Target-responsive aptamer-cross-linked hydrogel sensors for the visual quantitative detection of aflatoxin B$_1$ using exonuclease I-Triggered target cyclic amplification

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Abbreviations:
aflatoxin B$_1$ (AFB$_1$, PubChem CID: 186907)
Zearalenone (ZEN, PubChem CID: 5281576)
Fumonisin B$_1$ (FB$_1$, PubChem CID: 2733487)
deoxynivalenol (DON, PubChem CID: 40024)
T-2 toxin (T-2, PubChem CID: 5284461)
Aflatoxin G$_1$ (AFG$_1$, PubChem CID: 14421)
Aflatoxin B$_2$ (AFB$_2$, PubChem CID: 2724360)
and Aflatoxin M$_1$ (AFM$_1$, PubChem CID: 15558498)
Ammoniumpersulfate (APS, PubChem CID: 62648)
HRP (PubChem CID: 9865515)
Agarose (PubChem CID: 11966311)
N,N,N',N'-tetramethylethylenediamine (TEMED, PubChem CID: 8037)
3,3',5,5'-tetramethylbenzidine (TMB, PubChem CID: 41206)
H$_2$O$_2$ (PubChem CID: 784)

ABSTRACT

For the on-site detection of aflatoxin B$_1$ (AFB$_1$), a DNA hydrogel was prepared as a biosensor substrate, while an AFB$_1$ aptamer was used as the recognition element. An AFB$_1$-responsive aptamer-cross-linked hydrogel sensor was constructed using an enzyme-linked signal amplification strategy; AFB$_1$ binds competitively to the aptamer, causing the hydrogel to undergo cleavage and release horseradish peroxidase (HRP). The addition of exonuclease I (ExoI) to the hydrogel causes the release of AFB$_1$ from the aptamer, promoting additional hydrogel cleavage to release more HRP, ultimately catalysing the reaction between 3,3',5,5'-tetramethylbenzidine and H$_2$O$_2$. The hydrogel sensor exhibited an outstanding sensitivity (limit of detection, 4.93 nM; dynamic range, 0–500 nM), and its selectivity towards seven other mycotoxins was confirmed. The feasibility and reliability were verified by measuring the AFB$_1$ levels in peanut oil (recoveries, 89.59–95.66 %; relative standard deviation, <7%); the obtained results were comparable to those obtained by UPLC-HRMS.

Introduction

The aflatoxins are a class of compounds containing the difuran and coumarin skeletons, and they are produced by fungal strains such as Aspergillus flavus, A. nomius, and A. parasiticus (Xie, Wang, & Zhang, 2019). These compounds have been detected worldwide in agricultural planting, harvesting, storage, transportation, and processing, as well as in agricultural products, foodstuffs, and animal feeds (Liu, Zhao, Lu, Ye, Wang, Wang, et al., 2020). Among them, aflatoxin B$_1$ (AFB$_1$) is the most toxic owing to its strong carcinogenicity, mutagenicity, immunosuppression, and potential to induce liver damage (Fan, Xie, & Ma, 2021). Therefore, the International Agency for Research on Cancer of the World Health Organization has classified AFB$_1$ as a Class IA dangerous substance and a Class I carcinogen (Xie, Wang, & Zhang, 2019). In addition,
the U.S. Food and Drug Administration (FDA) has set 20 μg/kg as the permissible limit for AFB1 in foods (Lerdseri, Thunkhamrak, & Jakmuneek, 2021; Xuan, Liu, Ye, Li, Tian, & Wang, 2020). The European Commission has set a 2 μg/kg limit for AFB1 in some cereals and their derivative products (Commission, 2006). In addition, China’s national food safety standard GB 2761–2017 stipulates an AFB1 limit of 0.5–20 μg/kg in foods (Xuan, Liu, Ye, Li, Tian, & Wang, 2020). Thus, to permit the on-site detection of AFB1 during food and feed production and processing, especially in underdeveloped areas, it is necessary to develop a portable qualitative AFB1-detection method with a high sensitivity and specificity.

