Sensors and Actuators B 260 (2018) 42–47

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

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

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ARTICLE INFO

Article history:
Received 6 November 2017
Received in revised form
15 December 2017
Accepted 18 December 2017
Available online 19 December 2017

Keywords:
Microcantilever array
Nanomechanical sensor
Aptamer
Microcystin-LR
Surface stress

ABSTRACT

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 toxin. 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 micocantilever 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 $\mu g\,L^{-1}$, and the cantilever deflection had a good linear relationship within the concentration range of 1–50 $\mu g\,L^{-1}$. 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.

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 $\mu g\,kg^{-1}$ [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 $\mu g\,L^{-1}$ 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,

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https://doi.org/10.1016/j.snb.2017.12.112
0925-4005/

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 layer, 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-mercapto-1-hexanol (MCH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). MC-LR aptamer [40] with the sequence of 5′-(SH)-$(CH_2)_6$-GGC GCC AAA CAG GAC CAC CAT GAC AAT TAC CCA TAC CAC CTC ATT ATG CCC CAT CTC CGC-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)-$(CH_2)_6$-GGG CGC GCT AAA AGT AGG GGG ATT GAT AAG GGT AAA TCA TGT ATA TCG GTG TAC TCG CCG-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 $MgCl_2$. 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% H_2SO_4):V(30% H_2O_2) = 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