# Ultra-Sensitive Analysis of Microcystin LR Using Microchip Based Detection System

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For the detection of cyanobacterial toxin, an Enzyme-linked immunosorbent assay (ELISA) was integrated into a PDMS microchip. The conjugates of microcystin-LR (MCLR) and keyhole limpet hemocyanin (KLH) were adsorbed on the surface of polystyrene beads and these MCLR-KLH polystyrene beads were introduced into a microchamber. MCLR on the surface of polystyrene beads reacted with horseradish peroxides (HRP) conjugated anti-MCLR monoclonal antibody (mAb) which had a competitive reaction with MCLR in water sample. After the enzyme substrate 3,3,5,5-tetramethyl benzidine (TMB) was injected into the chamber and catalyzed by HRP, the color change was detected with a liquid-cord waveguide. This integration shortened the conventional ELISA analysis time from several hours to about 30 min with only 4.2 μL MCLR sample consuming which was useful for the environmental analysis. More over, troublesome operations required for ELISA could be replaced by simple operations. The microchip based detection system showed a good sensitivity of 0.05 μg/L and maintained good reliability through its quantitative range with low coefficients of variation (2.5-10.5%).

**Key Words :** Microchip, Microcystin, Sensitive analysis

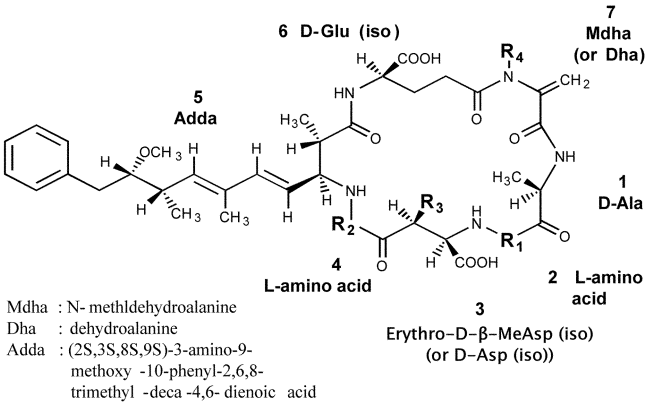
## Introduction

Algal blooms, in particular cyanobacterial blooms, are a major issue for water authorities, causing significant taste and odor problems. The knowledge that many of these blooms are toxic has changed the concern from a purely aesthetic problem to one that affects human health.

Over 20 different cyclic peptide hepatotoxins termed microcystins,[1] have been isolated from cyanobacteria (blue-green algae).[2,3] While *Microcystis* is the most studied genus, species in the genera *Anabaena*, *Nodularia*, *Nostoc*, and *Oscillatoria* also contain these toxins.[4,5,6] Microcystins are a family of cyclic polypeptides produced by different species of cyanobacteria (blue-green algae), which can form blooms in lakes and water reservoirs.[6] Their basic structure is a cyclic heptapeptide and their structural variations give rise to more than 50 types of microcystins known today (Figure 1).[6] The most extensively studied form is microcystin-LR that contains L-leucine and L-arginine in the two main variant positions. Toxic cyanobacterial blooms have been reported in many countries.[6,7] Toxic waterblooms cause the death of domestic animals and wildlife and human illness. Cyanobacterial toxins are toxic to zooplankton and fish[8] and can be accumulated in fish and aquatic animals.[9] The structures and functions of the toxins are classified into three groups, neurotoxin, hepatotoxin, and lipopolysaccharide. *Microcystis aeruginosa*, which is the most common toxin-producing cyanobacteria found worldwide, mainly produces microcystin LR, RR and YR.

Many studies showed that these microcystins and nodularin inhibit in vitro activity of protein phosphatase in a cytosolic fraction of mouse liver.[10,11] Liver is reported as the target organ that shows the greatest degree of histopathological change when animals are poisoned by these cyclic peptides. The cause of death in mice is at least partially known and is concluded to be hypovolemic shock caused by interstitial hemorrhage.[12]

There are many kinds of detection methods for micro-




| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | MW |
|---|---|---|---|---|---|
| Microcystin - LR | Leu | Arg | $CH_3$ | $CH_3$ | 994 |
| Microcystin - YR | Tyr | Arg | $CH_3$ | $CH_3$ | 1044 |
| Microcystin - RR | Arg | Arg | $CH_3$ | $CH_3$ | 1037 |

**Figure 1**. Structure of microcystins. A characterisitic of microcystins and related cyanobacterial toxins is the hydrophobic amino acid Adda which contains in position 5 two conjugated double bonds. Numbers represent the positions of the corresponding amino acid.

cystin, and among them the protein phosphatase inhibition assay is a sensitive one.[13] The detected amounts in raw and treated waters were estimated to be 0.12-0.87 and 0.09-0.18 μg/L, respectively.[14] Although this method has been widely used in research, there is reluctance in adopting if for the routine monitoring of microcystins because of the requirement to use radioactivity which necessitates speccialised laboratory equipment and regulations.