At present, the market products available for the detection of aflatoxin focus mainly on chromatography-based (Er Demirhan & Demirhan, 2022; Wu, Ye, Xuan, Li, Wang, et al., 2021; Sarwat, Rauf, Majeed, De Boeve, De Saeger, & Igbal, 2022; Xuan, Ye, Zhang, Li, Wu, & Wang, 2019) and immunnoassay-based (Li, Wang, Sun, Ji, Ye, Li, et al., 2021; Yan, Zhu, Li, He, Yang, & Li, 2022; Wang, Zhang, Luo, Qin, Jiang, Qin, et al., 2021) solutions (Li, Wang, Sun, Ji, Ye, Li, et al., 2021; Yan, Zhu, Li, He, Yang, & Li, 2022). Although chromatography is highly sensitive and exhibits a good reproducibility, its detection cost is high, it requires large-scale instruments and professional operators, and the sample pretreatment process is relatively complicated (Lerdseri, Thunkhamrak, & Jakmuneek, 2021; Xiang, Ye, Shang, Li, Zhou, Shao, et al., 2021). Therefore, this approach is unsuitable for on-site testing. In addition, although the immunnoassay-based approach offers some advantages over chromatography (Lerdseri, Thunkhamrak, & Jakmuneek, 2021; Xiang, et al., 2021), antibodies are required as recognition elements, which presents new challenges. For example, antibody-based approaches are expensive, a poor thermal stability, and difficulty in terms of the transportation and storage, thereby hindering the development and application of immunological technologies (Ni, Zhuo, Pan, Yu, Li, Liu, et al., 2020). To address these issues, in the early 1990s, Ellington and Szostak obtained a nucleic acid sequence after multiple rounds of Systematic Evolution of Ligands by Exponential Enrichment (SELEX), which they named an “aptamer (Ellington & Szostak, 1990).” Aptamers are of interest because they can be easily prepared and modified, and they are also known to exhibit a strong thermal stability, low immunogenicity properties, almost no batch-to-batch variation, and facile storage and transportation; as such, they are expected to replace antibodies for various applications (Ni, et al., 2020). In recent years, there have been many reports on the application of aptamers in the detection of mycotoxins, such as in target-responsive DNA smart hydrogel sensors (Liu, Huang, Ma, Jia, Gao, Li, et al., 2015; Sun, Li, Chen, Wu, Ji, et al., 2019) and ochratoxin (Liu, et al., 2015) can be used as cross-linking agents to hydrogels to produce colorimetric aptamers (AuNPs). When a target is detected, the hydrogel is cleaved and the AuNPs are released, thereby turning the supernatant a red colour. Although this method can be used to determine the concentration of a target substance, its sensitivity is generally poor, and it is difficult to meet the threshold of the limit of detection for most applications. To overcome these shortcomings, Huang (Huang, et al., 2014) and Liu (Liu, et al., 2015) replaced AuNPs with platinum nanoparticles (PtNPs), and found that the released PtNPs catalysed the decomposition of H2O2 to O2, which then causes the pigment to move on a microfluidic chip, and results in concentration of the target for facile detection. In addition, Tang (Tang, Huang, Lin, Qiu, Guo, Luo, et al., 2020) used the air pressure generated by PtNPs to catalyse the decomposition of H2O2 into O2, which promoted the discharge of water. The concentration of the target was then determined by weighing the obtained water with an analytical balance. Whether performed with a microfluidic platform or an analytical balance, this method relies on the device being air-tight, and the instrument exhibiting a high precision. Furthermore, the design of the microfluidic platform requires the participation of different professionals, which largely limits its popularisation and application.

