Simultaneous Detection of Ochratoxin A and Fumonisin B1 in Cereal Samples Using an Aptamer–Photonic Crystal Encoded Suspension Array

Sun Yue,†,‡ Xu Jie,†,‡ Li Wei,† Cao Bin,† Wang Dou Dou,† Yang Yi,† Lin QingXia,‡ Li JianLin,*,† and Zheng TieSong*,†

†Department of Food Science and Nutrition, Nanjing Normal University, Nanjing 210097, China

ABSTRACT: A simple, new aptamer–photonic crystal encoded suspension array was designed to simultaneously quantify and qualitify ochratoxin A (OTA) and fumonisin B1 (FB1) in cereal samples. The aptamers of OTA and FB1 were immobilized on the surfaces of photonic crystals by chemical bonding. When the target mycotoxins appear in a sample, the fluorescence-labeled complementary DNA of the aptamer dissociates from their double DNA hybrid and results in an obvious decrease in fluorescence intensity of the microsphere. The difference value of fluorescent intensities for each kind of silica photonic crystal microsphere (SPCM) quantitatively conveys the concentration of mycotoxin, and the structure colors or reflectance peak positions of the SPCMs determine the kind of mycotoxin detected. The reaction conditions including the immobilization method for aptamers, hybridization, and incubation conditions have been optimized. This method displayed a wide linear detection range (0.01–1 ng/mL for OTA and 0.001–1 ng/mL for FB1) and a low limit of detection (0.25 pg/mL for OTA and 0.16 pg/mL for FB1). The recovery rates in the spiked cereal samples ranged from 81.80% to 116.38% for OTA and 76.58%–114.79% for FB1. The positive detection results in the naturally contaminated cereal samples were in agreement with those of classic enzyme-linked immunosorbent assay (ELISA). This simple suspension array scheme displays a great application potential for the high throughput screen assay of mycotoxins.

Mycotoxins are toxic secondary metabolites released and produced by various species of fungi which often appear in contaminated cereals. About 25% of the world’s agricultural commodities are contaminated by mycotoxins to a certain degree and lead to an estimated annual loss of 1 billion metric tons of food products equivalent to about 5 billion dollars per year.‡ Many of these toxins or their metabolites have been reported to be carcinogenic, teratogenic, nephrotoxic, and mutagenic.³,⁴ Most mycotoxins are chemically stable even when cooked at quite high temperature such as those reached during baking bread or breakfast cereal production.⁵ More importantly, the previous studies indicated that multiple mycotoxins might coexist in a single sample and could cause increased toxicity by additive and synergistic effects.⁶–⁸ They may usually pose a serious threat to human and animal health when they enter the food chain through contaminated cereals or feedstuffs.⁹,¹⁰ A sensitive, rapid, cost-effective, and accurate detection method for mycotoxins is one important prevention measure to keep our food chain free from mycotoxin contamination.

Currently, chromatographic techniques, immunochemical assays, electrochemical or other biosensor techniques, and microarrays have been developed to quantitatively or semi-quantitatively or qualitatively detect mycotoxins. Chromatographic techniques based on the physicochemical apparatus have become classic methods for analysis of mycotoxins, especially, liquid chromatography combined with immunoaffinity column (IAC) cleanup and mass spectrometry (LC-MS/MS) has become increasingly popular.¹⁰ However, these chromatographic methods require expensive instruments and complex pretreatment procedures, are time-consuming, and are unsuitable for analysis of large numbers of samples.⁸,¹¹–¹⁵ Though electrochemical (such as amperometric, impedanced, potentiometric, or conductometric) biosensors and other optical biosensors based on specific antibodies or aptamers of mycotoxins as affinity ligands are sensitive, simple, and fast analytical methods,¹⁶ these methods are difficult to realize high throughput detection.⁴ Immunochemical assays, typically enzyme-linked immunosorbent assay (ELISA), lateral flow devices (LFD), and dipstick tests have been widely applied in mycotoxin determination as screening methods. The main disadvantages of immunochemical assays are the high volume consumption of reagents, single mycotoxin assay, and the cross-reactivity with structurally related toxins or matrix compo-

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nents. Microarrays are high throughput detection techniques and have been used to simultaneously assay multiplex mycotoxins based on immunological principles or detect multiplex mycotoxin biosynthesis genes in foods. Though these detection systems enable mycotoxin analysis standard parallel performance and only need nanoliter (nL) analysis reagents, they are complex, expensive, and not easy to be widely used for on-site screening mycotoxin analysis.

