

# One-Step Ultrasensitive Bioluminescent Enzyme Immunoassay Based on Nanobody/Nanoluciferase Fusion for Detection of Aflatoxin B<sub>1</sub> in Cereal

Wenjie Ren,<sup>†</sup> Zhenfeng Li,<sup>‡</sup> Yang Xu,<sup>\*,†</sup> Debin Wan,<sup>‡</sup> Bogdan Barnych,<sup>‡</sup> Yanping Li,<sup>†</sup> Zhui Tu,<sup>†</sup> Qinghua He,<sup>†</sup> Jinheng Fu,<sup>†</sup> and Bruce D. Hammock<sup>\*,‡,§</sup>

<sup>†</sup>Key Laboratory of Food Science and Technology, and Sino–German Joint Research Institute, Nanchang University, 235 Nanjing East Road, Nanchang 330047, China

<sup>‡</sup>Department of Entomology and Nematology and UCD Comprehensive Cancer Center, University of California, Davis, Davis, California 95616, United States

## S Supporting Information

**ABSTRACT:** Nanoluciferase (Nluc), the smallest luciferase known, was used as the fusion partner with a nanobody against aflatoxin B<sub>1</sub> to develop a bioluminescent enzyme immunoassay (BLEIA) for detection of the aflatoxin B<sub>1</sub> in cereal. Nanobody (clone G8) against aflatoxin B<sub>1</sub> was fused with nanoluciferase and cloned into a pET22b expression vector, and then transformed into *Escherichia coli*. The nanobody fusion gene contained a hexahistidine tag for purification by immobilized metal affinity chromatography, yielding a biologically active fusion protein. The fusion protein G8-Nluc retained binding properties of the original nanobody. Concentration of the coelenterazine substrate and buffer composition were also optimized to provide high intensity and long half-life of the luminescent signal. The G8-Nluc was used as a detection antibody to establish a competitive bioluminescent ELISA for the detection of aflatoxin B<sub>1</sub> in cereals successfully. Compared to classical ELISA, this novel assay showed more than 20-fold improvement in detection sensitivity, with an IC<sub>50</sub> value of 0.41 ng/mL and linear range from 0.10 to 1.64 ng/mL. In addition, the entire BLEIA detection procedure can be completed in one step within 2 h, from sample preparation to data analysis. These results suggest that nanobody fragments fused with nanoluciferase might serve as useful and highly sensitive dual functional reagents for the development of rapid and highly sensitive immunoanalytical methods.

**KEYWORDS:** aflatoxin B<sub>1</sub>, nanoluciferase, one-step ultrasensitive bioluminescent immunoassay

## INTRODUCTION

Mycotoxins are a problematic toxic group of small organic molecules that are produced as secondary metabolites by several fungal species that colonize crops.<sup>1</sup> They lead to contamination at both the field and postharvest stages of food production with a range of cereals. Aflatoxins are a group of naturally occurring mycotoxins, produced mainly by *Aspergillus*. More than 20 types of aflatoxins and some Metabolites have been identified, among which four aflatoxins (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>) occur naturally (Figure S1). Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is the most toxic and one of the most potent carcinogens in nature,<sup>2</sup> classified as a class 1 carcinogen to humans.<sup>3</sup> Because of the high toxicity and carcinogenicity of aflatoxins, many countries have set up strict maximum limits for AFB<sub>1</sub> or total aflatoxins permissible in food and agriproducts with the range from 2 to 20 µg/kg.<sup>4</sup> Numerous analytical methods have been developed for aflatoxin determination, including chromatography-based instrumental analytical methods<sup>5</sup> and immunoassay-based rapid methods (e.g., ELISA, lateral flow strips, and sensors).<sup>6–8</sup> In past decades, numerous antibodies against aflatoxins have been developed by polyclonal and monoclonal techniques and applied in various immunoassay-based rapid methods for the detection of aflatoxins. With the advent of molecular engineering and phage display technology, recombinant antibodies produced in host bacteria or other cell lines have

become more useful reagents compared to conventional animal-derived antibodies. Aflatoxin-specific recombinant antibodies such as Fab and ScFv have been utilized in ELISAs and biosensors.<sup>9,10</sup> Recently, a novel kind of recombinant antibody derived from camelids or related species, called nanobodies, have attracted much more attention. Lacking the light chain seen in monoclonal antibodies, these recombinant heavy-chain-only antibodies retain the antigen binding capacity of antibodies, but are much smaller in size, averaging approximately 15 kDa. Nanobodies are promising reagents for the next generation of immunoassays because of their high thermostability and high expression yields compared to the conventional recombinant antibody fragments. Currently, nanobody technology has been widely used in the clinical diagnostic field targeting protein biomarkers and an increasing number of nanobodies have been isolated against small molecules for detection in rapid immunoassays.<sup>11</sup> An aflatoxin-specific nanobody was successfully obtained from an immune alpaca phage-display VHH library constructed by our laboratory. In our previous study, it has been applied in a

**Received:** January 28, 2019

**Revised:** March 12, 2019

**Accepted:** March 18, 2019

**Published:** March 18, 2019

colorimetric immunoassay<sup>12</sup> and immunoaffinity column preparation,<sup>13</sup> proving to be a powerful analytical reagent to recognize AFB<sub>1</sub> in various foods.

