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Rapid detection of four mycotoxins in corn using a microfluidics and microarray-based immunoassay system

Yiqi Chen[a], Xiangrui Meng[a], Yunzeng Zhu[a], Minjie Shen[a], Ying Lu[a], Jing Cheng[a,b,*], Youchun Xu[a,b,*]

[a] Department of Biomedical Engineering, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Tsinghua University School of Medicine, Beijing 100084, China
[b] National Engineering Research Center for Beijing Biochip Technology, Beijing 102206, China

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ABSTRACT

Mycotoxins threaten human health seriously because they usually exist in food, fodder and commodities. In this study, a rapid and sensitive immunoassay system for commonly encountered mycotoxins was established based on microfluidics and protein microarrays. Four mycotoxins (T-2 toxin, aflatoxin B1, ochratoxin A, and zearalenone) can be automatically detected in a custom-made microdevice within 30 min under the assistance of a prototype of the instrument with a fluid control system and an imaging system. Once the microdevices are fabricated, they are small-sized and user-friendly. Standard curves for each of the studied mycotoxins were generated with a good logistic correlation ($R^2 > 0.98$). Working ranges from 0.1 to 20 ng/ml were employed in the immunoassay being the limit of detection achieved between 0.03 and 1.24 ng/ml. These values were calculated when the four mycotoxins were present in samples at the same time. Samples of spiked water and field corn were tested to assess the performance of our microfluidic-based detection technique for the mycotoxins. Recovery rates of mycotoxins from spiked water and corn samples were accessed and the results ranged from 80% to 110%, where the intra-assay coefficients of variation were under 15%. In summary, the system can realize rapid and reliable detection of multiple contaminants in actual samples automatically.

1. Introduction

Mycotoxins are harmful secondary metabolites produced by fungi like *Aspergillus* and *Fusarium*, which universally contaminate food, fodder and the environment [1–3]. Many mycotoxins are extremely toxic and have been proven teratogenic, mutagenic or carcinogenic [4]. According to surveys in China [5], most commonly found mycotoxins in corn include aflatoxins (AFTs, especially aflatoxin B1 (AFB1)), ochratoxin A (OTA), T-2 toxin (T-2), and zearalenone (ZEN). These four mycotoxins were chosen as the targets of this study because of their high detection rates in corn samples and their severe damage to human health. AFB1 is classified as Group 1 carcinogen by the International Agency for Research in Cancer and has been verified to have potential to cause liver cancer [6]. OTA is listed as a carcinogen in Group 2B and has the ability to inflict hepatic and renal intumescence [7]. ZEN affects reproductive system of mammals, and T-2 is toxic to the immune system [8].

Due to the severity of the health risks caused by mycotoxins, many international organizations and countries, including European Union and WHO, have set standards and permissible limits for mycotoxins in food products at ppb level (parts per billion or μg/kg) [9,10]. Since mycotoxins are stable molecules, they can remain active long time and pose a threat to the health of human and farm animals. Therefore, the establishment of a sensitive, multiplexed and rapid analytical method for the screening of mycotoxins in cereals is one of the most important needs in agrofood analysis.

Currently, methods based on high-performance liquid chromatography (HPLC) or mass spectrometry have been advocated by international organizations as standard methods for the analysis of samples suspected of contaminated by mycotoxins [11,12] for high accuracy and sensitivity, being the most authoritative methods. However, HPLC-based techniques are time-consuming, and they require expensive instruments and highly skilled personnel. Thin-layer chromatography [13] and immunochromatographic assays (also named lateral-flow immunoassays) [14,15] can detect more than one mycotoxin in parallel, and there are some commercial devices available [15]. Yet, most

* Corresponding authors at: Department of Biomedical Engineering, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Tsinghua University School of Medicine, Beijing 100084, China.
E-mail addresses: jcheng@tsinghua.edu.cn (J. Cheng), xyc2012@tsinghua.edu.cn (Y. Xu).

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of them have limits in sensitivity and numbers of indexes. Enzyme-linked immunosorbent assay (ELISA) [16,17] and surface plasmon resonance (SPR) [18] can detect mycotoxins too, but these techniques involve complicated operations or expensive apparatus.

Being multiplexed, specific and high-throughput makes protein microarray a suitable and powerful technique for mycotoxin detection. Wang et al. [19] successfully developed an immunochip for simultaneous detection of six different types of mycotoxins. However, the process of analysis is time-consuming (about 4 h) and non-automatic. To overcome the shortage of traditional immunochip, microfluidics technology can be combined to promote the rate of reaction and the degree of automation.

With this thought, a custom-made instrument which combining microfluidics and microarray was developed. A programmable syringe pump and a rotary valve were used to manipulate the solutions involved in the micro-channel and chamber covering the protein microarray. Four mycotoxins (T-2, AFB1, OTA, and ZEN) in spiked samples and field corn samples were successfully detected by our microdevice within 30 min, demonstrating the immunoassay system described in this study can be used in on-site sample analysis and shorten the response time for taking remedial measures against fungal contamination.

2. Experiment

2.1. Reagents

The four mycotoxin conjugates, T-2-BSA (bovine serum albumin), AFB1-BSA, OTA-BSA, ZEN-BSA and their murine monoclonal antibodies (mAbs) were purchased from Huaan Magnech Bio-Tech (Beijing, China). Mycotoxins T-2, AFB1, OTA, and ZEN were purchased from Fermentek (Jerusalem, Israel). Stock solutions were prepared in methanol or acetonitrile (analytical reagent grade) and stored at − 20 °C.

