Analytical Methods

Development of a quantum dot nanobead-based fluorescent strip immunosensor for on-site detection of aflatoxin B\textsubscript{1} in lotus seeds

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A B S T R A C T

Owing to the serious threat of aflatoxin B\textsubscript{1} (AFB\textsubscript{1}) to public health, development of a reliable method for accurate determination of it is extremely necessary and urgent. In this study, a simple, rapid and highly-sensitive quantum dot nanobeads (QB) based lateral flow fluorescent strip immunosensor was developed for on-site detection of AFB\textsubscript{1} in edible and medicinal lotus seeds. Carboxylated QBs were used as the fluorescent markers to prepare the fluorescent probe through coupling QBs with anti-AFB1 antibodies. Bovine serum albumin (BSA)-AFB\textsubscript{1} antigens and goat anti-mouse IgG antibodies were coated on the nitrocellulose (NC) membrane to prepare the test (T) and control (C) lines, respectively. Qualitative analysis of AFB1 was realized by naked eye, and the quantitative determination was achieved with a portable strip reader. Results showed that the newly-developed test strip sensor could achieve rapid detection of AFB\textsubscript{1} within 15 min, allowing a limit of detection (LOD) of 1 ng/mL (2 μg/kg) and a linear range of 1–19 ng/mL (2–38 μg/kg). Recovery rates from the fortified lotus seeds with low, medium and high spiking concentrations (2.5, 5 and 10 μg/kg) ranged from 94.0% to 116.0% with relative standard deviations less than 10%. All the results were confirmed by a standard LC-MS/MS method. The QBs-based fluorescent strip immunosensor with high sensitivity, easy operation, and low cost provided a preferred solution for rapid, on-site screening and highly-sensitive quantitation of AFB\textsubscript{1} in a large number of lotus seed samples.

1. Introduction

Lotus seeds are the dry mature seeds of *Nelumbo nucifera* Gaertn (Zhang et al., 2012). With well-known edible and medicinal values, as well as remarkable nutritional functions, they have been widely traded and used globally (Kaur et al., 2019). However, due to improper operation or environmental conditions in the cultivation, harvest, processing and storage processes, lotus seeds are easily contaminated by mycotoxins, especially aflatoxins (AFs) (Liu et al., 2019; Wei et al., 2019; Zhao et al., 2020).

AFs are the secondary metabolites produced by *Aspergillus* fungi of the *Aspergillus* genus (Bräse et al., 2009), which have obtained worldwide concern due to their severe hepatotoxic, carcinogenic, teratogenic, mutagenic toxic effects against human health (Kumar et al., 2017; Martins et al., 2020). Of these, AFB\textsubscript{1} is the most toxic and has been classified as a Class 1A human carcinogen by the International Agency for Research on Cancer (IARC, 1993). Strong toxicity and high incidence rate of AFB\textsubscript{1} in lotus seeds have seriously affected their quality, safety and effectiveness, as well as physical and health threats to the consumers. Consequently, a maximum residue level of 5 μg/kg is officially regulated for AFB\textsubscript{1} in lotus seeds in China (China Pharmacopoeia Commission, 2020). To meet with the strict regulation, development of reliable methods for accurate and sensitive determination of AFB\textsubscript{1} at μg/kg level in lotus seeds is of great importance and urgency.

Over the last few decades, the strategies in addressing this issue were mainly focused on chromatographic and spectrographic techniques (Liao et al., 2020; Liu et al., 2019; Oplatowska-Stachowiak et al., 2016; Wang et al., 2020; Wei et al., 2019; Zhao et al., 2020). Although are sensitive and precise, they have been limited in professional laboratories relying on sophisticated and heavy instruments, skilled person, tedious pre-treatment step, and high cost- and time-consumption (Chen et al, 2020; Fang et al, 2020). As a promising candidate, fluorescent strip (FS) immunosensor provides a preferred solution owing to its outstanding simplicity, rapidity, portability, low cost, and small time- and sample-consumption (Qin et al., 2019; Shi et al., 2020). This technique integrates antibody as the specific sensing element for directly capturing the target, as well as an appropriate physicochemical transduction mechanism to convert the recognition events to readable fluorescent
signals for qualitative and quantitative analysis (Wang et al., 2019; Zhang et al., 2020; Zhu et al., 2019). While, for achieving enhanced fluorescent signal intensity to improve the detection sensitivity, various nanomaterials have been introduced into FS immunosensor (Shirshahi and Liu, 2021; Xing et al., 2020). Among these, quantum dot nanobeads (QBds) have addressed special attention. By embedding numerous quantum dots (QDs) in the polymer nanobeads, QBds integrate the excellent optical properties of QDs, while, displaying 1000-times higher fluorescent intensity (FI) and stronger tolerance against solution pH and ion strength than the corresponding QDs (Hu et al., 2017; Li et al., 2019). In addition, the use of QBds as the carrier can significantly increase the speed of flow measurement chromatography to shorten the detection time (Huang et al., 2019; Tan et al., 2020; Wu et al., 2020). More importantly, a variety of capture probes can be fixed on the surface of the QBds for the simultaneous determination of multiple targets of interest (Rong et al., 2019; Xiao et al., 2017). However, the combined use of QBds and FS immunosensor for the detection of AFB₁ in edible and medicinal lotus seeds has not been reported.

