Short communication

Aptamer-based Colorimetric and Chemiluminescence Detection of Aflatoxin B1 in Foods Samples

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Abstract

We developed a new biosensor for the detection of aflatoxin B1(AFB1) based on the interaction of gold nanoparticles (AuNPs) with the aptamer. Aggregation of AuNPs was induced by desorption of the AFB1 binding aptamer from the surface of AuNPs as a result of the aptamer target interaction leading to the color change of AuNPs from red to purple. The linear range of the colorimetric aptasensor covered a large variation of AFB1 concentrations from 80 to 270 nM and the detection limit of 7 nM was obtained. Also, the catalytic activity of the aggregated AuNPs greatly enhanced the chemiluminescence (CL) reaction, where the detection limit was determined at 0.5 nM with a regression coefficient of R^2 = 0.9921. We have also shown that the sensitivity of detection was increased by employing CL and using the catalytic activity of aggregated AuNPs, during luminol–hydrogen peroxide reaction. Therefore the proposed nanobiosensor was demonstrated to be sensitive, selective, and simple, introducing a viable alternative for rapid screening of toxin in foods.

Keywords: Aptamer, colorimetric, chemiluminescence, aflatoxin B1

1. Introduction

Aflatoxins (AF) are toxic compounds which are produced as secondary metabolites by the fungi of *Aspergillus flavus* and and *Aspergillus parasiticus* growing on a variety of agricultural products.[1] Aflatoxins are a group of compounds containing molecular structure of a coumarin and a double-furan ring. These toxins are known to be carcinogenic, mutagenic, teratogenic and immunosuppressive. Among the 18 identified aflatoxins, the major ones are AFB1, AFB2, AFG1 and AFG2, of which AFB1 was classified as a Group 1 carcinogen by IARC of WHO in 1993.[1,2]

AFB1 is found widely in agricultural products, such as peanuts, corn, soybean, and rice. Thus it can enter animal or human food chain and result in food safety problems.[3,4] Therefore, developing a detection method for AFB1 in agricultural products with high specificity and sensitivity, high throughput and rapidity has great practical significance.

During the past few years, some well-established methodologies and analytical techniques have been studied for discrimination and quantization of AFB1 in many different foodstuffs, such as thin-layer chromatography (TLC), liquid chromatography (LC), immunoaffinity chromatography (IAC), high-performance liquid chromatography (HPLC),[5] liquid chromatography–mass spectrometry (LC–MS),[6] LC–MS/MS,[7] enzyme-linked immunosorbent assay (ELISA)[8] and electrochemical immunosensor.[9] Although these methods are sensitive, more rapid, simple, and cost-effective approaches are still requested by the food industry. More recently, optical biosensors coupled with gold nanoparticles (GNPs) provided a promising platform for this purpose based on their remarkably high absorption coefficient and strongly distance dependent optical properties.[10,11] The color change in this case, which arises from the inter particle plasmon coupling during GNP aggregation or redispersion of an GNP aggregate,[12] is perhaps one of the most powerful and simple nanosensing methods available.[13] The methods demonstrated that sensitive detection of specific targets can be accomplished simply and rapidly without complex instrumentation.[14]

Aptamers are mainly specific oligonucleotides (ssDNA or RNA) for targets that are obtained by an in vitro

