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p-Bromophenol-Enhanced Bienzymatic Chemiluminescence Competitive Immunoassay for Ultrasensitive Determination of Aflatoxin B_1

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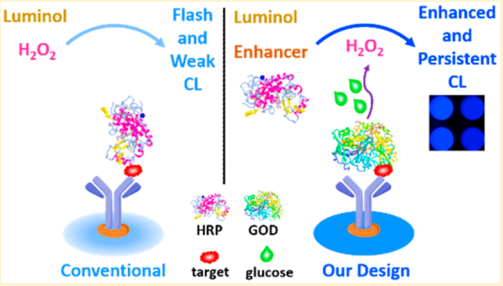
Supporting Information

ABSTRACT: Aflatoxin B_1 (AFB_1) contamination is one of the most critical global issues in food safety. The high carcinogenic nature necessitates rapid and specific methods for the determination of AFB_1 in foodstuffs at ultratrace levels. Here, we report an enhanced bienzymatic chemiluminescence competitive immunoassay for ultrasensitive and high-throughput determination of AFB_1. In this assay, protein G was first coated on the wells of a microplate for recognizing the Fc fragment of anti-AFB_1 mAbs to reduce the antibody dosage and guarantee high immunological reaction efficiency. The target AFB_1 competed with glucose oxidase labeled AFB_1 for the limited anti-AFB_1 mAbs in the wells of the microplate. *p*-Bromophenol was employed as an enhancer to obtain intense and long-lasting chemiluminescence. The utilization of an enhancer and bienzymatic catalysts effectively improved the detection sensitivity. The developed method offered a good linearity over 5 orders of magnitude, a detection limit of 5 pg L^{-1}, and a relative standard deviation of 1.9% for AFB_1. The application of the developed method to the analysis of grain samples gave quantitative recoveries from 94.0% to 97.0%. The developed method provides a universal platform for high-throughput, ultrasensitive, and high specific detection of pollutants or nutrients in foods.



Aflatoxin B_1 (AFB_1), a group I carcinogen, is one of the most potent hepatotoxins, carcinogens, teratogens, and mutagens.[1,2] AFB_1 contamination is one of the most critical global issues in food safety. Many countries have established regulations to govern the AFB_1 level in foodstuffs to avoid overexposure of humans and animals to AFB_1. For example, the National Food Safety Standards of China (GB 2761-2017) give the maximum allowable level (MAL) of AFB_1 as 5−20 $\mu g\ kg^{-1}$ in grains.[3] The Codex Alimentarius Commission (CAC) sets a limit of 0.5−15 $\mu g\ kg^{-1}$ for aflatoxins in a variety of nuts, grains, dried figs, and milk.[4]

The early monitoring of possible contamination of AFB_1 is essential since AFB_1 is hard to destroy due to its potent heat-stable property.[5] High-performance liquid chromatography (HPLC), liquid chromatography−mass spectrometry (LC-MS), and liquid chromatography−tandem mass spectrometry (LC-MS/MS) are conventional validated analytical methods for the determination of AFB_1.[6−8] However, these techniques suffer from the requirements of professional laboratory conditions, time-consuming sample pretreatment, and expensive instrumentation, which limits the on-spot detection and point-of-care application. The specific, rapid, simple, low-cost, and user-friendly nature makes immunoassays, such as enzyme-linked immunosorbent assays (ELISA),[9] photoelectrochemical immunoassay,[10] fluorescence polarization immunoassays,[11] and immunochromatographic assays[12] attractive for rapid and on-site detection of AFB_1. In particular, ELISA as a classical analytical technique enables significant improvement of the efficiency of routine surveillance in food safety, and enzyme-based ELISA kits are popular for the rapid detection of AFB_1. Chemiluminescence enzyme immunoassay (CLEIA) exhibits higher sensitivity and signal-to-noise ratio than conventional ELISA, thereby attracting much attention in food safety analysis.[13−15] Nevertheless, a common horseradish peroxidase (HRP)−luminol−H_2O_2 system also gives limited sensitivity and usually shows flash-type signal,[16] making it difficult to early screen or determine AFB_1 at low contamination levels. It is, therefore, an urgent need to develop a feasible and reliable point-of-care detection assay to achieve ultrasensitive determination of AFB_1 in foods.

