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A simple aptamer-based fluorescent assay for the detection of Aflatoxin B_1 in infant rice cereal

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ABSTRACT

A fluorescent assay for the rapid, sensitive and specific detection of Aflatoxin B_1 (AFB_1) was developed in this study. Initially, a DNA/DNA duplex was formed between a fluorescein-labeled AFB_1 aptamer and its partially complementary DNA strand containing a quencher moiety, resulting in fluorescence quenching due to the close proximity of fluorophore and quencher. Upon the addition of AFB_1, an aptamer/AFB_1 complex was generated to release the quencher-modified DNA strand, thus recovered the fluorescence of fluorescein and enabled quantitative detection for AFB_1 by monitoring fluorescence enhancement. Under optimized conditions, this assay exhibited a linear response to AFB_1 in the range of 5–100 ng/mL with a detection limit down to 1.6 ng/mL. Trials of this assay in infant rice cereal with satisfactory recovery in the range of 93.0%–106.8%, demonstrate that the new assay could be a potential sensing platform for AFB_1 determination in food.

1. Introduction

Mycotoxins are the toxic secondary metabolites produced by a variety of molds, which could cause serious problems to human health through contamination of various foods and animal feeds (Atar, Eren, & Yola, 2015; Mata et al., 2015; Zhu et al., 2015). In recent years, aflatoxins, as the most predominant and toxic mycotoxins, have raised global concern by being frequently detected in agricultural products and causing subsequent economic losses (Pietri, Fortunati, Mulazzi, & Bertuzzi, 2016; Zhang et al., 2016). Among several types of aflatoxins (B_1, B_2, G_1, G_2, M_1, and M_2), Aflatoxin B_1 is known as the most potent carcinogen, and has been classified as a group 1 carcinogen by the International Agency for Research on Cancer (Lee, Her, & Lee, 2015). Some agriculture products, such as peanuts, corn, and cereals, are especially vulnerable to contamination by AFB_1 (Chen et al., 2014; Iqbal, Rabbani, Asi, & Jinap, 2014; Zhang et al., 2016). Therefore, the European Commission has regulated a maximum contamination level for AFB_1 (2 μg/kg) and total aflatoxin (4 μg/kg) in all cereals and cereal-derived products to protect consumers' health and prevent food safety issues (Commission, 2010). Given of the low permissible limit and severe toxicity of AFB_1, rapid, sensitive and specific analytical methods for AFB_1 determination are vitally important.

Analytical methods based on thin layer chromatography (TLC) (Var, Kabak, & Gök, 2007), high-performance liquid chromatography (HPLC) (Herzallah, 2009; Yazdanpanah et al., 2013), and liquid chromatography combined with mass spectrometry (LC–MS) (Abia et al., 2013; Warth, Sulyok, & Krska, 2013) have been developed for the detection of AFB_1. However, these typical instrumental methods usually require special equipments and professional personnel, as well as time-consuming sample pretreatment (Shim et al., 2007). Meanwhile, rapid detection methods based on immunoassays have been widely used for AFB_1 measurements in food and agricultural products (Mozaffari Nejad, Sabouri Ghannad, & Kamkar, 2014; Sheng et al., 2014; Xu et al., 2014). These antibody-based detection methods, while highly selective, are often limited by high cost and easy-to-denature during storage, making them difficult for real-time and on-site detection (Huang, Zhao, Chen, Shi, & Liang, 2012). As an alternative method to antibody for small molecules recognition, aptamer is a single-stranded DNA or RNA oligonucleotides, which could bind to a target with very high affinity and specificity. With the advantages of less

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expensive, facile synthesis, ease modification, and high stability under non-physiological conditions (Jayasena, 1999), aptamer-based biosensors have been developed for the detection of mycotoxin, such as ochratoxin A (OTA) and fumonisin B_1 (FB_1) (Z. Guo, Ren, Wang, & Wang, 2011; Kuang et al., 2010; Wu et al., 2012, 2013; Yang, Lates, Prieto-Simón, Marty, & Yang, 2013). In 2012, specific aptamers to AFB_1 had been patented by Neoventures Biotechnology Inc. (Canada) (Patent: PCT/CA2010/001292), after which studies on aptasensor for AFB_1 determination have emerged and successfully applied to agricultural products (Castillo et al., 2015; Evtugyn et al., 2014; Seok, Byun, Shim, & Kim, 2015; Shim, Kim, Mun, & Kim, 2014; Wang et al., 2016). For example, an aptamer-based dipstick assay based on a competitive reaction of the biotinylated form of aptamer and cy5-modified DNA probes has been recently developed for detection of AFB_1, achieving a LOD of 0.32 nM (Shim, Kim, et al., 2014). Fluorescent nitrogen-doped carbon dots (N,C-dots) were assembled on aptamer modified gold nanoparticles (Aptamer/AuNPs) for sensitive detection of AFB_1 (Wang et al., 2016). Previously, our group reported an ultrasensitive aptasensor based on configurational changes of the aptamer specific to AFB_1 immobilized in PCR tubes (Guo et al., 2014). However, the reported aptamer assays for AFB_1 based on real-time quantitative polymerase chain reaction (RT-qPCR) are time-consuming, either require complicated sample preparation or long incubation time in rigorous conditions. Therefore, simple, rapid and low-cost methods for quantifying AFB_1 levels in food and animal feeds are still challenging.

