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Short communication

Detection of microcystins in environmental samples using surface plasmon resonance biosensor

Chenlin Hu[a,b], Nanqin Gan[a], Yuanyuan Chen[c], Lijun Bi[c], Xianen Zhang[c], Lirong Song[a,*]

[a] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Donghu Nanlu 7, Wuchang, Wuhan 430072, PR China
[b] Graduate School of Chinese Academy of Sciences, Beijing 100049, PR China
[c] Analytical Biotechnology of State Key Laboratory of Macromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, PR China

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ABSTRACT

An indirect inhibitive surface plasmon resonance (SPR) immunoassay was developed for the microcystins (MCs) detection. The bioconjugate of MC-LR and bovine serum albumin (BSA) was immobilized on a CM5 sensor chip. A serial premixture of MC-LR standards (or samples) and monoclonal antibody (mAb) were injected over the functional sensor surface, and the subsequent specific immunoreaction was monitored on the BIAcore 3000 biosensor and generated a signal with an increasing intensity in response to the decreasing MCs concentration. The developed SPR immunoassay has a wide quantitative range in 1–100 $\mu g\,L^{-1}$. Although not as sensitive as conventional enzyme-linked immunosorbent assay (ELISA), the SPR biosensor offered unique advantages: (1) the sensor chip could be reusable without any significant loss in its binding activity after 50 assay-regeneration cycles, (2) one single assay could be accomplished in 50 min (including 30-min preincubation and 20-min BIAcore analysis), and (3) this method did not require multiple steps. The SPR biosensor was also used to detect MCs in environmental samples, and the results compared well with those obtained by ELISA. We conclude that the SPR biosensor offers outstanding advantages for the MCs detection and may be further developed as a field-portable sensor for real-time monitoring of MCs on site in the near future.

1. Introduction

Microcystins (MCs) are a family of cyclic heptapeptides produced mainly by some freshwater cyanobacteria such as *Microcystis*, and are hazardous to humans and animals due to their hepatotoxicity and tumor-promoting activity [1,2]. Among the MCs variants, MC-LR is the most common and well investigated. To reduce the adverse effects on humans and animals, World Health Organization (WHO) has established a maximum permissible concentration of 1 $\mu g\,L^{-1}$ of MC-LR in drinking water [3]. It is therefore necessary to develop a rapid and reliable method for MCs detection in environmental monitoring. Recently, a series of biochemical and physicochemical methods have been developed for MCs detection such as protein phosphatase inhibition assay (PPiA), enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC) and liquid chromatography with mass spectrometry (LC/MS) [4]. However, these methods require either complex operation or expensive materials and devices. To circumvent these drawbacks, several research groups have made the attempts to develop biosensors for MCs detection, such as molecularly imprinted polymer (MIP)-based sensor [5], electrochemical immunosensors [6–9], and evanescent wave all-fiber immunosensors [10,11]. There have been the reports that described the application of test strips for rapid MCs analysis *in situ* [12,13]; however, it is still challenging to develop a simple, rapid, and sensitive method for MCs analysis.

Recently, the optical biosensor based on the surface plasmon resonance (SPR) has received more and more attention because of its own outstanding advantages: allowing rapid and real-time analysis, owning reusable sensor chip, and requiring low amounts of reagents without chemical labels. And it has been successfully and widely employed to detect small molecules of biomedical, food and environmental interest [14]. In this paper, we developed an indirect inhibitive SPR immunoassay for the MCs detection. The sensor chip was firstly functionalized by the amine-coupling method, and then the MCs detection was performed with indirectly measuring the inhibition by MCs in samples. The regeneration of the sensor chip was investigated. Moreover, the SPR biosensor was also used to detect MCs in environmental samples, the results were discussed and compared with the conventional ELISA.

* Corresponding author. Tel.: +86 27 68780806 fax: +86 27 68780871.
E-mail address: lrsong@ihb.ac.cn (L. Song).

