A Ratiometric Fluorescent Aptamer Homogeneous Biosensor Based on Hairpin Structure Aptamer for AFB1 Detection

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Abstract

On the basis of aptamer (Apt) with hairpin structure and fluorescence resonance energy transfer (FRET), a ratio fluorescent aptamer homogeneous sensor was prepared for the determination of Aflatoxin B_1 (AFB1). Initially, the Apt labeled simultaneously with Cy5, BHQ2, and cDNA labeled with Cy3 were formed a double-stranded DNA through complementary base pairing. The fluorescent aptamer sensor demonstrates a weak fluorescence emission of Cy3 and a high fluorescence emission of Cy5 due to the quenching effect of BHQ2. The double-stranded DNA structure will be disintegrated in the presence of AFB1, resulting the removal of Cy3 and the close of Cy5 with BHQ2. The fluorescence signal of Cy3 and Cy5 were restored and quenched respectively. Thus, the ratio change of F_{Cy3} to F_{Cy5} was used to realized the detection of AFB1 with wider detection range and lower limit of detection (LOD). The response of the optimized protocol for AFB1 detection was wider linear range from 0.05 ng/mL to 100 ng/mL and the LOD was 12.6 pg/mL. The sensor designed in this strategy has the advantages of simple preparation and fast signal response. It has been used for the detection of AFB1 in labeled corn and wine, indicating it had good application potential in practical samples.

1. Introduction

According to the statistics, about 25% of the agricultural products are contaminated with mycotoxins during crop growth, storage or processing in the world [1]. Fungal contamination of food is a global concern, most of the toxic substances produced by pollution have strong carcinogenic effects on human beings [2]. Aflatoxin is the most important and toxic mycotoxin among many mycotoxins and AFB1 is the most toxic aflatoxin. Many crops such as peanuts, corn and grains are vulnerable to AFB1 contamination, which makes AFB1 a serious threat to food safety and human health [3]. At present, more than 100 countries and regions have set limits for AFB1 in various foods ranging from 1 to 20 μg/kg [4]. Therefore, there is an urgent need to establish a fast, safe, accurate and capable of on-site detection of AFB1 method.

At present, the methods used for AFB1 detection mainly include thin layer chromatography (LC), high-performance liquid chromatography (HPLC), high performance liquid chromatography-mass spectrometry (HPLC-MS) [4]. Although these methods have good accuracy and high sensitivity, they have disadvantages such as tedious sample pretreatment process, expensive instruments, highly trained professionals and so on, which limit their application for rapid and on-site screening of AFB1 [5]. Due to these disadvantages of traditional methods, fluorescence detection which has the advantages of high sensitivity, simple operation and simultaneous detection of multiple toxins, has attracted the attention of researcher [6]. With the rapid development in recent decades, various fluorescent biosensors have emerged in an endless stream [7]. In order to enhance the specific recognition of the sensor and the target, aptamer as target recognition element compared with antibody, enzyme, *etc*.

Aptamer has stronger affinity with the target, the simpler and cheaper synthesis process and better stability. What's more, the aptamer is not easy to be limited by the storage environment, temperature, and its resistance to degeneration [8]. Li *et al.* reported a new aptamer fluorescence sensor, whcih can sensitively detect Pb^{2+} [9]. Yang *et al* designed a novel fluorescence based protein detection method using aptamer probe as recognition molecule and cationic conjugated polymer (CCP) as reporter molecule [10]. At present, the fluorescence aptasensors prepared by using aptamer instead of antibody has been widely used in the detection of mycotoxins, protein, cell [11].

Most of the fluorescence aptasensors require a tedious and time-consuming process of embedding the probe or some recognition molecules on the surface of the material used to prepare the sensor, which will greatly increase the preparation time of the sensor. In addition, the separation and purification process involved in fixing the probe and identifying the molecules will lead to the signal damage of the sensor and affect the performance of the sensor. To solve this problem, many researchers have chosen to use homogeneous sensors [12–13], which is kept in a solution from preparation to detection without any separation operation. Fluorescence resonance energy transfer (FRET) is a commonly used method for quenching fluorescent dyes in fluorescence sensors. The FRET process requires a distance between the quenching material and the fluorescence probe within 10 nm. When the quenching material and the fluorescence probe are dispersed in the system, the FRET process is weak. It almost has no effect on the signal of fluorescent probe, which is convenient for the application of homogeneous detection in fluorescent adaptor sensor. Lu[14] *et al.* provided a simple, homogeneous and sensitive strategy mediated FRET for the histone analysis. The homogeneous sensor has been widely used in the detection of mycotoxins, protein, cell[15].