The Enzyme-Linked Immuno Sorbent Assay (ELISA) technique is currently the most promising method for rapid sample screening for microcystins because of its sensitivity, specificity and ease of operation. Using the monoclonal antibody produced by Nagata *et al.*,[15] a more sensitive competitive ELISA method has been developed by Ueno *et al.*[16] with detection limit of 0.05 μg/L for water samples. This method has been successfully applied to detect microcystins in drinking water in China.[14]

However, the conventional ELISA needs a long time and involved troublesome procedure and many expensive reagents. To overcome these drawbacks, integration to the immunoassay system seems to be effective. There have been some papers about microchip immunoassays, but most were based on separation of the free form and the complex of the antigen and the antibody by microchip-based capillary electrophoresis[17,18,19] there have been very few reports in which the antigen-antibody reaction was performed on a microchip. A heterogeneous immunosorbent assay is used widely and is superior to liquid-phase separation because of its easy and clear separation of the free form and the complex. Integration of the heterogeneous immunoassay system, which is based on the same principle as the conventional immunosorbent assay, has only been reported by Kitamori *et al.*[20,21] But they used the more complex glass microchip instead of our convenient, reproducible and ease-making PDMS microchip whose advantages have been reported previously.[22,23,24] At the same time, we believe it is the first time to detect the microcystin-LR using ELISA integration PDMS microchip.

For integrated analytical system, a detection method with high sensitivity in microspace is indispensable. Liquid core waveguide (LCW), which is coated with Teflon® AF2400 (Dupont), can provide a long optical path length by total internal reflection to constrain the light propagation within a liquid medium (water, n = 1.33) that has a higher refractive index than the cladding (n = 1.29) of the waveguide. For the modern analysis, the small volume for analysis is always required for in-field usage. The microchip application can approach this, getting the determination volume down to micro liter scale. Here, we connected the LCW with PDMS microchip to obtain our high sensitivity and low sample volume analysis.

In this paper, we report the integration of an ELISA into a PDMS microchip using the liquid-cord waveguide as a detector. Microcystin-LR, the most frequently found cyanobacterial toxin in aqueous environment, was assayed with this system.

## Experimental Section

**Intrgration of ELISA and Microchip.** Schematic illustrations of our enzyme-linked immunoassay in a PDMS microchip for the detection of microcystin-LR (MCLR) are shown in Figure 2. MCLR-KLH polystyrene microparticles (diameter, 56 $\mu$m) were introduced into the microchamber and selected as the reaction solid phase. Different concentrations of MCLR sample were mixed with anti-MCLR-mAb-HRP beforehand for the antigen-antibody conjugation. After the conjugation, the mixture was injected into the chamber and the left free anti-MCLR-mAb-HRP would still have the chance to react with the MCLR antigens onto the surface of the polystyrene beads. The lower concentration of MCLR sample, the more free anti-MCLR-mAb-HRP could attach onto the surface of polystyrene beads also *via* antigen-antibody conjugation. Following washing step, only the polystyrene beads still stayed into the chamber due to the structure design; then HPR complex would catalyze the introduced substrate (TMB), and the color change from transparent to blue was detected by liquid core waveguide. For low concentration of MCLR sample, the absorbance signal would theoretically higher than that of the high concentration one, which was good for the real samples detection for their low concentration in nature.

**Microchip Fabrication.** The chip was composed of three parts (Figure 3), the transparent PDMS microchip (30 mm × 20 mm) acted as the reaction chip, the frit and the black PDMS microchip (40 mm × 20 mm) with 5 cm length of optical tubing (OD: 508 μm, ID: 254 μm), which worked as the detection chip; the thickness of both chip were about 4 mm and the height of microstructures was 100 μm. We fabricated two chips respectively as reported previously.[19-21]

A PDMS microchip with polystyrene beads is shown in Figure 3(c). In this chip, all reactions and washing