selection process called systematic evolution of ligands by exponential enrichment (SELEX). Compared with antibodies, aptamers are easy to be synthesized, modified and fixed, repeatedly used, and preserved long-term.[15–16] Therefore, aptamers have been widely used as recognition elements to fabricate aptamer-based biosensors, which have been applied in protein research,[17] mycotoxins,[18–21] drug testing[22] and medical diagnosis.[23] Recently, Neoventures Biotechnology Inc. (Canada) has patented a specific aptamer to AFB1 (Patent: PCT/CA2010/001293) that has been used as molecular recognition probe for detection of AFB1. The method of detection has been based on a chemiluminescence competitive assay using hemin G quadruplex horseradish peroxidase-like DNAzyme (HRP-DNAzyme) linked to AFB1 aptamer. The assay allowed detection of AFB1 with LOD 0.35 nM and it has been validated in spiked corn samples.[24] Nan *et al.* reported an approach based on real time-quantitative polymerase chain reaction (PCR) for detection of AFB1 with a limit of detection of 25 fg · mL^{-1}.[25] Very recently, an electrochemical aptasensor for detection of AFB1 developed through layer coating of cystamine, PAMAM G4 dendrimers and DNA aptamers specific to AFB1 has been reported by Castillo and co-authors. This biosensor achieved high sensitivity (LOD 0.4 nM) and it was also tested for quantifying AFB1 levels in contaminated peanuts.[26]

In this work, a colorimetric and chemiluminescence detection method of AFB1 using unmodified AuNPs as indicator and its aptamer as specific recognition element were developed (Scheme1). The detection could be performed by monitoring the color change of the AuNPs even with naked eyes and by enhancement of chemiluminescence intensity.

2. Experimental

2. 1. Reagents

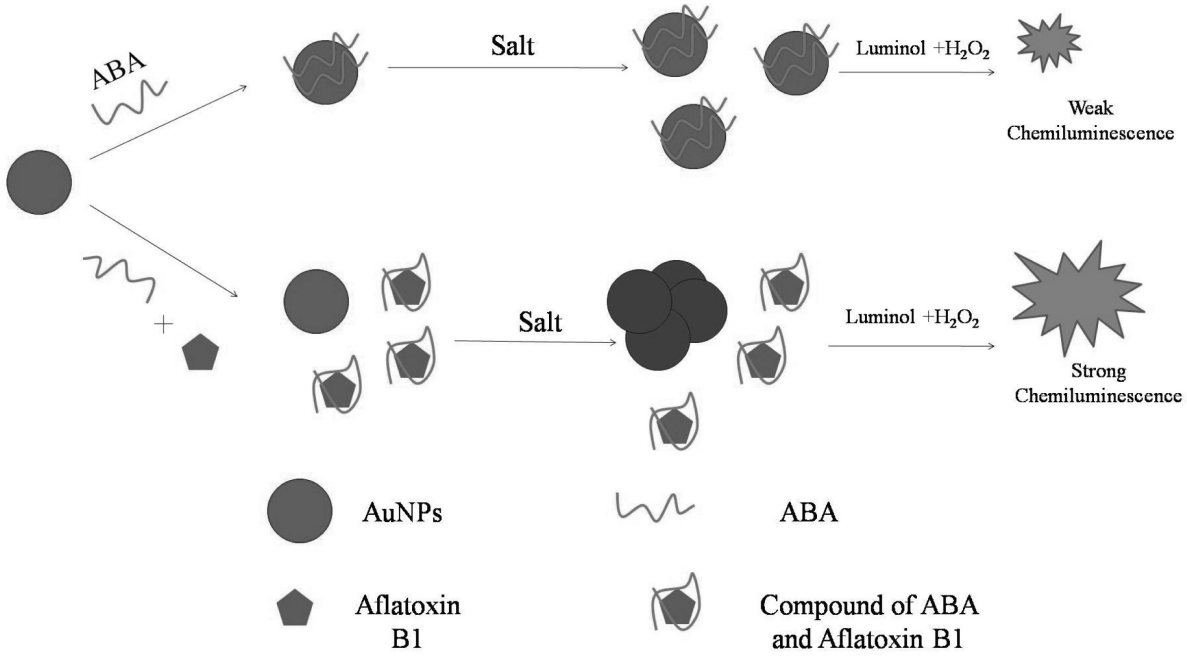
Hydrogen tetrachloroaurate(III) tetrahydrate and sodium citrate were purchased from Merck and all other commercially available substances were purchased from Aldrich, Merck and Acros and used without further purification. The 49-mer AFB1 aptamer oligonucleotide (5-GTTGGGCACGTGTTGTCTCTCTGTGTCTCGTGCC-CTTCGCTAGGCCCACA-3) was synthesized by Shanghai Generay Biotech Co (Shanghai, China). The aflatoxin B1 (AFB1) standard sample was purchased from Sigma–Aldrich, Co. All other reagents were of analytical reagent grade and ultra-pure water (Milli-Q plus, Millipore Inc., Bedford, MA) was used throughout the experiments.