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2. Experimental

2.1. Materials and sample preparation

MC-LR with >95% purity (HPLC-grade), the (MC-LR)–BSA bioconjugate, and anti-(MC-LR) monoclonal antibody (mAb) were prepared in our laboratory, the cross-reactivity of mAb with MCs variants has been tested in our previous work [15]. *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), ethanolamine, and Tween-20 were purchased from BIAcore (Amersham Pharmacia Biotech, Uppsala, Sweden). All other chemicals used in the experiment were of analytical grade. All the solutions were prepared using deionized water (18.2 MΩ cm), which was obtained from a Milli-Q System (Millipore, Bedford, MA).

Natural water samples and fish tissue extractions were collected, pretreated, and extracted according to our previous procedure [16].

2.2. Instrumentation

The SPR immunoassay was performed with a BIAcore™ 3000 instrument (BIAcore, Uppsala, Sweden). The molecular weight (MW) of BSA and (MC-LR)–BSA were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) instrument (Bruker Daltonic Reflex™ III mass spectrometer, Bruker Doltonic, Bremen, Germany).

2.3. Immobilization protocol

Before the immobilization, the molecular weight (MW) of BSA and (MC-LR)–BSA were determined to be 66,354 and 68,708, respectively. The difference of MW between BSA and (MC-LR)–BSA (2354) indicated that the molar ratio of MC-LR to carrier protein BSA was 2.4 in this work, which was suitable for further immobilization. Then the CM5 sensor chip was functionalized by the EDC–NHS amine coupling as follows: the sensor chip was activated twice by mixing equal volumes of NHS (0.1 M) and EDC (0.4 M) and injecting the mixture over the sensor chip for 10 min at a flow rate of 10 μL min^{-1}. (MC-LR)–BSA (100 μg mL^{-1}) was dissolved in sodium acetate buffer (10 mM, pH 4.3) and injected over the sensor chip surface at a flow rate of 5 μL min^{-1}. NaOH (25 mM) was injected to wash the surface, and capping of the unreacted sites was achieved by injection of ethanolamine (1 M, pH 8.0) for 7 min.

2.4. SPR immunoassay and indirect competitive ELISA (ic ELISA)

The SPR immunoassay was performed in the indirect inhibitive immunoassay format. MC-LR standards or samples (40 μL) were incubated with an equal volume of mAb for 30 min at room temperature. The equilibrated mixture was then injected over the functional surface at a flow rate of 5 μL min^{-1}; the remaining free mAb was captured by (MC-LR)–BSA on the surface. Real-time monitoring will be displayed in a sensorgram as the optical response (RU) versus time (in seconds). To convert the response back to baseline, the sensor chip was regenerated by injecting NaOH (40 mM) at a flow rate of 20 μL min^{-1}. The next sample was analyzed at random. A typical sensorgram for the real-time detection of MCs could be seen in Fig. 1.

To evaluate the reliability of the SPR immunoassay, MCs in the same samples were detected by the indirect competitive ELISA (ic ELISA) and the SPR immunoassay. The ic ELISA was performed according to our previous report [16].