Furthermore, the conventional fluorescent aptasensors were designed based on the changes of the "signal on" or "signal off" of single probe. The conventional fluorescent aptasensors were easily affected by the environmental and instrument factors in detection of the actual sample, resulting in the degradation of the sensor performances[16]. Yang *et al.*[17] prepared a simple and rapid fluorescence homogeneous sensor for AFB1 detection by using the adaptation system of BHQ2-labeled complementary chain and Cy3 labeled fluorescent probe. However, the signal ratio is not sensitive to changes affected by environmental and instrumental. To improve the stability and accuracy of the sensor, the ratiometric fluorescent aptasensor was selects two signal probes with different excitation and emission wavelengths. The final signals are determined by the fluorescence signal ratio of the two probes [18–19]. Therefore, the ratiometric fluorescent aptasensor using two signal probes has become a new research hotspot.

In this paper, a simple, rapid and sensitive ratio homogeneous fluorescent aptasensor was constructed by using aptamer with hairpin structure labeled simultaneously with Cy5, BHQ2 and cDNA labeled with Cy3 to detect AFB1. The AFB1 aptamer with hairpin structure was used as recognition element, and the labeled BHQ2 whcih can quench the fluorescent dyes Cy3 and Cy5. The aptamer labeled with Cy5 and BHQ2 was paired with the cDNA labeled with Cy3 to form a double strand through complementary

pairing. At this time, Cy3 was close to BHQ2, leading to fluorescence quenching. A high fluorescence emission of Cy5 emerged due to the distance of BHQ2. The hairpin structure would be opened in the presence of AFB1.The fluorescence intensity of Cy3 was increased due to it was far away from BHQ2, and the fluorescence intensity of Cy5 was decreased due to Cy5 approaching BHQ2. AFB1 was detected by comparing the ratio of F_{Cy3} to F_{Cy5} before and after adding AFB1.

2. Materials And Methods

2.1. Materials

The AFB1 aptamer (Apt) and complementary DNA (cDNA) were synthesized by Sangon Biotechnology Co., Ltd (Shanghai, China). The sequences of the oligonucleotides are as follows: Apt: 5'-BHQ$_2$-TGC ACG TGT TGT CTC TCT GTG TCT CG TGC-Cy5-3', cDNA: 5'-GCA CGT CTG GAC ACG AGA CAA CAC GTG CA-Cy3-3'. AFB1, ochratoxin A (OTA), fumonisin B$_1$ (FB$_1$), deoxynivalenol (DON), and zaralenone (ZEN) were purchased from Sigma Co., Ltd (USA). Sodium chloride (NaCl), Magnesium chloride (MgCl$_2$), Ethanol absolute (C$_2$H$_6$O) and hydrochloric acid (HCl) were purchased from Kermel Chemical Reagent Co., Ltd (Tianjin, China). Tris (hydroxymethyl) aminomtethane was purchased from Shanghai Macklin Biochemical Co., Ltd (China). Ethylene diamine tetraacetic acid disodium salt (EDTA) was purchased from Luoyang Chemical Reagent Factory (Henan, China). 50 mM Tris-HCl buffer (pH 7.4, 50 mM Tris, 0.2 M NaCl and 1 mM EDTA) were used in this paper. All oligonucleotides were diluted with 50 mM Tris-HCl buffer, and all reagents shall be fully mixed before use. All the water used in the experiment was sterilized ultra-pure water (18.2 MΩ).

2.2. Preparation of Cy5-Apt-BHQ2/cDNA-Cy3 sensor

20 μL cDNA (1 μM) was mixed with 18 μL Apt (1 μM) with stirring, the mixture was heated to 95 °C in PCR and kept for 5 minutes, then slowly cooled to 4 °C, Cy5-apt-BHQ2 / cDNA-Cy3 sensor was prepared and stored at 4 °C until use.

2.3. Detection of AFB1

10 μL AFB1 (200 ng/mL) was mixed with 38 μL of prepared Cy5-Apt-BHQ2/cDNA- Cy3 sensor solution, and adding 20 mmol/L Tris-HCl (pH 7.4) buffer solution to 200 μL. Then the mixed solution was incubated at 37 °C with a water bath thermostatic oscillator for 40 minutes. In this paper, excitation wavelength scanning method was used to detect fluorescence intensity. The fluorescence intensity was measured at the excitation wavelength scanning mode by Hitachi F-7100 fluorescence spectrophotometer (Japan). All measurements were all carried out at room temperature. The fluorescence cell was a quartz fluorescence cell with a slit of 2 mm, and the emission spectrum of Cy3 probe was recorded in the excitation wavelength of 530 nm and in the wavelength range of 550 nm-650 nm. The emission spectra of the Cy5 probe were recorded in the excitation wavelength of 635 nm and the wavelength range of 650 nm-750 nm. The scanning voltage was 950 V, and the excitation and emission slit widths were set to 5 nm. All measurements were made at room temperature. Fluorescence

spectrophotometer was used to record the fluorescence spectra of mixed solution before and after adding AFB1 at excitation wavelength of 530 nm (Cy3) and 635 nm (Cy5), the ratio value of fluorescence intensity of Cy3 (F_{Cy3}) to fluorescence intensity of Cy5 (F_{Cy5}) was compared before and after AFB1 added.All the test data were performed three times.