Herein, we report a bienzymatic chemiluminescence competitive immunoassay (BCCI assay) for rapid, highly specific, and ultrasensitive determination of AFB_1 in grains. In this BCCI assay, protein G was coated on the plate first to recognize the Fc

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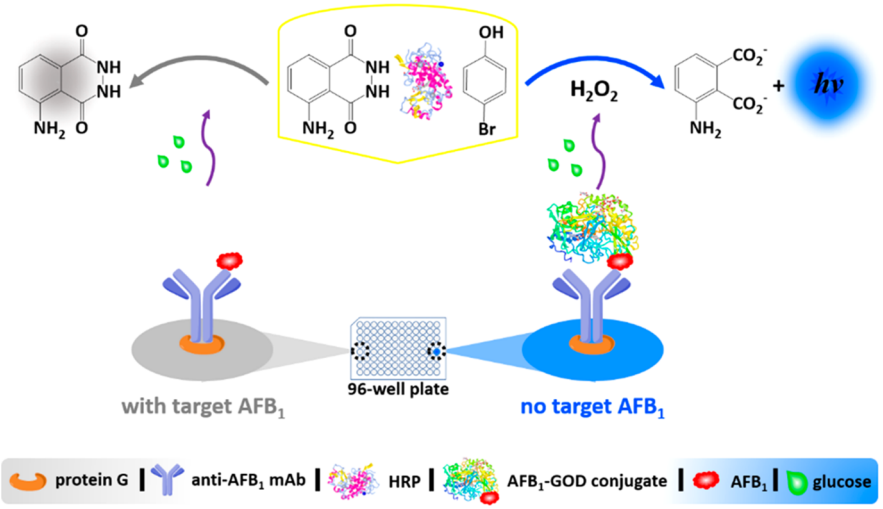
Scheme 1. Schematic Representation of BCCI Assay for Ultrasensitive Detection of AFB_1



fragment of the monoclonal antibody against aflatoxin B_1 (anti-AFB_1 mAb). Then, AFB_1 was linked to glucose oxidase (GOD) as the competitor of target AFB_1 for competitive binding to anti-AFB_1 mAb, in which GOD catalyzed the oxidation of glucose with oxygen into hydrogen peroxide (H_2O_2). In our chemiluminescence (CL) system, the enhancer *p*-bromophenol accelerated the cyclic transformation of HRP and the electron transfer between luminol and radicals, resulting in an intense and long-lasting CL signal. The efficient bienzymatic catalysis with GOD and HRP and the enhancement of the CL signal with *p*-bromophenol are advantageous for ultrasensitive determination of AFB_1.

EXPERIMENTAL SECTION

Materials and Reagents. AFB_1 standards (purity >99%) were purchased from the Meizheng Group (Beijing, China). Horse radish peroxidase (HRP, ≥300 units mg^{-1}), glucose oxidase (GOD, 100,000−250,000 units g $solid^{-1}$), dicyclohexylcarbodiimide (DCC), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) and *β*-D-glucose were purchased from Solarbio Life Science (Beijing, China). Luminol, O-(carboxymethyl)hydroxylamine hemihydrochloride (CMO), Tween-20, *p*-bromophenol, and pyridine were purchased from Aladdin (Shanghai, China). *N*,*N*-dimethylformamide (DMF), tetrahydrofuran (THF), chloroform, and ethyl acetate were purchased from Sinopharm (Beijing, China). Anti-AFB_1 mAb ascites (2A8, 24.684 mg mL^{-1}) was provided by Zhanhui Wang at the Beijing Advanced Innovation Center for Food Nutrition and Human Health, China Agricultural University. A certified reference material (GBW(E)100386) (maize) (Academy of State Administration of Grain, Beijing, China) was used for method validation.

A phosphate buffer (PB) (pH 6.5, 0.2 mol L^{-1}) was prepared by thoroughly mixing a Na_2HPO_4 solution (315 mL, 0.2 mol L^{-1}) and NaH_2PO_4 solution (685 mL, 0.2 mol L^{-1}). Phosphate buffered saline (PBS) (pH 7.0, 7.4, and 8.6; 10 mmol L^{-1}) was prepared via dissolving 8.0 g of NaCl, 0.2 g of KCl, 0.2 g KH_2PO_4, and 2.9 g of $Na_2HPO_4{\cdot}12H_2O$ in ultrapure water, adjusting pH to 7.0, 7.4, and 8.6, and then bringing to a volume of 1 L with ultrapure water. A phosphate-buffered solution containing Tween-20 (PBST) was prepared by adding 0.05% (v/v) Tween-20 to PBS (pH 7.0, 10 mmol L^{-1}) for immediate use.

