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# Aptamer fluorescence signal recovery screening for multiplex mycotoxins in cereal samples based on photonic crystal microsphere suspension array

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ABSTRACT

We design a novel high throughput photonic crystal microsphere (PHCM) suspension array for multiplex mycotoxins in cereal samples based on the aptamer fluorescence signal recovery. The hybridization duplex strand DNA from mycotoxin aptamer and anti-aptamer respectively labeled with fluorescence dye and quencher was immobilized on the carboxylated surfaces of PHCMs. When the corresponding mycotoxin targets bind to their aptamers, the fluorescence recovery signal intensity of PHCMs reported the concentration of mycotoxins. The different kinds of mycotoxins were distinguished by the structure colors of PHCMs. The fluorescence signal intensity on the PHCMs is nearly higher 100 times than that of solid glass beads. The detection system presents an ultrasensitive, high selectivity, and small volume reagent screening for multiplex mycotoxins with a dynamic linear detection range of 0.1 pg/mL–0.1 ng/mL for AFB1/OTA and 0.1 ng/mL–10 ng/mL for FB1 and a limit of detection (LOD) of 15.96 fg/mL, 3.96 fg/mL and 11.04 pg/mL for AFB1, OTA and FB1, respectively. The recovery rates in spiked cereal samples were well consistent with that of the traditional ELISA. The designed system provides a new high throughput aptamer-based suspension array for small molecule screening in parallel.

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## 1. Introduction

Mycotoxins are toxic low-molecular-weight compounds which produced by the metabolism of fungi species and often enter into the food chain when the agricultural commodities are harvested, stored, transported and processed in the absence of proper conditions. The most prevalent toxin-producing genera isolated from food and feed are *Aspergillus*, *Fusarium* and *Penicillium* [1–3], which could typically produce aflatoxins, fumonisins and ochratoxin A(OTA), respectively. Most mycotoxins are suspected human carcinogenic agents and have been demonstrated to result in mutagenicity, teratogenicity and immunosuppression [4]. Especially aflatoxin B1 (AFB1) has been listed as Group 1 carcinogen by the International Agency for Research in Cancer and responsible for liver cancer in human beings and animals [5].

Generally, mycotoxins are chemically and thermally stable and very difficult to remove once they contaminate the food chain. Therefore, mycotoxins may also be found in beer or wine and animal products resulting from the use of contaminated cereals or livestock eating contaminated feed [6]. To protect consumer health and reduce financial loss, many countries have set maximum limits tolerable for the mycotoxins in food and feed [7]. However, until now, a worldwide priority requirement is to develop the sensitive, accurate and cost-effective analysis methods to monitor the occurrence of such contaminants and evaluate the risk of food and feed [4].

Mycotoxigenic fungi are usually capable of producing several toxic metabolites, or several species can be present simultaneously, producing different toxins in the same sample, which leads to the fact that multiplex mycotoxins always coexist and display additional or synergistic toxic effects [5,8–11]. Additionally, mycotoxins show chemical and structural diversity and uneven

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distribution in cereals. These characteristics of mycotoxins make multiplex mycotoxin analysis method urgent requirement, especially for onsite screening large of samples. Obviously, multiplex mycotoxin analysis method is superior to a single mycotoxin assay protocol in analysis time and costs for the same analytical system. Currently, chromatographic techniques, immunoassays, biosensors, and non-destructive methods have been used to develop the multiplex mycotoxin analysis. Both gas chromatography and liquid chromatography can separate a huge number of analytes and coupled with mass spectrometry techniques enabling the use of multimycotoxin detection [12]. However, these techniques not only require complex sample pretreatment (such as immunoaffinity or solid phase extraction columns and derivatization) but also expensive instruments and skilled operators, which limit their application in practice [13]. Generally, chromatographic detection methods are mainly devoted to confirmatory analyses. Immunochemical methods mainly include enzyme-linked immunosorbent assay (ELISA), lateral flow devices (LFD) [7], dipstick tests [14], fluorescence polarization immunoassays (FPIA) [15] and chemiluminescent immunoassay [13,16]. Immunoassays for mycotoxins require mycotoxin artificial antigens and antibodies which are complicated production process and the different batches display a different titer of antibody. Biosensor techniques based on electrochemistry [17,18], optical label-free surface plasmon resonance (SPR) [19,20] and protein microarrays [5,21,22] also used for mycotoxin detection. Though these techniques have a lot of advantages, they are still cumbersome and difficult to integrate with sample pretreatment.

Microsphere suspension array technique is a high throughput, rapid and flexible detection method and has been used for analysis of multiplex mycotoxins [10,13,23–28]. The most typical microsphere suspension array commercially available is Luminex 200 system from Luminex Corporation (Austin, Texas, USA). The key technique in this system is the microsphere encoding with fluorescent dyes and decoding with a specialized flow cytometer. Compared with the common solid microarray, the system for multiplex mycotoxins shows the obvious advantages in dynamic linear detection range, limit of detection (LOD) and cost. More importantly, the microsphere suspension array may be easy to integrate with sample pretreatment by designing the multifunctional microsphere (such as magnetic microspheres modified with mycotoxin antibody). Unfortunately, fluorescent dye encoding microspheres may produce fluorescent bleaching and encoding overlap. In additional, encoding with fluorescent dye and decoding with flow cytometer make the optical path system very complicated, which limit the further application of the system.

