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Development of aflatoxin B_1 aptasensor based on wide-range fluorescence detection using graphene oxide quencher

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ABSTRACT

Aflatoxin B_1 (AFB_1) is a carcinogenic substance produced by fungi of genus *Aspergillus*, especially *Aspergillus flavus*. Few nanograms of AFB_1 that permeated through the skin is sufficient to cause liver cancer and stunted growth. In this study, a rapid aptamer-based assay for AFB_1 was developed using the fluorescence quenching property of graphene oxide (GO) and a fluorescein amidite (FAM)-modified aptamer specific to AFB_1. The aptamer, modified with the fluorescence dye FAM on its 5′-end, was used as a probe. Once bound by AFB_1, a conformational change of the aptamer was caused that led to its interaction with the well-known fluorescence quencher GO, resulting in a decrease of the fluorescence intensity of the system. In the absence of AFB_1, the fluorescence intensity remained unchanged. The aptamer-based AFB_1 assay process was conducted through 3 steps within 40 min. The aptamer was incubated with AFB_1 before the addition of GO. The amount of AFB_1 present was measured by the change in fluorescence intensity. The detection system was evaluated with standard solutions of AFB_1 of various concentrations. The results showed that the fluorescence intensity decreased linearly as the concentration of AFB_1 gradually increased. Although the assay was specific to AFB_1, there was slight interference by other types of aflatoxin. When the assay was applied to a real sample, the limit of detection was 4.5 ppb, which was within the wide detection range of up to 300 ppb with good linearity. Thus, this biosensor is considered to be competitive with the conventional detection methods in the field owing to its wide detection range and assay rapidity.

1. Introduction

Aflatoxin B_1 (AFB_1), one of the most common mycotoxins, is a highly toxic substance produced by fungi such as *Aspergillus flavus* [1]. It is classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC), and only few nanograms are enough to cause liver cancer and stunted growth by permeation through the skin [2]. The European Union established the maximum permitted level of AFB_1 in nuts, dried fruits, cereals, and spices to be 12 ppb, whereas that in infant foods was set at 0.1 ppb [3].

Chromatographic methods such as high-performance liquid chromatography (HPLC) and gas chromatography have been generally used to quantify AFB_1 in foods [4]. Although these methods provide reliable qualitative and quantitative results, they require well-trained professionals, high-cost instruments and materials, and long times for analysis. Immunogenic detection methods such as the enzyme-linked immunosorbent assay have shown high sensitivity [5], but the stability, productibity, and high cost of the antibodies limit the usability of the methods. To overcome such limitations, numerous detection methods using other types of probes instead of antibodies have been studied recently [6–9].

Aptamers are short sequences of DNA or RNA that specifically bind to a variety of target molecules, ranging from small molecules to proteins [10]. Aptamers are economical, and easily synthesized, modified, and reproduced, and show good stability against thermal stress [11,12]. Therefore, in many studies, aptamers have been applied as probes for detecting numerous types of targets [13–17]. An aptamer that binds specifically to the AFB_1 molecule was used as a probe in this study.

Graphene oxide (GO) is a single-layer, two-dimensional material that possesses extraordinary electrical, thermal, and mechanical properties, and has been used for a variety of applications such

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as sensors and nanocomposites [18]. It can be used as an excellent anchor for biomolecules, owing to its large surface area (up to 2630 m^2/g) and unique sp^2 (sp^2/sp^3)-bonded network [19]. These properties along, with its great fluorescence quenching ability, prompted us to use GO as a platform for aptamers in this study.

Fluorescence sensors have many advantages, such as their low cost, high sensitivity, simplicity, and rapidity. During the past decade, numerous methods with high sensitivity based on fluorescence detection have been introduced, including DNA sequencing, DNA fragment analysis, and a variety of fluorescence immunoassays. Fluorescence is more sensitive than absorption because of the way it is measured. Absorbance is measured by comparing the difference in intensities between the reference and the sample. On the other hand, fluorescence was measured directly from the sample itself, which provides higher sensitivity and simplicity [20]. Compared with other analytical methods such as HPLC and mass spectrometry, the assay methods using direct measurement of fluorescence from samples require only a simple device that can be portable. Thus, sensitivity and portability, the most important attributes of biosensors, can be satisfied by the fluorescence sensor.