Therefore, in this study, a simple and easy-to-construct CdSSe@ZnS QBds-based FS immunosensor was developed in Scheme 1 for on-site rapid detection of AFB₁ in lotus seeds. The nitrocellulose (NC) membrane was previously fixed on the polyvinylchloride (PVC) backing pad to construct a strip. Then, anti-AFB₁ monoclonal antibodies (mAbs, primary Abs) labeled with QBds (QBds-mAbs) were sprayed and dried on the conjugate pad of the NC membrane as the fluorescent reporters. The bovine serum albumin-AFB₁ (BSA-AFB₁) antigen conjugates were fixed on the test (T) line for recognizing the reporters. While, the goat anti-mouse IgG antibodies (secondary Abs) was immobilized on the control (C) line to capture the QBds-mAbs-AFB₁ complexes for the positive sample, as well as the unbound QBds-mAbs from the conjugate pad regarding the negative sample, to further confirm the performance (valid or invalid) of the immunosensor. The visualization of the color changes on the T and C lines by naked eye can achieve rapid qualitative analysis (positive or negative sample). And a portable strip reader was introduced to record the FI values of QBds on the T (FI_T) and C (FI_C) lines excited by the light source for on-site quantitative detection of AFB₁. The integrated use of mAbs with high affinity and specificity against target AFB₁ and the CdSSe@ZnS QBds with stable and strong fluorescence...
signals largely improved the method selectivity and detection sensitivity. Under the optimal conditions, this developed QBs-FS immuno-sensor exhibited ultra-low limit of detection (LOD) of 2 μg/kg for AFB1 in lotus seeds, as well as small analytical time of <15 min for one run. Hence, it offers great potential for rapid, cost-effective, and highly-sensitive quantitative detection of trace contaminants in food safety testing, and environmental pollution monitoring.

2. Materials and methods

2.1. Reagents and chemicals

Surface-carboxylated CdSSe@ZnS QBs (excitation wavelength of 300–450 nm and emission wavelength of 610 nm) was obtained from Beijing Nanogen Biotechnology Co., Ltd. (Beijing, China). Murine monoclonal antibodies of AFB1 (anti-AFB1: Abs) and bovine serum albumin (BSA)-AFB1 were all acquired from Shandong Lvdv Bio-science & Technology Co., Ltd. (Shandong, China). 25 μg/mL of AFB1 standard solution was supplied by Pribolab Company (Singapore) and kept at −20 °C in the dark. Goat anti-mouse IgG Abs were provided by Beijing Biodragon ImmuneTechnologies Co., Ltd. (Beijing, China). N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC) were all purchased from Beijing Bailingwei Technology Co., Ltd. (Beijing, China). Nitrocellulose (NC) membrane was supplied by Germany Sartorius Co., Ltd. (Germany). ABP-S370 absorbent paper was purchased from Huaiyuan Tongcheng Paper Products Co., Ltd. (Shanghai, China). 0.5%*300 polyvinylchloride (PVC) sheet was sheet purchased from Dongguan Jinlei Biological Technological Co., Ltd. (Guangdong, China) and Ahlstrom 8951 glass fiber membrane was acquired from Shanghai Jieyi Biotechnology Co., Ltd. (Shanghai, China). 0.2% BSA and 0.2% Tween-20 in 0.1 M Tris-HCl were mixed as the sample diluent. The QBs blocking solution was prepared by dissolving 10 mg/mL of BSA with 100 mM glycine. Other reagents were of analytical grade or better. All solutions were prepared with ultra-pure water from a Milli-Q purification system.

2.2. Apparatus

An HM3030 type dot film instrument (Shanghai Kinhio Tech.Co., Ltd. China) and ZQ2002 automatic cutting machine (Shanghai Kinhio Tech.Co., Ltd. China) were all used to prepare the test strips. A fluorescence quantitative chromatography detection card reader from Suzhou Hemai Precision Instrument Co., Ltd. (Suzhou, China, excitation wavelength of 365 nm and emission wavelength > 600 nm) was used for recording the fluorescence intensity from the T and C lines. Confirmation of AFB1 in positive samples was performed on an ultra-fast liquid chromatography (Shimadzu, Kyoto, Japan) system coupled to an Applied Bio-system Sciex 5500 QTRAP® hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA) equipped with the electrospray ionization (ESI) source.