2.5. Application of the fluorescence sensor in actual samples

To study the feasibility of the designed sensor, the spiked corn flour samples were tested. The processing method of corn flour referred to Seok's [18] processing method, 1 g corn meal sample was put into a 10 mL flask, and adding 1 mL analytically pure methanol was mixed with AFB1 in different known concentrations, eventually. The mixture was left in a fume hood for 12 hours to completely evaporate the solvent, then extracted at room temperature with methanol/water (2:8, v/v) for 15 minutes. After centrifugation at 10000 rpm for 15 min, the extract was filtered through a 0.45 mm injection filter. The filtrate was diluted with 50 mM Tris-HCl (pH 7.4) to the concentration of 0.1 ng/mL, 1 ng/mL, and 10 ng/mL. The prepared sensor was used to detect the spiked samples, and the fluorescence spectra of Cy3 and Cy5 were recorded after the addition of the fluorescence sensor to the samples.

After removing the foam from the wine samples by nitrogen blowing, the spiked wine samples were detected. The toxin concentration was diluted with Tris-HCl (pH 7.4) until 0.1 ng/mL, 1 ng/mL and 10 ng/mL with different known concentrations of AFB1. By recording the fluorescence spectra of Cy3 and Cy5 of the fluorescence sensors after adding samples, the labeled samples were detected. All the test data were performed three times

The contaminated feed (corn peanut meal) was ground to powder (1.0 g) and the extraction solvent was added after stirring for 30 min. The supernatant was treated to detect AFB1. All results were compared by HPLC. All the test data were performed three times

3. Results And Discussion

3.1. The design principle of the aptasensor for AFB1 detection

Ratiometric homogeneous fluorescent aptamer based on the hairpin structure of Apt and the characteristic that BHQ2 can simultaneously quench the fluorescence probe Cy3 and Cy5, a fluorescence aptamer homogeneous sensor was designed for the detection of AFB1. The detection principle was illustrated in Figure 1. The Apt labeled with Cy5 and BHQ2 and cDNA labeled with Cy3 form a double-stranded DNA through complementary base pairing. At this moment, Cy3 was close to BHQ2, resulting in fluorescence quenching. Due to the opening of the hairpin structure, Cy5 was far away from BHQ2 leads to the recovery of the fluorescence signal. The double-stranded DNA structure will be disintegrated in the presence of AFB1, resulting the removal of Cy3 and the close of Cy5 with BHQ2. At this time, a weak

fluorescence emission of Cy3 and a high fluorescence emission of Cy5 due to the quenching effect of BHQ2.By comparing the ratio change of F_{Cy3} to F_{Cy5} before and after adding AFB1, the detection of AFB1 was realized. Therefore, the ratiometric fluorescent aptamer homogeneous sensor can be used for AFB1 detection theoretically.

3.2 The feasibility of the ratiometric fluorescent aptamer homogeneous sensor

The feasibility of the prepared Cy5-Apt-BHQ2/cDNA-Cy3 sensor was analyzed in the absence and presence of AFB1. The result was shown in Figure. 2. The initial fluorescence intensity of the sensor showed that the ratio of F_{Cy3}/F_{Cy5} was 1.1 in the absence of AFB1 (Red line). After adding 10 ng/mL AFB1 for incubation for 40 minutes, the value of the F_{Cy3}/F_{Cy5} was increased 6 times compared to without AFB1, indicating the feasibility of the prepared sensor for AFB1 detection (Blue line). The double-stranded DNA structure will be disintegrated in the presence of AFB1, resulting the removal of Cy3 and the close of Cy5 with BHQ2. Due to the fluorescence quenching effect of BHQ2, the ratio of F_{Cy3}/F_{Cy5} increased to 6.6 when adding 10 ng/mL AFB1. The results were shown that the ratiometric fluorescent aptamer homogeneous sensor can be used to detect AFB1, sensitively.

3.3 Optimization of the experiment parameter

In order to get the optimal performance of the prepared sensor, the ratio value between Apt and cDNA should be optimized. The background signal will be affect by the change of the ratio value Apt to cDNA Because changing the ratio value of Apt to cDNA would affect the ratio of the initial signal F_{Cy3} to $F_{Cy5}[(F_{Cy3}/F_{Cy5})_0]$, $(F_{Cy3}/F_{Cy5}/(F_{Cy3}/F_{Cy5})_0$ was used to reflect the optimization results[18]. As shown in Figure. 3, $(F_{cy3}/F_{cy5})/(F_{cy3}/Fc_{y5})_0$ was growing with the increasing the proportion between Apt and cDNA from 0.7 to 0.9. When the proportion between Apt and cDNA was greater than 0.9, $(F_{cy3}/F_{cy5})/(F_{cy3}/F_{cy5})_0$ started to decrease. This was because the double-strand DNA formed by Apt and cDNA has reached a saturation state, and the redundant Apt reduces the sensitivity of the sensor and the signal change was no longer obvious. Therefore, 0.9 was selected as the optimal ratio value between Apt and cDNA.

