Label-free aptamer-based detection of microcystin-LR using a microcantilever array biosensor

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**A R T I C L E   I N F O**

**A B S T R A C T**

Cyanotoxins, produced by cyanobacteria, are a series of widely present toxins frequently found in fresh water during algal blooms. They are extremely hazardous and persistent, which makes them a serious threat to human and animal health. In this paper, we developed an aptamer-based microcantilever array sensor to detect microcystin-leucine-arginine (MC-LR), one of the most concerned liver toxins. For this assay, an easily synthesized thiol-modified aptamer with specific recognition for MC-LR was used as a probe. The aptamer was covalently and directionally immobilized on the gold surface of a microcantilever by one-step immobilization via its thiol group, which simplified the conventional preparation of microcantilever array sensors. Interactions between the immobilized aptamer and MC-LR successfully changed the surface stress of the microcantilever, resulting in a bending conformation. The detection range was from 1 to 500 µg L⁻¹, and the cantilever deflection had a good linear relationship within the concentration range of 1–50 µg L⁻¹. Additionally, this sensor could identify MC-LR from other congeners. Thus, the aptamer-based microcantilever sensor operated in stress mode could achieve simple, rapid, real-time, label-free and quantitative detection of MC-LR, making it a convenient and economical approach for MC-LR detection. The aptamer-based microcantilever array sensor has great potential for detecting various cyanotoxins while new aptamers specific for cyanotoxins are available, which may be developed to monitor the environment and protect life health.

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1. Introduction

Microcystins are a group of bioactive cyclic heptapeptide compounds mainly produced by cyanobacteria, and are widespread cyanotoxins of concern. When water blooms occur, blue-green algae produce microcystins, which are later released into water upon cellular cracking and death [1]. Direct contact with water containing microcystins (e.g., swimming) may cause allergies and acute gastroenteritis. Microcystin is a serious hepatotoxin, which can strongly inhibit the protein phosphatase activity and lead to liver damage, bleeding, and necrosis [2]. Additionally, long periods of exposure to even very low concentration of microcystins have been reported to promote tumor formation. A study showed that the rate of liver cancer increases by 5–8 folds in residents who drink shallow water and river water containing low concentrations of microcystin as compared to those who drink deep well water [3]. Additionally, animals that are exposed to or drink water containing microcystins may suffer from diarrhea, vomit, polypnea and even death. Microcystins can be enriched through food chains [4] and are genotoxic [5]. The harmfulness of microcystin to water environment and human health has become one of the most pressing global environmental problems. There are more than 80 known microcystin congeners up to now, and most of them have a lethal dose, 50% (LD50) of about 50–600 µg kg⁻¹ [6]. Among these congeners, microcystin-leucine-arginine (MC-LR) is known to be the most common and toxic, making it a deep concern to researchers. The provisional upper limit for MC-LR recommended by World Health Organization (WHO) is 1 µg L⁻¹ in drinking water [7].

Due its great harm to human and animal health, it is extremely important to detect MC-LR in drinking water. Currently, there are some common methods [8] for MC-LR detection, including biological assay [9], high performance liquid chromatography (HPLC) [10], enzyme-linked immunosorbent assay (ELISA) [11] and protein phosphatase inhibition assay (PPIA) [12]. Although each of these techniques have advantages, they also encounter major limitations. For instance, biological assays may be used to monitor toxic symptoms but are very time-consuming, have sensitivity issues and are prone to false positives. HPLC is widespread, quantitative,
highly sensitive and may distinguish between microcystin congeners. However, it is expensive, time-consuming, and requires laboratory expertise and complicated sample pretreatment. ELISA and PPIA are sensitive, easy, rapid and effective detection methods but they cannot identify microcystin congeners and cannot be used for routine screening. Additionally, molecules need to be labeled for the ELISA technique, which is often difficult for small molecules and may reduce the sensitivity; in addition, the process to produce antibodies is complicated, and some antibodies are difficult to obtain, especially for small molecules and toxic targets [13]. Therefore, the development of simple, rapid, label-free methods to detect microcystins with high sensitivity is much needed.