Recently, construction of antibody-reporter (enzyme/photo-protein) fusions based on recombinant DNA technology has shown a significant advantage in immunoassays. Fusions directly linking recombinant antibody fragments with the reporter enzyme like alkaline phosphatase are excellent probes for immunoassays and have been shown to be promising reagents for detection of protein antigens and small chemicals, like *Bacillus anthracis*,<sup>14</sup> dermonecrotxin,<sup>15</sup> and *O,O*-diethyl organophosphorus pesticides.<sup>16</sup> AP-nanobody fusions have also been utilized for the detection of small molecules, such as ochratoxin A,<sup>17</sup> tetrabromobisphenol A,<sup>18</sup> and parathion.<sup>19</sup> Another group of reporters are fluorescent proteins (i.e., GFP and RFP), which have been used for construction of fusion proteins with antibody fragments.<sup>20–22</sup> The one-step immunoassay based on antibody–reporter fusions can reduce long assay times, which is a drawback for traditional immunoassays requiring secondary or tertiary antibody enzyme conjugates. Another important group of photoproteins are luciferases. These enzymes from a variety of organisms catalyze a light-emitting reaction and are widely utilized as reporter proteins. The first-generation protein species is firefly luciferase (Fluc),<sup>23</sup> which requires ATP for the oxidation of luciferin. Other popular luciferases are derived from the sea pansy *Renilla reniformis* (Rluc)<sup>24</sup> and the marine copepod *Gaussia princeps* (Gluc).<sup>25</sup> They can oxidize their substrate coelenterazine without ATP or Coenzyme A, which simplifies their use in a number of reporter applications. Genetic fusions between Coelenterazine-based luciferases and antibody fragments have been applied in clinical diagnostics and utilized as analytical tools.<sup>26,27</sup> Nanoluciferase (Nluc) derived from the deep-sea shrimp *Oplophorus gracilirostris* is the newest commercially available luciferase enzyme. It is only 19.1 kDa in size and produces high intensity, glow-type luminescence. Nluc is one of the smallest luciferases in the world,<sup>28</sup> exhibiting several excellent physical properties superior to traditional luciferases; it is a thermostable enzyme which retains activity after 30 min of incubation at 55 °C and is active over a broad pH range (fully active in the pH 7–9 range, and also retains significant degree of activity at pH 5–7). Furthermore, its monomeric nature facilitates its use as transcriptional reporter or fusion partner. Nluc also contains no post-translational modifications, including disulfide bonds, making it a powerful luminescent probe, which can be used as a fusion partner with antibodies or other binding proteins/peptides for developing immunoassays.<sup>29–31</sup>

Although the aforementioned luciferase enzymes have been available to researchers for over a decade, their application as reporters in immunoassays have been limited because of some inherent caveats such as substrate stability and the bioluminescence signal half-life. Various substrates and buffer systems have been developed to alleviate some of these problems. However, many of these reagents are expensive. Recently, new coelenterazine substrates have been developed and are highly sensitive for the detection of luciferase, as low as the attomole level. However, the combination of different coelenterazine substrates with this latest luciferase Nluc has not yet been well studied. A few studies have reported on the application of nanobody-Nluc fusions for the one-step detection; their potential had not been revealed so far. In our study, we developed a novel fusion protein as a powerful

dual functional reagent and its matching substrate system. Then an ultrasensitive one-step competitive bioluminescent enzyme immunoassay (BLEIA) based on nanobody-Nluc fusion was developed and used for quantification of AFB<sub>1</sub> in cereals.

