Quantum bead-based fluorescence-linked immunosorbent assay for ultrasensitive detection of aflatoxin M₁ in pasteurized milk, yogurt, and milk powder

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ABSTRACT

Herein, we reported a novel direct competitive fluorescence-linked immunosorbent assay (dcFLISA) for the ultrasensitive detection of aflatoxin M₁ (AFM₁) in pasteurized milk, yogurt, and milk powder using 150-nm quantum dot beads (QB) as the carrier of competing antigen. Large QB were applied to decrease the binding affinity of the competing antigen to antibody and enhance the fluorescent signal intensity. The aflatoxin B₁ molecule was used as the surrogate of AFM₁ to label with BSA on the surface of QB because of its 63% cross reaction to anti-AFM₁ mAb. The binding affinity of the competing antigen to mAb was tuned by changing the labeled molar ratios of aflatoxin B₁ to BSA. Through combining the advantages of QB as the carrier of the competing antigen, including low binding affinity to mAb and highly fluorescent signal output, the proposed dcFLISA exhibited an ultrahigh sensitivity for AFM₁ detection, with a half-maximal inhibitory concentration of 3.15 pg/mL in 0.01 M phosphate-buffered saline solution (pH 7.4), which is substantially lower than that of the traditional horseradish peroxidase-based ELISA. The proposed method also exhibited very low detection limitations of 0.5, 0.6, and 0.72 pg/mL for real pasteurized milk, yogurt, and milk powder, respectively. These values are considerably below the maximum permissible level of the European Commission standard for AFM₁ in dairy products. In summary, the proposed dcFLISA offers a novel strategy with an ultrahigh sen-

sitivity for the routine monitoring of AFM₁ in various dairy products.

Key words: quantum beads, fluorescence-linked immunosorbent assay, competing antigen, tuning affinity, aflatoxin M₁

INTRODUCTION

Aflatoxin M₁ (AFM₁) is a hydroxylated metabolite of aflatoxin B₁ (AFB₁; Iqbal et al., 2015; Ali et al., 2017). When dairy cattle are fed AFB₁-contaminated feeds, AFB₁ is converted into AFM₁ by the cytochrome P450-associated enzyme in the liver, which is subsequently secreted into the milk (Bahrami et al., 2016; Wu et al., 2016). Aflatoxin M₁ can be found in various dairy products, such as pasteurized milk (Amoli-Diva et al., 2015; Karczmarczyk et al., 2016), milk powder (Huang et al., 2014; Iqbal et al., 2015), and yogurt (Iqbal et al., 2017; Hassan et al., 2018), because of its relative stability during heat treatment (Gizachew et al., 2016). Aflatoxin M₁-contaminated dairy products could pose serious risks to milk consumers (Hassan and Kassaify, 2014), especially to infants (Zheng et al., 2017; Barukčić et al., 2018), because of the carcinogenicity, genotoxicity, and immunosuppression of AFM₁. The European Commission stipulates a maximum permissible level of 50 pg/mL for AFM₁ in milk and dried or processed dairy products (Flores-Flores and González-Peñas, 2018).

High-performance liquid chromatography with fluorescence detector (FLD; Cao et al., 2018; Rodríguez-Carrasco et al., 2018) and ELISA (Schwartzbord et al., 2017; Chen et al., 2018) are the 2 most popular methods for the quantitative detection of AFM₁ in milk and dairy products. High-performance liquid chromatography-FLD has high sensitivity, good accuracy, and reliability (Shuib et al., 2017); however, it generally requires a sophisticated instrument, highly professional technicians, and tedious sample preparation (Mao et

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al., 2015). Thus, it is unsuitable for routine screening analysis of samples in large numbers. Compared with HPLC-FLD, ELISA has higher throughput with fewer sample pretreatment procedures (Ossa et al., 2015). Nevertheless, the sensitivity of conventional ELISA involving horseradish peroxidase (HRP) catalytic tetramethylbenzidine as signal output generally ranges from 0.1 to 10 ng/mL (Swierczewska et al., 2012), which is insufficient to reach the European Union standard for the rapid screening and detection of AFM₁.