Aptamers are synthetic single-stranded oligonucleotides, which are selected by SELEX (systematic evolution of ligands by exponential enrichment) [14,15]. As probe molecules, aptamers, also called chemical antibodies, have several advantages over existing technologies such as conventional protein antibodies. They can recognize and bind to a wide variety of specific targets with high affinity and specificity, including small molecules [16,17], proteins [18,19], cells [20] and microorganisms [21] and even toxic targets. Aptamers can be obtained by chemical synthesis and purification, and thus their use as probes is cost-effective and exhibits minimal differences in activity between different batches [22]. While protein antibodies are produced by animal immune systems, which cannot be controlled and can result in activity differences between different batches, aptamers have high stability and can be used under a wide variety of assay conditions. In addition, aptamers can be easily chemically modified to improve the stability, affinity and specificity. For instance, aptamers can be linked with a thiol group to be immobilized on a gold microcantilever surface, while the immobilization procedure of protein antibodies is much more complex and may reduce the activity of the antibody [23]. What’s more, the spatial structure of aptamers changes when they bind with targets, which have great potential to be applied in many kinds of biosensors [24].

Microcantilever sensing technology is a highly sensitive and label-free biochemical molecular analysis and nanomechanical sensing technology that is performed in real time and in situ. This technology has been applied in the detection of small molecules [25–28], proteins [29,30], genomics [31], and microbiology [32,33]. For a microcantilever sensor working in stress mode, the binding reaction, between the probe molecules and the target molecules on the microcantilever surface, changes the surface stress, which causes the bending of the microcantilever. When aptamers are immobilized on the cantilever surface, the cantilever can detect binding reactions between aptamers and their targets. Aptamer was first used as receptor molecules in the context of a microcantilever sensor by Savran et al. [34]. The combination of aptamers and microcantilever sensor has been widely used in recent years. This aptasensor has been mostly applied for medical treatment, like drug abuse monitoring [35–37], disease diagnosis [38,39], and etc. The aptamer-based sensor was reported with high sensitivity and selectivity, and a detection limit of μM–nM level. What’s more, the aptamer-functionalized microcantilever sensor can be regenerated by heating after sensing experiment and no change in sensitivity and selectivity in subsequent experiments was found [35].

The detection of microcystins by aptamers on the microcantilever sensor platform has not yet been studied. In this study, the MC-LR specific aptamer was immobilized on the cantilever via its thiol groups with one-step immobilization, and MC-LR was detected quantitatively on a homemade cantilever array sensor platform in stress mode. Microcantilever deflections generated by different concentrations of MC-LR were recorded in real time. Further, selectivity for MC congeners of this aptasensor was analyzed, and the utility of tap water samples was also tested.

2. Materials and methods

2.1. Materials and reagents

Microcystin-LR, –YR, –LA, and 6-mercaptop-1-hexanol (MCH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MC-LR aptamer [40] with the sequence of 5′-(SH)-(CH₂)₆-GGC GCC AAA CAG GAC CAC CAT GAC AAT TAC CCA TAC CAT ATT ATG CCC CAT CTC CGG-3′ was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). An aptamer that does not bind with MC-LR specifically [40] was used as reference, and the sequence is 5′-(SH)-(CH₂)₆-GCG GCC CGT AAA AGT AGG GCG ATT CAT AAG GTG ATA TCG GTG TAC TCG CGG-3′. All other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The binding buffer (pH = 7.5) contained 50 mM Tris-HCl, 150 mM NaCl and 2 mM MgCl₂. The microcantilever array (Micromotive GmbH, Mainz, Germany) used contained eight silicon cantilever beams (500 μm long, 90 μm wide and 1 μm thick). The topside of the microcantilever had a thin film of titanium (2 nm) covered with a 20 nm layer of gold.