In this study, we report a novel fluorescent assay for the detection of AFB_1 using a fluorophore-labeled AFB_1 aptamer and its partially complementary DNA strand (denoted as cDNA) that is covalently modified with a quencher. In the absence of AFB_1, the aptamer naturally binds to cDNA, bringing the fluorophore and quencher into close proximity to induce highly efficient fluorescence quenching. After introducing AFB_1, the aptamer prefers to form an aptamer/AFB_1 complex rather than the DNA/DNA duplex, triggering the release of cDNA, which is accompanied by the increase of fluorescence. The whole sensing procedures are simple, rapid, and shows high specificity to AFB_1, which offers great potential for on-site analysis. Measurement of AFB_1 levels in spiked infant rice cereal samples is also achieved, demonstrating the practicability of this approach.

2. Experimental

2.1. Materials and reagents

Aflatoxin B_1 (AFB_1), Ochratoxin A (OTA), Aflatoxin M_1 (AFM_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1), Aflatoxin G_2 (AFG_2), Zearalenone (ZEA) and Fumonisin B_1 (FB_1) were purchased from the National Standard Reference Center (Beijing, China). Other chemical reagents such as sodium chloride (NaCl), potassium chloride (KCl), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), and anhydrous calcium chloride ($CaCl_2$) were purchased from Shanghai Chemical Reagent Company (Shanghai, China). Infant rice cereal samples (Nestle and Heinz) were purchased from local supermarket. Water was purified using a Milli-Q purification system. Tris-HCl buffer (10 mM Tris, 120 mM NaCl, 5 mM KCl, 20 mM $CaCl_2$, at pH 7.0) was used for fluorescent assay.

The oligonucleotides used in this work were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China) and purified with HPLC. The sequence of AFB_1 aptamer was according to Patent: PCT/CA2010/001292 with modification (Guo et al., 2014). The sequences were as follows:

AFB_1 Aptamer: 5′-GT TGG GCA CGT GTT GTC TCT CTG TGT CTC GTG CCC TTC GCT AGG CCC-FAM-3′

Partially Complementary DNA (cDNA):cDNA1: 5′-TAMRA-GGG CCT AG-3′cDNA2: 5′-TAMRA-GGG CCT AGC-3′cDNA3: 5′-TAMRA-GGG CCT AGC G-3′cDNA4: 5′-TAMRA-GGG CCT AGC GA-3′cDNA5: 5′-TAMRA-GGG CCT AGC GAA-3′

2.2. Apatmer-based fluorescent assay for AFB_1 detection

AFB_1 aptamer and cDNA were dissolved and diluted with Tris-HCl buffer. Then, 500 μL of AFB_1 aptamer solution (10 nM) was mixed with 500 μL of cDNA at molar ratios of 1:1, 1:2, 1:3, 1:4 and 1:5 (unless stated otherwise, a molar ratio of 1:2 was used for AFB_1 analysis). The mixture was heated to 88 °C for 5 min and incubated at room temperature for 30 min. Then, 500 μL of AFB_1 standard solution of different concentrations was added to the mixture, giving a final reaction volume of 1.5 mL. After votexing, the fluorescence intensity at excitation/emission wavelength of 495/520 nm was recorded by F-7000 fluorophotometer (Hitachi, Japan). Each concentration was measured for five times. Considering aflatoxin is a highly fluorescent substance (Ex/Em: 360/420 nm for AFB_1), the background fluorescence signal of AFB_1 might overlap with the fluorescence signal of FAM modified aptamer. To avoid the influence of background fluorescence signal of aflatoxins, we used background correction method by measuring the fluorescence of the control, which contains AFB_1 and the buffer, but without FAM modified AFB_1 aptamer and cDNA.

2.3. Specificity analysis

To prove the ability of this method for highly selective detection of AFB_1 over other mycotoxins, 40 ng/mL of OTA, ZEA, FB_1, AFM_1, AFB_2, AFG_1 and AFG_2 were measured at the same conditions as AFB_1.

2.4. Analysis of AFB_1 spiked infant rice cereal samples

The practicability of this assay was verified by quantitative detection of AFB_1 in two brands of infant rice cereal samples that were spiked with known concentration of AFB_1. Each sample was spiked with AFB_1 at concentrations of 5, 10, 20 μg/kg, respectively. Each sample was accurately weighed (2.00 ± 0.05 g) and 10 mL of extraction solution (methanol: water, 6:4) was then added to extract AFB_1 from the sample. The entire mixture was vortexed for 5 min using a Vortex-Genie 2 (Scientific Industries, USA) and then centrifuged at 10,000*g* for 10 min. The supernatant was collected, filtered through a 0.45 μm filter, and diluted with Tris buffer (1:5, v:v) for recovery experiments. Five repeats were measured for each sample to assess the accuracy of the assay.

3. Results and discussion

3.1. Design of the aptamer-based fluorescent sensing platform

Schematic illustration for the design of fluorescent assay for AFB_1 detection was displayed in Fig. 1A. In this sensing system, a AFB_1 aptamer was labeled with Carboxyfluorescein (FAM) and its partially complementary DNA was modified with Carboxytetramethylrhodamine (TAMRA) quenching group. Without introducing AFB_1, the hybridization of AFB_1 aptamer and cDNA resulted in a close proximity for FAM and TAMRA quenching group. In this case, the fluorescence of FAM was effectively quenchend. Upon the addition of AFB_1, structural switch of AFB_1 aptamer was induced, leading to the formation of AFB_1/aptamer complex (Seok et al., 2015). Thereby, the cDNA was dehybridized from AFB_1 aptamer and recovered the fluorescence of FAM. To confirm that the presence of AFB_1 could result in dehybridization of aptamer/cDNA