2.3. Preparation of the QBs-mAbs probes

The water-soluble QBs were synthesized by using a microemulsion method in Beijing Nanogen Biotechnology Co., Ltd. (Beijing, China). The size distribution and morphology of the QBs were characterized by a high-resolution transmission electron microscope (FEI Tecnai G2 F30, Oregon, USA).

The QBs-mAbs probes were prepared by coupling the amino group of anti-AFB1 mAbs with the carboxyl group of the QBs using the active ester method (Li et al., 2019). First, a 10 mM 2-(N-morpholine) ethanesulfonic acid (MES, pH 6.0) buffer system was prepared by diluting 100 μL of QBs (1 μmol/L) solution with 10 μL of 2-(N-morpholine) ethanesulfonic acid (500 mM, pH 6.0). After the addition of 2 μL of 20 mg/mL EDC/NHS solution, the mixture reacted at 37 °C for 15 min, followed by centrifugation at 10,000 r/min for 10 min to discard the supernatant. Then, the precipitates were resuspended with the addition of 10 mM MES (pH 6.0) buffer to the final volume of 0.5 mL for activating the QBs by ultrasonic processing. Immediately, 100 μL of anti-AFB1 Abs (100 μg/mL) were added to the activated QBs to react at 37 °C for 1 h. Subsequently, 10 μL of QBs blocking solution was added to block the excess binding sites for 30 min at 37 °C, followed by centrifugation at 10,000 r/min for 10 min. After discarding the supernatant, the precipitates were resuspended in 100 μL 0.1 M Tris-HCl (pH 8.0) for mixing by ultrasonic processing ultrasound. Then, the anti-AFB1 mAbs were immobilized onto the surface of QBs to obtain the QBs-mAbs probes that were stored at 4 °C for future use.

2.4. Fabrication of the QBs-FS immunosensor

The detection principle of the QBs-FS immunosensor was shown in Scheme 1, which was composed of sample pad, conjugate pad, NC membrane, and absorbent pad. The sample pad was saturated with 30 mL of the treatment solution containing 0.5% (w/v) BSA, 0.1% polyvinyl pyrrolidone (PVP)-10, 0.9% sodium chloride (NaCl), and 1% Tween-20 in Tris-HCl (0.1 M pH 8.0) and dried at 60 °C for 2 h. And the conjugate pad was prepared in advance by coating 30 mL of the treatment solution containing 4% sucrose, 1% trehalose, 0.2% Tween-20, and Tris-HCl (0.1 M pH 8.0) on a 30*20 cm NC membrane, and dried at 60 °C for 2 h. The BSA-AFB1 (0.5 mg/mL) conjugates and goat anti-mouse IgG Abs (0.2 mg/mL) were dispensed onto the surface of the NC membrane at densities of 4 μL/cm, respectively, and then dried at 37 °C for 12 h to prepare the T and C lines. The above-obtained QBs-mAbs probes were sprayed on the conjugate pad to be used as the fluorescent reporters. Finally, the sample pad, conjugate pad, NC membrane, and absorbent pad were sequentially laminated and pasted onto a PVC backing card overlapping 1 mm on top of each other. The assembled card was cut into 4 mm wide strips by using an automatic programmable cutting machine, and sealed in a plastic bag with desiccant gel for storage at room temperature before use.

2.5. Sample solution preparation

Fourteen batches of normal crude lotus seed samples were collected from different origins in Hebei, Hunan, Fujian, Jiangxi, Hubei, and Liaoning Provinces of China, and labeled as S1-S14. All samples were ground to pass through a 40-mesh sieve. 5 g of sample powder was accurately weighed with the addition of 0.4 g NaCl and extracted with 10 mL of methanol–water (70:30, v/v) for 5 min on a vortex shaker. After centrifugation at 3000 r/min for 10 min, the supernatant solution was collected stored at −20 °C as the negative (AFB1-free) sample solution.

Different volumes of AFB1 standard solution were added into the above negative sample solution to prepare the fortified lotus seed extracts as the working solutions containing 0, 0.2, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/mL of AFB1 for specificity and accuracy evaluation.

2.6. QBs-FS immunosensor detection of AFB1 in lotus seeds

Eighty microliter of the above-prepared working sample solution was dropped added into the well of the sample pad. After a 15-min incubation period, the color changes on the T and C lines were visualized with a fluorescent reader to record the Fl1 and Fl2 to calculate the Fl1/Fl2 ratio. Each analysis was repeated 3 times.