2. 2. Apparatus

Absorption spectra were studied on a Perkin-Elmer lambda25 spectrometer. All chemiluminescence measurements were carried out on a Perkin-Elmer LS55 luminescence spectrometer with turn-off xenon lamp.

2. 3. The synthesis of Citrate-protected AuNP Solution

Fifty milliliters aqueous solution of hydrogen tetrachloroaurate(III) tetrahydrate (1 mM) was heated to boiling while being stirred in a round-bottom flask with a reflux condenser. Then 10 mL of trisodium citrate (38.8 mM) was added into the solution rapidly and the solution was boiled for another 10 min while the color of the solu-



Scheme 1. Schematic of the mechanism of the colorimetric and chemiluminescent detection of AFB1 utilizing aptamer and unmodified AuNPs.

tion changed from yellow to wine red. After that, heating of the solution was stopped but was stirred until it cooled down to room temperature,[27] then the AuNP solution was stored in refrigerator at 4 °C. The TEM image was used to determine diameter of the synthesized AuNPs and their dispersion state. According to Beer's law by using the absorption coefficient ($2.7 \times 10^{8} M^{-1}cm^{-1}$) at 520 nm,[28] the concentration of the AuNP solution was calculated to be about 4.4 nM. The diameter of the synthesized AuNPs was about 13 nm.

2. 4. Preparation of Peanut and Rice Samples

The fresh peanut and rice samples purchased from a local market of agriculture products were finely ground with a laboratory mill, and 5 g of each sample was placed in a 50 mL centrifuge tube. Then, 15 mL of methanol–water (60:40, *v/v*) was added, and the samples were extracted by shaking for 100 min. After centrifugation at 3500 rpm for 5 min at room temperature, 0.3 mL of supernatant was transferred to a 1.5-mL tube and diluted with 0.7 mL of pure water. The resulting solution was used as blank sample, and the spiked samples with different amounts of AFB1 were used as analytical samples. The results of the proposed biosensor were validated using a standard HPLC method.

3. Results and discussion

3. 1. Colorimetric Method for Detecting Aflatoxin B1

Since the analyte generated alteration of the LSPR properties of AuNPs and the consequent color changes from dispersed state to aggregation under different conditions was the basis of a colorimetric detection method,[29,30] the aggregation of AuNPs under different conditions was investigated and the corresponding UV–Vis absorption spectra of AuNPs were illustrated in Fig. 1. Generally, when exposed to salt medium (NaCl), the as-prepared citrate-protected AuNPs had the tendency for aggregation because the electrostatic repulsion among the nanoparticles was screened by the opposite ion of the salt.[31] As shown in Fig. 1, when appropriate amount of NaCl (aq.) was added, the absorption of AuNPs at 520 nm which was characteristic of dispersive state of AuNPs decreased remarkably, and a broad absorption band at around 700 nm appeared. These changes indicated that AuNPs aggregation was formed under salt-induction. However, after the addition of ssDNA (AFB1 aptamer or random DNA), only a similar characteristic SPR absorption band of AuNPs

