery analyses in our experiment, which may be due to the sample-handling operations (extraction, dilution) or the characteristics of the sensitive chemiluminscent analytical method used in our experiment (or both).

3.4 *Application of the proposed method in natural samples*

Several water and fish tissue samples were tested using both icELISA and the proposed method. Before MC detection, the fish tissue samples underwent a set of solid-phase extraction (SPE) protocols described in section 2. Because the MC concentrations in the SPE extracts of fish liver sample were outside the working range of both icELISA and the proposed method, the SPE extracts should be diluted to perform the MC detection. All the results of the comparative study are summarized in tables 4 and 5; ELISA in this work has proved to be highly reproducible (mean RSD 3.7%, maximum 8.3% not shown in tables 4 and 5). The results indicated that there was a reasonable agreement for water and liver tissue samples between the two methods, but the results for the fish muscle tissue samples were unsatisfactory. The concentration of MCs determined in the muscle by the proposed method was much lower than those determined by the icELISA. This trend may be due to the matrix effect of the type of samples analysed. Work is in progress to evaluate these in our laboratory.

Table 4. Concentrations of MCs in different water samples determined by icELISA and proposed method.

Water sample code[a]	icELISA ($\mu g\,L^{-1}$)	Proposed method ($\mu g\,L^{-1}$) mean ± SD[b]
I	0.37	0.45 ± 0.09
II	0.35	0.24 ± 0.07
III	2.5	2.80 ± 0.18

[a]Samples I and II were from Fu Baowan of Dianchi Lake on 26 April 2005, and 23 April 2006, respectively and sample III was from an experimental pool named Guangqiao (Wuhan, China) on 10 June 2006.
[b]Standard deviation calculated from the average of triplicate analyses for each sample.

Table 5. Concentrations of MCs determined by icELISA and the proposed method in the fish tissue samples.

Sample code	Dilution of SPE extracts	icELISA ($\mu g\,L^{-1}$)	Proposed method ($\mu g\,L^{-1}$) mean ± SD[b]
1	1 : 100	1.68	2.19 ± 0.14
2	1 : 100	1	1.67 ± 0.04
3	1 : 1	2.5	0.60 ± 0.04
4	1 : 1	2.44	0.46 ± 0.03

[a]Samples I and II were liver tissues, and samples 3 and 4 were muscle tissues; MCs extracted from the samples were dissolved in a 1-mL aliquot of ultrapure water, and SPE extracts were measurably diluted to perform the MCs analysis.
[b]Data are mean ± SD from triplicate analyses for each sample.

4. Conclusions

The present work demonstrated that a novel chemiluminescent immunoassay was developed for the detection of MCs. The performance of the immunoassay in terms of sensitivity and linear range was improved due to the employment of gold nanoparticles and the CL detection method. In this work, a wide linear range (0.05–10 $\mu g\,L^{-1}$) with an LOD of 0.024 $\mu g\,L^{-1}$ was obtained by the new method, which is more sensitive than conventional icELISA. The recovery for water and fish tissues was investigated, and, as expected, a satisfactory recovery was obtained for water samples (90–103%), but not for fish tissues (58–76%), mainly due to the extraction procedure. The new method was applied to MC analyses in natural samples, and there was a reasonable agreement between the new method and conventional icELISA. These results suggest that the new method was suitable for MC detection in natural samples, and an alternative for conventional ELSA for MCs detection in natural samples.

Acknowledgements

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