2.2. Instrument

The detection experiments were carried out on a homemade microcantilever array sensor platform in stress mode (Fig. 1) [41]. Eight semiconductor lasers were used as the light sources for the microcantilever array, which were switched on and off by sequential control. The lasers were temperature controlled and had a stable output. The beams were exported from optical fibers which were coupled to the eight lasers respectively, and the other ends of the fibers were aligned and sealed in V-grooves manufactured from Si wafer to keep a same interval as the microcantilevers. The beams were focused on the tip of the microcantilevers by aspherical lens, and a quadrant photodiode detector was used to detect the deflection signals of the eight microcantilevers. A 200-μL liquid cell was used to mount the microcantilever array. A peltier element was used as a temperature controller with an accuracy of 0.02 °C under the bottom of liquid cell.

2.3. Microcantilever array functionalization

The modification procedures of microcantilever array were performed in microplate wells and a new well was used after each step. A new microcantilever array was immersed in piranha solution (V(98% H₂SO₄):V(30% H₂O₂) = 3:1, 200 μL) for 5 mins before use, rinsed four times with deionized water and dried under nitrogen gas. Then, the microcantilever array was immersed in 200 μL of 1 μM aptamer solution diluted in buffer for 3 h at room temperature. After that, the microcantilever array was washed with binding buffer three times, and backfilled by 1 mM MCH for 30 mins to strengthen the binding response [42]. Finally, the microcantilever array was immobilized with aptamers and MCH was washed with binding buffer three times and used in the experiment immediately. The reference aptamer was immobilized on a new microcantilever array with the same procedures.

2.4. Deflection measurement

The functionalized microcantilever array was placed into the liquid cell. The binding buffer was injected into the cell and all air bubbles were drained. A constant flow rate (1 μL·s⁻¹) was kept after the cell was filled with buffer. The temperature of the liquid cell was maintained at 25.00 ± 0.02 °C, and the ambient temperature was maintained at 25.0 ± 0.2 °C. The microcantilever array was equilibrated in flowing buffer. A quadrant photodiode detector was then used to measure the microcantilever array deflections. After stable...
signal curves of the microcantilever array were obtained, MC-LR samples at different concentrations were introduced into the liquid cell. The deflection data was recorded and shown in real time. A new cantilever array was used for each detection experiment.

3. Results and discussion

3.1. Detection of MC-LR by microcantilever array

To initiate MC-LR detection, we used a MC-LR specific DNA aptamer, which has been selected by Ng et al. [40]. When the MC-LR-specific aptamer was immobilized on the microcantilever surface, the specific binding between aptamer and MC-LR induced a surface stress change on the microcantilever, resulting in microcantilever bending (Fig. 2). We measured the microcantilever array deflection, and thus the detection of MC-LR could be realized.

The functionalized microcantilever array was mounted in the liquid cell, which maintained a constant flow rate of 1 μL s⁻¹. After stable signal curves were obtained, 100 μg L⁻¹ of MC-LR in buffer was introduced into the liquid cell. Fig. 3 shows real-time deflections generated by the specific binding of MC-LR and the MC-LR specific aptamer for eight microcantilevers in an array. Deflections were defined by the bend of the microcantilever, where bending toward the gold side was considered positive (bending up) and bending toward the silicon side was considered negative (bending down). After the injection of the MC-LR sample, positive deflections were observed in all eight microcantilever signals. The deflections were stable after about 70 min. Deflections of eight microcantilevers showed good consistency of deflections in an array for the detection of MC-LR (123.0 ± 8.7 nm at 85 min), which is an important characteristic for microcantilever array sensing. DNA aptamers are single strand oligonucleotides with stable three-dimensional space structure. When the aptamers bind with the targets, the aptamers undergo adaptive conformational changes, creating a specific binding site for the targets. The intermolecular interactions between aptamers and targets include complementation in shape, stacking interactions between the nucleobases of aptamers, electrostatic interactions between charged groups, hydrogen bonding, and etc [43,44]. When the immobilized aptamers on the microcantilever surface bind with MC-LR molecules in the buffer, these effects may change the sur-
face stress of the microcantilever during the detection experiment, resulting in a positive deflection of the microcantilever.