3.4. Detection of AFB1

Under the optimal experimental parameters, F_{Cy3}/F_{Cy5} was measured by analyzing AFB1 solution with different concentrations. The detected spectrogram was shown in Figure. 4(A), the fluorescence intensity of the fluorescence sensor Cy3 (F_{cy3}) increased and the fluorescence intensity of Cy5 (F_{cy5}) decreased with the increasing concentration of AFB1 added (0, 0.05, 0.1, 0.5, 1, 10, 50, 100, 500 ng/mL). A good linear relationship was obtained between 0.05 ng/ mL and 100 ng/ mL (Figure. 4B), the linear equation

was $$\frac{F_{Cy3}}{F_{Cy5}} = 2.34 Lg C_{AFB1} + 4.60 (R^2 = 0.994)$$. The LOD was calculated to be 12.6 pg/mL (3σ rule). The result indicated the prepared ratiometric homogeneous fluorescent aptasensor had a wider linear range and lower detection limit for AFB1 detection.

In order to study the specificity of the prepared fluorescence aptasensor to different toxins. Under the same experimental condition, 10 ng/mL of AFB1, 100 ng/mL of interference toxins such as OTA, FB1, DON, ZEN and mixture of AFB1 with all interference toxins were detected and results were shown in Figure. 5. After incubating the prepared aptasensor with a single interfering toxin for 40 minutes, the ratio value of fluorescence intensity of the two dyes (F_{Cy3}/F_{Cy5}), remained unchanged even though its concentration was 10 times higher than that of AFB1. However, as long as AFB1 was present, the value of F_{Cy3}/F_{Cy5} of fluorescence intensity could be significantly increased after incubation for detection, whether AFB1 was existed alone or AFB1 was mixed with interfering toxins, indicating that the designed aptasensor had good specificity.

The detection of the designed aptasensor was compared with other AFB1 aptasensors, and the results are shown in Table 1. It can be seen that the aptasensor designed in this experiment had a good linear range and a lower LOD compared with most sensors. This result indicated that the dual-signal change of the ratio sensor can effectively improve the sensitivity of the fluorescence aptasensor, and the homogeneous detection method improved the stability of the sensor.

Table 1
Comparison of other sensors developed for AFB1 detection

Methods	Linear range (ng/mL)	LOD(ng/mL)	Reference
Electrochemistry	0.6-313	0.6	[19]
ELISA	0.24−2.21	0.13	[20]
Liquid Chromatography	0.05−2	0.108	[21]
Electrochemistry	0.05-20	0.016	[22]
Fluorescence	0.1-0.8	0.07	[23]
Fluorescence	0.05-100	0.05	[24]
Fluorescence	0.015-15	0.003	[25]
Fluorescence	0.05-100	0.0126	This work

3.5. Detection of the actual samples

In order to further verify the accuracy and practicability of the ratio homogeneous fluorescent apatasensor, the spiked corn flour samples and spiked wine samples were tested, and the scalars were 0.1 ng/mL, 1 ng/mL, and 10 ng/mL, respectively. The detection results were shown in Table 2. The recovery of the detection results were 95-110% and 93-108%, respectively. The relative standard deviation (RSD) were not more than 10%. HPLC and the prepared fluorescence sensor were used to detect the

contaminated feed. The results showed that the prepared sensor had certain accuracy and practicability which can be used as an alternative to traditional detection methods.

Table 2
Detection of AFB1 in corn meal and wine samples (n=3)

Sample	Spiked amount (ng/mL)	Measured by HPLC (ng/mL)	Found amount (ng/mL)	Recovery (%)	RSD (%)
Corn	0	ND	ND	-	-
	0.1	0.09	0.11	110	9.3
	1	1.1	0.95	95	7.1
	10	10.47	9.56	96	6.9
wine	0	ND	ND	-	-
	0.1	0.097	0.093	93	8.2
	1	1.03	0.98	98	6.2
	10	10.2	10.84	108	7.3
ND: Not detected.					