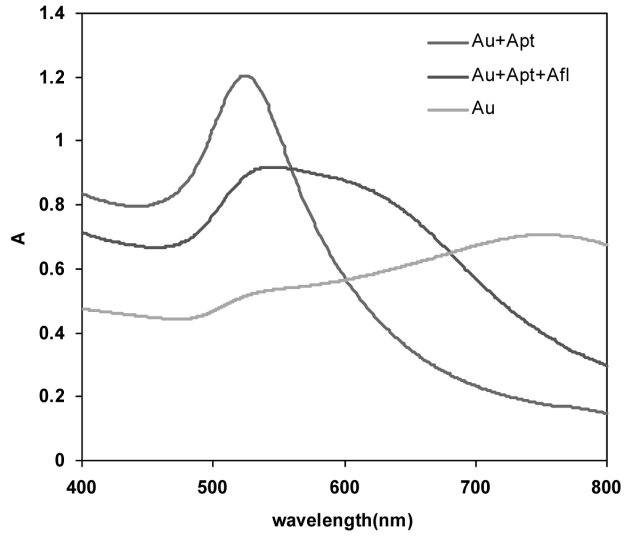


Figure 1. UV-Vis absorption spectra of AuNPs under different conditions

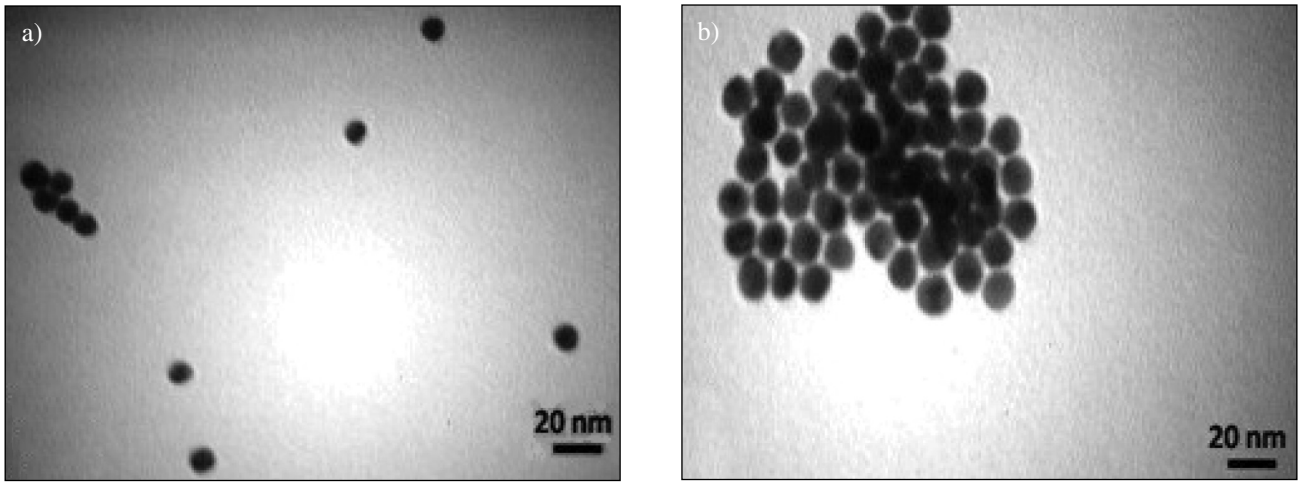


Figure 2. TEM images of AuNPs in the presence of 30 mM NaCl and 200 nM aptamer before (A) and after (B) addition of AFB1.

at about 520 nm appeared (Fig. 1). This phenomenon suggested that the aptamer AFB1 could serve as a stabilizer and protected AuNPs from salt-induced aggregation.[32] The state of AuNPs was further confirmed by the TEM image (Fig. 2A) from which we could observe that AuNPs remained dispersed in the presence of AFB1 aptamer. On the contrary, when both AFB1 aptamer and the target molecules were added, aggregation of AuNPs happened again (as shown in the TEM image of Fig. 2B), and the color of AuNPs changed from red to purple blue. At the same time, the absorption of AuNPs at 530 nm decreased accordingly in the UV–Vis spectra (Fig. 1), and a new absorption peak at about 660 nm appeared which may be ascribed to the absorption of aggregated AuNPs. It was presumed that this aggregation process of AuNPs was induced by the decrease of the electrostatic repulsion between AuNPs compared with that of the AFB1-protected AuNPs, originated from the target-binding event of AFB1 by formation of aptamer–AFB1 supramolecular complex. Utilizing this analyte-induced AuNPs aggregation and the corresponding color and LSPR changes, AFB1 could be conveniently determined by colorimetric method.