In this study, we developed a simple and rapid biosensor for AFB_1 based on the fluorescence-quenching ability of GO, and using a fluorescein amidite (FAM)-labeled AFB_1-specific aptamer as the probe. As shown in Fig. 1, when the labeled aptamer binds to an AFB_1 molecule, its conformation changes, allowing the aptamer-AFB_1 complex to interact with GO, which then quenches or decreases the fluorescence intensity. In the absence of AFB_1, there is no interaction of the probe with GO and the fluorescence intensity therefore should remain unchanged.

2. Materials and methods

2.1. Materials and reagents

Potassium persulfate ($K_2S_2O_8$), concentrated sulfuric acid (c-H_2SO_4), phosphorus pentoxide (P_2O_5), permanganate ($KMnO_4$), and AFB_1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Graphite powder was obtained from Kanto Chemical (Kanto, Japan). Hydrochloric acid (HCl), 30% hydrazine solution (NH_2NH_2), and hydrogen peroxide (H_2O_2) were purchased from Samchun Chemicals (Seoul, Korea). The 0.1-μm filter papers were purchased from Whatman GmbH (Dassel, Germany). Methanol, ethanol, and isopropanol were purchased from Merck KGaA (Darmstadt, Germany). The FAM-modified AFB_1 aptamer (5′-FAM-AAA AAA AAA AGT TGG GCA CGT GTT GTC TCT CTG TGT CTC GTG CCC TTC GCT AGG CCC ACA-3′, PCT/CA2010/001292, 2010) was synthesized by Bioneer (Daejeon, Korea). Ultrapure water was purchased from Welgene (Gyeongsan, Korea). An AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) mixture was purchased from Romer Labs (Union, MO, USA). The GO and solvents were diluted with deionized (DI) water that had been purified with a Milli-Q system (Millipore, Billerica, MA, USA). The T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) was used for the incubation steps. The Synergy H1 Multi-Mode Reader (BioTek Synergy H1, Winooski, VT, USA) was used for measuring fluorescence intensity.

2.2. Preparation of graphene oxide

GO was synthesized from natural graphite powder using a modified Hummers' method [21,22]. In brief, 10 mL of concentrated sulfuric acid was added to a 250 mL round flask, and P_2O_5 (2 g), $K_2S_2O_8$ (2 g), and graphite powder (2 g) were subsequently added. The mixture was stirred at 85 °C for 5 h and then cooled to room temperature, after which DI water (100 mL) was added slowly into the flask. The mixture was stirred for 14 h to allow for complete peroxidation of the graphite and was then filtered through a 0.1-μm filter and dried at room temperature. The peroxidized graphite was then added to chilled c-H_2SO_4 (100 mL), following which $KMnO_4$ (10 g) was added slowly under stirring for 1 h. Then, the solution was stirred vigorously at room temperature for 24 h. DI (500 mL) water and 30% H_2O_2 (20 mL) were then sequentially added. In this step, the color of the solution changed from dark brown to yellow. The GO solution was filtered and washed with 10 wt% HCl (1 L) and DI water to remove excess ions and other impurities. Finally, the pure GO was dried at 60 °C in a dry oven.

2.3. Optimization of aptamer concentration

To optimize the aptamer concentration, the GO concentration was fixed as 20 μg mL^{-1}, whereas that of the aptamer was differentiated as 0.25, 0.3, 0.35, 0.4, and 0.45 μM. After mixing the GO with each aptamer concentration and incubating at 37 °C, respectively, the fluorescence was measured every 5 min with the multi-plate reader. At the same time, the fluorescence of a control without GO was measured for each concentration of aptamer to compare the quenching efficiencies. The results were processed as follows: $\Delta I = 100\% \times (F_0 - F_1)/F_0$ (F_0 = fluorescence intensity of aptamer only; F_1 = fluorescence intensity of the aptamer with GO).

