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Simultaneous determination of trace Aflatoxin B_1 and Ochratoxin A by aptamer-based microchip capillary electrophoresis in food samples

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ABSTRACT

An aptamer-based microchip capillary electrophoresis coupled with laser induced fluorescence (MCE-LIF) detection method for fast determination of Aflatoxin B_1 (AFB_1) and Ochratoxin A (OTA) was developed. Aptamers that are specific to these two mycotoxins were first hybridized with their aptamer complementary oligonucleotides. The double strand DNA that comes in contact with mycotoxin-containing environment would be unwound into separate aptamer-mycotoxin complex and aptamer complementary single strand. Different types of oligonucleotides can be separated in MCE and detected under the aid of fluorescent dye SYBR gold in LIF detection unit. Under the optimal conditions, on-chip aptamer-mycotoxin conjugates analysis was achieved within 3 min with extremely low LODs (0.026 ng/mL for AFB_1 and 0.021 ng/mL for OTA). Specificity study indicated that other major mycotoxins would not cross-react with these two aptamers, demonstrating the good selectivity of the proposed method. Quantification of trace AFB_1 and OTA in real food samples was carried out and satisfactory recoveries were obtained. It is demonstrated that this method is fast, facile and specific for Simultaneous determination of trace AFB_1 and OTA from foodstuffs.

1. Introduction

Mycotoxins (aflatoxins, ochratoxins, deoxvnivalenol, zearalenone, fumonisins, trichothecene, citrinin, etc.) are secondary metabolites that are produced by molds and fungus during their growth [1,2]. Aflatoxin B_1 (AFB_1) is produced by *Aspergillus flavus* and *A. parasiticus* and it has been considered as the most toxic and mutagenic aflatoxin among all the aflatoxins (B1, B2, G1, G2, M1, and M2) or even among all the mycotoxins. Its contamination of foodstuffs and animal feeds poses moderate carcinogenic risks to human and animal health [3,4]. AFB_1 has been designated as a group I carcinogenic contamination in food by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) [5]. Ochratoxin A (OTA) is another toxic secondary metabolite that belongs to ochratoxin family (OTA, OTB, and OTC), produced by several mold fungi in the genus of *Aspergillus ochraceus* and *Penicillium verrucosum* [6,7]. OTA has been classified as group IIB toxin and shows the highest nephrotoxic and hepatic effect among all the ochratoxins [8,9]. AFB_1 and OTA are metabolized slowly when ingested and are frequently detected in agricultural commodities such as grains, peanuts, barley, wheat, corn, nuts, when preserved under inappropriate environments (hot and humid conditions) [10–13].

In order to prevent food commodities and animal feeds recall from AFB_1 and OTA contamination and subsequent economic losses, many countries have set extremely low regulation limits for both of these mycotoxins. In more than 99 countries the Maximum Residue Levels (MRLs) of AFB_1 and of total AFs in food are set as 5 and 10–20 ng/g, respectively, whereas the occurrences of them are 2 and 4 ng/g, respectively, in the European Union (EU) regulations [14]. Stricter MTLs, as low as 0.1 ng/g for AFB_1, are set for infant formulas and infant foods in the EU [15]. MRLs for OTA that have been set by the European Commission, under several EC regulations are from 0.5 to 10 ng/g in food commodities such as cereals and cereal products, raisin, roasted and soluble coffee, wine, grape juice, and infant formulas and infant foods [16,17].

Till now, immunologic and chromatographic analysis are two major approaches for AFB_1 and OTA quantification. Enzyme-linked immunosorbent assay (ELISA) has been the mainstream mycotoxin quantification method [18,19]. Antibodies are the most frequently used molecular recognition agents for mycotoxin determination by

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ELISA, however, their poor stability in transportation and storage necessitates high levels of control in transportation and storage. Moreover, ELISA generally requires multiple incubation and washing steps. Chromatographic-based methods including thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and HPLC-mass spectrometry (HPLC-MS) have been developed for AFB_1 and OTA quantification [18,20–25]. These methods often require experienced personnel and are laborious for routine analysis. To overcome these disadvantages, microchip capillary electrophoresis-laser induced fluorescence (MCE-LIF) method offers an excellent alternative [26]. MCE excels in the miniaturization of multiple functional units unto a micron scale channel and has displayed outstanding analytical efficiency in separation and quantification biological samples [27–29]. Among them, DNA or RNA MCE detection has long been proved possible and feasible [30,31].

Aptamer has been seen as an upgraded version of antibody. Nucleic acid aptamers are single stranded DNA or RNA probes that are created *in vitro* by systematic evolution of ligands by exponential enrichment (SELEX) process from a large random sequence pool [32–35]. It also outperforms antibody for aptamer can be entirely edited in a test tube, cheap in production, easy and ultrafast in synthesis, and displays little or no immunogenicity in therapeutic applications [36,37].