Novel signal transducers have also been introduced to improve the sensitivity of immunoassay for AFM₁ detection (Chauhan et al., 2016). Zhang et al. (2013) used antibody-labeled gold nanoparticles as a probe and dynamic light scattering as a signal output to establish a sensitive immunoassay for AFM₁ detection in milk with a limit of detection (LOD) of 27.5 pg/mL. Karczmarczyk et al. (2016) reported a surface plasmon resonance immunosensor and employed the antibody-labeled gold nanoparticles as a signal amplifier for the sensitive detection of AFM₁ in milk with LOD of 18 pg/mL. Nguyen et al. (2013) used electrochemical signals as a signal transducer to develop an aptasensor for AFM₁ detection, and LOD reached as low as 1.98 pg/mL. Sharma et al. (2016) reported a fluorescence resonance energy transfer-based aptasensor for AFM₁ detection; the developed sensor showed a high sensitivity for AFM₁ detection with a LOD of 5 pg/mL in milk. Although the novel signal transducers have great contributions to the sensitivity of the immunoassay, these methods are not compatible with the conventional ELISA platform, resulting in many obstacles in the industrialization process (Song et al., 2018).

Designing a competing antigen with a low binding affinity to antibodies is considered another important strategy to improve the sensitivity of competitive ELISA (Zou et al., 2016). In our previous study, we demonstrated that using a large nanomaterial as a carrier of the competing antigen is more conducive to tuning the binding affinity of the competing antigen to antibodies than using a small protein (Xiong et al., 2017a). In the present study, we used highly luminescent quantum dot beads (QB) with an average diameter of 150 nm as a substitute for HRP to construct a direct competitive fluorescence-linked immunosorbent assay (dcFLISA) for the ultrasensitive detection of AFM₁ in pasteurized milk, milk powder, and yogurt. We tuned the binding affinity of the competing antigen to anti-AFM₁ mAb by controlling the labeled molar ratios of AFB₁ on the surface of the QB because the AFB₁ molecular shows 63% cross reaction to anti-AFM₁ mAb. In addition, the QB exhibited very strong fluorescence signal, which was approximately 1,000 times brighter than that of

the corresponding quantum dots. The detection performance of the developed method was evaluated based on specificity and accuracy by analyzing AFM₁-spiked dairy products.

MATERIALS AND METHODS

Aflatoxin M₁, AFB₁, poly(maleic anhydride-alt-1-octadecene), poly(methyl methacrylate), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, SDS, and BSA were purchased from Sigma-Aldrich Chemical (St. Louis, MO). All other reagents were of analytical grade and purchased from Sinopharm Chemical Corp. (Shanghai, China). Chemicals and materials were used without further purifications. Ultrapure water was obtained from a Milli-QA apparatus (Molsheim, France). The 96-well microplates (high binding, white/black) were obtained from Costar Inc. (Cambridge, MA).

Preparation of QB and BSA-Coated QB

The QB were synthesized in accordance with our previously reported method with some modifications (Duan et al., 2017). In brief, 2.5 mg of octadecylamine-coated CdSe/ZnS quantum dots, 5 mg of poly(methyl methacrylate), and 5 mg of poly(maleic anhydride-alt-1-octadecene) were fully dissolved in 65 μ L of chloroform. Then, 250 μ L of 3.5 mM SDS aqueous solution was added into the organic phase. The mixture of organic and aqueous phases was continuously emulsified with an ultrasonicator for 2 min (working, 9.9 s; pausing, 5 s) at 76 W. The mini-emulsion was incubated at 50°C to evaporate the chloroform. The QB were obtained after stirring constantly for 4 h and then collected by centrifugation at $1,200 \times g$ for 15 min (room temperature). The carboxy group-modified QB were obtained by hydrolyzing polymaleic anhydride on the surface of QB by dissolving the QB in NaOH solution (pH 11) for 24 h.