3. 2. Optimization of Experimental Conditions

In this work, the net absorption ratio between 660 nm and 520 nm, $\Delta(A_{660}/A_{520})$, in the presence and the absence of AFB1, was used to optimize the experimental conditions. The $\Delta(A_{660}/A_{520})$ value was mainly influenced by the concentrations of AFB1, concentration of NaCl, binding time of aptamer and AFB1, incubation time after adding AuNPs, reaction time after adding NaCl and the size of AuNPs. Firstly, the effect of the concentration of NaCl was studied in the concentration range of 20–120 mM. The results showed that $\Delta(A_{660}/A_{520})$ reached the maximum value when the concentration of NaCl was 40 mM. Thus, 40 mM was chosen for this work on the basis of higher sensitivity.

Secondly, the effect of the concentration of aptamer was investigated in the concentration range of 0–400 nM. The $\Delta(A_{660}/A_{520})$ reached the maximum value when the concentration of aptamer was 200 nM which was regarded as an optimum concentration in this experiment. Thirdly, the effect of binding time of AFB1-aptamer over the range of 0–15 min was studied, and $\Delta(A_{660}/A_{520})$ increased until the binding time was 10 min and it was chosen for the following experiments. Subsequently, the incubation time after adding AuNPs was studied over the range of 0–15 min and the 5 min demonstrated to be efficient incubation time because the results showed that the interaction could be completed within 5 min. Then, the reaction time after adding NaCl over the range of 0–30 min was investigated. It was found that the $\Delta(A_{660}/A_{520})$ increased substantially as the reaction time increased up to 10 min, therefore 10 min was chosen as an optimum reaction time.

3. 3. Selectivity

The selectivity of this colorimetric biosensor to AFB1 was evaluated by measuring the absorption ratio value, $\Delta(A_{660}/A_{520})$ in the presence of some common interferents such as aflatoxin B2 (AFB2), aflatoxin G1(AFG1), aflatoxin G2(AFG2), ochratoxin A (OTA) and aflatoxin M2(AFM2). As can be observed in Fig. 3, upon the addition of AFB1, there was a noticeable change in UV–Vis adsorption spectra, while no or just a little spectral change in the absence (blank) or presence of the common interferents occurred. The data derived from Fig. 3 showed that the adsorption ratio value, $\Delta(A_{660}/A_{520})$, in the presence of AFB1 was considerably larger than those of blank or the common interferents. All results indicated that our assay approach had a high specificity to AFB1.