Preparation of AFB_1−GOD Conjugates. The AFB_1−GOD conjugates were synthesized according to a previous report with some modification.[17−19] First, 1 mg of AFB_1 and 2 mg of CMO were dissolved in pyridine. The mixture was vortexed and incubated at room temperature in the dark for 24 h. After removing the pyridine with nitrogen, the oximation product (AFB_1-oxime) was dissolved in pH 8.0 ultrapure water. The solution was adjusted to pH 2.0 with 1 mol L^{-1} HCl and set at 4 °C for 15 min for precipitation. The precipitate was collected via centrifugation and repeatedly extracted with 0.5 mL of chloroform and 0.5 mL of ethyl acetate, respectively, five times. The supernatant was collected via centrifugation after each extraction and mixed with 5 mL of ultrapure water (pH 2.0) and set overnight. The extracted AFB_1−oxime in chloroform was collected via drying with nitrogen.

Then, 721 *μ*g of AFB_1−oxime, 1.1 mg of NHS, and 11 mg of DCC were dissolved in 1 mL of anhydrous THF, and the solution was gently shaken in the dark at room temperature for 1 h. The precipitate was removed via centrifugation, while the supernatant extract (esterification product of AFB_1, AFB_1−ester) was dried with nitrogen on a water bath at 55 °C and dissolved in DMF to a final concentration of 1 mg mL^{-1} for AFB_1−ester. The AFB_1−ester solution was added dropwise into the GOD solution (10 mmol L^{-1} PBS, pH 7.4) with a certain mole ratio. After continuous gently shaking at 4 °C for 4 h, the product was dialyzed in a 10 mmol L^{-1} PBS (pH 7.0) buffer at 4 °C for 72 h. The obtained AFB_1−GOD conjugate solution (containing 50% glycerol, v/v) was stored at −20 °C for further use.

Procedures for the BCCI Assay. The high-binding 96-well assay plate was first coated with protein G (20 *μ*g mL^{-1} in PBS (pH 8.6, 10 mmol L^{-1}), 100 *μ*L/well) at 4 °C overnight. After washing three times with PBST and two times with PBS (pH 7.0, 10 mmol L^{-1}), the plate was blocked with 1% BSA (w/v in PBS (pH 7.0, 10 mmol L^{-1}), 300 *μ*L/well) at room temperature for 1 h and subsequently washed three times with PBST and two times with PBS (pH 7.0, 10 mmol L^{-1}). Then, anti-AFB_1 ascites (3.1 *μ*g mL^{-1} in PBS (pH 7.0, 10 mmol L^{-1}), 100 *μ*L/well) was added to the wells and set at room temperature for 2 h. After washing three times with PBST and two times with PB, a mixture of 50 *μ*L of AFB_1−GOD (0.25 *μ*g mL^{-1}) and 50 *μ*L AFB_1

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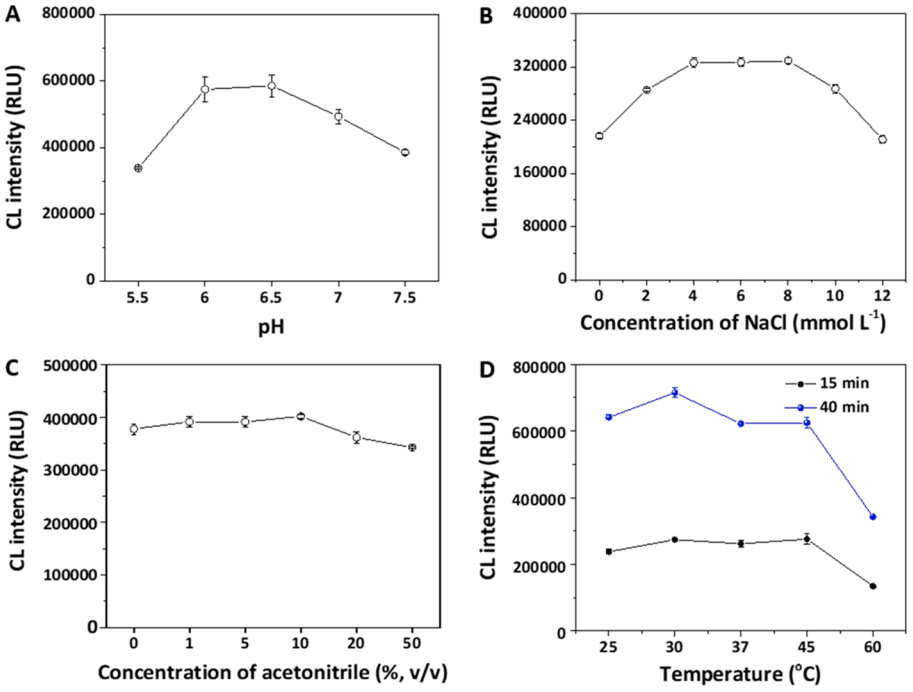