Thus, we herein report the construction of an AFB1-responsive DNA smart hydrogel sensor using a high-affinity and high-specificity AFB1 aptamer as the cross-linking agent and recognition element; this construction is combined with an enzyme cascade signal amplification strategy. During hydrogel formation, horseradish peroxidase (HRP) becomes encapsulated inside the hydrogel, and it remains encapsulated until the hydrogel undergoes cleavage. Upon the addition of AFB1, aptamer binding takes place, resulting in cleavage of the hydrogel and the release of HRP. Subsequently, the addition of exonuclease I (ExoI) specifically recognises and cleaves the AFB1-aptamer complex, thereby releasing AFB1. AFB1 again competes with the aptamer for binding, resulting in further hydrogel cleavage and the release of additional HRP, which catalyses the generation of oxygen free radicals from H2O2 outside the hydrogel system. These free radicals promote the colour development of 3,3′,5,5′-tetramethylbenzidine (TMB), thereby achieving the highly-sensitive and highly-specific visual and quantitative detection of AFB1. Finally, this method is applied for the detection of AFB1 in peanut oil samples, and its accuracy and consistency are compared with those obtained by UPLC-HRMS to determine its feasibility for use in the detection of AFB1 in underdeveloped areas.

Materials and methods

Chemicals and materials

Exol and 10 × Exol reaction buffer (pH 7.5) were purchased from Thermo Fisher Scientific (Shanghai, China) and were stored at ~20 °C. Ammonium persulfate (APS, PubChem CID: 62648) was purchased from Kulaibo Technology Co., Ltd (Beijing, China), N,N,N′,N′-tetramethylethylene diamine (TEMED, 100 %, PubChem CID: 8037) was purchased from Merck Investment (China) Co., Ltd. Standard solutions such as AFB1 (PubChem CID: 186907), Zearalenone (ZEN, PubChem CID: 5281576), Fumonisin B1 (FB1, PubChem CID: 2733487), deoxynivalenol (DON, PubChem CID: 40024), T-2 toxin(T-2, PubChem CID: 5284461), Aflatoxin G1 (AFG1, PubChem CID: 14421), Aflatoxin B2 (AFB2, PubChem CID: 2724360), and Aflatoxin M1 (AFM1, PubChem CID: 15558498) were purchased from Biopure (Tulln, Austria) and was stored at ~20 °C. Working solutions were prepared by dilution with HPLC grade methanol, and then stored in vials at 4 °C and were renewed weekly. HRP (PubChem CID: 9866551), TMB(PubChem CID: 41206)and H2O2 (PubChem CID: 784) were purchased from Solarbio Science & Technology Co., Ltd (Beijing, China), while Acr-Bis(29:1) and acrylamide were purchased from Beijing Coolibo Technology Co., Ltd and was stored at ~20 °C prior to use. Furthermore, 10 × Tris-borate-EDTA (TBE) buffer was purchased from Sangan Biological Engineering Technology & Services Co., Ltd (Shanghai, China), phosphate-buffered saline (PBS) solution was purchased from HyClone Laboratories Inc (USA), and agarose (PubChem CID: 11966311) and the TAE (Tris base, acetic acid, and EDTA) buffer solution were purchased from Soleilbio Technology Co., Ltd. (Beijing, China). NHS activated magnetic beads was purchased from Beaver ( Suzhou, China).

All oligonucleotides were synthesised by Sangon Biotech Co., Ltd. (Shanghai, China). Their sequences are shown below:

| Strand A | 5′-Acrydite-TTTGGTGCGGCTTAGGGA-3′ |
| Strand B | 5′-Acrydite-TTTACCGTGCCCAAC-3′ |
Preparation of polymers PS-A and PS-B

Referring to the method of Si (Si, Li, Wang, Zheng, Yang, & Li, 2019) 25 % acrylamide (4 µL) was mixed with PBS buffer solution (12 µL), followed by 4 mM of an acrylamide-labelled Strand A or Strand B solution (8 µL), which was mixed and degassed in a vacuum dryer for 10 min. Subsequently, a 5 % solution of APS (1 µL, the solvent is ultrapure water) and a 5 % solution of TEMED (1 µL, the solvent is ultrapure water) were added and mixed evenly. After degassing in a vacuum dryer for 18 min, the polymers were purified using a 100 kDa ultrafiltration centrifuge, redissolved in PBS buffer, and stored at 4 ◦C for later use. The PS-A and PS-B polymers were quantified using NanoDrop spectrophotometry and characterised by means of 2 % agarose gel electrophoresis.

