Talanta 189 (2018) 442–450

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Direct fluorescence anisotropy approach for aflatoxin B1 detection and affinity binding study by using single tetramethylrhodamine labeled aptamer

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ARTICLE INFO

Keywords:
Aptamer
Fluorescence anisotropy
Fluorescence polarization
Aflatoxin
Affinity ligand
Binding affinity

ABSTRACT

The discovery of aptamers for aflatoxin B1 (AFB1), one of toxic carcinogens, has allowed to develop aptamer-based sensors and assays for aflatoxin. In this work, we reported a direct fluorescence anisotropy (FA) assay for investigation of aptamer-AFB1 binding and detection of AFB1 with the aptamer having single tetramethylrhodamine (TMR) label on a specific site. From a series of labeling sites of a 50-mer aptamer, we screened out the aptamer with TMR labeling at the 26th T, capable of generating good and large FA-decreasing response to AFB1. By using the T26-labeled 50-mer aptamer probe in FA analysis, we determined the affinity and selectivity of aptamer, and identified the crucial region of aptamer and optimum experimental conditions for strong binding. The aptamer could be further truncated to as short as 26 nucleotides in length, and this shorter aptamer possessed a simple stem-loop secondary structure and retained good binding affinity. Nucleotides in the loop region of the aptamer were conserved and important for affinity recognition. We achieved FA detection of AFB1 with a detection limit about 2 nM by using the TMR-labeled aptamer probe. The cross reactivity of aflatoxin B1, aflatoxin B2, aflatoxin M1, aflatoxin M2, aflatoxin G1, and aflatoxin G2 with aptamer were estimated to be 100%, 61%, 23%, 21%, 6.3%, 6.5%, respectively. The aptamer probe presented good selectivity over other mycotoxins and showed potential in complex sample analysis. This study of affinity binding between aptamer and aflatoxins will be helpful for developing other aptamer-based assays and sensors for aflatoxins.

1. Introduction

Aflatoxins, secondary metabolites of *Aspergillus flavus* and *A. parasiticus*, have attracted great concern since they were discovered with the outbreak of Turkey-X disease in the UK [1]. Aflatoxin B1, B2, G1, G2, M1 and M2 are the most common aflatoxins, and aflatoxin B1 (AFB1) has been recognized as the most toxic and powerful human carcinogen [2,3]. AFB1 exposure may cause numerous healthy effects on human and animals, such as immunosuppression, anemia, reduced growth rate of animals, liver damage, and even liver cancer in human beings [4–6]. Various food products, such as corn, wheat, peanuts, soybeans, cooking oil, alcoholic beverages, poultry feeds, and etc., are susceptible to AFB1 contamination, which also leads to great economic losses. Considering the toxicity and frequent contamination of AFB1 in food materials, rapid and sensitive detection of AFB1 is important in food safety and environmental analysis. Typical methods for AFB1 quantitative analysis include chromatographic methods and immunoassays [6–9], but they meet some limitations in high cost, time consuming processes, or poor stability of antibodies.

Aptamers, artificial nucleic acid ligand, show advantages in biological sensing and assays [10–13]. Compared with antibodies, aptamers can be chemically synthesized in vitro with low cost, precisely labeled with functional groups, and show good thermostability. Aptamer based assays have been applied to many targets, like proteins, metal ions, small molecules, cells, virus, and etc [12–18]. Different aptamers against AFB1 have been reported [19,20], and since then the aptamer-based assays for AFB1 have attracted broad and growing attentions, and have been developed with different methods, such as fluorescence [20–23], colorimetry [24], electrochemistry [25], surface plasma resonance (SPR) [26], pH meter [27], surface-enhanced Raman scattering (SERS) [28], photoelectrochemistry [29], real time quantitative polymerase chain reaction (RT-qPCR) methods [30], and etc. Among these

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https://doi.org/10.1016/j.talanta.2018.07.036
Received 11 April 2018; Received in revised form 7 July 2018; Accepted 11 July 2018
0039-9140/

assays, a 50-mer DNA aptamer (5′-GTT GGG CAC GTG TTG TCT CTC TGT GTC TCG TGC CCT TCG CTA GGC CCA CA-3′) has been frequently used for detection of AFB1 [19]. The aptamer-AFB1 binding is the basis of versatile assays for AFB1, but the characterization of affinity binding between this aptamer and AFB1 and investigation of key factors influencing the affinity binding are still limited.