The mycotoxin-BSA conjugates were printed onto the epoxy group-modified glass substrate as probes. Cy3-labeled goat anti-Mouse IgG was purchased from Sigma-Aldrich (Shanghai, China). Cy3-labeled BSA (Cy3-BSA) was purchased from Bioss (Beijing, China). IgG produced in mouse was purchased from Genia (Beijing, China). The corn sample was provided by the Academy of State Administration of Grain (Beijing, China).

The antibody solutions were prepared by serial dilution in 0.01 mol/L PBS (pH = 7.4) purchased from Beijing Solarbio Science & Technology Corp (Beijing, China). PBST (PBS with 0.05% Tween-20) was used as washing buffer. Water in the experiments was purified by a Milli-Q system (Millipore, Beijing, China).

2.2. Design and fabrication of the microdevice

The microdevice consisted of three layers from top to bottom: polymethyl methacrylate (PMMA) layer, adhesive layer and glass layer (Fig. 1A). The top PMMA layer was a 1.5 mm thick plate, which was much easier to be machined than PDMS. The microfluidic channel and chamber were engraved in this layer using a laser engraver (Trotec, Shanghai, China) (Fig. 1B). A double-sided adhesive layer (QL-9970-025, Wuxi Bright Technology, Wuxi, China) was located between the glass layer and the PMMA layer. The surface of the glass slide was modified with epoxy group (CapitalBio, Beijing, China). All the probes were printed on the glass slide before the three layers were bonded together (Fig. 1C).

2.3. The automated and integrated microfluidic system

The schematic of the self-contained instrument for flow control and signal detection is presented in Fig. 2A. The system contains a fluid control system and an imaging system. A laptop with a custom-made LabVIEW program (National Instruments, Austin, TX) is set up to send commands and collect data from the imaging system (Fig. 2B).

As showed in Fig. 2A, four tubes with different reagents were stocked in the instrument, including the mixture of the first antibody solution and sample, the first washing buffer, the Cy3-labeled secondary antibody solution and the second washing buffer. These tubes were connected to a multi-channel modular valve positioner (Hamilton, Bonaduz, Swizerland), that could switch each tube to the inlet as planned. A syringe pump (PSD/4, Hamilton) drives and regulates the flow of the fluids. A switching power supply (S-25W-24V, Share Electronic, Leshan, China) was used to power the pump and the valve. During the whole process, the corresponding solutions were driven into the microdevice and flow through the reaction chamber repeatedly. Such an arrangement could accelerate the rate of immunoreaction and wash to reduce the required time. The flow rate was fixed at 300 ml/h and 500 ml/h for incubation and wash. Once the 500-μl glass syringe was filled, the waste was expelled to a waste tube.

All the fluorescent dyes used in the detection were Cy-3 (Emmision wavelength is 532 nm and excitation wavelength is 570 nm). A laser (532 nm, W532FS-60 mV, Pavilion, Suzhou, China) with an expanding beam was used to excite the fluorescent signal. At the same time, the results were captured by a digital camera (1280 × 960, MV-BS30U, Crevis, Korea) with a set of magnifying lens and a filter (570 ± 15 nm, Bodian Optical, Beijing, China). Double cemented lens (f' = 50 mm, Daheng Optics, Beijing, China) were used to eliminate aberration in the optical system.

2.4. Printing and immobilization of the probes

All the BSA-Ags and control probes were printed on the epoxy group-modified glass slides, including four BSA-Ags (T-2-BSA, AFB1-BSA, OTA-BSA and ZEN-BSA) and three controls (Cy3-BSA as the printing control, 0.5 mg/ml mouse-IgG as the positive and hybridization control, and printing buffer as the negative control).

Two types of the microdevices were contact printed. First, different concentrations of each Ag solution were contact printed on the glass slides with eight replicates to optimize the spotting concentrations (Fig. S2A). Second, once the spotting concentrations of the Ags were determined, the four Ags of the optimal concentration and the control probes were printed on the microdevices for detection (Fig. S2B).

A printing robot (Personal Arrayer™ 16, CapitalBio, Beijing, China) was used to spot the probes onto the specific area of the slides where the reaction chamber was located. The volume of each probe was around 0.8 nl. After spotting, the epoxy group-modified glass slides were dried under vacuum in an oven overnight. The epoxy groups of the slides would react with the free amino groups of the protein, covalently linking the protein to the glass substrate.

2.5. Optimization of antigen and antibody concentrations

The concentrations of the four antigens were determined by printing varying concentrations of each antigen on the optimization microdevices listed in Fig. S4. To optimize the concentrations of the four monoclonal antibodies for competitive immunoassay, the microdevices printed with different concentrations of Ags were incubated individually in the corresponding antibody solution. A series of concentrations of each antibody were tested (Fig. S4).

The detailed operating procedure of the chip is as follow: before use, the channel and reaction chamber of microdevices were blocked with PBST (30 μl) containing 2% BSA and incubated for 1 h, minimizing non-specific binding of the Ags to the microdevices. With the aid of the programmed syringe pump, the used buffer was pumped into the waste tube. Next, 50 μl of solution containing corresponding monoclonal antibodies was aspirated into the microdevice and incubated for 10 min. After this first hybridization step, the microdevice was washed again. Then, 50 μl of Cy3-labeled anti-Mouse IgG solution (diluted with PBS by 1:400) was incubated in the reaction chamber for 10 min, which was followed by a washing step.