Preparation of the AFB1-responsive aptamer-cross-linked hydrogels

Polymers PS-A and PS-B (2 µL each, 200 µM solutions) were mixed, placed in a metal bath, and incubated at 55 ◦C for 5 min, and then at 27 ◦C for 5 min; this double incubation process was repeated twice. Subsequently, HRP (1 µL, 8 mg/L) and the AFB1 aptamer (2 µL, 190 µM) were added and mixed, and the resulting mixture was placed in a metal bath for incubation at 55 ◦C for 5 min, and then at 27 ◦C for 5 min. This incubation process was repeated 6 times to yield a 3D cross-linked DNA hydrogel embedded with HRP, as characterised by 20 % PAGE gel electrophoresis.

AFB1 detection based on amplification of the Exol enzyme signal

Under the optimal conditions, referring to the method described in Tang’s study (Tang, et al., 2020), 20 U exonuclease Exol was introduced into the system to further amplify the signal and improve the sensitivity of the method. More specifically, for detection of the target AFB1, an aliquot (50 µL) of the AFB1 sample solution containing 20 U Exol (methanol content < 10 %) was added to a centrifuge tube containing the DNA hydrogel and reacted at 150 rpm and 25 ◦C for 1.5 h (Ma, Hao, Huang, He, Yan, Tian, et al., 2016). Subsequently, an aliquot (25 µL) of the supernatant was employed for the colour-generating reaction with TMB (100 µL). After 20 min, a 1 M HCl solution (100 µL) was used to quench the reaction. The absorbance of the supernatant was measured at 650 nm before quenching with hydrochloric acid, and at 453 nm after quenching.

Evaluation of the detection performance

Under the optimal conditions, AFB1 was added to the system at concentrations of 0, 100, 200, 300, 400, and 500 nM to investigate the visual detection performance of the hydrogel sensor. The specificity of the method was verified by the analysis of a 100 nM AFB1 solution and 1 µM solutions of AFB1, structural analogues, such as zearalenon (ZEN), fumonisin B2 (FB2), deoxynivalenol (DON), T-2 toxin (T-2), aflatoxin G1 (AFG1), aflatoxin B2 (AFB2), and aflatoxin M1 (AFM1).

Procedure for real sample processing

According to the method described by Xuan (Xuan, Ye, Zhang, Li, Wu, & Wang, 2019), AFB1 immune affinity magnetic beads were used on an automatic purification apparatus to extract and purify AFB1 from peanut oil. More specifically, the peanut oil sample (0.25 mL, 0.221 g), a 0.5 % PBST solution (0.45 mL), and the immune affinity magnetic beads (150 µL) were mixed in the reaction well. A 0.5 % PBST solution (2 mL) was evenly divided into two cleaning wells, and PBS (1 mL) was placed in another cleaning well. The extraction procedure involved the AFB1 present in peanut oil being captured by the AFB1 antibody fixed on the magnetic beads. After cleaning three times, the AFB1 fixed on the magnetic beads was eluted with methanol, and the volume was fixed to 1.2 mL. After filtration through a 0.2-µm filtration membrane, an aliquot was taken for quantitative analysis by UPLC-HRMS, while the other aliquot was subjected to the developed method for AFB1 detection.