## MATERIALS AND METHODS

**Chemicals and Reagent.** Restriction enzymes *NotI*, *Sall*, *NcoI*, and *SfiI*, Phusion High-Fidelity DNA Polymerase, and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA, USA). Invitrogen Bolt 4–12% Bis-Tris Plus, disposable 5 mL polypropylene columns, 96 flat bottom white polystyrol (442404), bovine serum albumin (BSA), protease inhibitor cocktail (EDTA-free, 100×), B-PER bacterial protein extraction, and HisPur Ni-NTA resin were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Skim milk powder was purchased from Magermilchpulver (Billerica, MA, USA). Black 96-well (E18073CV) and white 96-well (E180538G) microplates were purchased from Greiner Bio-One. Coelenterazine substrates (CTZ-native, CTZ-400a, and CTZ-H) were purchased from NanoLight Technology, Anti-6X His tag antibody (HRP) was purchased from Abcam. Fumonisin B<sub>1</sub> (FB<sub>1</sub>), aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), ochratoxin A (OTA), deoxynivalenol (DON), and zearalenone (ZEN) were purchased from Fermentek (Jerusalem, Israel). pNPP was purchased from American Sigma. Primers used for polymerase chain reaction (PCR) amplification were synthesized and purified by Invitrogen.

**Construction of Recombinant Plasmid, Expression, and Purification.** All the nucleotide sequences of primers used are listed in Table S1 (Supporting Information). The nanoluciferase encoding gene was first amplified by PCR with primers 1 and 2. After digestion with *NotI* and *Sall* and purification with a PCR cleanup kit, the resulting nanoluciferase gene was subcloned into a modified pET22b vector digested by *NotI* and *Sall*. The resulting positive vector was confirmed by DNA sequencing. The gene for the nanobody containing the upper hinge at the C-terminal (G8) was amplified with primers 3 and 4 and subcloned into the pET22b vector carrying the nanoluciferase gene using *NcoI* and *SfiI* as described above. The constructed vector carrying G8-Nluc was confirmed by DNA sequencing, and the full length of the protein sequence of the fusion is shown in Table S1. The vector of nanoluciferase and the vector of G8-Nluc fusion protein were transformed into *E. coli* BL21(DE3) competent cells. The transformed cells were grown on Luria-Bertani (LB) agar plates containing ampicillin and single colonies were used to inoculate 5 mL of LB broth containing ampicillin and incubated at 37 °C, 220 rpm overnight. The culture was used to inoculate 200 mL of LB broth containing ampicillin and incubated at 37 °C, 220 rpm until the OD<sub>600</sub> (optical density of a sample measured at a wavelength of 600 nm) reached approximately 0.5 (more than 3 h). To induce expression of the target proteins, isopropyl β-D-1-thiogalactopyranoside was added to the culture at a final concentration of 0.1 mM. After incubation for 12 h at 20 °C and 180 rpm, cells were harvested by centrifugation at 5000g for 10 min. The periplasmic extract was obtained by using Bacterial Protein Extraction Reagent (B-PER): 4 mL of B-PER and 2 μL of lysozyme were added for each gram of cells. After the cells were lysed thoroughly for 30 min at room temperature, the supernatant was collected by centrifugation at 15 000g for 5 min. Nanoluciferase and G8-Nluc fusion each contain a hexahistidine tag at their C-terminals and were purified by using HisPur Ni-NTA resin according to the manufacturer's protocol. The eluted fractions were analyzed by SDS-PAGE and stained according to a standard protocol. The concentration of the nanoluciferase and G8-Nluc fusion were determined by absorbance at 280 nm using a Nanodrop.

**Catalytic Activity of the Fusion Protein.** Ten microliters of G8-Nluc solution in 10 mM phosphate buffered saline (PBS) was added per well (white 96-well plate) and mixed with 100 μL of solutions of different coelenterazine substrate analogues (CTZ-native, CTZ-400a, and CTZ-H). After mixing, the bioluminescent signal was measured by a Tecan 1000 plate reader in the luminescence mode.

Horseradish peroxidase (HRP) was 2-fold serially diluted with 10 mM PBS ( $3.97 \times 10^6$  to  $1.2 \times 10^4$  fmol). Ten microliters of HRP serial dilutions were mixed separately with 100  $\mu$ L of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution at 37 °C for 10 min. The reactions were stopped with 50  $\mu$ L of 2 M  $H_2SO_4$  and the absorbance was read at 450 nm. Similarly, 10  $\mu$ L of 2-fold serial dilutions of AP enzyme ( $2.9 \times 10^5$  to  $2.8 \times 10^2$  fmol, in 1 $\times$  PBS) were separately mixed with 100  $\mu$ L of BBTP fluorescent substrate in corresponding buffer, incubated at 37 °C for 30 min, and the fluorescent intensities were measured at 550 nm after excitation at 450 nm. The nanoluciferase and G8-Nluc fusion proteins were 2-fold serially diluted in 10 mM PBS, from  $1.5 \times 10^4$  to 14.7 fmol for nanoluciferase and  $1.06 \times 10^4$  to 10.35 fmol for G8-Nluc, respectively. Ten microliters of nanoluciferase or G8-Nluc fusion serial dilutions were separately mixed with 100  $\mu$ L of CTZ-H chemiluminescent substrate in white 96-well plates and the bioluminescence intensity was measured with Tecan 1000 within 60 s after mixing.