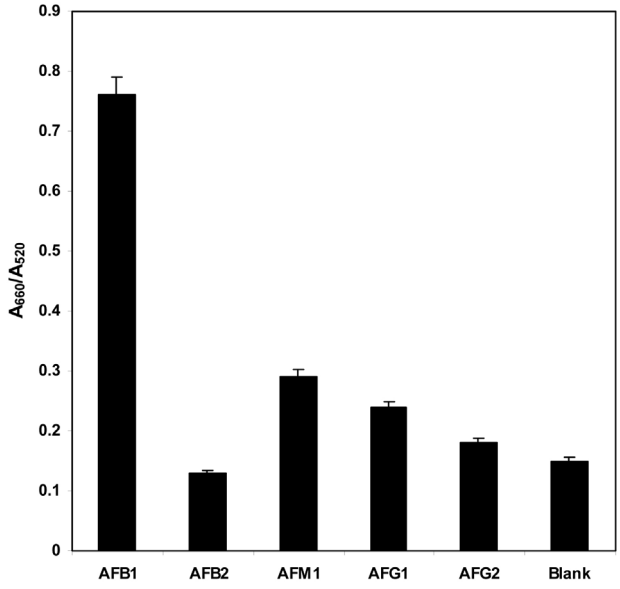
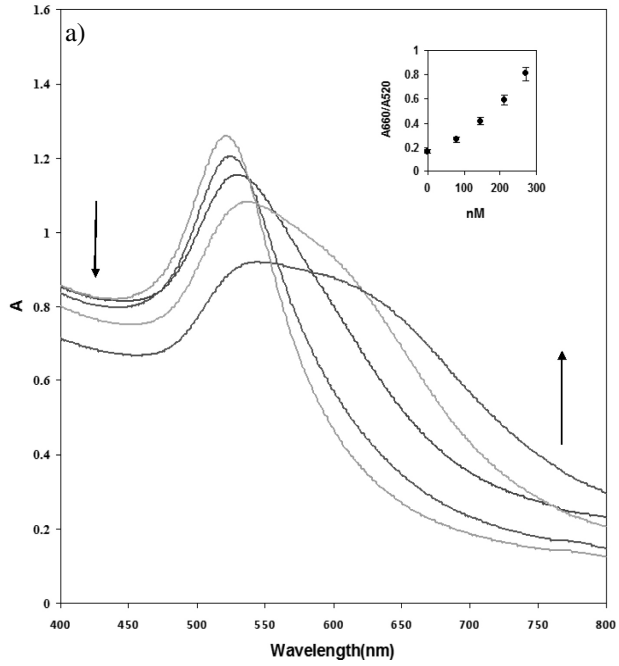


Figure 3. $\Delta(A_{660}/A_{520})$ ratio of AuNPs in the presence of different toxins in the concentration of 40 nM.

3. 4. Analytical performance of the colorimetric aptasensor for AFB1

Under the above-mentioned optimized experimental conditions, a series of different concentrations of AFB1 was respectively added. As shown in Fig. 4A, with the increase of AFB1 concentration, the absorption peak of AuNPs at 520 nm decreased gradually, whereas the absorption at 660 nm increased accordingly, indicating that more AuNPs aggregates were generated and the corresponding color of AuNPs changed from red to purple blue (Fig. 4B). It further confirmed the speculation that with the increasing concentration of AFB1, more aptamer would interact with AFB1, leading to much more severe aggregation of the less protected AuNPs in the same salt-induction situation. The limit of detection for this colorimetric



b)

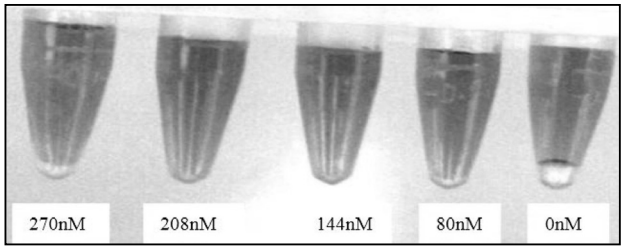


Figure 4. (A) UV-Vis absorption spectra of the AuNPs under different concentrations of AFB1, (inset) The linear calibration curve of $\Delta(A_{660}/A_{520})$ ratio to AFB1 concentration. (B) The corresponding color changes of AuNPs with different concentrations of AFB1.

analytical method was determined as 7 nM (3 *S/N*). The results showed that the $\Delta(A_{660}/A_{530})$ ratio increased linearly with AFB1 concentration in the range of 80 nM to 270 nM (Fig. 4A). It was speculated that this relatively high sensitivity of the colorimetric method could be ascribed to the color sensitivity and LSPR changes of the AuNPs from dispersive to aggregated state, as well as the high binding affinity between aptamer and AFB1.[30]

3. 5. Principle of Detection of AFB1 Using the CL Method

As shown in Scheme 1 and Fig.5, in the absence of the AFB1, the unfolded aptamer could adsorb on the AuNPs surface and prevent their salt-induced aggregation. The dispersed AuNPs induce a weak CL signal for the luminol–H_2O_2 system. In the presence of the AFB1, the aptamer binds to AFB1 strongly and results in the aggregation of the AuNPs at high salt concentration. The aggregated AuNPs induce a strong CL signal.[33–35]