Results and discussion

Preparation of the hydrogel biosensor and its AFB1 detection mechanism

To prepare the hydrogel biosensors, two acrydite-modified nucleic acids were initially prepared, namely Strand A and Strand B, which can be paired with the complementary bases at both ends of the cross-linking agent (i.e., the AFB1 aptamer), which is located within the hydrogel. Under the catalysis of APS and TEMED, Strands A and B were copolymerised in the presence of the acrylamide monomer to form polymers PS-A and PS-B, respectively. The aptamer was then linked to PS-A and PS-B to form DNA hydrogels. During hydrogel formation, HRP was added, resulting in its embedding inside the hydrogel. When the target AFB1 is present, it binds competitively to the nucleic acid aptamer, resulting in the cleavage and collapse of the hydrogel. The addition of Exol then leads to the specific recognition and cleavage of the AFB1-aptamer complex, thereby releasing AFB1, which then again competes with the aptamer for binding, resulting in further hydrogel cleavage and the release of a large amount of HRP. The released HRP then catalyses the generation of oxygen free radicals from H2O2 outside the hydrogel, and these free radicals then promote the colour change of TMB. More specifically, a blue solution was obtained in the presence of AFB1, yielding an absorbance peak at 650 nm. After termination of the reaction using hydrochloric acid, the solution turned yellow giving an absorbance peak at 453 nm. The intensities of these peaks (i.e., the peak absorbances) were found to vary depending on the content of AFB1 present, thereby confirming the visual and quantitative detection of AFB1. The principle of operation of the aptamer-cross-linked hydrogel sensors is illustrated in Fig. 1.

Characterisation of PS-A and PS-B and the mechanism of hydrogel formation

The characterisation of polymers PS-A and PS-B was carried out by means of 2 % agarose gel electrophoresis, and the results are shown in Fig. 2A. Compared with Strand A (lane 1) and Strand B (lane 3), the migration rates of polymers PS-A (lane 2) and PS-B (lane 4) were lower, thereby indicating that the molecular weights of PS-A and PS-B were significantly higher than those of their predecessors, namely Strands A and B, respectively. This is consistent with the results of the study by Ma (Ma, et al., 2018), indicating that polymers PS-A and PS-B were successfully prepared. In addition, the observation of lanes 5, 6, and 7 also showed that 2 % agarose gel electrophoresis was not suitable for characterising the mechanism of hydrogel formation.

Therefore, 20 % non-denaturing polyacrylamide gel electrophoresis (PAGE) was used to characterise the mechanism of formation of the AFB1-responsive aptamer-cross-linked hydrogel (Ma, et al., 2016). As shown in Fig. 2B, comparisons between Strand A (lane 1) and PS-A (lane 3), and Strand B (lane 2) and PS-B (lane 4) showed that nucleic acids were retained in the injection port after polymer formation. Furthermore, the migration rate of Strand B (PS-B) was higher than that of Strand A (PS-A), which also indicates that the molecular weight of Strand B (PS-B) is lower than that of Strand A (PS-A). By comparing PS-A (lane 3), PS-B (lane 4), the aptamer (lane 5), PS-A + aptamer (lane 6), PS-B + aptamer (lane 8), and PS-A + aptamer + PS-B (lane 11), the positions of the binding bands of the various component could be easily identified, as indicated in the figure. Theoretically, according to the
molecular weight, the mobility of the PS-B + aptamer band should be higher than that of the PS-A + aptamer band, but in fact, the opposite result was obtained. This suggests that the combination of PS-B with the aptamer leads to a change in the aptamer conformation, which then alters the mobility. In addition, the brightness of the aptamer-containing bands decreased after the addition of AFB$_1$, while the brightness of the aptamer and PS-B + aptamer bands decreased significantly. It was therefore inferred that the PS-B/aptamer hybridisation region and the AFB$_1$ binding site intersect, which is consistent with the results of the study by Ma (Ma, et al., 2016). Moreover, based on the observation of lane 10 (i.e., PS-A + PS-B), in the absence of the aptamer, no interactions took place between PS-A and PS-B.