The detection principle of the QBs-FS immunosensor has been presented in Scheme 1. The added sample solution liquid is drawn toward the absorbent pad by capillary forces. As regard to the negative (AFB1-free) sample, most of the QBs-mAbs probes from the conjugate pad bind to the BSA-AFB1 antigen on the T-line and the other probes bind with the goat anti-mouse IgG Abs on the C line, resulting in bright colors, as well
as higher FI<sub>T</sub> value than FI<sub>C</sub> value. Here, the FI<sub>T</sub>/FI<sub>C</sub> value was measured as B<sub>0</sub>. While, regarding the positive (containing AFB<sub>1</sub>) or spiked sample, the QBs-mAbs probes will competitively capture the target AFB<sub>1</sub> with the BSA-AFB<sub>1</sub> antigen on the T line through the antigen–antibody reaction, and further bind with the goat anti-mouse IgG Abs on the C line, leading to bright color on the C line, as well as decreased FI<sub>T</sub> value. At this time, the FI<sub>T</sub>/FI<sub>C</sub> value is counted as B. If no color is visualized or no fluorescence is detected on the C line, the FS immunosensor is considered invalid.

The content of AFB<sub>1</sub> was quantitatively calculated according to a linear regression equation that was established by plotting the FI<sub>T</sub>/FI<sub>C</sub> value against the spiked AFB<sub>1</sub> concentration (c). And the limit of detection (LOD) to assay the sensitivity of the QBs-FS immunosensor by the fluorescent reader was defined as the concentration of AFB<sub>1</sub> in the solution that induce 10% decrease of the FI<sub>T</sub>/FI<sub>C</sub> value compared with that induced by solution without AFB<sub>1</sub>.

2.7. UFLC-MS/MS confirmation

The reliability and practicality of the newly-developed QBs-FS immunosensor was further confirmed by UFLC-ESI-MS/MS analysis based on two essential parameters, including retention time and fragment ion of AFB<sub>1</sub>. The separation of AFB<sub>1</sub> was carried out on a Waters ACQUITY BEH C<sub>18</sub> column (2.1 × 100 mm, 1.7 μm) held at 35 °C. The mobile phase was consisted of methanol (A) and water (B), both containing 0.1% formic acid (v/v) at a flow rate of 0.2 mL/min and the following gradient elution procedure: 60% B for 0–0.5 min, 5% B for 0.5–4.5 min, 60% B for 4.5–10 min. The most intense ion-pair information for multiple reaction monitoring (MRM) was optimized as follows: m/z 313.1 → 285.2 and 313.1 → 269.1 for AFB<sub>1</sub>. ESI source was performed at +5000 V. The conditions of mass spectrometer detector were performed as follows: nebulizer gas (GS1), 50 psi and auxiliary gas (GS2), 50 psi; curtain gas (CUR), 35 psi; capillary temperature, 550 °C.

3. Results and discussion

3.1. Characterization of QBs

The size distribution and morphology of fluorescent CdSSe@ZnS QBs were characterized by using TEM analysis. Fig. 1 indicated that QBs were tightly encapsulated in the well-isolated polymer nanobeads with relatively uniform and regular quasi-spherical shapes of 50–100 nm. These phenomenon suggested that the fluorescent CdSSe@ZnS QBs were standard enough for the establishment of QBs-FS immunosensor.

![TEM images of QBs with different magnifications.](image)

3.2. Optimization of the labeled amount of mAbs on the QBs

The successful conjugation of anti-AFB<sub>1</sub> mAbs with QBs to form the QBs-mAbs probes is crucial for ensuring the sensitivity and wide detection range of the established FS immunosensor. Herein, the QBs coated with diverse amounts of mAbs were fixed on the conjugate pad, and the FI<sub>T</sub> and FI<sub>C</sub> values on the C and T lines after adding a series of spiked lotus seed sample solutions with different concentrations of AFB<sub>1</sub> were recorded to calculate the FI<sub>T</sub>/FI<sub>C</sub> value for establishing the FI<sub>T</sub>/FI<sub>C</sub>-c regression equation. Fig. 2A showed that with increasing the added content of AFB<sub>1</sub> in the range of 0–40 ng/mL, the FI<sub>T</sub>/FI<sub>C</sub> value all gradually decreased. The increase of the coated amount (50–300 μg/mL) of mAbs on the surface of QBs would widen the detectable content range of AFB<sub>1</sub> from 0 to 10 ng/mL to 0–40 ng/mL, as well as the detected range of FI<sub>T</sub>/FI<sub>C</sub> value from 0.67 ± 0.19 to 30.52 ± 5.82. All these demonstrated that the FS immunosensor was successfully established for reliable detection of AFB<sub>1</sub>.