Figure 1. Factors affecting the immunoreaction between anti-AFB_1 mAb and AFB_1: (A) pH, (B) NaCl concentration, (C) acetonitrile content, and (D) temperature. The error bars represent one standard deviation (n = 3).

standard solution or sample extract in 0.2 mol L^{-1} PB (containing 10% (v/v) acetonitrile and 5 mmol L^{-1} NaCl, pH 6.5) was added to the wells and allowed to proceed for 30 min. After another washing three times with PBST and two times with ultrapure water, the plate was incubated with glucose (50 mmol L^{-1}, 100 μL/well) for 30 min. Subsequently, a luminous solution (containing 20 μmol L^{-1} HRP, 50 mmol L^{-1} p-bromophenol, and 10 mmol L^{-1} luminol at pH 11.5, 100 μL/well) was added, and after 25 min, the chemiluminescence (CL) intensity was recorded on a Cytation 3 multimode microplate reader (BioTek, VT).

Grain Samples. Seven different grain samples, including wheat flour, oats, northeast China rice, Jiangsu rice, millet, corn flour, and corn grit, were collected from local supermarkets. The sample extract was prepared according to the National Food Safety Standards of China (GB5009.22-2016) with minor modification.[20] Briefly, 5.0 g of the finely ground sample was extracted with 20 mL of 84% (v/v) acetonitrile/water solution. After ultrasonic processing for 20 min, the samples were centrifuged at 10,000g for 15 min. The supernatants were filtered through a 0.22 μm filter membrane and stored at 4 °C. An eight-times dilution was made just before analysis.

RESULTS AND DISCUSSION

Design of the BCCI Assay. In a conventional CL immunoassay for the detection of AFB_1, AFB_1 is linked to HRP for competitive binding to the detection antibody with target AFB_1; thus, the concentration of HRP is directly related to the concentration of the target, which determines the CL intensity. In addition, there is a flash-type short-lived CL emission when the H_2O_2/HRP ratio is too high.[21] In this work, we developed a BCCI assay with an enhanced and persistent CL signal. Scheme 1 shows the principle of this BCCI assay for ultrasensitive determination of AFB_1. Protein G is first coated on the high-binding 96-well plate to effectively recognize the Fc fragment of anti-AFB_1 mAb. AFB_1−GOD is prepared as the

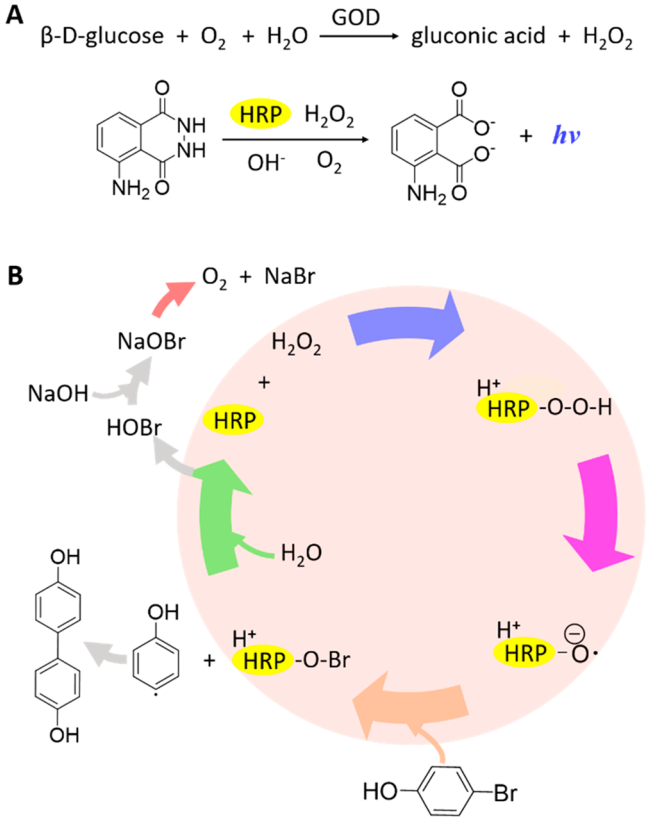

Figure 2. Possible mechanism of the developed BCCI assay: (A) Bienzyme-catalyzed chemiluminescence. (B) Cyclic transformation of HRP in the presence of p-bromophenol in the BCCI assay.