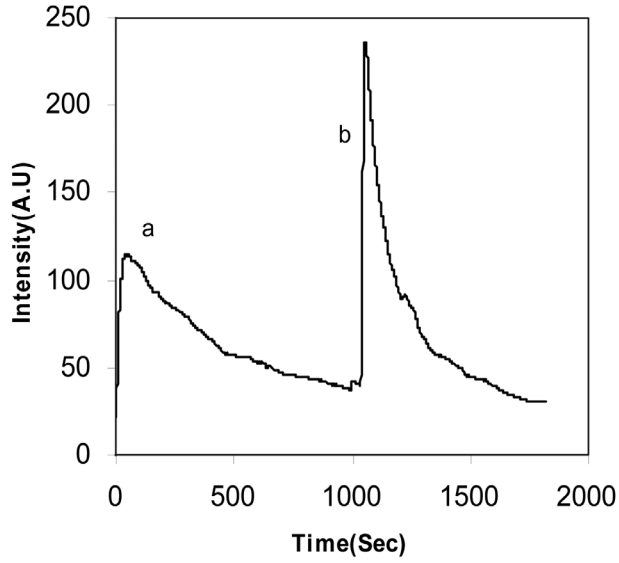


Figure 5. The effect of AFB1 on the luminol-AuNPs-H_2O_2 CL reaction (a) AuNPs in the prescence of aptamer (aptamer 200 nM; AFB1 0 M; NaCl 40 mM), (b) AuNPs in the presence of aptamer and AFB1 (aptamer 200 nM; AFB1 20 nM; NaCl 40 mM).

3. 6. Analytical performance of the CL aptasensor for AFB1

Under the optimized conditions, experiments were carried out by adding increasing amounts of AFB1 to the CL aptasensor to examine whether the CL change could be used for AFB1 quantification. As shown in Fig. 6, the CL intensity increased with the increase in AFB1 concentration and showed a linear relationship in the AFB1 concentration range 10–100 nM. The detection limit of AFB1 was 0.5 nM with a regression coefficient of $R^2 = 0.9921$, as shown in Fig. 6. Hence, the sensitivity of this system was increased 10-fold compared with the AuNP based colorimetric method for detection of AFB1.

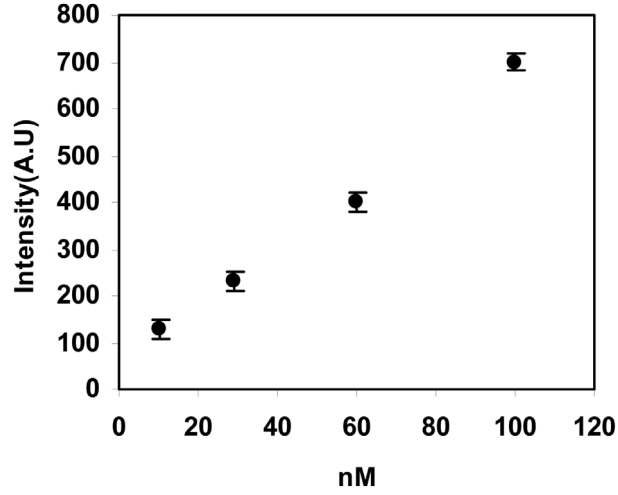


Figure 6. Calibration curve for CL method.

Table 1. Comparison of the characteristics of this aptasensor with those of the previously reported for AFB1

Detection technique	Recognition probe	Steps required	Limit of detection	Time	Ref.
Aptasensor based on real time PCR	Aptamer	>4	5 fg/ml	>2 h	24
Immunochromato-graphy	Antibody	1	0.5 ng/ml	15 min	36
Conductometric biosensor based on acetylcholinesterase	Enzyme	3	50 ng/ml	5 min	37
Aptasensor based on ELAA	Aptamer	4	0.11 ng/ml	>20 min	25
ELISA	Antibody	6	4 pg/ml	110 min	38
Colorimetric injection immunoassay (SIIA)	Antibody	6	0.1 ng/ml	<30 min	39
This work	Aptamer	2	2 ng/ml(7 nM) with colorimetric 0.1 ng/ml (0.5 nM) with CL	<10 min	–