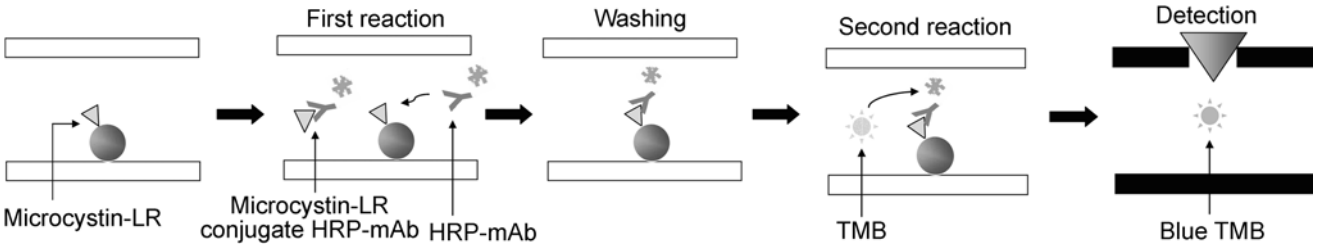



**Figure 2**. Schematic illustrations of PDMS microchip-based ELISA for the detection of microcystin-LR.

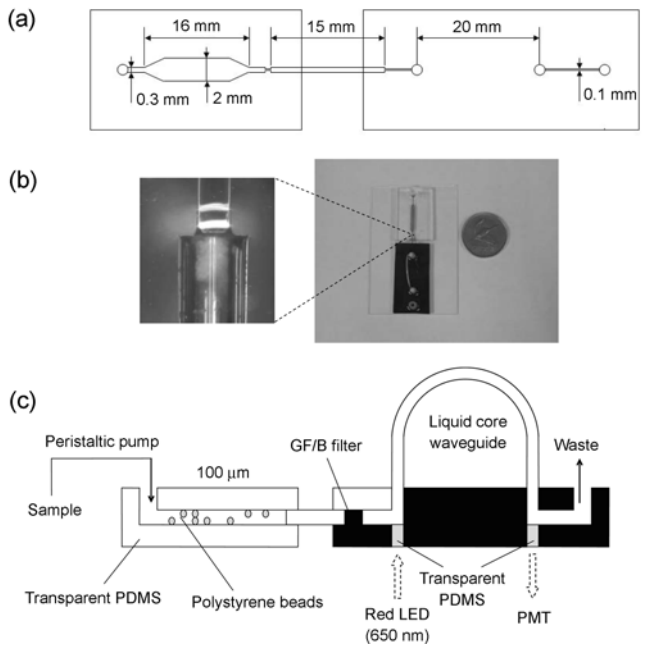



**Figure 3**. PDMS microchip for enzyme-linked immunosorbent assay. (a) Dimension (b) Photo (c) Schematic diagram.

operations were performed by injection of solution through the inlet hole using microsyringe pump or peristaltic pump. Each reaction or washing was achieved simply by controlling both the pumps. The required operations were much easier than the liquid handling operations in the conventional assay, that is, many pipetting and liquid discarding steps. Pump control was very simple and caused relatively small variation in operations. We concluded that this integrated system provided simple operations with relatively small error than manual handing.

The frit was made by filling 1 cm length of capillary (OD: 366 μm, ID: 248 μm) with about 0.5 mm length of GF/B filter, which can retain the microparticles down to 0.1 μm. Then the frit was inserted into the microchannel to connect the reaction chamber and detection chip. Because the width of microfluidic channel was only 100 μm and smaller than the ID of capillary, the filling filter would stay in the capillary and avoid the leakage of 56 μm polystyrene beads from chamber.

**General Reagents.** Phosphate buffer (PB; 0.01 M, pH 7.4 at 25 ºC) was made from phosphate buffered saline tablet (Sigma Chemical Company, USA) and deionized water. 56 μm MCLR-KLH-Polystyrene beads solution, BSA solution and 3,3,5,5-tetramethyl benzidine (TMB) solution were prepared in the laboratory.

**Antigen and Antibody-HRP Conjugate.** The MCLR antigen and HRP conjugate monoclonal antibody were also prepared in the laboratory. The production procedure of the monoclonal antibody of MCLR has already been reported.[25]

**Apparatus.** In the construction of homemade detection system, the light source was red LED (650 nm, U-JIN LED CO., LTD. Korea). Photo multiplier tube H5784-04 (Hamamatsu, Japan) was used as the sensitive light detector. All the solutions were pumped into the system by 4 channel peristaltic pump (Ismatec, Switzerland). The PMT data acquisition system was Autochro Data Module (Younglin Instrument, Korea). For antigen-antibody reaction, we used infuse-only dual syringe pump (Harvard Apparatus Model, USA), syringe (Norm-Ject, 1 mL) and connect tubing (TYGON® S-54-HL Microbore Tubing).