3.2. Relationship between deflections and the concentrations of MC-LR

To test the relationship between the deflection value and MC-LR concentrations, we measured the average deflection of the microcantilever array for different concentrations of MC-LR (1 μg L\(^{-1}\) to 500 μg L\(^{-1}\); Fig. 4). A new microcantilever array chip was used for each experiment. Deflection values at 85 min were 12, 30, 46, 109, 123 and 170 nm for the concentrations of 1, 10, 20, 50, 100 and 500 μg L\(^{-1}\) MC-LR, respectively. Deflections of the microcantilevers increased with the increase of the MC-LR concentrations. Reference experiments were performed by using one microcantilever array modified with reference aptamer (reference) and another microcantilever array modified only with 6-mercapto-1-hexanol (blank reference). A relatively small deflection was detected after the injection of 500 μg L\(^{-1}\) MC-LR sample by the reference microcantilever array. Meanwhile, no obvious deflection was observed when 500 μg L\(^{-1}\) MC-LR was detected by the microcantilever array of blank reference. This indicated that the specific binding between the immobilized MC-LR specific aptamer and the MC-LR generated the deflections of the microcantilevers. These results suggest that the microcantilever array functionalized with the MC-LR specific aptamer can detect MC-LR quantitatively in a simple, label-free, and rapid way.

Fig. 5 shows the calibration curve for the microcantilever deflection as a function of MC-LR concentration. On this curve, each point represents the average deflection and the error bar represents the standard deviation for the eight microcantilevers in an array. The data-derived calibration curve fit a Langmuir curve and had a dissociate constant (Kd) of 31.5 μg L\(^{-1}\) (about 31.7 nM), which is similar to the Kd previously reported by from Ng et al. (50 ± 12 nM) [40]. We additionally observed that the deflection increased linearly with MC-LR concentration in the range of 1–50 μg L\(^{-1}\) (inset in Fig. 5). The linear fit equation was \(y = 2.11x + 9.01\), with a correlation coefficient of 0.999. These experimental results show that the combination of MC-LR specific aptamer and microcantilever array sensor could be used for the quantitative detection of MC-LR.

The bending of the microcantilever is dependent upon the specific interaction between the immobilized aptamer and MC-LR. Molecular conformational changes on the microcantilever surface will have a great impact on the deflection [45]. After immobilization on the gold surface of the microcantilever, the aptamer molecules are gathered and try to expand on the surface, which leads a downward bending of the microcantilever [46]. When the immobilized DNA aptamer binds with MC-LR, the conformation of the aptamer changes [43,44]. This may make the aptamer structure orderly and reduce the crowded conditions between molecules and the compressive stress on the microcantilever surface, resulting in upward bending of the microcantilever (Fig. 2). This should be the main reason that upward bendings of the microcantilevers were recorded when MC-LR was detected. However, the detection limit of aptamer-based microcantilever sensor method is lower than that of the electrochemical assay method. Since the aptamer and MC-LR molecule are both negatively charged, the microcantilever motion may also be affected by electrostatic repulsive forces. When MC-LR molecules bind with aptamers on the microcantilever surface, there is an extra repulsion because of the presence of the negatively-charged MC-LR molecules. This repulsion interaction may weaken the upward bending of the microcantilever, which may be one reason why this assay exhibits lower sensitivity than that of the aptamer-based electrochemical assay method [40]. Having a better understanding of the interplay between electrostatic forces and microcantilever motion may help to improve the sensitivity of a biosensor operated in stress mode when a molecule with positive charge is detected.

