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An aptamer-based immunoassay in microchannels of a portable analyzer for detection of microcystin-leucine-arginine

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ABSTRACT

The rapid detection of microcystin-leucine-arginine (MC-LR), the most highly toxic among MCs, is significantly important to environmental and human health protection and prevention of MC-LR from being used as a bioweapon. Although aptamers offer higher affinity, specificity, and stability with MC-LR than antibodies in the immunodetection of MC-LR due to steric hindrance between two antibodies and limited epitopes of MC-LR for use in a sandwich immunoassay, no sandwich immunoassay using an aptmer has been developed for MC-LR detection. This study is aimed at developing an aptamer-antibody immunoassay (AAIA) to detect MC-LR using a portable analyzer. The aptamers were immobilized onto the glass surface of a microchamber to capture MC-LR. MC-LR and horseradish peroxidase (HRP)-labeled antibody were pulled into the microchamber to react with the immobilized aptamer. The chemiluminescence (CL) catalyzed by HRP was tested by a photodiode-based portable analyzer. MC-LR at 0.5–4.0 μg/L was detected quantitatively by the AAIA, with a CL signal sensitivity of 0.3 μg/L. The assay took less than 35 min for a single sample and demonstrated a high specificity, detecting only MC-LR, but not MC-LA, MC-YR, or nodularin-R. The recovery of two spiked real environmental samples calculated as 94.5–112.7%. Therefore, this AAIA was proved to be a rapid and simple method to detect MC-LR in the field by a single analyst.

1. Introduction

Cyanobacteria hepatotoxins-microcystins (MCs), a group of 800- to 1000-Da cyclic peptides with five non-protein and two protein amino acids, is mainly produced by *Microcystis aeruginosa* [1]. The widespread bloom-forming microcystis [2] is an increasing concern in regard to water contamination worldwide [3] due to the eutrophication of water and global warming [4]. MCs have been recognized as a contributory factor to human health problems [5–7] and a serious threat in biological warfare [8].

Microcystin-leucine-arginine (MC-LR), the most common and most toxic MC, contains protein amino acids leucine and arginine [9] shown in Fig. 1. Acute poisoning by MC-LR can cause skin irritation, vomiting, diarrhea, and functional and structural disturbances and damage to the liver [10]. Even a low level of MC-LR in the human body long-term causes primary liver cancer [11]. Poisoning with MC-LR and other MCs can occur through drinking or having skin contact with contaminated water [12] and consuming poisoned aquatic or agricultural products [12,13]. More importantly, MC-LR will pose a serious threat if it is used as a biological warfare agent [8]. Recent studies have demonstrated that even trace amounts of MCs (1 μg/L, 1.0×10^{-9} mol/L) in water significantly interrupt cellular process [14], and the World Health Organization (WHO) recommends a limit of 1.0 μg/L MC-LR in drinking water [15]. Therefore, an effective method for detecting

Abbreviations: AAIA, aptamer-antibody immunoassay; HRP, horseradish peroxidase; APTES, 3-Aminopropyl triethoxysilane; MCs, microcystins; CL, chemiluminescence; MC-LR, microcystin-leucine-arginine; ELISA, sandwich enzyme-linked immunosorbent assay; RLU, relative light units

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MC-LR is extremely important to prevent MCs from being used as bioweapons and to environmental and human health protection.

Although some methods can be used to detect MCs in water, including high-performance liquid chromatography and mass spectrometry [16] for known or novel MCs in the laboratory [17], it has not been reported a simple and rapid portable analyzer for MC-LR detection in the field [18–20]. Recently, some miniature immunoassays for MCs [21,22] based on the binding affinity between antibody and antigen [23] such as immusensors [24] have been developed for compact and portable analysis [25]. However, the selection of a proper antibody/antigen complex and the stability of antibody binding to MCs have prevented effective application of these immunoassays, because there are only a limited number of specific antigenic epitopes for MCs. Furthermore, the steric hindrance of MCs poses a difficulty in developing a sandwich enzyme-linked immunosorbent assay (ELISA) to capture and detect the small MC cyclic peptides. Thus, although many competitive immunoassays using a single antibody have been used for MC-LR detection [26–29], the large biomolecules such horseradish peroxidase (HRP) used to label antibodies to MC-LR may interfere with the affinity and specificity to the small size targets. In addition, antibodies to MC-LR, such as MC8C10, exhibit lower cross-reactivity with other four-arginine MCs but a high cross-reactivity with nodularin-arginine [30].

An aptamer is a small ssDNA/RNA that recognizes various targets molecules, including cells, proteins, peptides, and amino acids [31]. Compared to antibodies, aptamers offer higher affinity, specificity and stability with targets under a variety of conditions and thus conjugate with some biological molecules more easily [32]. Recently, RNA [32] or ssDNA [33,34] aptamers for the detection of MC-LR, such as RC4, RC6, RC22, and HC1, have shown a similar weaker or increased affinity to other MCs [33]. These aptamers have two or more selectivities for binding at positions 2 and 4 to MCs congeners' seven-cyclic peptide structure. Therefore, aptamers are advantageous over antibodies for immunodetection of MC-LR.