The recommended limit of AFB<sub>1</sub> in lotus seed in the Chinese Pharmacopoeia (2020 Ed.) is 5 μg/kg, which is equal to 2.5 ng/mL regarding the spiking level for lotus seed in this study. Fig. 2A displayed that, when the coated amount of mAbs was up to 200 or 300 μg/mL and the concentration of AFB<sub>1</sub> was set at 0–2.5 ng/mL, the FI<sub>T</sub>/FI<sub>C</sub> value did not present significant change, which was not enough for sensitive detection of AFB<sub>1</sub>, at 2.5 ng/mL. When 50 μg/mL or 100 μg/mL of mAbs was coated onto the surface of the QBs, the FI<sub>T</sub>/FI<sub>C</sub> values exhibited remarkable decreasing trends for AFB<sub>1</sub> in the concentration ranges of 0–10 ng/mL and 0–20 ng/mL, respectively, which could achieve sensitive detection of AFB<sub>1</sub>. Regarding 100 μg/mL of mAbs, the FI<sub>T</sub>/FI<sub>C</sub>-c curve showed a much wider detectable content range of AFB<sub>1</sub> in the fortified lotus seed sample than that of 50 μg/mL of mAbs. Therefore, 100 μg/mL of anti-AFB<sub>1</sub> mAbs was verified as the coated amount onto the surface of QBs.

3.3. Optimization of the QBs-FS immunosensor

In this QBs-FS immunosensor, the labeled QBs-mAbs probes and the BSA-AFB<sub>1</sub> antigen were sprayed on the conjugate pad and the T line, respectively. This would affect the sensitivity and reproducibility between different batches of immunosensors. To obtain the optimal sensitivity and strong FI signals on both T and C lines, a classical “checkerboard titration” method was performed with different QBs-mAbs spraying contents under a series of concentration of BSA-AFB<sub>1</sub> conjugates on the T line for various combinations. The values of FI<sub>T</sub>, FI<sub>T</sub>/FI<sub>C</sub>, and the competitive inhibitory rate (CIR) on both lines were introduced to confirm the optimal parameters. Here, the competitive inhibition rate was calculated by (1 - B/B<sub>0</sub>) × 100%, where B<sub>0</sub> and B represented FI<sub>T</sub>/FI<sub>C</sub> of the negative sample and a spiked lotus seed extract sample with 2.5 ng/mL of AFB<sub>1</sub>, respectively.

Table 1 indicated that with increasing the spray amount of QBs-mAbs fluorescent probes from 2 μL/cm to 6 μL/cm on the conjugate pad, the FI<sub>T</sub> and FI<sub>C</sub> values gradually increased. Similarly, the increase of the spray content of BSA-AFB<sub>1</sub> from 0.25 mg/mL to 1.0 mg/mL on the T line resulted in the enhancement of FI<sub>T</sub> values. When 2, 4 or 6 μL/cm of QBs-mAbs probes were sprayed on the conjugate pad, the FI<sub>T</sub>/FI<sub>C</sub> value gradually increased with increasing the BSA-AFB<sub>1</sub> in the range of 0.25–1.0 mg/mL. And when the coated content of BSA-AFB<sub>1</sub> was set at 0.25, 0.5 or 1.0 mg/mL, the highest FI<sub>T</sub>/FI<sub>C</sub> value was obtained when 4 μL/cm of QBs-mAbs probes were sprayed. In addition, it was found that the CIR (1 - B/B<sub>0</sub>) values in the fourth (44% ± 1%) and seventh groups (42% ± 3%) were higher than that in the fifth group (38% ± 6%), but, the FI<sub>T</sub> and FI<sub>C</sub> values in the fourth (2930 ± 179 a.u. and 23858 ± 1493 a.u.) and seventh groups (2194 ± 54 a.u. and 23489 ± 1942 a.u.) were lower than that in the fifth group (4346 ± 364 a.u. and 44682 ± 2867 a.u.). Therefore, the following optimal conditions were achieved: 4 μL/cm of QBs-mAbs complex were sprayed on the conjugate pad as the fluorescent probes to capture the targets in samples, and 0.5 mg/mL of BSA-AFB<sub>1</sub>.
antigen was spotted on the T line to competitively binding with the fluorescent probes.