Photonic crystal encoded microcarrier is a new generation suspension array technique, which overcomes the disadvantages of fluorescent dye microsphere system and shows a great potential in practical application [29–31]. The photonic crystal microspheres (PHCMs) are composed of spatially periodically ordered lattices and exhibit brilliant structural colors. Unlike fluorescent dye encoding microspheres, the PHCMs encode microcarrier with their structural colors which are very stable during the whole analysis process. The three-dimension (3D) porous structure microcarrier with the unique optical physical properties could accommodate more biomolecule probes in their internal surfaces and improve sensitivity of detection. Recently, we have established new suspension arrays for multiplex mycotoxins based on protein microarray [32] and chemiluminescent immunoassay [33,34]. These results indicated that the new suspension array could be used as high sensitive, high throughput and cost-effective platform for multiplex mycotoxin detection.

Aptamers are single-stranded DNA or RNA molecules that can capture their target molecules with high specificity and affinity through their distinct nanoscale shapes [35]. Due to their synthesis simplicity, chemical stability, convenient regeneration, easy storage and modification, aptamers are promising to replace antibodies for multiplex mycotoxin detection. All kinds of aptamer techniques such as chromatographic [36], optical [37], electrochemical [38], enzyme [39] and fluorescence [40,41] methods have been developed to detect mycotoxins. However, the limitations of these methods are only used to detect single or two mycotoxins and difficult to engineering, standardized application in practice. In addition, previous hybridization duplex strand DNA aptamer fluorescence methods based on mycotoxins displacing anti-aptamer sequences [42], which may lead to the false negative result because of the double DNA incomplete dissociation. In this work, we designed a new aptamer suspension arrays for screening multiplex mycotoxin. The hybridization duplex strand DNA from mycotoxin aptamer and anti-aptamer respectively labeled with fluorescence dye at the 3’end and quencher at the 5’end was immobilized on the carboxylated surfaces of PHCMs. When the corresponding mycotoxin targets bind to their aptamers, the fluorescence recovery signal intensity of PHCMs reported the concentration of mycotoxins. The aptamer suspension array detection system could provide a new high throughput, cheap and sensitive detection platform not only for multiplex mycotoxin assay, but for other molecules.

## 2. Experimental section

### 2.1. Experimental materials

AFB1, AFB2, AFG1, AFG2, FB1, OTA standard substances were purchased from Pribolab (Singapore). (3-aminopropyl)triethoxysilane(APTES), 3-glycidoxypropyltrimethoxysilane (GPTMS), Dimethyl sulfoxide (DMSO), Tris(hydroxymethyl) aminomethane (Tris), succinic acid, glutaraldehyde, *p*-Phenylene (PDITC), and *N,N*-dimethylformamide (DMF) were bought from Sigma-Aldrich (Shanghai, China). N-Hydroxysuccinimide (NHS) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were from TCI Chemistry Co. (Shanghai, China). Pyridine, dichloromethane, absolute ethanol and sodium bicarbonate were obtained from Nanjing Chemistry Reagents Co. (Nanjing, China). AFB1, FB1, and OTA ELISA kits were bought from Hongdu Biotech Co., Ltd (Shandong, China). Aptamers and anti-aptamers for AFB1, FB1and OTA were as follows:

AFB1-aptamer, 5′-$NH_2$-$(CH_2)_6$-AGT TGG GCA CGT GTT GTC TCT CTG TGT CTC GTG CCC TTC AGG CCC ACA-Cy3-3′, AFB1anti-aptamer, 5′-BHQ2-TGT GGG CCT AGC GA-3′;

FB1-aptamer,5′-$NH_2$-$(CH_2)_6$-TCT AAC GTG AAT GAT AGA TTA ACT TAT TCG ACC ATA CAC GTC TGC ATT ACC TTA TTC GAC CAT ATT CCA TTA CGC TAA TTA ACT TAT TCG ACC ATA-Cy3-3′, FB1anti-aptamer, 5′-BHQ2-TAT GGT CGA ATA AGT TAA-3′;

OTA-aptamer, 5′-$NH_2$-$(CH_2)_6$-GAT CGG GTG TGG GTG GCG TAA AGG GAG CAT CGG ACA-Cy3-3′,OTA anti-aptamer, 5′-BHQ2-TGT CCG ATG C-3′.

These aptamers and anti-aptamers were synthesized and modified by Sangon Biotech Co., Ltd. (Shanghai, China). The $SiO_2$ PHCMs were prepared by our lab and characterized according to our previous method [32] (see Fig. S1). The glass beads (diameter in range of 212–300 μm) were bought from sigma-Aldrich (Shanghai, China). Cereal samples were purchased from a local market in Nanjing (China). All other chemicals were of analytical grade.

### 2.2. The modification of PHCM surfaces

The surfaces of PHCMs were firstly hydroxylated with piranha solution (30% hydrogen peroxide and 70% sulfuric acid (v/v)) for 12 h. The four kinds of modification methods were used to immo-

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