4. Conclusions

In summary, combining Apt with hairpin structure labeled with Cy5 and BHQ2 and cDNA labeled with Cy3 to detect AFB1, a simple, rapid and sensitive ratio homogenous fluorescent aptasensor was constructed. The ratio value of fluorescence intensity between Cy3 and Cy5 (F_{Cy3}/F_{Cy5}), and LgC_{AFB1} showed a good linear relationship within the concentration range of AFB1 from 0.05 ng/ mL to 100 ng/mL, and the LOD was 12.6 pg/mL. The recovery rates of AFB1 in spiked corn meal samples and wine were 92-110% and 88-112%, respectively. Furthermore, the adequate determination of AFB1 in corn, wine samples designed in this paper confirmed the applicability of this strategy compared with HPLC. The aptasensor designed in this paper is simple in preparation, fast in signal response, which has the application prospect of realizing fast detection of AFB1 on site.

Declarations

Ethics Approval

Not applicable.

Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Authors' Contributions

Beibei Feng: concept design, editing, Review modification, editing, and analysis data. *Fei Zhao:* Performed the experiments and modification. *Min Wei*: Corresponding author, concept design, Review writing and editing. *Yong Liu*: Contributed reagents, materials. *Xinyu Ren*: Contributed reagents. *Zhiguang Suo*: Review modification, editing, and Analyzed and interpreted the data.

Data Availability

Not applicable.

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Figures
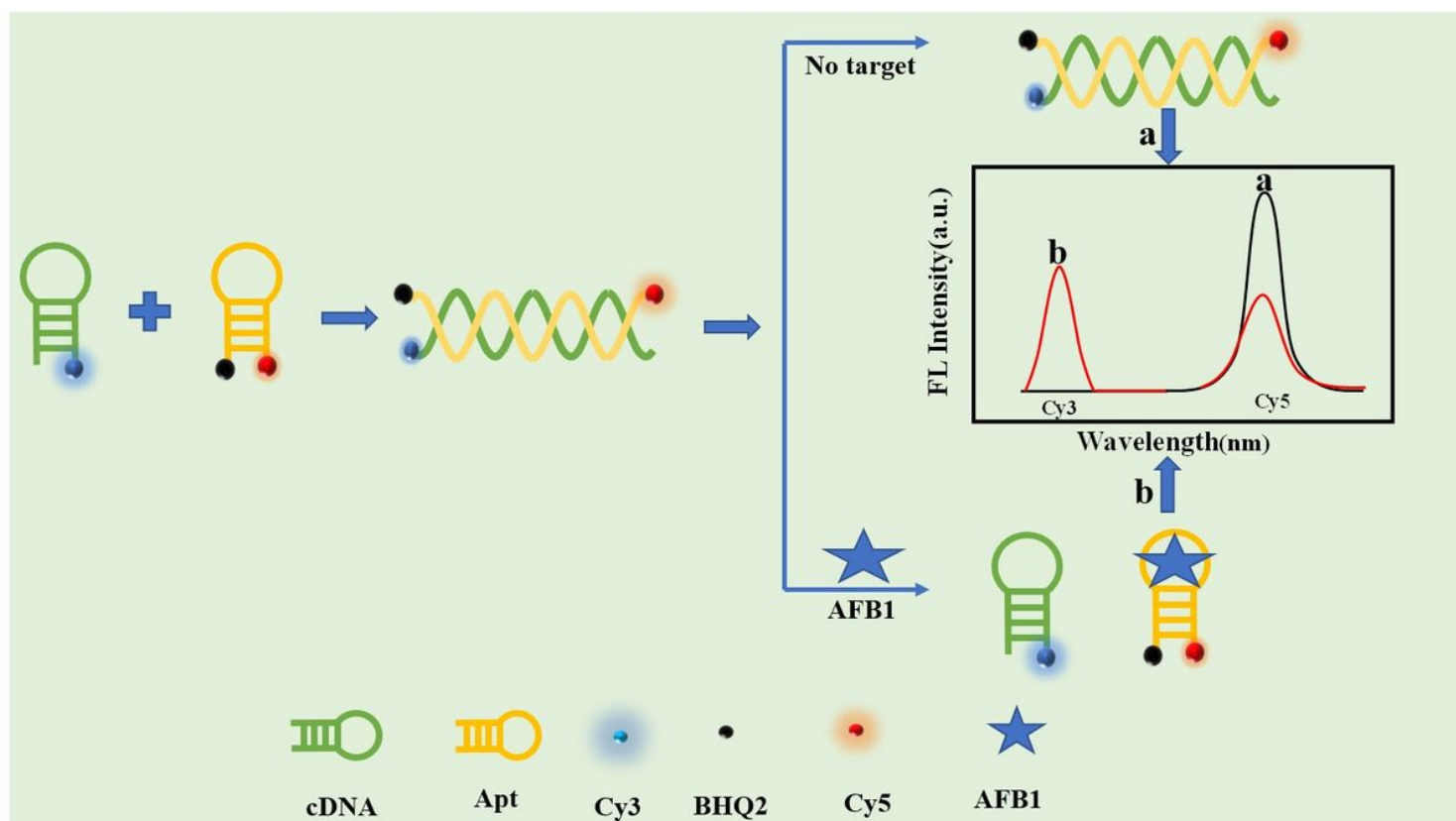
Figure 1