Then, the immunoreaction time for the newly-established QBs-FS immunosensor was taken into consideration. Considering the antigen–antibody reaction, the immunological kinetics analysis was introduced for optimizing the response time. 80 μL of the spiked sample

\[ \text{Competitive inhibition rate} = \left( 1 - \frac{B}{B_0} \right) \times 100\% \]

where \( B_0 \) and \( B \) represent \( \frac{F_{I_T}}{F_{I_C}} \) of the negative sample and a fortified lotus seed extract with 0.5 ng/mL AFB1, respectively.

Fig. 2. Effects of (A) different amounts of mAbs labeled QBs, and (B) immunoreaction time of the QBs-FS immunosensor on \( F_{I_C} \), \( F_{I_T} \) and \( \frac{F_{I_T}}{F_{I_C}} \).

Table 1
Optimization of the sprayed amount of QBs-probes and concentration of BSA-AFB1 antigen by using a checkerboard titration method (n = 3).

<table>
<thead>
<tr>
<th>Group</th>
<th>Amount of QB-mAbs probe (μL/cm)</th>
<th>Concentration of BSA-AFB1 antigen (mg/mL)</th>
<th>( F_{I_T} ) (Negative sample)</th>
<th>( F_{I_C} ) (Negative sample)</th>
<th>( \frac{F_{I_T}}{F_{I_C}} ) ratio (B)</th>
<th>( \frac{F_{I_T}}{F_{I_C}} ) ratio (B0)</th>
<th>Inhibition rate (%) (1 - B/B0) × 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.25</td>
<td>3935 ± 140</td>
<td>19836 ± 2611</td>
<td>5.04 ± 0.62</td>
<td>3.28 ± 0.20</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.25</td>
<td>6965 ± 213</td>
<td>41827 ± 1177</td>
<td>6.01 ± 0.35</td>
<td>4.63 ± 0.57</td>
<td>23 ± 10</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>0.25</td>
<td>11586 ± 1490</td>
<td>60990 ± 6493</td>
<td>5.28 ± 0.16</td>
<td>4.36 ± 0.15</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>0.5</td>
<td>2930 ± 179</td>
<td>23858 ± 1493</td>
<td>8.15 ± 0.35</td>
<td>4.54 ± 0.11</td>
<td>44 ± 1</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>0.5</td>
<td>4346 ± 264</td>
<td>44682 ± 2867</td>
<td>10.29 ± 0.27</td>
<td>6.43 ± 0.59</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>0.5</td>
<td>6862 ± 912</td>
<td>64438 ± 5387</td>
<td>9.44 ± 0.51</td>
<td>8.03 ± 0.57</td>
<td>15 ± 6</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>1.0</td>
<td>2194 ± 54</td>
<td>23489 ± 1942</td>
<td>10.70 ± 0.64</td>
<td>6.21 ± 0.33</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>1.0</td>
<td>3800 ± 325</td>
<td>45523 ± 1820</td>
<td>12.02 ± 0.68</td>
<td>9.50 ± 0.46</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>1.0</td>
<td>6174 ± 622</td>
<td>65456 ± 6470</td>
<td>10.61 ± 0.36</td>
<td>8.85 ± 0.34</td>
<td>17 ± 3</td>
</tr>
</tbody>
</table>

Fig. 3. Effects of (A) the content of methanol, (B) pH, (C) dilution fold, and (D) 2- and 5-fold dilution of sample extract solution on \( F_{I_C} \), \( F_{I_T} \) and \( \frac{F_{I_T}}{F_{I_C}} \). Competitive inhibition rate was defined as \( (1 - B/B_0) \times 100\% \), where \( B_0 \) and \( B \) represent \( \frac{F_{I_T}}{F_{I_C}} \) of the negative sample and a fortified lotus seed extract with 0.5 ng/mL AFB1, respectively.
solution was dropped onto the sample pad to measure the FLc and FLr values during the 25 min observation period at a 1-min interval. As shown in Fig. 2B, the FLc and FLr values increased, while the FLr/FLc value decreased within 10 min, and then reached constant after 10 min. The relative standard deviation (RSD), which was calculated for the FLc, FLr, and FLr/FLc value of each adjacent 3 min, was 1.14% at 10 min and 0.18% at 15 min. Finally, 15 min was selected as the detection time. These results indicated that the immunoreaction time of the QBs-FS immunosensor was short, and 15 min was determined for quantitative analysis.

3.4. Optimization of sample extract

In addition, the content of methanol, acidity/alkalinity (pH), and dilution fold conditions of the sample extract would influence the sensitivity and reproducibility of the newly-established QBs-FS immunosensor. So, they were also optimized according to the immunological kinetics analysis by using the negative and spiked lotus seed extract sample with 2.5 ng/mL of AFB1.