Till now, few methods could achieve simultaneous detection while guaranteeing trace quantification of AFB_1 and OTA among both the immunologic and chromatographic assays. In this paper we take full advantage of aptamer as a mycotoxin recognition molecule and as a DNA probe. In recent years, MCE-LIF detection of aptamer have been widely applied on biological samples [38–41]. The whole idea was to detect target analytes that are difficult, or even impossible (e.g. antibiotics, proteins, toxins) to be witnessed on MCE/CE system. By converting signal from analyte to aptamer, the potential of MCE application is significantly broadened. As different length pairs of aptamer reflect different analytes, simultaneous detection of more than one analytes is feasible when the resolution of aptamers is guaranteed. Another merit concerning of this aptamer-based MCE detection lies in the ultrahigh specificity on the target molecules. Thanks to the specificity of aptamer to its target, the problem cross reaction of aptamer to the target analog is alleviated. In this respect, a novel aptamer-based AFB_1 and OTA-MCE detection method was therefore established. As AFB_1 and OTA do not exhibit natural fluorescence under 488 nm laser excitation, we built a signal conversion from mycotoxin (cannot be excited under the MCE-LIFD) to aptamer (strong fluorescence yield under SYBR gold development), and by doing so the aptamer-based mycotoxin detection makes what otherwise invisible visible. The detection strategy was illustrated in Fig.1. When binding together with its aptamer, AFB_1 or OTA, the molecular weight of which is less than 500, is too small to bring any detectable weight and charge change to its aptamer. As the molecular weight of aptamer-mycotoxin complex is approximately close to that of the free aptamer, increasing the molecular weight difference of aptamer-mycotoxin complex from its free aptamer is therefore a novel strategy to distinguish the former from the latter. Here we employed the partially complementary DNA of aptamers (C-aptamer) as a weight distinguisher of free aptamer. Once meeting target molecules, aptamer with C-aptamer hybridization double strand DNA (dsDNA) is prone to be split into C-aptamer single strand DNA (ssDNA) and aptamer-target complex [42]. In this case dsDNA and aptamer-mycotoxin complex would exhibit different electrophoretic mobility (migration time) in MCE-LIF detection. By achieving aptamer-AFB_1 and aptamer-OTA MCE separation, AFB_1 and OTA will be quantified in an unprecedentedly fast manner.

To the best of our knowledge, aptamer-based MCE-LIF for simultaneous detection of AFB_1 and OTA has not been previously described. Hydroxyethyl cellulose (HEC) was used as sieving agent to realize a better aptamer-mycotoxin resolution. Another key parameter to realize aptamer-based MCE-LIF detection is the addition of the nucleotide fluorescent dye in the electrophoretic buffer. In this work, we chose SYBR gold as staining agent for its ultrahigh sensitive single/double strand DNA staining property [43,44]. Optimization of the aptamer-based MCE-LIF detection was carried out by interrogating key factors that affected the overall performances. The optimized assay was further validated by analyzing foodstuff samples that were spiked with known concentrations of AFB_1 and OTA. It is demonstrated that the proposed method displays satisfactory sensitivity, stability, and selectivity for simultaneous detection of AFB_1 and OTA. These advantages enable aptamer-based MCE-LIF detection a potential application in food safety regulation and consumer protection.

2. Materials and methods

2.1. Chemicals and solvents

All chemicals used in this work were of analytical grade or higher. Solutions were filtered against 0.22 μm membrane filters before use. Water used in this work was double distilled water (ddH_2O) (Ulupure, Chengdu, China). Sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium dodecyl sulfate (SDS), tris (hydroxymethyl) aminomethane (Tris), eathylene diamine tetraacetic acid (EDTA), HEC (250,000 Da) were purchased from Tianjin Damao Chemical Reagent Factory (Tianjin, China). TE buffer (10.00 mM Tris−HCl, pH 8.2, 10 mM EDTA) was used as electrophoretic running buffer. AFB_1 and OTA, deoxvnivalenol (DON) and zearalenone (ZEA), fumonisin B1 (FB1), trichothecene T-2 toxin (T-2), citrinin (CIT) were kindly provided by Tongwei Group Co., Ltd. (Chengdu, China). All oligonucleotides (Table S1) used in this work were synthetized in Sangon Biotech (Shanghai, China) according to previous works [45,46]. SYBR Gold and Ultra Low Range DNA Ladder were purchased from Thermo Fisher (Shanghai, China). Rice, corn, peanut, barley, sesame oil, olive oil, peanut oil, and soybean oil were purchased from local markets.

2.2. Apparatus and microfluidic chip

The tests were carried out on a microchip electrophoresis coupled with confocal laser induced fluorescence detection system with excitation wavelength at 488 nm and emission wavelength at 523 nm (Shandong Normal University, Jinan, China), while a six-path high voltage power supply which is variable in the range of 0–5000 V (Shandong Normal University, Jinan, China) was employed for MCE sample loading and separation. The schematic layout of the glass microchip is shown in Fig. 1B. All four reservoirs were 3.0 mm in diameter and 1.5 mm in depth. The cross channels in the bottom glass substrate were etched 25.0 μm in depth and 60.0 μm in width. The sample injection channel was of 10.0 mm in length and the effective separation channel was of 35.0 mm from the injection cross to window of detection.

2.3. Aptamer MCE-LIF analysis

Microchip was stored in 3 M NaOH solution with its channel filled up with 1 M NaOH. During every test, microchip was subsequently conditioned with 10 μL of 1 M NaOH, H_2O, SDS solution, H_2O and TE buffer solution for 1 min, respectively. Sample reservoir was filled with sample solution and the rest three reservoirs with the running buffer. Four platinum electrodes were perpendicularly in contact with four reservoirs. Sample was then introduced into the intersection by pinched injection mode: 400 V at S reservoir, 250 V at Buffer (B) reservoir, 400 V at Buffer Waste (BW) reservoir,

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