2.4. Optimization of solvent condition

To optimize the solvent condition, the final aptamer concentration was fixed as 0.45 μM. AFB_1 (15 ppb) was spiked onto rice seeds and extracted with 50% (v/v) methanol, ethanol, or isopropanol. The extracts (45.5 μL) were incubated with the aptamer (10 μM, 4.5 μL) for 30 min at 45 °C. Then, GO (20 μg mL^{-1}, 50 μL) was added and the mixture was incubated for 10 min at 37 °C. Finally, the fluorescence intensities were measured with the multi-plate reader. Methanol solutions at concentrations of 0%, 10%, 30%, 50%, and 70% (v/v) were prepared and used to dissolve a standard solution of AFB_1 as well as to extract AFB_1 from spiked rice seeds. The exactly same GO-aptamer reaction process was then performed with the extracts and standard solutions, and the results of the extract and standard solution in the same concentration of methanol were compared.

2.5. Aptamer-mediated AFB_1 assay

The aptamer (10 μM, 4.5 μL) and AFB_1 (45.5 μL) were mixed and incubated for 30 min at 45 °C. GO (20 μg mL^{-1}, 50 μL) was added and the mixture was incubated for 10 min at 37 °C. Finally, the fluorescence intensity was evaluated three times with the multi-plate reader.

2.6. Selectivity test

Four different aflatoxins (viz., AFB_1, AFB_2, AFG_1, and AFG_2) were examined to confirm the selectivity of the biosensor. AFB_1 and AFG_1 were evaluated at the concentration of 200 ppb, whereas the respective concentration of AFB_2 and AFG_2 was 50 ppb. The procedure of the selectivity test was the same as the assay process.

2.7. Real sample test with rice seeds

Rice seeds were spiked with AFB_1 at the concentrations of 0, 5, 10, 15, 20, 30, 40, 50, 60, 200, and 300 ppb, respectively. AFB_1 was first diluted in absolute ethanol and then spiked onto the rice seeds by vortexing for 5 min. The spiked rice seeds were dried completely, and AFB_1 on the rice seeds was then extracted with the optimized concentration of methanol. The extracts were examined with the assay procedure described in Section 2.5.

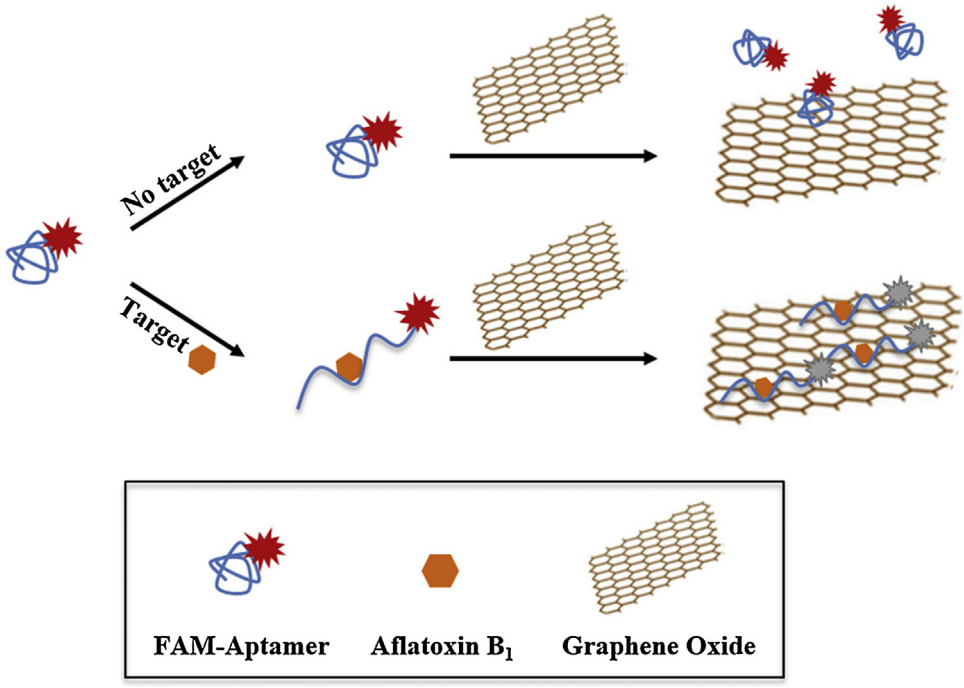

Fig. 1. Schematic illustration of the AFB_1 detection principle using graphene oxide (GO) and a 5′ FAM-modified aptamer.