Table 2. Results of the proposed approach for peanut and rice samples based on aptasensor and validation by HPLC

Sample	Spiked (nM)	Found aptasensor[a] (nM)	Recovery(%)	HPLC	Recovery(%)
peanut	5.0	(5.40[a] 0.10)	108	(5.20[a] 0.10)	104
	10.0	(9.95 0.10)	95	(9.970.10)	97
Rice	5.0	(4.70 0.30)	94	(5.100.10)	102
	10.0	(10.30 0.10)	103	(9.950.20)	95

[a] Results are based on four measurements

Table 1 shows the comparison of the sensitivity, rapidity and simplicity of this method with other aptamer- or antibody-based methods. Some aptasensors has been reported for AFB1 analysis. The previous studies showed that aptasensor based on real PCR could detect 25 fg/ml of AFB1 but required time-consuming (>2 h) and complicated steps (>4; pre-incubation, washing and real time PCR, etc). The sensivity of CL and colorimetric methods suggested in this work was also comparable to those of other immunosensors except ELISA based method which requires complicated experimental steps (6 steps). However, compared with the currently used immunosensors, the results in this work clearly indicate an excellent sensivity for detection of AFB1.

The proposed method was used to detect AFB1 in agricultural products and the accuracy was studied by the recovery experiment. The prepared blank and spiked samples were tested by replacing AFB1 standard samples and the concentration of AFB1 in peanut and rice samples was determined from the calibration curve. The results were compared with those obtained by the HPLC method. As shown in Table 2, percentage recovery of the spiked AFB1 samples in the range 94.0 % to 108.0 % was obtained, indicating acceptable accuracy of the proposed detection sensor for AFB1 in real samples. This analysis demonstrated that the established biosensing system could be applied for AFB1 determination in real agriculture products.

4. Conclusions

The paper introduced a novel analytical technique to detect AFB1 using aptamer as a recognition element and AuNPs as an indicator for the first time. This method showed a good sensitivity and selectivity towards AFB1 detection and avoided the interference from common interferents such as aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), ochratoxin A (OTA) and aflatoxin M1 (AFM1). Through optimization of the amount of aptamer, AuNPs, salts and incubation time, the linear range of the colorimetric aptasensor covered a large variation of AFB1 concentrations from 80 to 270 nM and the detection limit of 7 nM was obtained. The sensivity of a novel biosensor suggested in this work was also comparable to other apatasensors and immunosensors except ELISA based method which requires complicated experimental steps (6 steps).

5. Acknowledgements

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6. References

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Povzetek

Razvili smo nov biosenzor za detekcijo aflatoksina B1(AFB1), ki temelji na interakciji zlatih nanodelcev (AuNP) z aptamerjem. Agregacijo AuNP delcev je inducirala desorpcija AFB1-vezavnega aptamera s površine AuNP delcev zaradi tarčne interakcije aptamera. To je povzročilo spremembo barve AuNP delcev iz rdeče v vijolično. Linearno območje kolorimetričnega aptasenzorja je zajelo širok razpon koncentracij AFB1 od 80 do 270 nM; dosegli smo mejo zaznave 7 nM. Katalizna aktivnost agregiranih AuNP delcev je zelo povečala intenzivnost kemiluminiscenčne (CL) reakcije, pri kateri smo določili mejo zaznave 0,5 nM in regresijski koeficient R^2 = 0,9921. Pokazali smo, da se občutljivost detekcije poveča ob uporabi CL zaradi katalizne aktivnosti agregiranih AuNP delcev pri reakciji med luminolom in vodikovim peroksidom. Za predlagani nanobiosenzor smo pokazali, da je občutljiv, selektiven in preprost, kar predstavlja uporabno alternativo za hitro presejalno analizo toksina v živilih.