**Optimisation of the concentration of each component**

The strength of the hydrogel depends on the polymer and aptamer concentrations, wherein a higher concentration leads to a stronger hydrogel. In contrast, low concentrations of the polymer and the aptamer render the hydrogel prone to cracking, which prohibits the complete encapsulation of HRP, thereby rendering it difficult to detect AFB$_1$. Therefore, to form a hydrogel with a suitable strength and sensitivity, two ratios of PS-A:PS-B:aptamer were initially determined to be suitable, namely 1:1:0.5 and 1:1:1. More specifically, during optimisation, these ratios were found to enable a sufficient amount of HRP embedding into the hydrogel to explore the optimal concentrations of polymers PS-A and PS-B. As shown in Fig. 3A, with a polymer concentration of 200 $\mu$M and a PS-A:PS-B:aptamer ratio of 1:1:1, macroscopic hydrogels were formed. The photographic images shown in this figure indicate that the zero boundary of each macroscopic hydrogel was present at a polymer concentration of 200 $\mu$M and an aptamer concentration between ~100 and 200 $\mu$M.

Furthermore, if the concentration of HRP is too high, complete
embedding was not achieved, resulting in a large background signal that interferes with the detection results. Conversely, if the concentration of HRP is too low, the cleavage response signal is also low, resulting in a poor sensitivity of the detection method. Thus, to optimise the concentration of HRP, the polymers and the aptamer (200 μM each) were combined in a 1:1:1 ratio, and the HRP concentration was varied across six samples. As shown in Fig. 3 B, at HRP concentrations of 2, 6, and 8 mg/L, the background signal value is relatively small. However, upon increasing the HRP concentration to 10 mg/L, the background signal is more than double that obtained at 8 mg/L. Furthermore, at a concentration of 15 mg/L, a higher background signal was observed once again, and so an HRP concentration of 8 mg/L was considered to be optimal.

Optimisation of the aptamer concentration was then performed in the concentration range of 180–220 μM, as shown in Fig. 3 C. It was found that not only the hydrogel cleavage signal formed in the presence of 180 μM aptamer (i.e., the signal corresponding to 2.5 μM AFB1), but also the associated background signal (i.e., the signal value corresponding to 0 μM AFB1) were the largest signals. The background signals for the remaining four concentrations (i.e., 190, 200, 210, and 220 μM) were relatively comparable (OD < 0.1), although a slightly superior result was obtained for an aptamer concentration of 190 μM, and so this was selected as the optimal aptamer concentration.

**Signal amplification strategy**

ExoI was introduced into the experiment to obtain superior sensitivity over a relatively short period of time. More specifically, according to a study by Tang (Tang, et al., 2020), ExoI can assist AFB1 in splitting the hydrogel and releasing additional HRP to further catalyse the colour development of TMB. Indeed, as shown in Fig. 3 D, the introduction of
ExoI increased the difference between the cleavage signal and the background signal by 1.56 times, which demonstrated that ExoI can assist AFB$_1$ in cleaving the hydrogel. The released HRP then catalyses the reaction between H$_2$O$_2$ and TMB. Prior to quenching the reaction with hydrochloric acid, the reaction signal initially increased sharply prior to stabilising after 75 min. As shown in Fig. 3E, the rate of increase in the signal value is most pronounced during the initial 20 min. Thus, to shorten the detection time as much as possible, 20 min was selected as the optimal colour development time. As a result of this process, the detection range of the sensor can be increased or decreased by adjusting the reaction time within a certain range.

Visual quantitative detection of AFB$_1$

Under the optimised conditions, a range of AFB$_1$ concentrations were investigated to demonstrate the quantitative detection of this toxin. As shown in Fig. 4A and 4B, upon increasing the concentration of AFB$_1$, the solution colour become more intense, and the corresponding absorbance value gradually increased, showing a positive linear relationship that can be fitted with the equation: $y = 0.0012x + 0.1453$ ($R^2 = 0.9943$). The limit of detection for AFB$_1$ based on this method was therefore estimated to be 4.93 nM (signal-to-noise ratio = 3).