Owing to its hydrophobic property, AFB1 is insoluble in water and easily soluble in organic solvent like methanol. The extract solution containing a certain concentration of methanol leads to higher extraction recovery of AFB1 from real samples. However, a too high concentration of methanol in the solution will affect the activity of anti-AFB1 Abs, and the immune response of antigen–antibody reaction, thus lowering the detection sensitivity and accuracy of the QBs-FS immunosensor. Herein, the effects of methanol concentration on FLc, FLr, FLr/FLc, and CIR values were investigated. Fig. 3A indicated that methanol with a concentration in the range of 50% to 80% had no significant effect on FLc and FLr. While, the CIR values increased when the content of methanol was lower than 70% and then declined sharply from 39% to 3% when the percentage of methanol increased to 80%. Therefore, 70% methanol was selected as the extraction solvent for AFB1 in the lotus seed samples.

Too acidic (low pH) or too alkaline (high pH) condition of the sample extract will influence the activity of Abs and affect all the affinity reaction of this immunosensor. To explore the effects of pH on performance of the immunosensor, the final pH values of sample solutions were adjusted to 5.0, 6.0, 7.0, 8.0, and 9.0. The samples were then tested with the QBs-FS immunosensor to obtain FLc, FLr, FLr/FLc, and CIR values of each solution. Fig. 3B exhibited that the FLc and FLr values for the negative sample gradually decreased with increasing pH value from 7.0 to 11.0, while, the CIR values gave an increasing–decreasing-increasing trend. And the CIR value for AFB1 concentration at 2.5 ng/mL was the highest (31% ± 5%) at pH 9.0. Therefore, when using the newly-established QBs-FS immunosensor to obtain strong fluorescence signals on the T-line for real detection, the sample solution should be adjusted to pH 9.0.

Considering the ultra-sensitivity of the newly-established QBs-FS immunosensor, the real sample extract should be properly diluted with suitable diluent to reduce the complex matrix interferences for meeting with the detection requirements. Here, the commonly-used 0.1 M Tris-HCl solution containing 0.2% BSA and 0.2% Tween-20 was preferred as the diluent for the negative and spiked lotus seed extract. Fig. 3C displayed that the FLr, FLr/FLc, and CIR values gradually decreased with increasing the sample dilution fold from 2 to 20. Further investigation revealed that the dilution of sample solution not only affected the FLr, FLr/FLc, and CIR values, but also influenced the detectable concentration range of target AFB1. Fig. 3D indicated that low concentration (<25 ng/mL) of AFB1 in the sample extraction solution after 2- and 5-fold dilution could be sensitively detected by the QBs-FS immunosensor. Interestingly, the detectable concentration range of AFB1 in the sample extraction solution after 5-fold dilution (0–25 ng/mL) was much wider than that after 2-fold dilution (0–10 ng/mL). Therefore, the real lotus seed sample solution extracted by 70% methanol should be further treated with 5-fold dilution using 0.1 M Tris-HCl for the immunosensor analysis.

Based on the above findings, the optimized experimental conditions of QBs-FS immunosensor for AFB1 quantitation were recommended as follows: the lotus seed extract containing 70% methanol was diluted 5-fold with 0.01 M Tris-HCl and adjusted to pH 9.0; 100 µg/mL of anti-AFB1 mAbs was pre-coated onto the surface of QBs; 4 µL/cm QBs-mAbs probes were sprayed on the conjugate pad; 0.5 mg/mL BSA-AFB1 antigen was spotted on the T line; after 15 min, the sensor was scanned by a portable fluorescent reader for quantitative analysis of AFB1.

3.5. Analytical performance of the QBs-FS immunosensor

After the QBs-FS immunosensor was successfully established, its analytical performance in terms of sensitivity, specificity and accuracy should be systematically evaluated.

Under the above optimal experimental conditions, the calibration curve of the QBs-FS immunosensor was constructed by plotting the value of FLr/FLc value against the various spiked concentrations of AFB1 in lotus seed samples (0–50 ng/mL). Fig. 4A presented the calibration curve as $y = (6.6774 + 0.3932)/[1 + (x/5.40941.4379)] - 0.3932$, where y is the value of FLc/FLr ratio and x is the spiked AFB1 concentration. A wide dynamic linear range from 1 to 19 ng/mL (2 to 38 µg/kg) with a reliable correlation coefficient ($R^2 = 0.997$) was obtained. The LOD was calculated on the basis of the corresponding 10% inhibitory concentration at 1 ng/mL (2 µg/kg), which was significantly lower (25-fold) than that (25 ng/mL) from the visual inspection on the T line of the immunosensor under an ultraviolet light source in Fig. 4B. Such a low LOD highlighted the important role of QBs as the signal amplification probes in the QBs-FS immunosensor for ultra-sensitive detection of AFB1 in complex lotus seed samples.