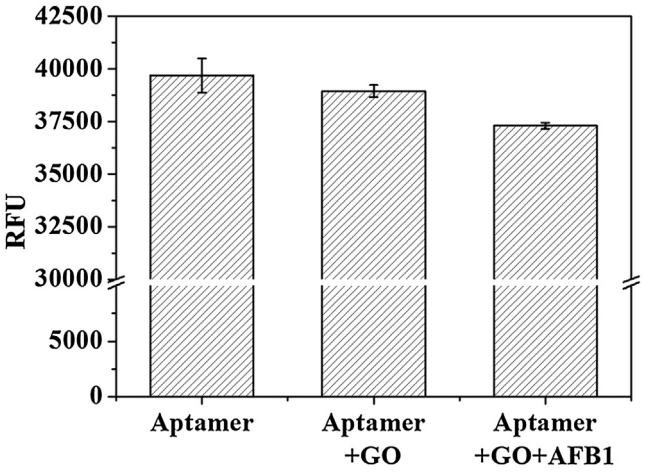

Fig. 2. Fluorescence emission spectra of the aptamer, the aptamer with GO, and the aptamer with GO and AFB_1 in methanol (n = 3).

3. Results and discussion

3.1. Examination of detection principle

The prepared GO showed a wide absorbance peak, as shown in Fig. S1a (see Supporting Information). The strong absorption peak at 230 nm and the shoulder peak at 300 nm corresponded to π-π^* transition of the C=C bonds and n-π^* transition of the C=O bonds of GO, respectively [23]. The Raman spectrum of GO showed a significant D band and 2D band (Fig. S1b). These results proved that GO was synthesized successfully. FAM showed maximum fluorescence emission at 520 nm with excitation at 483 nm [24–26]. The results implied that GO could absorb emitted energy from FAM because its absorbance spectrum included that of FAM [27,28].

When the FAM-modified aptamer was mixed with GO only, the fluorescence intensity decreased slightly, whereas addition of AFB_1 caused a significant decrease in the fluorescence intensity (Fig. 2). The secondary structure of the free aptamer was predicted to be folded (Supplementary Fig. S2), thus preventing it from attaching easily to GO (as long as the aptamer maintained its conformation) and resulting in the retention of total fluorescence. On the contrary, the binding of an AFB_1 molecule to the aptamer caused a conformational change of the latter into a secondary structure that was likely unfolded [29]. This resulted in the easy interaction of the aptamer–AFB_1 complex with GO, and a decrease in the total fluorescence intensity ensued as a result of GO absorbing the fluorescence emitted from the FAM-modified aptamer.

3.2. Optimization of the assay condition

To determine the optimal aptamer concentration for the biosensor, five different concentrations of aptamers were examined (Fig. 3a). The change of fluorescence intensity after GO addition kept increasing gradually with increasing aptamer concentration. The fluorescence intensity was also measured every 5 min after GO addition to determine the optimum incubation time for the assay. As shown in Fig. 3b, there was not much difference in intensity caused by GO after 10 min with 0.45 μM of aptamer. Therefore, the optimum assay condition was fixed as incubation of 0.45 μM of aptamer with GO for 10 min.