competitor of target AFB_1 for competitive binding to anti-AFB_1 mAb, in which the GOD can effectively catalyze the oxidation of glucose into H_2O_2. The CL signal depends on the concentration of the generated H_2O_2. GOD and HRP act as highly efficient

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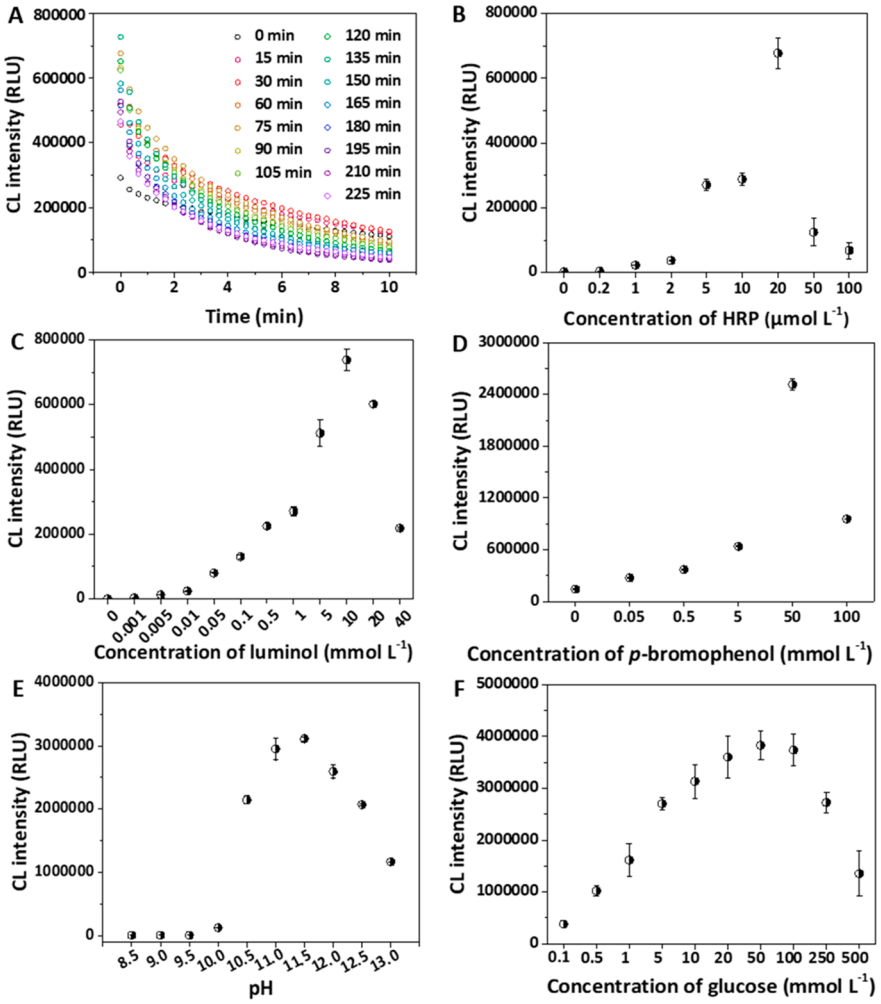

Figure 3. (A) Effects of preincubation time of glucose and GOD on the CL signal. Optimization of HRP concentration (B), luminol concentration (C), *p*-bromophenol concentration (D), pH (E), and glucose concentration (F) for the enhanced CL. The error bars represent the standard deviation (n = 3).