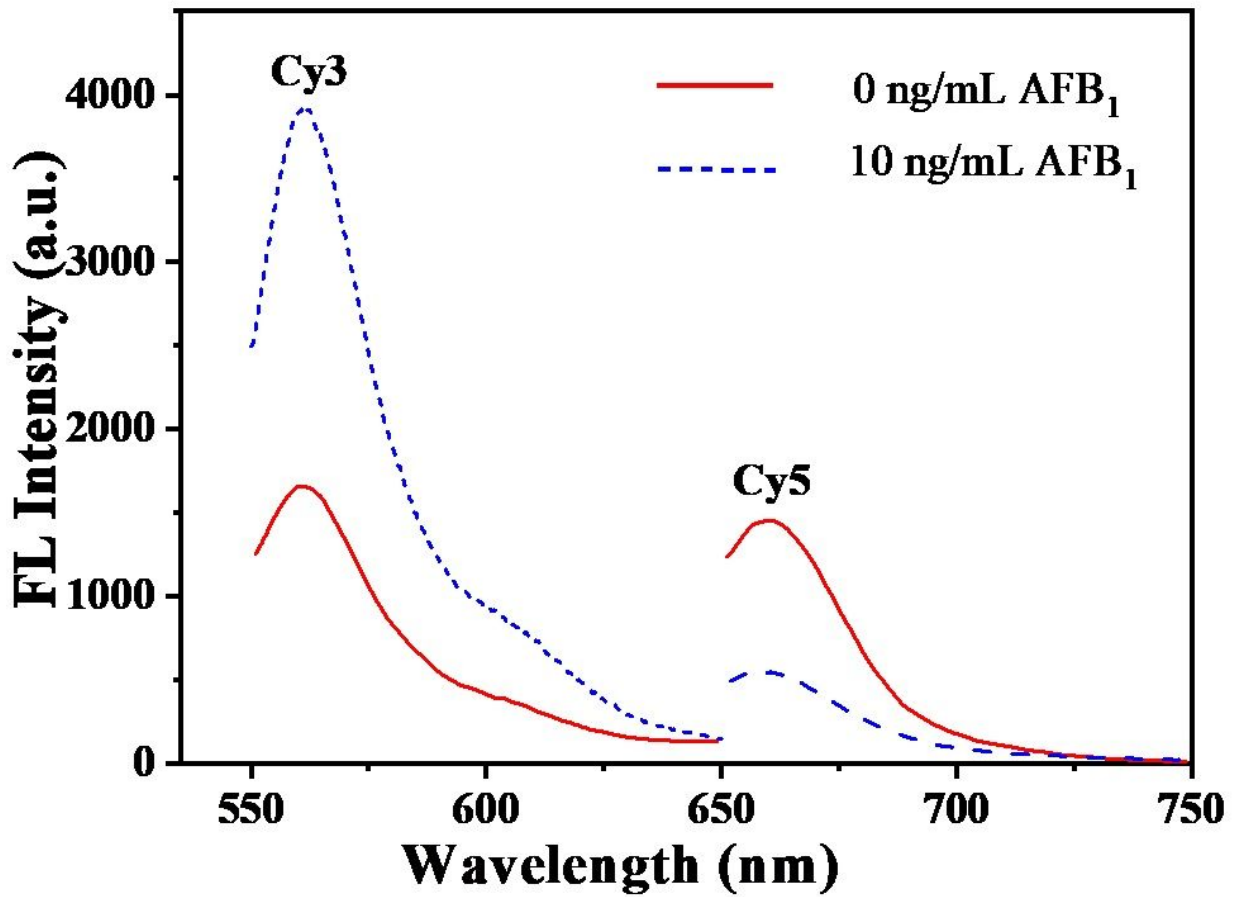Preparation of Cy5-Apt-BHQ$_2$ /cDNA-Cy3 sensor and its detection principle diagram of AFB1