**Analytical Procedure.** To maintain a stable pH value, the chip was washed with PBS buffer by the syringe pump (1.0 mL/hour) for 3 min before use. 25 μL MCLR sample was mixed with 5 μL anti-MCLR-anti-MCLR-mAb-HRP for 10 min reaction. At the same time, 5 μL polystyrene beads were injected into the chamber by syringe pump (1.0 mL/hour), and then 5 μL antigen-antibody mixture was also introduced into the chamber. After 10 min reaction, all of the solutions were washed away with PBS buffer by syringe pump (1.0 mL/hour) for 10 min. In the following diction, the continuous flow of PBS buffer was regarded as the detection background, and then 5 μL TMB was pushed by peristaltic pump (5.0 μL/min) into the chamber for 3 min catalytic reaction. Finally the absorbance signal was obtained with data acquisition software.

## Results and Discussion

**Rapid and Simple PDMS Microchip-Based ELISA.** With regard to the operation time, although the competitive reaction between MCLR sample and anti-MCLR-mAb-HRP happened immediately, but we always waited for 10 min to make sure the competition was completely. For the conjugation in the microchamber, it also needed only 10 min, and then the reaction chip took only 10 min including the washing step. The detection procedure was also easy to operate, if everything goes well, it will take less than 15 min to get the ideal absorbance signal. So the totally analysis time, compared with the conventional ELISA using the microtiter plate which would take up at least 3 hours, was greatly shortened. This effect seemed to be brought about by a reduction of the diffusion distance and an increase in the specific interface due to the spherical surface of solid phase,[20,21] so that antibody had more chance to react with plentiful antigens attached onto it.

**Small Sample Consuming.** In the conventional ELISA, because of performing on the plate, the precious reagent consuming is a big problem. For the microcystin detection, since algal growth depends on environmental factors, particularly on temperature and sunshine, and at the same time, it is not easy to extract the MCLR from the variants of microcystin, the small sample consuming is necessary and economical. In fact, in our experiment we at first mixed 25 μL MCLR sample with 5 μL HRP-mAb and then used only 5 μL of them; it meant that we only need 4.2 μL (5 μL × 5/6) of the MCLR sample. It is great advantage in the environmental analysis, and we believe that it may be the least MCLR sample consuming which has never been reported before.

**Sensitivity.** The MCLR sample determination was

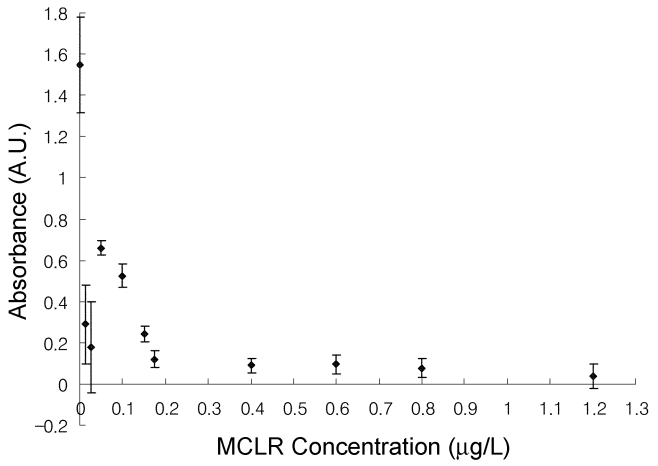



**Figure 4**. Calibration curve for MCLR with our PDMS microchip-based ELISA. Each point on the graph represented the mean values and error bars represented standard deviation values of three independent experiments.

performed with this PDMS microchip-based ELISA system. Figure 4 indicated that the calibration curve we got correlated properly with the conventional ELISA ones. Each point in Figure 4 represented the mean values and error bars represented standard deviation values of three independent experiments. In Figure 4, we could observe that all data points above 0.05 μg/L have small standard deviation, however below 0.05 μg/L, big error bars showed up and the average values also were off the trend. These results demonstrated that the sensitivity of our new method for the detection of microcystin was 0.05 μg/L (50 ppt), much higher than the standard of WHO in drinking water (MCLR < 1 ppb).[14] By adopting a sandwich immunoassay system, or by using antibody-coated beads, small amount of antigen molecules could be captured effectively and selectively and then detected with high sensitivity.

In our integrated PDMS microchip, we optimized the operations step with the pumps, which not only took the place of labor intensive and complicated liquid handing procedures such as washing and solution removal procedures, but the more important is to make sure the accuracy of the data. At the same time, the small sample consumption due to the integrated assay is meaningful in the application. With this sensitive detection system, we achieved the ideal detection limit of MCLR (0.05 μg/L), which is the same as the lowest one in the reported data.[14] Although the system still has many disadvantages, such as at present only suitable for standard MCLR solution and time-consuming chip fabrication, we believe that it is possible to develop portable, minimized, and convenient instrumentation based on our system for the real detection of microcystin in water samples. Since our integrated PDMS microchip detection requires so small sample volume and its quantitative range is within microcystin concentrations in water, it shows a big potential for routine use in the monitoring of microcystins in drinking water supplies.

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