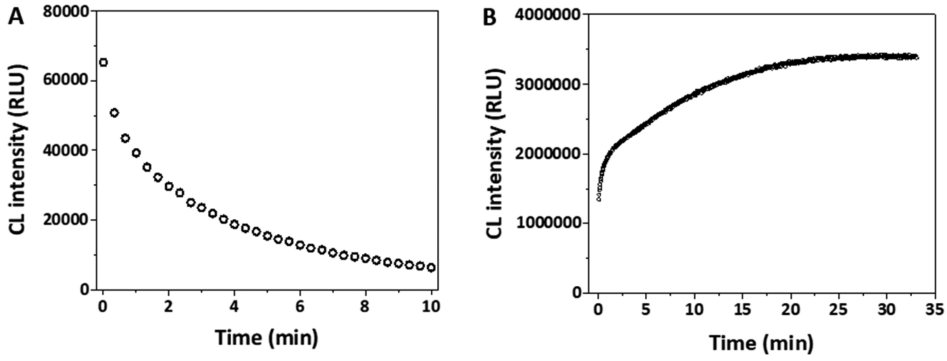

Figure 4. Kinetic curves of chemiluminescence (recorded after the first 9 s): (A) without *p*-bromophenol and (B) with *p*-bromophenol.

catalysts, while *p*-bromophenol serves as an enhancer of luminol chemiluminescence for an intense and long-lasting CL signal. Because of competition between target AFB_1 and AFB_1−GOD, the CL intensity and competitive inhibition rate (CIR = $(CL_0 - CL)/CL_0 \times 100\%$, where CL_0 and CL represent the CL intensities in the absence and presence of AFB_1, respectively) are related to the concentration of the target AFB_1.

Preparation and Characterization of AFB_1−GOD Conjugates. Generally, AFB_1 must be covalently linked to an immunogenic carrier molecule to elicit a strong immune response following immunization due to low molecular mass (312.27 Da).[22,23] Thus, AFB_1−GOD conjugates were prepared as the competitor of target AFB_1 in the proposed assay (Figure S1, Supporting Information). The electrophilic substitutive reaction of AFB_1 and CMO gave the oximation product of AFB_1 (AFB_1−oxime) with a yield of 87.7%. The TLC experiment confirms the formation of AFB_1−oxime and AFB_1−ester (Figures S2 and S3, Supporting Information). AFB_1−GOD

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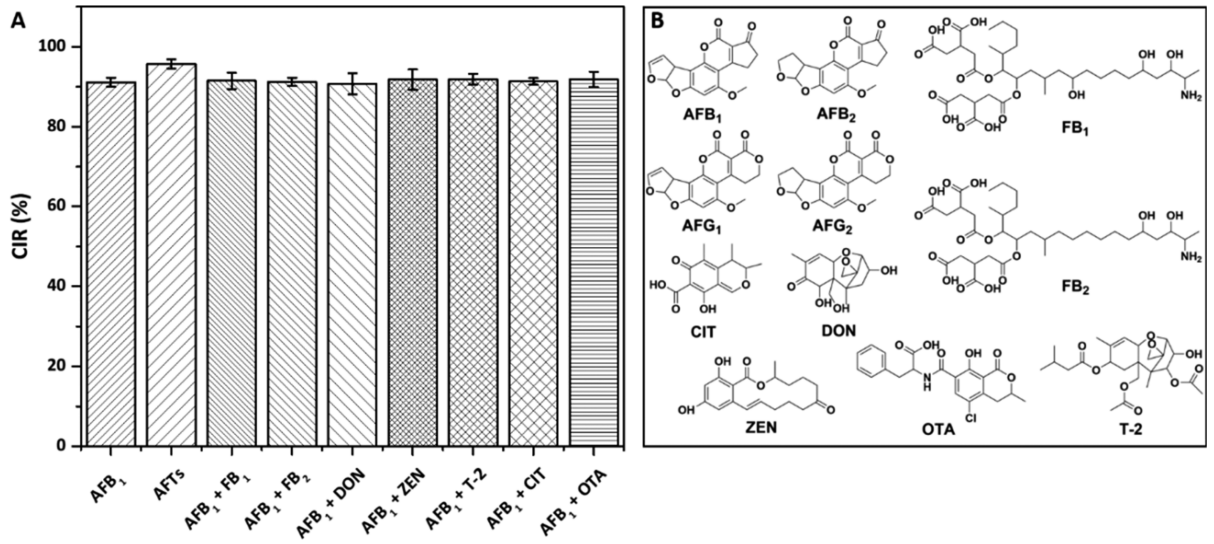

Figure 5. (A) Interference of other mycotoxins (AFTs = AFB_1 + ABF_2 + AFG_1 + AFG_2 (1.0 $\mu g\ L^{-1}$ each), FB_1 (5.0 $\mu g\ L^{-1}$), FB_2 (5.0 $\mu g\ L^{-1}$), DON (5.0 $\mu g\ L^{-1}$), ZEN (5.0 $\mu g\ L^{-1}$), T-2 (5.0 $\mu g\ L^{-1}$), CIT (5.0 $\mu g\ L^{-1}$), and OTA (5.0 $\mu g\ L^{-1}$)) on the determination of AFB_1 (1.0 $\mu g\ L^{-1}$) via the BCCI assay. (B) Chemical structures of aflatoxins and other seven mycotoxins.