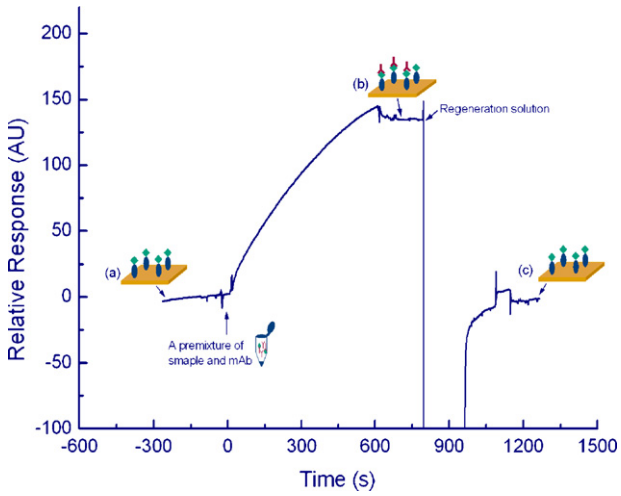

Fig. 1. The representative SPR response in real-time detection: PBS buffer was injected over the sensor chip surface immobilized with (MC-LR)–BSA, and a baseline of the SPR response was obtained (a); a premixture of samples and mAb was injected over the functional sensor chip for 10 min, and free mAb was captured by MC-LR on the sensor chip surface; the corresponding response was recorded (b); and the captured antibodies were removed using NaOH solution (40 mM) for the regeneration, the response returned to the baseline (c).

3. Results and discussion

3.1. The (MC-LR)–BSA bioconjugate immobilization

In the inhibitive SPR immunoassay, it is crucial to obtain a high bioconjugate immobilization level (amount), or rather a high hapten immobilization level because high amount of hapten generally means a potent capacity of antibody binding for the surface. The optimal immobilization level in this work was obtained through the preconcentration assay, as a result, a nearly saturated coverage level (approx. 9000 RU) was observed by using (MC-LR)–BSA solution (100 μg mL^{-1}) in 10 mM sodium acetate (pH 4.3). Subsequently, the capacity of mAb binding at the 9000 RU-immobilization level was further checked by injecting the mAb solution (15 μg mL^{-1}) over the functional surface, yielding a 218 RU-capacity of mAb binding, similar to the results in the other developed inhibitive immunoassays: 80 RU- and 300 RU-capacities of the antibody binding were generated at the same high bioconjugate immobilization level (10,000 RU) [17,18]. Actually, in a certain immunoassay, the capacity value of antibody binding on the surface should be evaluated rationally based on the used antibody affinity, working concentration, and especially, the effective hapten immobilization level. The surface with the high immobilization level did not necessarily yield a high capacity value of antibody binding. To be exact, only the high immobilization simultaneous with high molar ratio of hapten to carrier protein could contribute to the high surface antibody-binding capacity. Miura and co-workers previously reported the relative high capacity of antibody binding by using HBP–BSA bioconjugate with high molar ratio of HBP to BSA (n = approx. 54) [19], 22.5-fold more than that in this work (the molar ratio of MC-LR to BSA in this work, n = 2.4).

3.2. Detection of MCs using SPR biosensor and regeneration efficiency

According to the indirect inhibitive immunoassay format, MC-LR (0–100 μg L^{-1}) was incubated with mAb for 30 min; the mixture was then injected over the functional sensor chip. As observed in Fig. 2A, the binding response was inversely proportional to MC-LR concentration, which further supported the results obtained