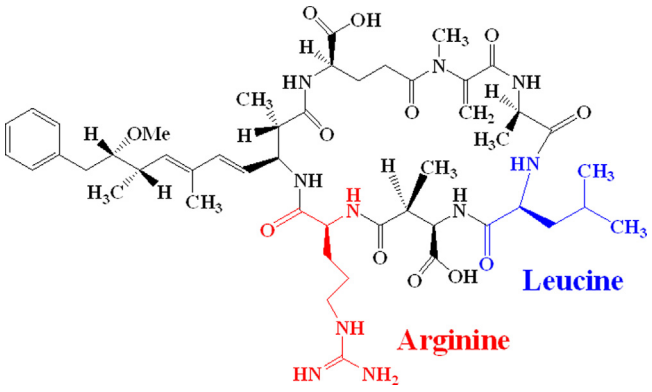

Fig. 1. Chemical structure of microcystin-LR (MC-LR). The two specific L-amino acids of MC-LRare represented with Blue (leucine, L) and Red (arginine, R).

In this study, we attempted to establish a sandwich ELISA using an aptamer to recognize distinct epitopes of MC-LR.

2. Materials and methods

2.1. Materials and reagents

Monoclonal antibodies to microcystin-LR (MC8C10), microcystins (MC-LR, -YR, and -LA), and nodularin-R were purchased from Beijing Puhuashi Technology Development Co., Ltd (Beijing, China). Super signal ELISA femto maximum sensitivity luminol substrates were obtained from Thermo Scientific (Pierce, Rockford, IL, USA). The HOOK HRP PLUS labeling kit for tagging monoclonal antibodies to MC-LR with HRP was purchased from Sangon (Shanghai, China). 3-Aminopropyl triethoxysilane (APTES), glutaraldehyde, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St Louis, MO, USA). Polythene tubing was obtained from Shenzhen Woer Heat-shrinkable material co., LTD (Shenzhen, China). Glass capillaries (length: 12.0 cm; inner diameter: 1.5 mm) were obtained from Sichuan University, West China Center of Medical Sciences (Chengdu, China). All other reagents were analytical grade, and all solutions were prepared with Ultra-pure water (resistance > 18.2 MΩ cm, Milli-Q, Billerica, MA, USA).

2.2. Aptamer preparation

To obtain an appropriate aptamer that can bind MC-LR with antibody, several ssDNA aptamers specific to MC-LR [35,36] (Table 1) were immobilized onto glass capillary surfaces with $-NH_2$ groups, and a linker $(-CH_2-)_6$ was added between the amino group and the aptamer. Aptamer buffer solution consisted of 50 mM Tris bases, pH 7.5, 75 mM NaCl, and 10 mM $MgCl_2$. The ssDNA aptamer sequences were synthesized by Sangon (Shanghai, China). The secondary structure of the ssDNA aptamers sequences were predicted using the Mfold Web Server [37] at web site: http://www.bioinfo.rpi.edu/ applications/mfold under true reaction conditions at 25 °C, 15.0 mmol/L $[Na^+]$, and 2 mmol/L $[Mg^{++}]$. The estimation of folding free energy change (ΔG) presents the trends of the predicted secondary structures.

2.3. Conjugation of aptamer to glass microchannels

The ssDNA aptamers were grafted onto the surface of glass capillaries through an amidation reaction in the following steps. The glass capillaries were first treated with 5% HNO_3 to remove organic contamination and to allow metal ions to desorb under ultrasonic treatment for 2 h and washed with triple distilled water. The glass capillaries were dipped into 4 mol/L NaOH solution in 95% ethanol to obtain a sufficient amount of SiOH groups under ultrasonic treatment at 60 °C for 2 h and treated at 200 °C for 2 h to remove the physically adsorbed water to expose the SiOH groups. Then the glass capillaries were soaked in a mixture of 25 ml methylbenzene and 5 ml APTES at 60 °C for 2 h and washed with methanol to remove unreacted APTES. The capillaries were

Table 1
Sequences and modification of ssDNA aptamers used in this study.

Aptamer name	ssDNA Sequences and modification, 5′-3′
Ap1[34]	NH2-$(CH_2)_6$-TTTTTGGGTCCCGGGGTAGGGATGGGAGGTATGGAGGGGTCCTTGTTTCCCTCTTG
Ap2[33]	NH_2-$(CH_2)_6$-GGCGCCAAACAGGACCACCATGACAATTACCCATACCACCTCATTATGCCCCATCTCCGC
Ap3[33]	NH_2-$(CH_2)_6$-CACGCAACAACACAACATGCCCAGCGCCTGGAACATATCCTATGAGTTAGTCCGCCCACA
Ap4[33]	NH_2-$(CH_2)_6$-CACGCACAGAAGACACCTACAGGGCCAGATCACAATCGGTTAGTGAACTCGTACGGCGCG
Negative control	NH_2-$(CH_2)_6$-AAAGCGAAGTTTGAGAAGTAGCCGTAGGGATGCAAAGTGTCGTACTGGGTGATATCTCCG