The BSA-coated QB (QB-BSA) were prepared by coupling the carboxyl group of QB with the amino group of BSA by the carbodiimide method (Shen et al., 2015). In brief, 100 μ L of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide solution (10 mg/mL), 10 mg of QB, and 100 mg of BSA were added to 5 mL of 0.01 M PBS buffer (pH 6.0). After reacting at room temperature for 30 min, 100 μ L of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide solution (10 mg/mL) was added again and then reacted for another 60 min under magnetic stirring. The mixtures were centrifuged at $12,000 \times g$ for 10 min (room temperature), and the precipitates

were washed with pure water for 3 times. Finally, the QB-BSA was resuspended with 1 mL of sodium carbonate solution (0.13 M, pH 8.6) for further use.

Preparation of Different AFB₁-Labeled QB-BSA Conjugates

The AFB₁ oxime was prepared by inserting a carboxyl into the cyclopentenone moiety of AFB₁ (Xiong et al., 2017b). In brief, 2 mg of AFB₁ and 3 mg of carboxymethoxylamine hemihydrochloride were added into 4 mL of pyridine. After reaction at room temperature for 6 h, the AFB₁ oxime was purified by thin-layer chromatography as described previously. Then, 8 of AFB₁-labeled QB-BSA (**QB-BSA-AFB₁**) conjugates were synthesized by coupling the carboxyl group of AFB₁ oxime with the amino group of BSA on the surface of QB through an activated ester method (Xiong et al., 2018). In brief, the carboxyl group of the AFB₁ oxime was activated by suspending AFB₁ oxime, dicyclohexylcarbodiimide, and *N*-hydroxysuccinimide in anhydrous tetrahydrofuran at a molar ratio of 1:4:2 at room temperature in the dark. After reacting under constant stirring for 90 min, the activated AFB₁ oxime solution was centrifuged at $2,500 \times g$ for 10 min (room temperature) to remove the precipitate and the supernatant was added to the QB-BSA solution with molar ratios of AFB₁ with BSA on the surface of QB at 1:20, 1:5, 1:2, 1:1, 10:1, 20:1, 50:1, and 100:1, respectively. The mixture was reacted overnight under constant stirring and the 8 QB-BSA-AFB₁ conjugates were obtained by centrifugation at $7,000 \times g$ for 10 min (room temperature). The precipitates were washed 3 times with wash buffer containing 20% ethanol and then resuspended in PBS containing 0.01% BSA with a QB concentration of 5.0 mg/mL. The final solutions were stored at 4°C until further use.

Procedure of QB-Based dcFLISA

The 96-well black microplates were first coated with 100 μ L of protein G (20 μ g/mL) in PBS solution at 4°C overnight. After washing 3 times with PBS containing 0.05% Tween 20 (**PBST**), the plates were blocked with 10 mg/mL of BSA solution (pH 7.4) for 2 h at 37°C. The plates were washed thrice with PBST buffer and then 100 μ L of the anti-AFM₁ mAb solution (0.75 μ g/mL) was added and incubated at 37°C for 1 h. After the plates were washed thrice with PBST buffer, 50 μ L of QB-BSA-AFB₁-2:1 (30 μ g/mL) and 50 μ L of the sample solution were added. After incubation at 37°C for 45 min, the microplates were washed with PBST 3 times and then washed with PBS once. The fluorescence signals from the QB were determined using

a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA) at an excitation wavelength of 450 nm and emission wavelength of 620 nm.

Sample Pretreatment

Pasteurized milk, milk powder, and yogurt samples that were confirmed AFM₁-free by LC-MS/MS were collected from a local market. Exactly 2 mL (g) of each pasteurized milk and yogurt sample was diluted with 8 mL of 0.01 M PBS buffer (pH 7.4), and then diluents were centrifuged at $7,000 \times g$ for 10 min (room temperature) to remove the upper fat layer. For the milk powder samples, the pretreatment process was the same except that 2 g of sample was dissolved in 10 mL of 0.01 M PBS buffer (pH 7.4). All pretreated dairy solutions were directly used for fluorescence immunoassay analysis.