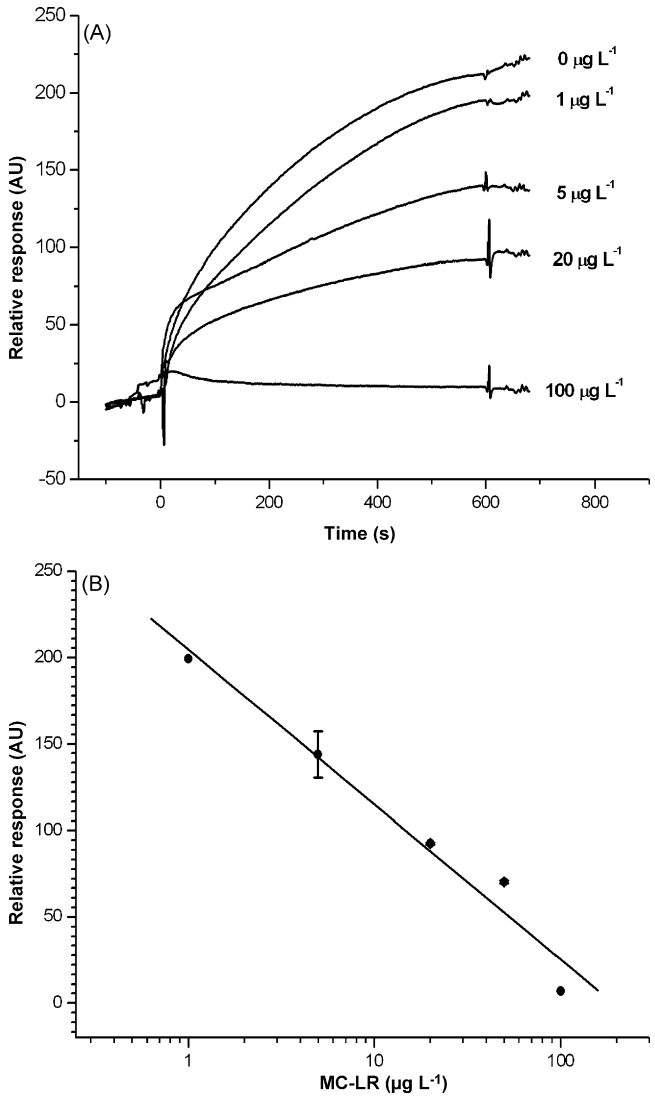

Fig. 2. (A) Sensorgram generated by injecting the solution mixture of monoclonal antibody and MC-LR at various concentrations (0, 1, 5, 20, and 100 μg L^{-1}) on the functional CM5 sensor chip and (B) standard curve for the detection of MCs by the SPR biosensor.

using the indirect inhibitive format. On the basis of these results, a dose–response curve was generated (Fig. 2B). The results indicated a good linearity (r^2 = 0.9667) in the wide quantitative range of 1–100 μg L^{-1}. And it has reached the permissive MCs concentration in drinking water (1 μg L^{-1}) by WHO [3]. In our previous work, sensitive time-resolved fluorescence (TRF) and chemiluminescence (CL) techniques were employed to improve the assay sensitivity, and exhibited the wide quantitative range of 0.01–10 and 0.05–10 μg L^{-1}, respectively [15,16], superior to the present SPR immunosensor sensitivity. However, the SPR immunosensor offered several important advantages over the above immunoassays: (1) compared to the ELISA, TRFIA, and CL immunoassays in our previous report, the SPR immunoassay could be rapidly accomplished in 50 min, whereas the immunoassays (ELISA, TRFIA, and CL immunoassays) required 2 h or more, (2) the entire SPR assay analysis is simple without any labeling and cumbersome washing that are indispensable for the above immunoassays, and (3) importantly the SPR sensor chip could be regenerated.

Furthermore, a comparison of the other biosensors for MCs detection is summarized in Table 1, in contrast to the other techniques such as the electrochemical and evanescent wave all-fiber biosensors for MCs detection [7,9,11–13], the present SPR immunosensor was not as highly sensitive as the biosensors above, which may be due to the different antibodies, assay strategies, and technique platforms. However, there is still further scope of the SPR immunoassay sensitivity improvement since antibody-amplification strategies have been successfully applied in other biochemical molecules analyses [21,22]. As an important parameter, the BIAcore analysis time could be 20 min, moreover, the whole analysis time including the preincubation and BIAcore analysis time was approximately 50 min, and could be also competitive with or better than other techniques in Table 1.

3.3. Regeneration efficiency

After one sample analysis, the antigen–antibody complex on the surface should be dissociated using a regeneration solution for the next analysis. In the present study, NaOH (40 mM) was found to be suitable for regeneration (Fig. 1). After 50 assay-regeneration cycles, the capacity of mAb binding for the functional chip was determined to be 179 RU compared with 218 RU-capacity of the original mAb binding, indicating that decrease in the binding capacity of the functional CM5 sensor chip was less than 20% of the initial binding capacity, which was an acceptable loss of binding capacity according to the validation parameters for the SPR biosensor [20].