Herein, we aimed to develop a direct fluorescence anisotropy (FA)/fluorescence polarization (FP) approach for systematic characterization of the aptamer-AFB1 binding and fabricating a simple and sensitive FA assay for AFB1 detection by using tetramethylrhodamine (TMR)-labeled aptamer. FA assay relies on the change of FA signals of fluorophore in the binding event, which is usually related with the change of rotation of fluorophore. The FA assay exhibits numerous advantages, such as simplicity, accuracy, reproducibility, rapidity, easy operation, homogenous assay format, and etc [31–34]. The use of aptamers has allowed to establish unique formats of FA assays for small molecules with dye-labeled aptamers, which usually are not available for antibody based FA methods [35–44]. Direct FA assays for small molecules are much simpler and more desired than the competitive FA assays, without requiring proteins or nanomaterials for signal generations and amplifications. Ruta et. al. have demonstrated a direct FA method for detection of tyrosinamide and argininamide based on the unique conformational flexibility of terminal labeled aptamer [35]. Taking advantage of target-binding induced changes of aptamer conformation and the intramolecular interaction between TMR and guanine (G) bases of aptamer, our group has developed direct FA assays for a few small molecules, e.g. ochratoxin A, adenosine triphosphate (ATP), cocaine, and vasopressin, using TMR-labeled aptamers [36–39].

To achieve FA sensing the aptamer-AFB1 interaction and developing direct FA analysis of AFB1 with aptamers, in this work we conjugated single TMR on a specific site of the 50-mer aptamer against AFB1, and tested the FA responses of a series of aptamer probes upon AFB1 binding. We found that TMR labeling on the 26th T base of the 50-mer aptamer allowed the aptamer probe to generate a remarkable and sensitive FA-decreasing response upon AFB1 binding. By using this responsive aptamer probe we fully investigated the affinity binding between the aptamer and AFB1, determined the binding affinity of aptamers, tested selectivity of aptamer binding, and identified the key sequence of the aptamer and experimental conditions for AFB1 recognition.

In our study, the 50-mer aptamer probe showed high binding affinity to AFB1, and the dissociation constant (K_d) reached tens nM level, which was also confirmed by isothermal titration calorimetry (ITC) experiments. The 50-mer aptamer could be shortened into a 26-mer DNA by truncating 24 nucleotides off the sequence, without disrupting the binding affinity to AFB1. The 50-mer and the 26-mer DNA aptamers share a simple stem-loop structure. We identified that the nucleotides in the loop region were crucial for affinity binding and the stable stem-loop structure of aptamer was important for maintaining strong binding affinity with AFB1. The aptamer showed similar high binding affinities to AFB1 and AFB2, and it interacted with AFM1 and AFM2 with relatively weaker affinity, while the aptamer bound to AFG1 and AFG2 with greatly reduced affinity. The aptamer did not bind to other mycotoxins, like ochratoxin A (OTA), ochratoxin B (OTB), fumonisin B1 (FB1), fumonisin B2 (FB2) and zearalenone (ZAE). By using the TMR-labeled aptamer probe, we achieved the direct sensitive FA detection of AFB1 ranging from 2 nM to 1 μM under optimized conditions. The direct FA assay also enabled the detection of AFB1 spiked in complex sample matrix, e.g. diluted human serum, urine, red wine, beer and corn flour samples.