RESULTS AND DISCUSSION

Principle of QB-Based dcFLISA for AFM₁ Ultrasensitive Detection

The detailed principle of the proposed dcFLISA for AFM₁ ultrasensitive detection is illustrated in Figure 1. In a traditional dcELISA system, HRP or alkaline phosphatase is used as the carrier of the competing antigen (Li et al., 2016), and the binding affinity of the competing antigen to the antibody can be tuned by decreasing the labeled molar ratios of hapten to HRP or alkaline phosphatase protein. However, the affinity-decreased magnitude of competing antigens is very limited because of the small size of the carrier protein. In the present study, large QB were introduced as the carrier of the competing antigens, which was prepared by capsuling numerous quantum dots inside a polymer matrix (Figure 1A). Under the same labeled amount of hapten molecules, the large QB used as the carrier of the competing antigen exhibited a lower binding affinity to antibodies because of their lower velocity of Brownian motion compared with conventional HRP protein at the same temperature (Millen et al., 2014). Given the decreased binding affinity of the competing antigen and the fluorescent signal amplification of QB, the sensitivity of proposed fluorescence immunoassay showed a remarkable enhancement (Figure 1B).

Characterization of QB and QB-BSA

Highly luminescent QB were synthesized in accordance with our previously reported method with some modifications (Shao et al., 2018). The average size and morphology of the prepared QB were analyzed

Figure 1. Schematic of 2 strategies was used to enhance sensitivity of fluorescence immunoassay. QD = quantum dots; PMAO = poly(maleic anhydride-alt-1-octadecene); PMMA = poly(methyl methacrylate); QB = quantum dot beads; AFB₁ = aflatoxin B₁; AFM₁ = aflatoxin M₁.

under a high-resolution transmission electron microscope (Figure 2A), indicating that the resultant QB are relatively uniform spherical beads with an average diameter ($\pm$ SD) of 150 ± 10 nm ($n = 50$). The inset in Figure 2A demonstrates that the numerous dark quantum dots are tightly encapsulated in the polymer matrix. The fluorescence intensities of the QB and the corresponding octadecylamine-coated quantum dots were determined to evaluate the signal enhancement of the resultant QB. As shown in Figure 2B, the maximum emission wavelength of the resultant QB was 613 nm (dissolved in ultrapure water), whereas that of the corresponding quantum dots was 610 nm (dissolved in chloroform). Meanwhile, the fluorescence intensity of the QB at 3.2 pM was almost the same as that of the corresponding quantum dots at 3.2 nM, indicating that the luminescence intensity of QB was approximately 1,000 times brighter than that of the quantum dots. The BSA protein was coated on the surface of the QB to decrease the nonspecific binding of the QB on the plate well. The QB-BSA were prepared using an active

ester method. Dynamic light scattering analysis using a particle size analyzer (Malvern Instruments Ltd., Worcestershire, UK) showed that the average hydrodynamic diameters of the QB and QB-BSA were at 163 and 188 nm (Figure 2C). Meanwhile, the surface potential of the QB-BSA increased from -34.8 to -8.2 mV; these results indicated that the BSA protein was successfully coated onto the surfaces of the QB. The amounts of BSA protein on the surface of each QB were calculated as 829 by determining the BSA concentration per milligram of QB using the BCA protein quantification kit (Thermo Fisher Scientific).