Figure 2

The fluorescence spectra of Cy5-Apt-BHQ2 / cDNA-Cy3 sensor before and after AFB1 incubation (The concentration of AFB1 is 10 ng/mL)
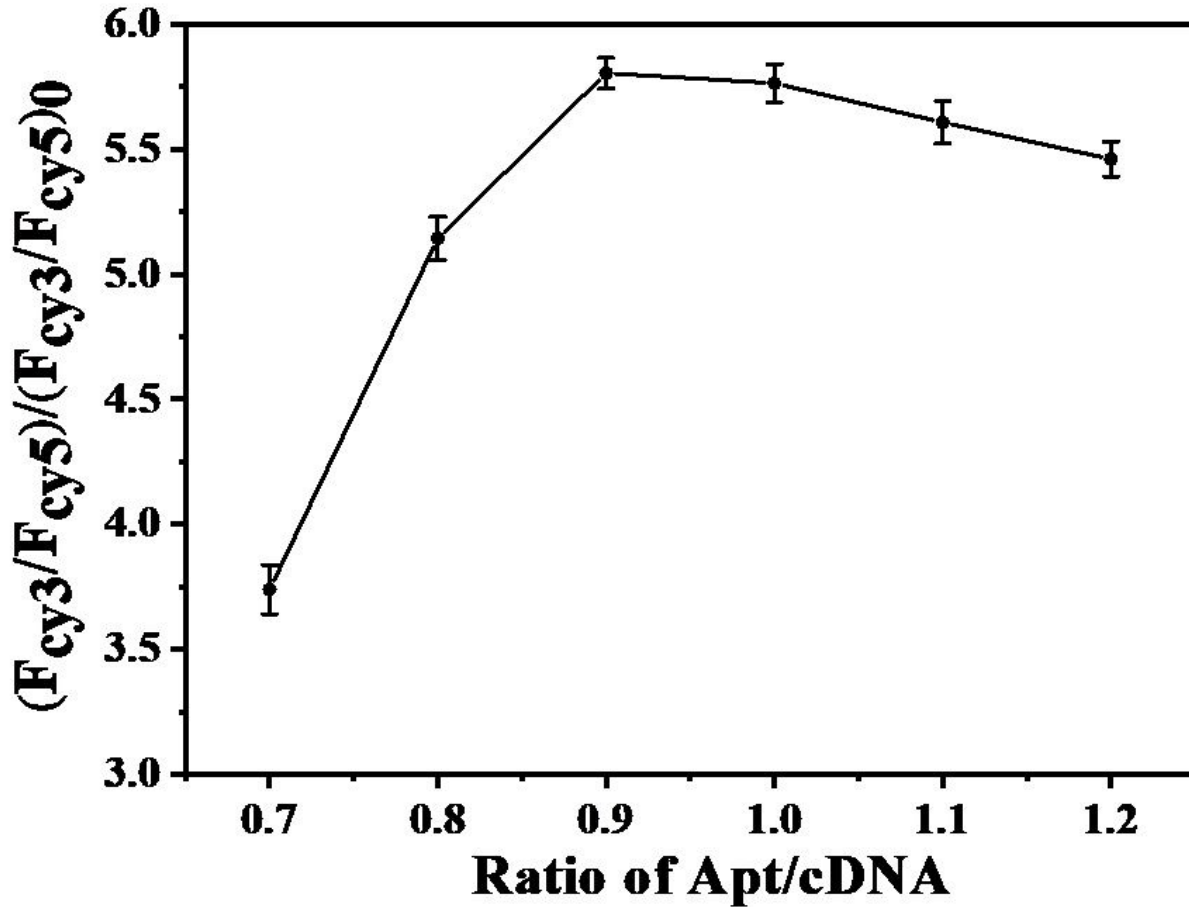
Figure 3

Effect of Apt and cDNA dosage ratio on Cy5-Apt-BHQ2 / cDNA-Cy3 sensor (AFB1 concentration is 10 ng/mL)
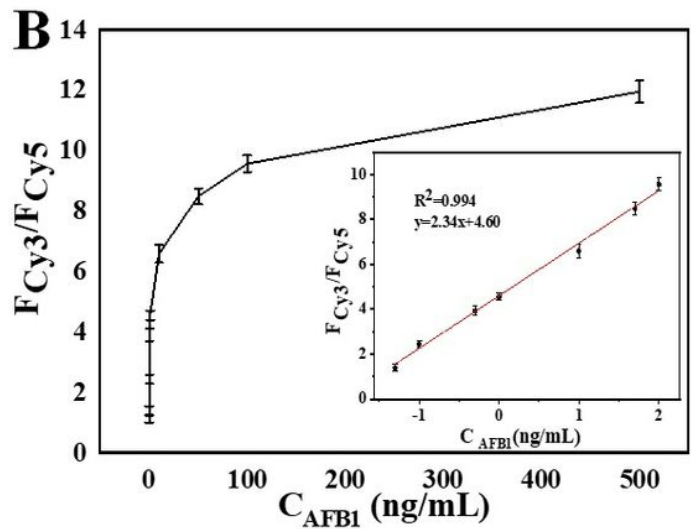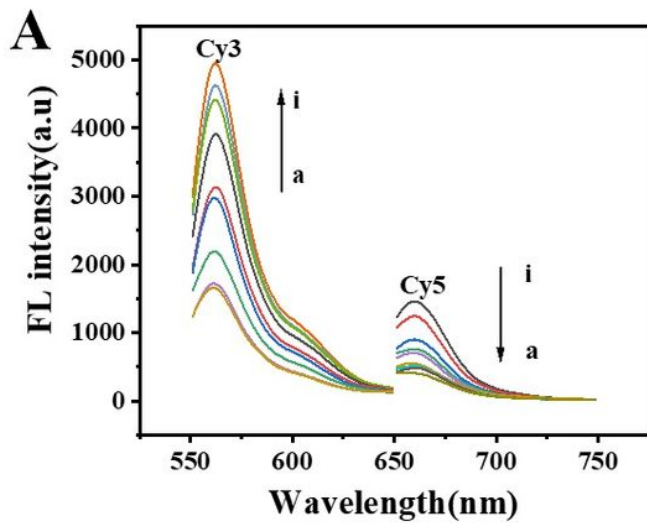
Figure 4