The solvent condition was another key parameter for the assay results. Methanol has generally been used for extracting AFB_1 from cereals. Thus, three different types of solvents (viz., methanol, ethanol, and isopropanol) were evaluated to determine the proper AFB_1 extraction condition for the assay. As shown in Fig. 4a, the fluorescence intensity decreased as concentration of AFB_1 in methanol increased, whereas the signal increased with increasing concentration of AFB_1 in isopropanol. With a longer alkyl chain, the slope of the plot increased from being negative to positive. The alkyl groups of alcohols can affect the trend depending on the concentration of aptamer. Finally, spiked rice seed extracts in different concentrations of methanol were compared to select a concentration of methanol that showed the best extraction efficiency. The AFB_1 samples extracted from spiked rice seeds with 0%, 10%, 30%, 50%, and 70% (v/v) methanol were compared with the standard solutions of

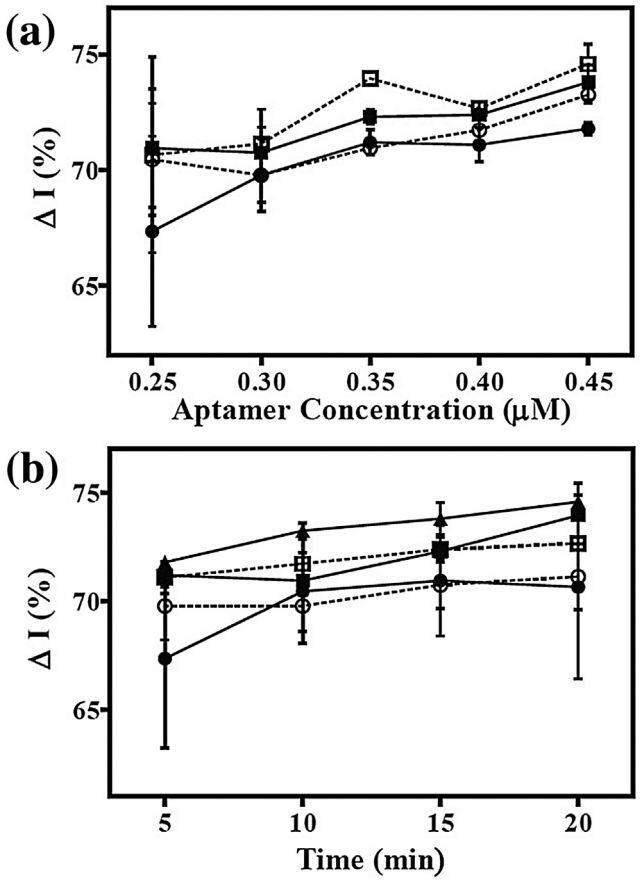

Fig. 3. Optimization of the aptamer concentration for the assay. (a) Plot of fluorescence intensity against the aptamer concentration at various incubation times: 5 min (-●-), 10 min (-○-), 15 min (-■-), and 20 min (-□-) (n = 3). (b) Plot of fluorescence intensity against incubation time with 0.25 (-●-), 0.3 (-○-), 0.35 (-■-), 0.4 (-□-), and 0.45 (-▲-) μM of aptamer (n = 3).