In the above-mentioned mainstream mycotoxin analysis methods, a lot of antibodies of toxins are traditionally used as specific molecular recognition units for improving the detection sensitivity and specificity. However, the synthesis of antibodies for hapten mycotoxins is complicated and time-consuming. The structure stability and immobilization procedures of antibodies can influence their biochemical activities and cause denaturation sometimes. Aptamers, as an alternative to antibodies, have recently attracted significant attention for molecule detection because they not only exhibit high affinity and selectivity but they also have a number of advantages over antibodies such as simple synthesis, chemical stability, easy storage, good reproducibility, easy modification, and convenient regeneration. Aptamers are single-stranded DNA (or RNA) that can capture a target molecule by using distinct nanoscale shapes and can be isolated from nucleic acid libraries via systematic evolution of ligands by exponential enrichment (SELEX). Aptamers have been used to enrich, clean up, and detect the ochratoxins A (OTA). However, the aptamer-based suspension array technique for simultaneous detection of multiplex mycotoxins has been rarely reported.

Recently, we reported antibody-based suspension arrays for multiplex mycotoxin analysis by the silica photonic crystal microsphere (SPCM) encoding technique, which showed a powerful advantage over common microarrays and suspension arrays. In this paper, we established a new aptamer-based suspension array for simultaneous detection of ochratoxins A (OTA) and fumonisin B1 (FB1) in cereal samples using SPCMs as the encoded carriers. Ochratoxins A (OTA) and fumonisin B1 (FB1) are selected as a proof-of-concept that shows the capacity of the array to adapt the aptamer for a multiplex mycotoxin assay. The sensitivity, specificity, repeatability, and reproducibility of the established system were evaluated, and the results of application in real cereal samples were compared with that of classic ELISA. The developed method displays the following advantages: (1) simple and flexible principle; (2) low volume consumption of reagents (2 μL reagent / each sample); (3) low limit of detection (pg/mL for OTA and FB1); (4) high throughput screen.

#### EXPERIMENTAL SECTION

**Experimental Materials.** Aflatoxin B1 (AFB1), FB1 standard substances, 3-glycidoxypropyl-trimethoxysilane (GPTMS), bovine serum albumin (BSA), tetraethoxysilane (TEOS), tolune, and glutaraldehyde were purchased from Sigma-Aldrich (Shanghai, China). OTA standard substances, 3-glycidoxypropyl-trimethoxysilane (TEOS), toluene, and glutaraldehyde were purchased from Yunuo Chemicals Ltd., China. H2O2 (30%), ammonia, absolute ethanol, and EDTA were bought from Nanjing Chemicals Ltd., China. The preparation of SPCMs was described in a previous study in our lab. Deionized water was produced with a Millipore purification water system. Cereal samples were purchased from a local market in Nanjing (China). All other chemicals were of analytical grade.

**Characteristics of SPCMs.** The surface characteristics of SPCMs were collected by scanning electron microscopy (SEM, Hitachi, S-300N). The bright-field microscopic images were collected by a common metalloscope when the SPCMs were put on the surface of a glass slide. Reflectance spectra of SPCMs were acquired using an Ocean Optics CCD S-2000 spectrometer. The characteristic results of SPCMs are shown in Figure S1 (Supporting Information).

**Modification of Microsphere Surfaces.** The SPCMs were activated with piranha solution (30% hydrogen peroxide and 70% sulfuric acid (v/v)) for 12 h. The SPCMs were washed with water and dried in an oven at 70 °C for 5 h. To modify the surfaces of SPCMs with epoxy groups, they were immersed in 0.5 mL of 1% GPTMS toluene solution and shaken on a shaking table at 37 °C for 8 h. Then these modified microspheres were washed with toluene and absolute ethanol solution three times, respectively. After that, they were dried in an oven at 110 °C for 1 h. To modify the surfaces of SPCMs with aldehyde groups, they were immersed in 0.5 mL of 5% APTES absolute ethanol solution and shaken at 37 °C at 8 g for 6 h and then washed with ethanol solution and dried in an oven at 70 °C for 5 h. After that, they were immersed in 2.5% glutaraldehyde--water solution and shaken at 37 °C at 8 g for 30 min and then washed three times with phosphate buffer solution containing Tween-20 (0.5% v/v) (PBST) and dried in an oven at 70 °C for 5 h.