Table 1. Analytical Results for Determination of AFB_1 in Grains

sample	AFB_1 determined (mean ± s, n = 3) ($\mu g\ kg^{-1}$)	recovery for 0.4 $\mu g\ kg^{-1}$ spiked AFB_1 (mean ± s, n = 3) (%)
flour	0.368 ± 0.007	94.3 ± 0.6
oats	0.336 ± 0.005	94.8 ± 1.2
northeast rice	0.223 ± 0.009	94.0 ± 1.5
Jiangsu rice	0.240 ± 0.006	94.3 ± 1.1
millet	0.016 ± 0.002	97.0 ± 0.8
corn starch	0.048 ± 0.003	97.0 ± 0.7
corn grit	0.321 ± 0.014	96.0 ± 0.3

with different coupling ratios was prepared through condensation between activated carboxyl groups on the AFB_1−oxime and amino groups on GOD. The prepared AFB_1−GOD displayed the characteristic absorption peak of AFB_1 at 363 nm, indicating successful conjugation of AFB_1 and GOD (Figure S4, Supporting Information). Also, no significant difference in catalytic activity between the prepared AFB_1−GOD and pure GOD was observed, but the AFB_1−GOD with higher coupling ratios displayed better detection performance (Figure S5, Supporting Information).

Factors Affecting the Immunoreaction between Anti-AFB_1 mAb and AFB_1. In this competitive immunoassay, instead of immobilizing the detection antibodies directly to the plate, protein G was coated on the plate first to capture the Fc region of the antibody,[24,25] allowing easy control of anti-AFB_1 mAb at a lower concentration to enhance the assay sensitivity. Also, AFB_1−GOD was utilized as the competitor of AFB_1 in the sample for binding to limited anti-AFB_1 mAb. Thus, the concentration of AFB_1−GOD and anti-AFB_1 mAb may affect the performance of the immunoassay. A competitive inhibition rate was used to obtain the optimal concentration of AFB_1−GOD and anti-AFB_1 mAb ascites. The results of the checkerboard-type titration show that 3.1 $\mu g\ mL^{-1}$ of anti-AFB_1 mAb ascites and 0.25 $\mu g\ mL^{-1}$ of AFB_1−GOD are cost effective for a higher competitive inhibition rate (Table S1, Supporting Information).

Other factors such as pH, ionic strength, organic solvent content, and temperature in the immunoreaction system could also affect the interaction between anti-AFB_1 mAb and AFB_1. The effects of pH, NaCl concentration, and acetonitrile concentration on the interaction of anti-AFB_1 mAb and AFB_1 were evaluated by monitoring the CL intensity (Figure 1). The results show that the optimal pH, NaCl concentration, and acetonitrile concentration are 6.0−6.5, 4−8 mmol L^{-1}, and 0−20% (v/v), respectively. Also, room temperature is suitable for antigen−antibody interactions (Figure 1D). Based on the above evaluation, the immunoreaction between anti-AFB_1 mAb and AFB_1 was performed in a 0.2 mol L^{-1} PB buffer (pH 6.5, containing 10% (v/v) acetonitrile and 5 mmol L^{-1} NaCl) at room temperature for 40 min.

Factors Affecting Chemiluminescence. In this BCCI assay, GOD catalyzes the oxidation of glucose to produce H_2O_2 for subsequent chemiluminescence in the presence of HRP, luminol, and *p*-bromophenol under an alkaline condition (Figure 2A), while *p*-bromophenol as an CL enhancer may accelerate the cyclic transformation of HRP (Figure 2B) and the electron transfer between luminol and radicals.[26,27] Specifically, the HRP cycle offers sustainable HRP and keeps the H_2O_2/HRP ratio suitable for persistent chemiluminescence of luminol.[21] Thus, pH, incubation time of glucose and GOD, and concentrations of luminol, HRP, and *p*-bromophenol were optimized. Based on the results in Figure 3, the solution (pH 11.5) containing 20 $\mu mol\ L^{-1}$ HRP, 50 mmol L^{-1} *p*-bromophenol, and 10 mmol L^{-1} luminol was chosen as the CL substrate solution due to the highest CL intensity. Moreover, 50 mmol L^{-1} of glucose for a 30 min incubation in the well was selected for the highest CL intensity.