Optimization of QB-Based dcFLISA

In the present study, anti-AFM₁ mAb exhibited 63% cross-reaction with the AFB₁ molecule (Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-16109>). Aflatoxin B₁ was suggested as the surrogate of AFM₁ in preparing the competing antigen to improve the sensitivity of the QB-based dcFLISA. The competing antigen (QB-BSA-AFB₁) was obtained by covalently coupling the carboxyl group of AFB₁ oxime to the amino group of BSA on the surface of QB-BSA by using an activated ester method. The 8 QB-BSA-AFB₁ conjugates with different binding affinities to anti-AFM₁ mAb were prepared by changing the labeled molar ratio of AFB₁ to BSA on the surface of the QB from 50:1 to 1:20, respectively. The plate wells were precoated with protein G to capture the fragment crystallizable region of mAb to improve the bioactivity of anti-AFM₁ mAb. The concentrations of anti-AFM₁ mAb and QB-BSA-AFB₁ conjugates were optimized through a checkerboard titration method, and the results are shown in Supplemental Tables S1 to S8 (<https://doi.org/10.3168/jds.2018-16109>). The effect of the labeled molar ratio of AFB₁ to BSA on the sensitivity of fluorescence immunoassay was observed in the competitive inhibition curves using the 8 QB-BSA-AFB₁ conjugates as competing antigens, which were generated by detecting 14 AFM₁ standard solutions prepared by spiking the AFM₁ stock solution to PBS (0.01 M, pH 7.4) to final concentrations of 0 (as negative control), 0.15, 0.31, 0.63, 1.3, 2.5, 5, 10, 20, 40, 80, 160, 320, and 640 pg/mL. As shown in Figure 3, the half-maximal inhibitory concentration (IC₅₀) value of the fluorescence immunoassay significantly decreased as the labeled molar ratio of AFB₁ to BSA was reduced. When QB-BSA-AFB₁-1:50 (equivalent to 1 BSA molecule labeled with 50 AFB₁ molecules) was used as the competing antigen, the IC₅₀ value was 59.1 pg/mL. When QB-BSA-AFB₁-2:1 (equivalent to 2 BSA molecules labeled with 1 AFB₁ molecule) was used as the competing antigen, the IC₅₀ value was achieved at a minimum value of 3.15

Figure 2. Characterization of the quantum dot beads (QB) and BSA-coated QB (QB-BSA). Transmission electron microscope image of QB (inset is the image of individual QB with high magnification; A), fluorescence intensity of QB and quantum dots (QD; B), and hydrodynamic diameter (d) of QB and QB-BSA (C).

Figure 3. Competitive inhibition curves of fluorescence immunoassay based on 8 different aflatoxin B₁-labeled BSA-coated quantum dot beads (QB-BSA-AFB₁) conjugates. (A) The half-maximal inhibitory concentration (IC₅₀) values of fluorescence immunoassay using QB-BSA-AFB₁ at 1:50, 1:20, 1:10, and 1:5 as a competing antigen were 59.1, 32.4, 15.1, and 7.3 pg/mL, respectively. (B) The IC₅₀ values of fluorescence immunoassay using QB-BSA-AFB₁ at 1:1, 2:1, 5:1, and 20:1 as a competing antigen were 5.85, 3.15, 4.65 pg/mL, and no signal, respectively. F₀ and F represent the fluorescence intensity of the negative control (AFM₁-free) and an AFM₁-spiked solution, respectively. AFM₁ = aflatoxin M₁. Vertical bars indicate SD (n = 3).

pg/mL, which is approximately 130-fold lower than that of the traditional HRP-based ELISA (IC₅₀ = 410 pg/mL, Supplemental Figure S1). When the labeled molar ratio of AFB₁ to BSA was decreased to 1:5, the IC₅₀ value slightly increased to 4.65 pg/mL. Therefore, the sensitivity of the proposed dcFLISA remarkably improved approximately 18.8 fold by decreasing the

labeled molar ratio of AFB₁ to BSA on the surface of the QB. However, when the labeled molar ratio of AFB₁ to BSA was further decreased to 1:20 (equivalent to 1 AFB₁ molecule labeled with 20 BSA molecules), no signals were detected on the plate wells, indicating that the affinity of the resultant competing antigen was too low to bind with anti-AFM₁ mAb (Supplemental Table S8). These results demonstrate that the large QB, as the carrier of the competing antigen, can tune the binding affinity of the competing antigen to mAb on a larger scale than the traditional carrier protein, and the sensitivity of the QB-based dcFLISA shows an 18.8-fold (59.1/3.15) enhancement by decreasing

the binding affinity of the QB-BSA-AFB₁ conjugate to anti-AFM₁ mAb.