**Immobilization of Aptamers and Hybridization Procedure.** The modified microspheres were immersed in TE solution (0.1 mol/L Tris-HCl solution with 0.01 mol/L EDTA) with 100 nmol/L aptamers of mycotoxins (2 μL / each microsphere) at 4 °C for 12 h. After that, the microspheres were washed with TE solution three times, blocked with 1% BSA PBS solution at 37 °C for 1 h, and washed with PBS solution three times. Then a certain concentration of FITC-labeled complementary DNA of aptamers in 5× saline sodium citrate (SSC) solution was added to hybridize at 37 °C for 2 h. Then the microspheres were successively washed with 1× SSC solution with 0.2% sodium dodecyl sulfate (SDS), 0.2× SSC solution, and 0.1× SSC solution and stored at 4 °C for next use.

**Detection of OTA and FB1.** The above-prepared hybridized microspheres which bound with probes of OTA and FB1 were respectively incubated with various concentrations of OTA and FB1 in binding buffer solution (0.01 mol/L, pH 8.0 Tris-HCl, 120 mmol/L NaCl, 20 mmol/L CaCl2, 5 mmol/L KCl, 20 mmol/L MgCl2) in one test tube at 37 °C for 1 h. After the microspheres were respectively washed with 0.1×...
According to ELISA kit instructions without diluting the traditional ELISA for validation assays was directly carried out controls were tested four times. Separated and diluted with 0.01 mol/L pH 8.0 Tris-HCl fume hood.

Fumigation of samples, and activation of SPCMs were carried out in a dark tube wrapped with aluminum foil, and fluorescence signal intensity was obtained immediately after the reaction and washing.

**Specificity.** AFB1, OTA, FB1, and FB2 were used to investigate the specificities of this method. One kind of toxin standard substance (1 ng/mL) was respectively added into test tubes which contained the above SPCMs bound to the aptamer probes and FITC-labeled complementary DNA of OTA and FB1. The detection procedures were the same as the above method.

**Spiked Sample Preparation and Assays.** Cereal samples including rice, corn, and wheat were pretreated as in the previous procedures. The spiked sample was generated from OTA- and FB1-contaminated ground cereal powder with 0.01, 0.1, and 1 ng/g standard solutions, respectively.

The samples were first shaken in 10 mL of methanol—water (6:4 v/v) solution for 2 h at 6g, and then the extracts were centrifuged at 3000g for 15 min. The supernatant solution was separated and diluted with 0.01 mol/L pH 8.0 Tris-HCl solution at a ratio of 1:10. The spiked samples and blank controls were tested four times.

**Detection for Real Cereal Samples.** The pretreatment, toxin extractions, and detection for naturally contaminated cereal samples were performed with the above method. The traditional ELISA for validation assays was directly carried out according to ELISA kit instructions without diluting the supernatant solutions.

**Safety Precautions.** Experimenters should be equipped with laboratory coat, safety glasses, gloves, and mouth-muffle and be strictly prohibited from direct contact with mycotoxins and contaminated samples. Extraction of mycotoxins, preparation of samples, and activation of SPCMs were carried out in a fume hood.

## RESULTS AND DISCUSSION

**Principle for Simultaneous OTA and FB1 Detection.** The principle for simultaneous detection of OTA and FB1 based on aptamer—SPCMs is shown in Scheme 1. The different aptamer probes for mycotoxins are bound to the surfaces of SPCMs which are used to encode the suspension arrays by their different structure colors or reflectance peak positions. The target (OTA and FB1) or extractions of cereal samples are introduced to the system, which induces a structural switch of the aptamer because of target binding and leads to the release of the fluorescence-labeled aptamer complementary DNA and a marked decrease in fluorescent intensity of each kind of SPCM. The difference value of fluorescent intensities for each kind of SPCM quantitatively conveys the concentration of mycotoxin, and the structure colors or reflectance peak positions of SPCMs confirms the kind of mycotoxin detected. The aptamer—SPCMs suspension arrays for simultaneous multiplex detection of mycotoxins combine the advantages of aptamer techniques with the SPCM-encoded suspension array technique, and these microspheres are easily handled during the entire detection. In addition, the toxins are captured by aptamer immobilized on the surfaces of SPCMs, which are easily recycled and safer to the environment.

Because the aptamers were found to be specific molecular recognition units, all kinds of aptamer-based methods have been developed to detect mycotoxins. Typically, aptamers, replacing the antibody, have been used to enrich and clean up mycotoxins, modified on the electrode for electrochemical detection, and designed for a strip test. Recently, a nanoparticle aptamer-based method was reported for multiplex detection of mycotoxins. These methods were sensitive and effective for rapid mycotoxin detection. Compared with these methods, the reaction reagents in our system only require 2 μL each sample and less than 1.5 h for the assay process not including the time of immobilization of the aptamer and hybridization of complementary DNA. More importantly, the proposed scheme is simple, flexible, and easy for high throughput screening or enriching multiplex mycotoxins in parallel.