Kinetics of CL. The time-dependent CL curves were examined with and without enhancer *p*-bromophenol to reveal the kinetic behavior of the BCCI assay. In the absence of *p*-bromophenol, CL intensity decayed rapidly (Figure 4A). In contrast, CL intensity in the presence of *p*-bromophenol increased in the first 20 min and then leveled off (Figure 4B). The results show that the present BCCI assay gave a much higher and more persistent CL intensity due to the introduction of an enhancer and high catalytic efficiency of the bienzymatic

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system, which is favorable for highly sensitive and reproducible detection.

Analytical Performance of the Developed BCCI Assay. Under optimal conditions, the competitive inhibition rate (CIR) linearly increased with AFB_1 concentration (C_{AFB1},ng L^{-1}) in the range of 0.017−3910 ng L^{-1}, giving a calibration function of CIR = 16.5lgC_{AFB1} + 41.6 with a determination coefficient (R^2) of 0.9953 (Figure S6). The half maximal inhibitory concentration (IC_{50}) and the detection limit (DL, 3s) were 3.38 ng L^{-1} and 5 pg L^{-1}, respectively, corresponding to 270.4 ng kg^{-1} and 0.4 ng kg^{-1} in grain, respectively. The linearity of the developed method spans over 5 orders of magnitude, which is highly advantageous for the determination of AFB_1. Compared with some previous methods,[28−36] the proposed BCCI assay displays a much lower DL and wider linear range for the determination of AFB_1 (Table S2, Supporting Information). The relative standard deviation for 11 replicate determinations of 100 ng L^{-1} AFB_1 is 1.9%.

As the monoclonal antibody was not characterized and published by the supplier, investigation on specificity was performed in this study. Besides structurally related analogs, aflatoxins (AFTs), the developed BCCI assay possesses high specificity as no significant interferences of other mycotoxins, including other seven common mycotoxins (fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), zearalenone (ZEN), deoxynivalenol (DON), ochratoxin A (OTA), citrinin (CIT) and trichothecenes (T-2)) on the determination of AFB_1 were observed (Figure 5).

Application to the Analysis of Grain Samples. The developed BCCI assay was validated by analyzing a certified reference material (GBW(E)100386) (maize) for AFB_1. The good agreement between the determined concentration of AFB_1 in GBW(E)100386 using our BCCI assay (27.7 ± 0.4 μg kg^{-1} (n = 5)) and the certified value (27 ± 3 μg kg^{-1}) demonstrates the accuracy of the developed BCCI assay. We then applied the developed BCCI assay to the determination of AFB_1 in various grain samples. The analytical results are listed in Table 1. The concentration of AFB_1 in these real grain samples ranged from 0.016 to 0.368 μg kg^{-1}, below the maximum allowable level set by CAC.[4] The recoveries for spiked AFB_1 in the grain samples were in the range of 94.0%−97.0%. The above results indicate that the developed BCCI assay is promising for ultrasensitive and specific determination of AFB_1 in real samples.

CONCLUSION

In summary, we have reported a BCCI assay for ultrasensitive determination of AFB_1. In the developed BCCI assay, GOD and HRP act as highly efficient catalysts, and *p*-bromophenol serves as the enhancer for chemiluminescence to produce an intense and long-lasting chemiluminescence signal. The developed BCCI assay is easily extended to other analytes by simple replacement of the corresponding antibody, showing great potential as a universal platform for ultrasensitive, high-throughput, and specific detection of pollutants or nutrients in foods.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b03579.

Schematic illustration for coupling of GOD and AFB_1; TLC monitoring of oximation of AFB_1; TLC monitoring of activation of AFB_1-oxime; absorption spectra of AFB_1, GOD, and different coupling ratios of AFB_1-GOD; comparison of catalytic ability of GOD in AFB_1-GOD with pure GOD for catalyzing glucose to produce hydrogen peroxide; performance of different coupling ratios of AFB_1-GOD for competitive immunoassay; plot of competitive inhibition rate against the logarithm of AFB_1 concentration; checkerboard-type titration of AFB_1-GOD and anti-AFB_1mAb ascites; comparison of proposed method with other developed methods for determination of AFB_1. (PDF)

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Notes

The authors declare no competing financial interest.

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