2. Materials and methods

2.1. Materials and reagents

Aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin M1 (AFM1), aflatoxin M2 (AFM2), ochratoxin A (OTA), ochratoxin B (OTB), fumonisin B1 (FB1), fumonisin B2 (FB2), zearalenone (ZAE) were purchased from Pribolab (Singapore). All DNA oligonucleotides were synthesized and purified by Sangon Biotech (Shanghai, China). The sequences of TMR labeled DNA probes were listed in Table S1, Table S2 and Table S3. How to conjugate TMR on the different positions of the aptamer sequence was shown in Fig. S1 in Supplementary material. The reaction between the amino group introduced on the aptamer sequence and the carboxyl group of TMR was applied in the conjugation procedure.

Human serum was obtained from Zhongke Chenyu Biotechnology (Beijing, China). Corn flour, red wine and beer samples were bought from the local market. Immunoaffinity columns (IAC) specific to AFB1 were purchased from Pribolab (Singapore). All solutions were prepared by using ultrapure water from a Purelab Ultra Elga Labwater system (Ultra Genetic). All other reagents were of analytical grade.

2.2. Fluorescence anisotropy analysis

Except for otherwise statements, fluorescence anisotropy (FA) analysis was performed in a fluorescence spectrometer (JASCO FP-8300 or HORIBA FluoroMax-4 spectrofluorometer). FA value was calculated by the following equation, $r = (I_{VV}\text{-}GI_{VH})/(I_{VV} + 2GI_{VH})$, where I_{VV} and I_{VH} corresponded to the emission intensities collected from the channels that are parallel (I_{VV}) and perpendicular (I_{VH}) to the vertical electric vector of the excitation, respectively. G-factor was used to correct the bias between different instruments. G-factor was calculated by the ratio of I_{HV} and I_{HH}, which corresponded to the emission intensities collected from the perpendicular (I_{HV}) and parallel (I_{HH}) channels to the horizontal orientation of the excitation, respectively. FA analyses of AFB1 using TMR-labeled aptamer probe were performed at 25 °C. AFB1 was incubated with 25 nM fluorophore labeled aptamer probes in binding buffer containing 10 mM HEPES (pH 7.0), 20 mM $MgCl_2$ and 0.1% Tween-20 (200 μL). After sample incubation for 10 min, FA measurements were conducted with excitation at 490 nm, and emission at 520 nm. Slits for the excitation and emission were both set at 5 nm. The average value collected by five times was used, and the relative standard deviation (RSD) was calculated. With FA analysis to AFB1, we also estimated the dissociation constants (K_ds) of dye labeled aptamers, which were determined by a non-linear regression analysis with GraphPad Prism software [35].

2.3. FA analysis of AFB1 in complex sample matrix

Prior to analysis, human serum, urine sample and red wine were centrifuged at 12000 rpm for 15 min, and then filtered with 0.22 μm membrane. After 20-fold (human serum and urine sample) or 10-fold (red wine) dilution by the binding buffer (10 mM HEPES (pH 7.0), 20 mM $MgCl_2$ and 0.1% Tween-20), the filtrate was used as complex sample matrix. For beer sample, it was taken into a beaker and sonicated for 2 h, and then cooled at 4 °C for 30 min. After that, the beer sample was filtered with a 0.22 μm membrane and diluted 2-fold by the binding buffer. Finally, various concentrations of AFB1 were spiked in these diluted samples and incubated with 25 nM A50-T26-TMR, and then the FA of A50-T26-TMR was measured.

For AFB1-positive corn flour samples, certain amounts of AFB1 were added in 1 g of corn flour to achieve final concentrations of 0, 10, 20, 50, 100 and 200 ng/g, respectively. Each spiked corn flour sample was extracted by 3 mL of methanol/water (7:3, v:v) for 2 min under intensely shaking and centrifuged at 12000 rpm for 15 min. Then the collected supernatant was filtered with a 0.22 μm membrane and diluted to 10 mL by ultrapure water. Afterwards, the diluted sample was put into an immunoaffinity column (IAC) of AFB1 and flowed past slowly. With this, AFB1 spiked in corn flour samples was captured and separated from other substances in extract. Then 1 mL of methanol was used to elute AFB1. The obtained solution was diluted 5-fold by the