**Optimization of the Detection Conditions.** Modified Conditions of Microsphere Surfaces. The microsphere surfaces were respectively modified with epoxy groups and aldehyde groups to immobilize the aptamers. The background fluorescence signals of different modification methods are shown in Figure 1a. For the modification with aldehyde groups, the high background fluorescence signal was produced over a long modification time (4 h) compared with short modification time (30 min). In contrast, the modification with epoxy groups resulted in a low background fluorescence signal even over a long reaction time (6 h). Therefore, modification with epoxy groups on the surfaces of SPCMs was used to immobilize the aptamers.

The optimal concentration of GPTMS was studied when 100 nmol/L OTA aptamer was hybridized with 100 nmol/L fluorescence-labeled complementary DNA (Figure 1b). From Figure 1b, as the concentration of GPTMS increased, the fluorescent signal intensity increased over a range of 1–5% and reached a plateau at 6%. This result was ascribed to an increase of epoxy groups on the surfaces of SPCMs which led to an increase in aptamer binding and fluorescent signal intensity. In consideration of the background fluorescence signal intensity of GPTMS, 5% GPTMS was taken as the suitable concentration for immobilization of aptamer.

**Optimal Concentration of Aptamers and Their Complementary DNA, Hybridization Time and Temperature, Incubation Time and Temperature of Toxin with Double DNA.** The concentration of aptamers and their complementary

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**Scheme 1.** Simultaneous Detection of OTA and FB1 Based on Aptamer—SPCMs Suspension Arrays

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DNA, hybridization time and temperature, and incubation time and temperature of toxin with double DNA can influence the signal intensity. The concentration of FITC-labeled aptamers in a range of $0.1^{-800} \text{nmol/L}$ were respectively immobilized on the surfaces of SPCMs and the fluorescence intensities of SPCMs were measured. The optimal concentration of aptamers was $400 \text{nmol/L}$ for OTA (Figure.S2a) and $200 \text{nmol/L}$ for FB1 (Figure.2b). Under the optimal immobilization concentration of aptamers, the suitable concentration of FITC-labeled complementary DNA were found to be $500 \text{nmol/L}$ for OTA (Figure.S2c) and $250 \text{nmol/L}$ for FB1 (Figure.S2d). Based on above results, the optimal hybridization time and temperature were $2 \text{h}$ (Figure.S2e) and $37^\circ \text{C}$ (Figure.S2f), respectively. The optimal incubation time and temperature of toxin with double DNA were found to be $1 \text{h}$ (Figure.S2g) and $37^\circ \text{C}$ (Figure.S2h), respectively.

**Specificity Evaluation.** High specificity is expected for the molecular recognition detection method. The two aptamers for OTA and FB1 were respectively immobilized on the surfaces of SPCMs. The difference values of fluorescent intensity were measured before and after OTA, FB1, FB2, and AFB1 were introduced to the system. The specificity of the designed method is shown in Figure 2. Only when the OTA and FB1 samples were introduced, the obvious difference values of fluorescent intensity were produced and the nonspecificity reaction signals were very low. Even for FB2, as a structure analogue of FB1, the signal fluorescent value was lower than 5% positive signal value. These results indicated that the aptamer—SPCM suspension array system could simultaneously distinguish OTA and FB1 in samples.