The specificity of the QBs-FS immunosensor was evaluated by testing AFB1 (2.5 ng/mL) and other three commonly-found mycotoxins, including deoxynivalenol (DON), ochratoxin A (OTA), and zearalenone (ZEA) at concentration of 100 ng/mL. Under the same experimental conditions, the FLc and FLr values at the C and T lines were recorded to calculate the FLr/FLc. Fig. 5 displayed that the FLr/FLc value of target AFB1 is the lowest (6.28 ± 0.43), while, that of the other three mycotoxins were all high without decreasing trends. Negligible cross-reactions among AFB1 and the other three mycotoxins suggested the excellent specificity and selectivity of the proposed QBs-FS immunosensor for AFB1 detection.

The accuracy of the proposed QBs-FS immunosensor was evaluated by analyzing three fortified lotus seed sample with low (2.5 µg/kg), medium (5.0 µg/kg), and high (10 µg/kg) concentration levels of AFB1. Table 2 showed that the average recoveries for the three spiked concentrations ranged from 94.0% to 116.0% with RSD < 8.5%, indicating good reliability of the developed QBs-FS immunosensor for accurate detection of AFB1 in lotus seeds.

3.6. Real sample analysis

The reliability, practicability and acceptance of the newly-developed QBs-FS immunosensor for AFB1 was demonstrated by analyzing 14 batches of real lotus samples collected from different sources in China. One analysis for the prepared sample solution took only 15 min by using the proposed QBs-FS immunosensor.

Table 3 displayed that 3 out of 14 samples were found to be AFB1-positive with the incidence rate of 21.4%. The residual level of AFB1 ranged from 4.72 µg/kg to 13.82 µg/kg, which in 2 samples exceeded the legal MRL (5 µg/kg) set by the Chinese Pharmacopoeia for lotus seed. And all the positive samples with AFB1 were confirmed by LC-MS/MS analysis in Fig. S1 (Supplementary Material) through the comparison of the peak retention time and fragment ion peak height ratio. The newly-developed QBs-FS immunosensor without false-negative or -positive results was reliable and accurate for practical qualitative and quantitative detection of trace AFB1 in complex lotus seed samples. The
comparison of the QBs-FS immunosensor with other methods for AFB$_1$ detection in Table S2 further validated satisfactory detection performance and analytical speed, and low cost of the QBs-FS immunosensor. Moreover, the results by using the current immunosensor could be quickly judged by naked eye, which was especially suitable for the onsite rapid assessment of large quantities of samples.

The above findings have validated that lotus seeds are susceptible to be contaminated with AFB$_1$ at high incidence rate and residue content, which will lower their edible and medical values, and eventually bring serious threatens to the consumers. Therefore, accurate identification and sensitive monitoring of AFB$_1$, as well as effective measures for preventing the contamination in lotus seeds and related products, are in urgent need.

4. Conclusion

In this study, QBs was used as the fluorescent probes to develop a new FS immunosensor for rapid and sensitive detection of trace AFB$_1$ in complex lotus seeds without resorting to large, expensive equipment. The newly-developed sensor could achieve rapid detection of AFB$_1$ within 15 min, allowing a limit of detection (LOD) of 1 ng/mL (2 μg/kg) and a linear range of 1–19 ng/mL (2–38 μg/kg), as well as good accuracy with recovery rates of 94.0%-116.0% in the fortified lotus seeds. The QBs-FS immunosensor showed superior performance in terms of both sensitivity and rapidity compared with previously reported assays for AFB$_1$. Compared with conventional laboratory methods based on large-scale instruments, such as liquid chromatography and liquid-mass spectrometry, the newly developed FS immunosensor is simple to prepare and operate with small detection time and cost for on-site rapid analysis of large amounts complex matrices. Compared with colloidal gold immunochromatographic test strips, it could provide quantitative data to avoid human eye recognition errors, displaying higher sensitivity and wider detection range. Combined with more mature and simpler sample pretreatment methods, the FS immunosensor can be promoted for the rapid and economic monitoring of more mycotoxins in foods, agricultural products, and Chinese medicinal materials.

CRediT authorship contribution statement

Boyu Jia: Methodology, Investigation, Validation, Writing - original draft. Xiaofang Liao: Software. Chaonan Sun: Formal analysis, Validation. Ling Fang: Formal analysis, Validation. Lidong Zhou: Conceptualization, Supervision, Data curation. Weijun Kong: Supervision, Conceptualization, Writing - review & editing, Resources, Project administration, Funding acquisition.
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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