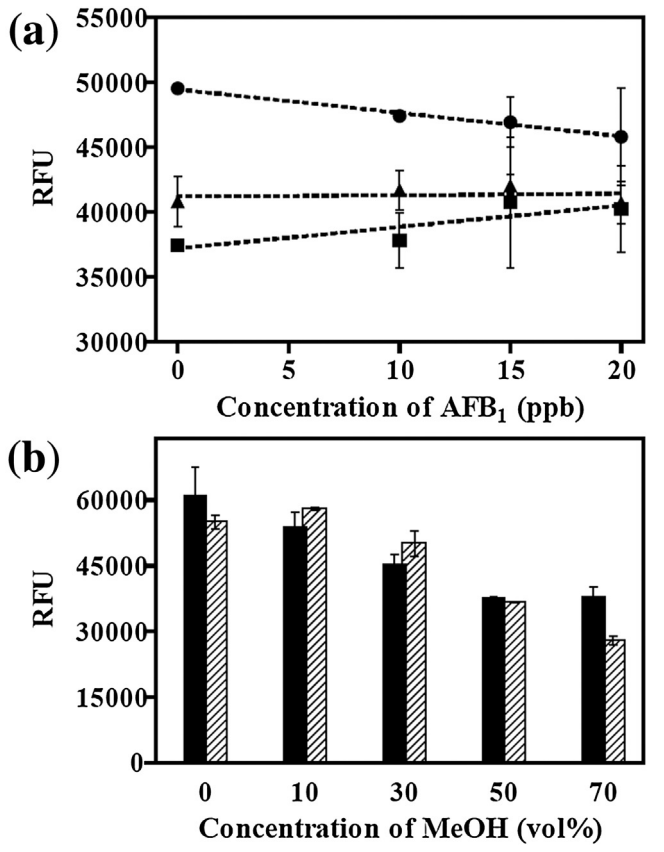

Fig. 4. Optimization of the extraction condition. (a) Changes of fluorescence intensity of the aptamers in 50% methanol (-●-), ethanol (-▲-), and isopropanol (-■-) against the concentration of AFB_1 (n = 3). (b) Comparison of fluorescence intensity between the standard solution (■) of AFB_1 and the extract solution (▨) from rice seeds in correspondent concentrations of methanol (n = 3).

AFB_1 dissolved in the respective concentrations of methanol. As shown in Fig. 4b, the sample extracted with 50% (v/v) methanol approached 97% of the result from the standard solution in 50% of methanol. The other extracts were differentiated around 10% or more in recovery rate. Therefore, 50% (v/v) methanol was used to extract AFB_1 in further assays. The optimized 3-step assay conditions were finally as follows: extraction of AFB_1 with 50% (v/v) methanol from rice seeds; incubation of the extract with 0.45 μM of the aptamer for 30 min; and addition of GO to the AFB_1–aptamer complex for 10 min before fluorescence measurement.

3.3. Aptamer-based quantitative assay of AFB_1

A quantitative assay of AFB_1 was performed under the optimum conditions. Fig. 5a shows a standard curve of the fluorescence intensities for different concentrations of AFB_1. Increase in the concentration of AFB_1 induced more interactions between the aptamer and GO, leading to a decrease in fluorescence intensity.

3.4. Selectivity test

AFB_2, AFG_1, and AFG_2 were selected to investigate the selectivity of the assay, because interference from other toxins with a similar structure would possibly appear. The mixture of AFB_1, AFB_2, AFG_1, and AFG_2 was assayed and the result was compared with that of AFB_1 at the same concentration as that in the mixture. As shown in Fig. 5b, although the mixture of aflatoxin showed decreased fluorescence relative to the blank, the decrease in fluorescence observed for AFB_1 alone was greater, implying that there was possible interference from the other types of aflatoxin. Despite this, AFB_1 was specifically detectable among the mixture.

3.5. Quantitative detection of AFB_1 on rice seeds

Based on the results from the quantitative assay of standard solutions of AFB_1, the applicability of the detection method was verified with spiked samples. Rice seeds were chosen as a model for cereals, in which AFB_1 is mostly detected. AFB_1 at a wide concentration range of 0–300 ppb was spiked on batches of ground rice seeds. The seeds were then treated with the optimum extraction condition, and the extract was recovered for assay. As shown in Fig. 6, the fluorescence decreased linearly with increasing concentration of spiked AFB_1, and the entire set of results showed high correlation between the wide concentration range of AFB_1 and fluorescence intensity ($R^2 = 0.9096$). The limit of detection of the method was 4.5 ppb, which is about one-third of the acceptable limit for AFB_1 in cereals (12 ppb according to European Union regulation). The performance of the assay was also compared with other currently available detection methods for AFB_1 (Table 1). Our biosensor showed a significantly wide detection range in a relatively short assay time. The result suggested the practicability of the assay in the field.

4. Conclusion

In this study, a simple biosensor for AFB_1 was developed using an aptamer and GO. The change of fluorescence against changing

Table 1
Comparison of the assay time and detection range of currently available methods for the detection of AFB_1.