A comparison of our AFB$_1$-responsive DNA smart hydrogel with some previously reported systems is presented in Table 1. Although the hydrogel constructions were essentially comparable between these systems, the embedding materials were different. In addition to the
nanoparticles mentioned in the introduction, target-responsive hydrogels have also been embedded with encapsulated enzymes (Mao, Li, Yan, Ma, Song, Tian, et al., 2017; Si, Li, Wang, Zheng, Yang, & Li, 2019; Ma, et al., 2016). For example, Tian and his colleagues (Tian, Wei, Jia, Ma, Song, Tian, et al., 2017; Si, Li, Wang, Zheng, Yang, & Ma, et al., 2016) used an amylase released during hydrogel decomposition of $H_2O_2$ to generate $O_2$, which promotes the movement of pigments on the microfluidic chip for the hydrogel cleavage to catalyse the redox reaction between $H_2O_2$ and $O_2$. Furthermore, Sun and his colleagues (Sun, Li, Chen, Wu, & Liang, 2020) used the HRP released by the hydrogel cleavage to catalyse the redox reaction between $H_2O_2$ and $O_2$. The generated $I_2$ was then used to etch gold nanorods (AuNRs), resulting in a wavelength shift, which allowed the content of a T-2 toxin to be estimated with a good sensitivity and accuracy. However, despite these advances, there are few reports on the use of AFB$_1$-responsive DNA smart hydrogels to encapsulate enzymes. Our method uses a cascade of enzyme reactions, and the introduction of ExoI achieves the desired cycle amplification and promotes the release of additional HRP to further catalyse the colour-generating reaction between TMB and $H_2O_2$, ultimately realising a dual signal amplification strategy. In addition, our system shows certain advantages in terms of its detection range, as indicated in the table.

**Detection selectivity**

To investigate the specificity of the developed hydrogel biosensor, we introduced additional toxins in combination with AFB$_1$, i.e., ZEN, FB$_1$, DON, T-2, AFG$_1$, AFB$_2$, and AFM$_1$. The concentration of AFB$_1$ was set at 100 nM, while the concentrations of the other toxins were set at 1 µg/kg. The results of the assay are shown in Fig. 4C, wherein the advantages of the aptamer-based approach can be clearly observed. More specifically, although the concentration of AFB$_1$ was only 10% that of each other toxin, its signal was 2–3 times more intense than those of the other toxins, confirming the specificity of the sensor toward our target toxin.

**Determination of AFB$_1$ contents in real samples**

Finally, the developed hydrogel biosensor was used to test peanut oil samples containing AFB$_1$. Thus, AFB$_1$ concentrations of 10, 20, and 40 µg/kg were added to the peanut oil samples; these concentrations are equivalent to 0.5, 1, and 2 times the 20 µg/kg limit specified by the FDA. Each sample was then pre-treated according to the experimental method described in Section 2.6, and the content of AFB$_1$ in each peanut oil sample was measured by both UPLC-HRMS and using our hydrogel biosensor. As outlined in Table 2, the average spiked recoveries obtained for the hydrogel biosensor and by UPLC-HRMS were approximately 89.59–95.66 % and 95.44–99.97 %, respectively, with RSD values of < 7 % in both cases. This comparison therefore confirms the accuracy and repeatability of our method, and indicate its potential for use in the detection of AFB$_1$ in real samples.