Analytical Performance of QB-Based FLISA

On the basis of the above results, the QB-BSA-AFB₁-2:1 conjugate was chosen as the competing antigen to develop a dcFLISA for AFM₁ detection in pasteurized milk, milk powder, and yogurt. Three matrix-matched calibration curves were constructed to decrease the interference from the matrices of dairy products. The AFM₁ standard solutions were prepared by adding AFM₁ stock solution in 5-fold diluted pasteurized milk,

Figure 4. Quantitative immunoassay of aflatoxin M₁ (AFM₁) by using the developed fluorescence immunoassay in spiked (A) milk, (B) yogurt, and (C) milk powder. Vertical bars indicate the SD ($n = 6$), where F_0 and F represent the fluorescence intensity of the negative control (AFM₁-free) and an AFM₁-spiked solution, respectively. (D) Cross-reaction (CR) of the direct competitive fluorescence-linked immunosorbent assay, calculated as $CR\% = [(IC_{50} \text{ AFM}_1)/(IC_{50} \text{ other mycotoxins})] \times 100\%$, where IC_{50} = half-maximal inhibitory concentration (Xiong et al., 2015). Only aflatoxin B₁ (AFB₁) exhibited 63% CR%; all of other mycotoxins have slight cross reaction with anti-AFM₁ antibody. LOD = limit of detection; OTA = ochratoxin A; DON = deoxynivalenol; ZEN = zearalenone; PAT = patulin; and FB₁ = fumonisins B₁.

Table 1. Precision assay of the proposed fluorescence immunoassay

Sample matrix	Aflatoxin M ₁ (μg/kg)		Recovery (%)	CV (%)
	Added	Recovered		
Milk	200	195.28	97.64	7.72
	100	98.58	98.58	8.07
	50	5.52	11.04	7.09
	30	28.38	94.61	7.99
	10	9.33	99.33	8.67
Yogurt	200	196.06	98.03	8.68
	100	98.70	98.70	4.97
	50	46.14	92.28	7.41
	30	29.12	97.07	9.19
	10	9.33	99.33	6.44
Milk powder	200	186.10	93.05	9.81
	100	96.98	96.98	3.20
	50	48.74	97.49	7.66
	30	30.96	103.2	5.58
	10	9.60	95.00	8.43

5-fold diluted yogurt, and 6-fold diluted milk power. The regression equations for the AFM₁ quantitative detection in diluted pasteurized milk, yogurt, and milk power are represented by $y = -16.54\ln(x) - 59.639$ ($0.15 \leq x \leq 20$ pg/mL, $R^2 = 0.9955$, Figure 4A), $y = -15.82\ln(x) - 55.513$ ($0.15 \leq x \leq 20$ pg/mL, $R^2 = 0.9912$, Figure 4B), and $y = -14.99\ln(x) - 45.309$ ($0.3 \leq x \leq 40$ pg/mL, $R^2 = 0.9945$, Figure 4C), respectively, where y is the competitive inhibition rate and x is the AFM₁ concentration. Error bars were based on 6 duplicate measurements at different AFM₁ concentrations. The IC₅₀ values of the QB-based dcFLISA for diluted pasteurized milk, yogurt, and milk powder were 1.28, 1.66, and 1.84 pg/mL, respectively, whereas the corresponding LOD values were 0.10, 0.12, and 0.12 pg/mL.