(A). Fluorescence spectra of AFB1 detected by Cy5-Apt-BHQ 2 / cDNA-Cy3 sensor in different concentration ranges. (a) 0 ng/mL☐(b) 0.05 ng/mL☐(c) 0.1 ng/mL☐(d) 0.5 ng/mL☐(e) 1 ng/mL☐(f) 10 ng/mL☐(g) 50 ng/mL☐(h) 100 ng/mL☐(i) 500 ng/mL (B). The relation curve and linear relation between Fcy3/Fcy5 and AFB1 concentration.
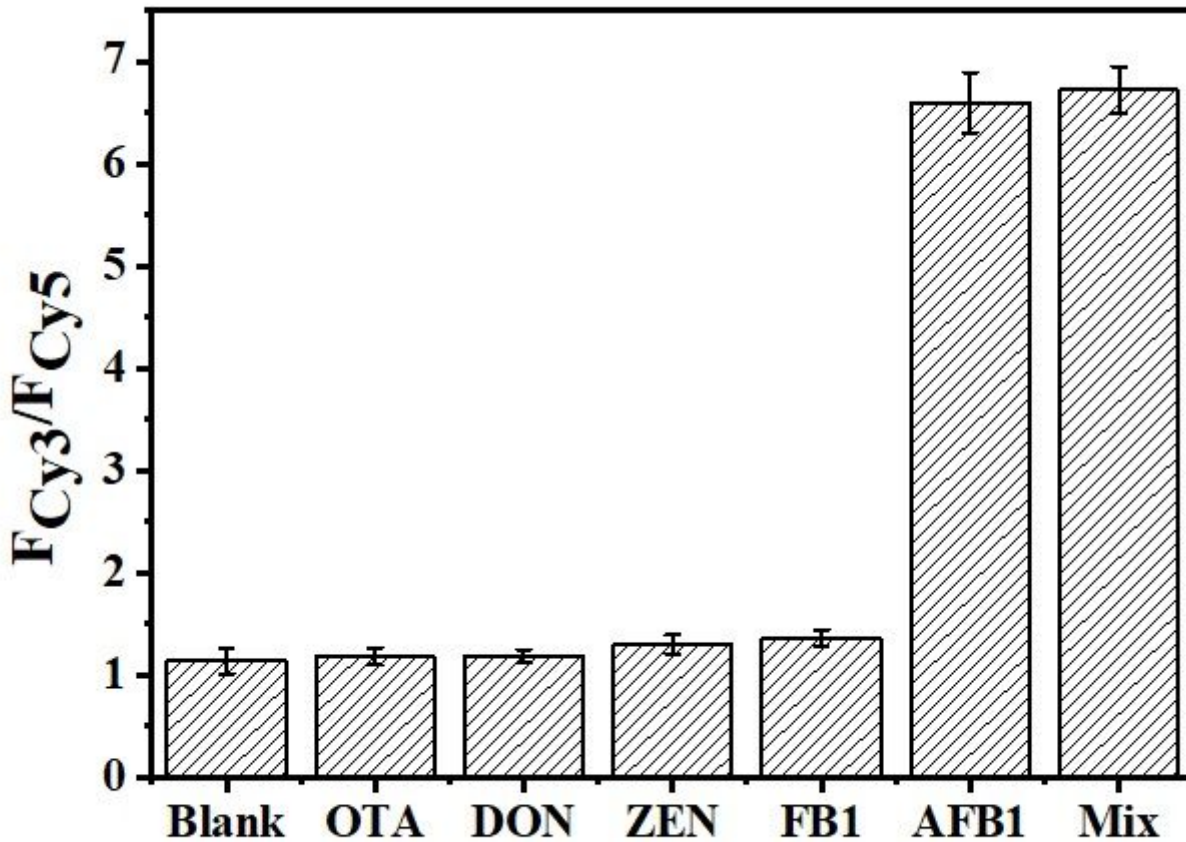

Figure 5

Histogram of the effects of different toxins on the fluorescence signal of Cy5-Apt-BHQ2 / cDNA-Cy3 sensor