### Table 1

| Embedded material | Signal amplification | detection method | detection limit (nM) | detection range (µM) | Recovery (%) | references |
|-------------------|---------------------|------------------|----------------------|----------------------|-------------|------------|
| PtNPs             | micro-fluidic chip  | AFB$_1$ splits the hydrogel and releases PtNPs. PtNPs catalyze the decomposition of $H_2O_2$ to generate $O_2$, which promotes the movement of pigments on the microfluidic chip | 1.77 | 0.25–40 | – | (Ma, et al., 2016) |
| AuNPs             | –                   | AFB$_1$ splits the hydrogel and releases AuNPs, which change the color of the supernatant from colorless to red. | 0.21 | 0.25–10 | – | (Ma, et al., 2016) |
| PtNPs             | Exo I               | AFB$_1$ splits the hydrogel to release PtNPs. PtNPs catalyze the decomposition of $H_2O_2$ to generate $O_2$, and the air pressure promotes the discharge of water, which is weighed by an analytical balance. | 9.4 | 31.2–6.2 | 91.5 % | (Tang, et al., 2020) |
| PtNPs             | Exo I               | AFB$_1$ splits the hydrogel, releases urease, and urease hydrolyzes urea, which changes the pH of the solution. | 0.1 | 0.2–2 | 82.26 % | (Zhao, Wang, Guo, Wang, Luo, Qu, et al., 2018) |
| HRP               | Exo I               | AFB$_1$ splits the hydrogel, releasing HRP. HRP catalyzes the color reaction between TMB and $H_2O_2$ and the concentration of AFB$_1$ in solution is obtained according to the change of color and absorbance value. | 4.93 nM | (8.395 µg/kg) | 89.59 % | This work |

### Table 2

Comparison between this experimental method and UPLC-HRMS in actual sample detection results.

| Sample | Added concentration (µg/kg) | Standard addition concentration (nM) | Developed sensor | UPLC-HRMS |
|--------|-------------------------------|--------------------------------------|------------------|-----------|
|        |                               |                                      | Detected (nM) | Recovery (%) | Average recovery (%) | RSD (%) | Detected (nM) | Recovery (%) | Average recovery (%) | RSD (%) |
| peanut oil | 0                             | 0                                    | ND              | –           | –                | –       | ND              | –           | –                | –       |
|         | 10                            | 5.8977                               | 5.78            | 97.97 %     | 89.59 %          | 6.61 %  | 5.37            | 91.08 %     | 95.44 %          | 3.25 %  |
|         |                              | 5.04                                 | 85.41           | 4.93 nM     | (8.395 µg/kg)    | 4.93 nM | 9.71            | 99.19 %     | 95.66 %          | 3.25 %  |
|         |                              | 5.04                                 | 85.41           | –           | –                | –       | 5.78            | 98.03 %     | –                | –       |
|         |                              | 20.96                                | 92.94           | 2.76 %      | 99.80 %          | 99.97 % | 11.77           | 98.95 %     | –                | –       |
|         |                              | 10.59                                | 89.80           | 2.10 %      | 98.16 %          | 98.16 % | 11.67           | 98.95 %     | –                | –       |
|         |                              | 11.33                                | 96.08           | 2.10 %      | 98.16 %          | 98.16 % | 11.93           | 101.16 %    | –                | –       |
|         |                              | 22.44                                | 95.14           | 2.05 %      | 98.38 %          | 98.38 % | 23.10           | 97.91 %     | –                | –       |
|         |                              | 22.07                                | 93.57           | 2.05 %      | 98.38 %          | 98.38 % | 23.51           | 99.64 %     | –                | –       |
|         |                              | 23.19                                | 98.28           | 2.05 %      | 98.38 %          | 98.38 % | 23.02           | 97.58 %     | –                | –       |
Conclusions

We constructed and tested a smart aptamer-based DNA hydrogel biosensor for the quick and accurate on-site detection of aflatoxin B1 (AFB1). An AFB1 aptamer with a high affinity and specificity was used as the cross-linking agent and recognition element of the hydrogel, and the cascade enzyme reaction signal amplification strategy was used to successfully construct the AFB1 responsive aptamer-cross-linked hydrogel sensor. The linear range of the sensor was ~ 0–500 nM, its limit of detection was 4.93 nM (signal-to-noise ratio = 3), and its accuracy and repeatability were comparable to those of UPLC-HRMS. Overall, our results indicate the potential of this sensor to be used for the highly sensitive on-site detection of AFB1 in underdeveloped areas.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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