The LOD for real dairy products was defined as 10% of the competitive inhibitory concentration multiplied by the dilution factor. Therefore, the LOD of the proposed method for pasteurized milk, yogurt, and milk powder were 0.5 (5-fold dilution), 0.6 (5-fold dilution), and 0.6 pg/mL (6-fold dilution), respectively. In summary, the LOD of our proposed dcFLISA for AFM₁ detection is superior to that of the traditional HRP-based ELISA and comparable to those of previously established approaches (Table 2). The proposed method entails fewer operation steps and less detection time than other methods because it does not require the HRP catalysis of the substrates for chromogenic reaction.

The specificity of the QB-based dcFLISA was evaluated by analyzing AFM₁, AFB₁, ochratoxin A, deoxyni-

Table 2. Performance comparison of recently reported methods for aflatoxin M₁ detection

Method	Probe	Sample	Limit of detection (pg/mL)	Reference
Direct competitive fluorescence-linked immunosorbent assay	QB-BSA-AFB ₁	Pasteurized milk, yogurt, and milk powder	0.5, 0.6, and 0.72	This work
Impedimetric immunosensor	Silver wire electrode	Milk powder	10	Bacher et al., 2012
Dynamic light scattering immunoassay	Gold nanoparticle labeled with antibody	Milk	377	Zhang et al., 2013
Electrochemiluminescent immunoassay	CdTe quantum dots-carbon nanotubes nanocomposite	Milk	3.0	Gan et al., 2013
Electrochemical biosensor	Fe ₃ O ₄ /polyaniline decorated electrode	Milk	1.98	Nguyen et al., 2013
Fluorescent immunoassay	Liposomes loaded with quantum dots	Milk	250	Beloglazova et al., 2013
Surface plasmon resonance biosensor	Gold nanoparticle labeled with secondary antibody	Milk powder	180	Karczmarczyk et al., 2016
Fluorescent immunoassay	Aptamers against AFM ₁ labeled with Cy5.5	Milk	84	Sharma et al., 2016
Fluorescent immunoassay	Fluorescein-labeled anti-AFM ₁ aptamer	Milk	5	Song et al., 2018

valenol, zearalenone, patulin, and fumonisin B₁. Figure 4D shows some cross reactions for structural analogs of AFB₁ molecules and a negligible cross-reaction for the other common mycotoxins. The accuracy and precision of the proposed dcFLISA were evaluated by determining AFM₁-spiked pasteurized milk, yogurt, and milk powder (Table 1). After a simple sample pretreatment, the 5-fold diluted pasteurized milk and yogurt and 6-fold diluted milk powder were fortified with different AFM₁ concentrations (200, 100, 50, 30, and 10 pg/mL). Each sample was determined by the proposed dcFLISA with 6 replicates. As shown in Table 2, the average recoveries of the pasteurized milk, yogurt, and milk powder ranged from 94.61 to 110.4% (CV ranging from 7.09 to 8.76%), 92.28 to 99.33% (CV ranging from 4.97 to 9.19%), and 93.05 to 103.2% (CV ranging from 3.2 to 9.81%), respectively. These results indicate that the accuracy of the QB-based dcFLISA is acceptable for AFM₁ quantification in real dairy products.

CONCLUSIONS

We proposed a simple and novel strategy for improving the sensitivity of dcELISA. Large QB were introduced as an alternative to HRP protein for enhancing the fluorescent signal intensity and tuning the binding affinity of the competing antigen to antibodies. Because of the increased fluorescent signal and decreased binding affinity of the competing antigen, the proposed dcFLISA exhibited an ultrahigh sensitivity for AFM₁ detection with an IC₅₀ value of 3.15 pg/mL, substantially lower than that of the traditional HRP-based ELISA. The proposed method also showed excellent performance in real pasteurized milk, yogurt, and milk powder samples, with LOD of 0.50, 0.60, and 0.72 pg/mL, respectively. Compared with the traditional HRP-based dcELISA, the developed QB-based dcFLISA only needs 45 min of detection time because it does not require the HRP catalysis of tetramethylbenzidine for chromogenic reaction. In summary, our designed QB-based dcFLISA can serve as a practical tool for the routine monitoring of AFM₁ in dairy products.

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