Though the existing methods [ELISA: 0.05 μg L\(^{-1}\) [11], electrochemical assay: 0.01 μg L\(^{-1}\) [40]] have higher detection limits, the detection limit (1 μg L\(^{-1}\)) obtained in this work is equal to the provisional upper limit for MC-LR recommended by WHO. Therefore, our method is still useful for MC-LR detection in water, and the quantitative detection by the combination MC-LR aptamer and microcantilever array sensor makes it a simple, rapid, real-time, label-free and cost-effective way. Moreover, the detection limit provided here (about 1 nM, the molecular weight of MC-LR is 995.2) is comparable to that of other small molecules (cocaine: 5 μM [35], oxytetracycline: 0.2 nM [36], kanamycin: 50 μM [37]) detected by aptamer-based microcantilever sensors in stress mode. Thus, further studies should focus on improving the detection limit. Additionally, several of the microcantilevers (2–4) in an array can
be used as references to subtract the nonspecific signal or noise in the deflection signal.

The detection limit of MC-LR was reported as low as 1 ng L\(^{-1}\) by using protein antibodies as probes on a piezoelectric-excited cantilever sensor in vibration mode [47]. However, the immobilization procedures of the antibodies were much more complicated than that of the aptamers in this work. Moreover, since antibodies are produced by animal immune systems, the procedures to obtain protein antibodies are much complex, especially for small molecules. Activity difference may also be found between different batches of antibodies when antibodies are used as probe. In this work, the MC-LR specific aptamer was obtained by chemical synthesis. Only one step is needed to immobilize the synthesized thiol-modified aptamer on the microcantilever. The direct binding of native sulphydryl of the MC-LR aptamer with gold layer may provide better stability of the connection between probe and microcantilever, higher repeatability of the detection results. The aptamer-based microcantilever sensor is a much simpler, rapid, and more cost-effective approach for MC-LR detection.

3.3. Selectivity of microcystin congeners by microcantilever array

Microcystin is a small molecule, which has dozens of congeners. Among these congeners, MC-LR is known to be the most common and toxic. General methods for microcystin detection, such as ELISA [11] and PPIA [12], cannot distinguish between different microcystin congeners. The aptamer used here has the ability to identify MC-LR from other microcystin congeners. In order to analyze the selectivity of the aptamer-based microcantilever array sensor, the functionalized microcantilever array was exposed to three microcystin congener (MC-LR, MC-LA, and MC-YR) samples of the same concentration (100 µg L\(^{-1}\)). Average deflections of the microcantilever array for all three microcystin congeners were shown in Fig. 6. Average deflections for detection of MC-LR, MC-LA, and MC-YR at 85 min were about 123, 49 and 34 nm, respectively. It is obvious that MC-LR induced much greater deflection than the other two congeners. It is possible to distinguish the response induced by MC-LR from that induced by MC-LA and MC-YR. This indicates that the aptamer-based microcantilever array sensor has good selectivity and is able to identify MC-LR from other congeners, which cannot be achieved using the ELISA and PPIA methods.

4. Conclusion

In this paper, a simple and label-free method for MC-LR specific detection using microcantilever array sensors was developed. To our knowledge, this is the first time that algal toxin was detected using the microcantilever sensor operated in stress mode. Thiol-modified aptamer was used as the sensing probe for MC-LR detection via one-step immobilization. The specific binding between MC-LR and aptamer on the microcantilever surface changed the surface stress, causing deflection of the microcantilever. The deflection had a linear relationship with the concentration of MC-LR in the range of 1−50 µg L\(^{-1}\). We therefore conclude that the aptamer-based microcantilever sensor is able to distinguish MC-LR from its congeners by the comparative detection of microcystin congeners. These results demonstrate that the aptamer-functionalized microcantilever is suitable for quantitative detection of MC-LR and provides a simple, rapid, label-free and highly selective method for MC-LR detection, which may be further extended to the detection of various kinds of cyanotoxins while corresponding aptamers are available. This study shows the feasibility of the microcantilever sensor being applied in environmental monitoring.

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References


Fig. 6. Average deflection of the microcantilever array for 100 µg L\(^{-1}\) MC-LR, MC-LA and MC-YR.


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