Method	Assay time (min)	Detection range(ppb)	LOD(ppb)	Reference
Immunochromatography assay	10	2.5–50	2.5	[30]
Electrochemical immunosensor	60	0.05–2	0.03	[31]
Real-time quantitative PCR	60	0.1–10	0.1	[32]
LC–ESI–MS/MS	7	0.02–20	0.007	[33]
Surface plasmon resonance biosensor	15	0.94–3.75	0.94	[34]
Aptasensor using GO quencher	40	4.5–300	4.5	This work

AFB_1, aflatoxin B_1; GO, graphene oxide; LC-ESI–MS/MS, liquid chromatography electrospray ionization-tandem mass spectrometry; LOD, limit of detection; PCR, polymerase chain reaction.

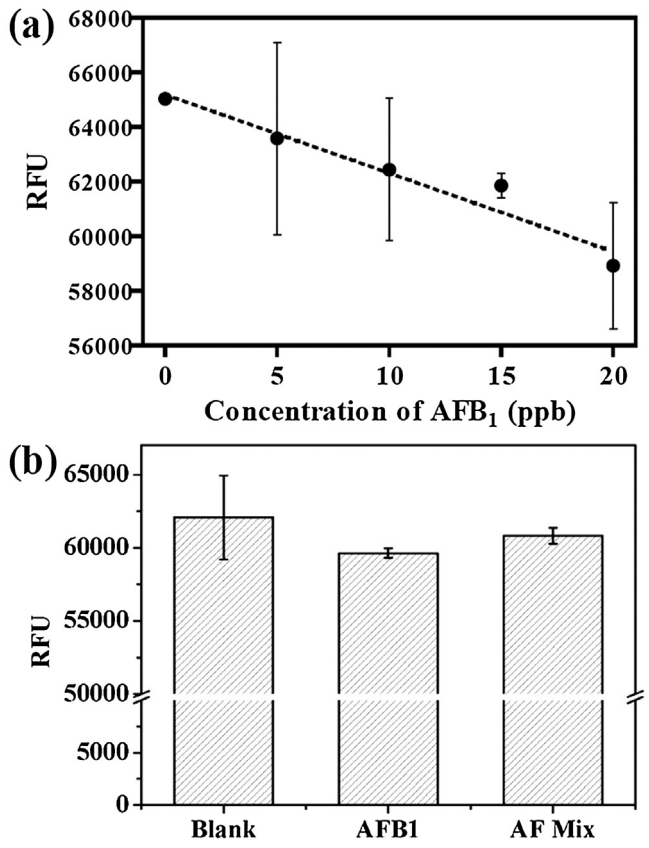

Fig. 5. (a) Standard curve of fluorescence intensities against the concentrations of AFB_1, performed under the optimum condition (n = 3). (b) Selectivity of the assay for AFB_1 against other types of aflatoxins. AF Mix: AFB_1, AFB_2, AFG_1, and AFG_2 (n = 3).

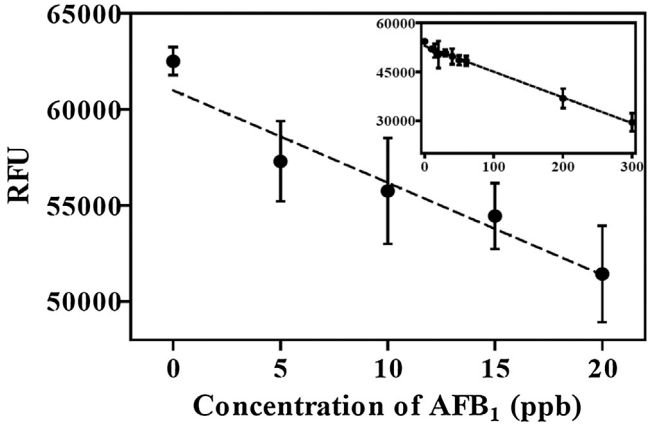

Fig. 6. Standard curve from rice seed extracts with different concentrations of AFB_1 (n = 3).

AFB_1 concentrations could be simply measured within 40 min. The use of an aptamer provides economic and time-saving advantages. Although the detection limit of the biosensor for a real sample was low (4.5 ppb), the wide detection range of 4.5–300 ppb has potential for its applicability in various fields. Furthermore, the assay can be improved for the practical sensor detection of other mycotoxins by simply replacing the aptamer sequences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2017.03.010.

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