**Sensitivity and Calibration Curves for OTA and FB1.** On the basis of the above optimal detection parameters, a series of concentrations of OTA and FB1 standard solutions were detected, and the calibration curves are shown in Figure 3. As the concentrations of OTA and FB1 standard solutions increased, the difference values of fluorescence intensities between before and after introduction of standard solution increased and reached a plateau at $100 \text{ng/mL}$ for OTA and FB1. These results were in agreement with the principle of the designed method. The detection linear dynamic range was $0.01-1 \text{ng/mL}$ for OTA and $0.001-1 \text{ng/mL}$ for FB1, and the linear equations were $y = 1054.35 + 322.89 \log x \left( R^2 = 0.986 \right)$, $y = 1491.68 + 410.88 \log x \left( R^2 = 0.994 \right)$, respectively. The limit of detection (LOD) from three times the standard deviation corresponding to the blank samples was calculated to be $0.25 \text{pg/mL}$ for OTA and $0.16 \text{pg/mL}$ for FB1, respectively. The LOD is much lower than that of the aptamer-based methods recently reported by Wu$^{33}$ ($0.02 \text{ng/mL}$ for OTA and $0.1 \text{ng/mL}$ for FB1), Chen$^{34}$ ($0.8 \text{ng/mL}$ for OTA), and Wang$^{32}$ ($0.18 \text{ng/mL}$ for OTA) and in the same level with that reported by Wu$^{35}$ ($0.1 \text{pg/mL}$ for OTA). The linear dynamic range is in the same level with that reported by Chen$^{34}$ ($1-100 \text{ng/mL}$ for OTA) and narrower than that reported by Wu$^{35}$ ($0.05-100 \text{ng/mL}$ for OTA, $0.1-500 \text{ng/mL}$ for FB1). The detection time is shorter than that reported by Wu$^{35}$ ($3.3 \text{h}$) and longer than that reported by Chen$^{34}$ (less than 10 min for qualitative and semiquantitative detection of OTA).

These wide linear dynamic detection ranges and high sensitivities for OTA and FB1 of the suspension arrays were...
mainly attributed to the three-dimensional porous and periodic structure of SPCMs which could observably enhance the fluorescent intensity factor. The fluorescence signal intensity was the 10 times higher than that obtained from the liquid system. The comparison of the calibration curves of SPCMs with the same size diameter of glass beads was performed under the same conditions, which further demonstrated the above result (Figure S3).

**Simultaneous Detection of OTA and FB1 in Cereal Samples.** The recovery rates were performed when the three levels of concentration of OTA and FB1 (0.01, 0.1, and 1 ng/mL) were spiked in rice, corn, and wheat samples, which are shown in Figure 4. The results indicated that the recovery rate was between 81.8 ± 6.28% to 116.38 ± 7.60% for OTA and 76.58 ± 5.96% to 114.79 ± 5.58% for FB1, respectively. Most recovery rates exceeded the 80% range, which was acceptable for the screening analysis.

The repeatability and reproducibility of the designed method were evaluated five times by examining the intra- and interassay variation coefficients with the signals for the same concentration of OTA and FB1. The intraassay coefficients were calculated to be 5.56% and 6.65% for OTA and FB1, respectively. The interassay coefficients were 10.39% and 10.56% for OTA and FB1, respectively.

To further evaluate the developed detection method for OTA and FB1, 21 naturally contaminated cereal samples were detected by this method and ELISA method. The results are shown in Figure 5 and Table 1S, Supporting Information. The results from the ELISA method indicated that there were 15 positive samples for OTA and 13 positive samples for FB1 in these cereal samples. However, the developed method showed that all these samples gave positive results for OTA and FB1. These negative results from ELISA might be ascribed to the low sensitivity of the ELISA kits. Figure 5 shows good correlation between the developed method and classic ELISA for the positive sample results which are consistent for OTA (Figure 5a) and FB1 (Figure 5b).

At present, the detection principle of suspension arrays for multiplex mycotoxins is mostly based on the reaction of antigen and antibody. For example, the Multi Analyte Profiling (MAP) technology system from Luminex Corporation (Austin, TX) has been commercialized and applied in mycotoxin analysis. The system requires the fluorescence dye beads for encoding the microspheres, a flow cytometer, and their software for extracting the data, which are expensive and complex for screening analysis. In addition, the stability and shelf life of antigen and antibody are obvious inferior to that of the aptamers. Although the present results indicate that this developed aptamer-based suspension array could replace the existing suspension array encoded by fluorescence dye, more validation work on large samples is needed and is underway in our lab.

### CONCLUSION

This work shows a new aptamer-based suspension array for simultaneous quantitative and qualitative detection of OTA and FB1. This developed method supplies a simple, sensitive, cost-effective, specific, and easily applied screening analysis. The recovery rates, repeatability, and reproducibility in the spiked samples shows that the method is reliable and has a great potential application for detection of OTA and FB1 in cereal samples. As the aptamers of other mycotoxins are screened, this aptamer-based suspension array may prove to be a better platform for on-site detection of multiplex mycotoxins.

### ASSOCIATED CONTENT

* Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

### AUTHOR INFORMATION

**Corresponding Authors**

*Tel.: +86 25 83598286; fax: +86 25 83598901; e-mail: jianlinli82003@aliyun.com.
*E-mail: tieszheng@sina.com.

**Author Contributions**

These authors contributed to this work equally and should be regarded as first co-authors.
Notes
The authors declare no competing financial interest.

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