3.4. Application of SPR immunoassay for environmental samples

In the present study, the reliability of the SPR immunoassay was evaluated by the conventional ELISA. The results are summarized in Table 2 [maximum relative standard deviation (RSD) for ELISA in the present study, 6.6%]. The results obtained by the biosensor and ELISA were in fairly good agreement, supporting the feasibility of the application of the SPR biosensor to detect

Table 1
Comparison of the biosensors and strips developed for the detection of MC-LR.

Analytical technique of the biosensor	Assay format	The working range (μg L^{-1})	The whole analysis time[a]	Reference
Piezoelectric sensor	Molecularly imprinted polymer-based specific recognition	NA[b]	Approx. 25 min	Chiallena et al. [5]
Electrochemical immunosensor	Protein phosphatase-based inhibition	NA[b]	NA[b]	Campàs and Marty [6]
Electrochemical immunosensor	Competitive immunoassay	10^{-4}–10^{2} (mAb); 10^{-1}–10^{2} (pAb)	Approx. 2 h 30 min	Campàs et al. [7]
Electrochemical immunosensor	Enzyme inhibition	37–188	Approx. 50 min	Campàs et al. [8]
Capacitive immunosensor	Immunoassay based on mAb	10^{-5}–1	Approx. 38 min	Loyprasert et al. [9]
Evanescent wave immunosensor	Indirect competitive immunoassay	NA[b]	Approx. 40 min	Long et al. [10]
Evanescent wave immunosensor	Indirect competitive immunoassay	0.1–10.1	Approx. 40 min	Long et al. [11]
Fluorescent immunochromatographic assay	Competitive immunoassay	0.125–2	Approx. 15 min	Kim et al. [12]
Electronical enzyme immunoassay strip	Competitive immunoassay	0.01–3.16	10 min 30 s	Zhang et al. [13]
SPR immunosensor	Indirect competitive immunoassay	1–100	Approx. 50 min	This work

[a] The whole analysis time was evaluated from each experiment section in the literatures.
[b] NA, not available.

Table 2
Microcystins determined by SPR biosensor and ic ELISA in environmental samples.

Sample code[a]	SPR biosensor ($\mu g\,L^{-1}$), mean ± SD[b]	ic ELISA ($\mu g\,L^{-1}$)
1	<1	0.49
2	<1	1.12
3	1.8 ± 0.3	1.66
4	2.4 ± 0.2	2.70
5	2.8 ± 0.1	2.50
6	14.5 ± 1.2	10.17

[a] Samples 1–3 were water samples, the pretreatment of samples was seen in Section 2. Samples 4–6 were fish tissues; MCs extracted from the samples were dissolved in 1 mL aliquot of ultrapure water; SPE extracts were diluted to perform the MCs analysis (if necessary).

[b] Data were mean ± SD from triplicate analyses for each sample.

MCs in environmental samples. Therefore, the SPR biosensor could be reliable and practical for the MCs analysis in environmental samples.

4. Conclusion

We have developed an inhibitive SPR immunoassay for MCs detection in environmental samples. The immunosensor chip was functionalized with (MC-LR)–BSA by amine coupling. Using this chip, a wide linear range ($1–100\,\mu g\,L^{-1}$) was achieved, which reached the permissive MCs concentration in drinking water ($1\,\mu g\,L^{-1}$) by WHO, meanwhile, only $40\,\mu L$ of samples were required and the whole assay can be analyzed in 50 min. Additionally, the functional sensor chip was favorable as its binding ability was not significantly affected after 50 assay-regeneration cycles. The SPR biosensor was applied to environmental samples, the results compared well with those by the conventional ELISA. This work has demonstrated that SPR immunosensor is simple, rapid, and suitable for the detection of MCs in environmental samples. Meanwhile, since less expensive portable SPR devices become increasingly popular and useful, presently, our further effort may be made to miniaturize the SPR biosensor to monitor field samples *in situ* for MCs detection.

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