**Formulation of Substrate Buffer for Coelenterazine-Based Bioluminescent Detection.** Coelenterazine-H is a suitable substrate for nanoluciferase. The 100 $\times$  stock solution (0.5 mg/mL) was prepared by dissolving coelenterazine-H in 75% methanol, 15% glycerin, and 100  $\mu$ M ascorbic acid. In biological luminescence measurement systems, the maximum luminescent intensity and the dynamic stability of the luminescence signal are critical for high-throughput detection. A series of luminescence assay buffers were formulated and their luminescence performance with nanoluciferase and the coelenterazine-H substrate was evaluated. All luminescence assay buffers contained BSA, EDTA- $Na_2$ , Triton X-100, and Tergitol NP-10. Ten millimolar PBS (pH 8) buffers containing different concentrations of the following additives were prepared: BSA (0.06, 0.12, 0.25, 0.5, and 1.0 mg/mL), EDTA- $Na_2$  (1.1, 2.2, 4.4, 8.8, and 17.6 mM), Tergitol NP-10 (0.25%, 0.5%, 1%, 2%, 4%, and 8%). One hundred microliters of 1 $\times$  coelenterazine-H substrate solutions in different luminescence assay buffers were loaded onto the white plate. The luminescence reactions were started by the addition of 10  $\mu$ L of the G8-Nluc (70 ng/mL) working solution and the luminescence intensity was recorded in 60 s intervals for 1200 s by using a Tecan 1000 instrument. The maximum intensity of luminescence ( $I_{max}$ ) was shown as a relative light unit (RLU). RLU and signal half-life were measured for reactions of CTZ-H with G8-Nluc fusion protein in a variety of luminescence assay buffers. After determining the optimal luminescence assay buffer, its characteristics were compared to two commercial luminescence assay buffers (Passive lysis buffer and Glo lysis buffer from Promega).

**Competitive ELISA Based on G8 Nanobody and G8-Nluc Fusion.** Optimal concentrations of G8 nanobody, G8-Nluc fusion, and coating antigen AFB<sub>1</sub>-BSA were determined by checkerboard titration. For this assay, a 96-well microtiter plate was coated with AFB<sub>1</sub>-BSA (2  $\mu$ g/mL in 10 mM PBS) at 100  $\mu$ L/well overnight at 4 °C; the liquid was removed, the plate was washed three times with 0.01% PBST, and the plate was blocked with 300  $\mu$ L/well skim milk at 37 °C for 1 h. After removal of the blocking solution, the plate was washed three times with PBST; 50  $\mu$ L/well of G8 nanobody (2.75  $\mu$ g/mL) or G8-Nluc fusion protein (3.1  $\mu$ g/mL) and an equal volume of serial concentrations of AFB<sub>1</sub> standard were added, and the plate was mixed and incubated at 37 °C for 30 min after the plate was washed three times with PBST. For the two-step ELISA, 100  $\mu$ L of HRP-labeled mouse anti-His tag secondary antibody (1:1000) was added, and incubated at 37 °C for 30 min. After washing three times with PBST, TMB substrate solution was added, and the plate was incubated at 37 °C for 7 min. The reaction was stopped by adding 50  $\mu$ L of 3 M  $H_2SO_4$ , and the absorbance was measured at 450 nm. The binding rate (%) of each standard concentration was calculated as  $B/B_0 \times 100\%$  ( $B$  is the  $OD_{450}$  in the presence of AFB<sub>1</sub> and  $B_0$  is in the absence of AFB<sub>1</sub>). Taking the logarithm of the AFB<sub>1</sub> standard concentration as the abscissa and the binding rate as the ordinate, an inhibition standard curve was obtained by plotting the binding rate against the AFB<sub>1</sub> concentration, which was analyzed using the program Origin 7.5 with the four parameters logistic formula listed below.

$$y = d + (a - d)/(1 + (x/c)^b)$$

**Competitive BLEIA Based on G8-Nluc Fusion.** The coating and blocking steps for BLEIA were the same as those used in the ELISA. In one-step BLEIA, the concentration of AFB<sub>1</sub>-BSA and the G8-Nluc were optimized by checkerboard titration. A 96-well high-binding white microtiter plate was coated with AFB<sub>1</sub>-BSA (1  $\mu$ g/mL in 10 mM PBS) overnight at 4 °C, followed by blocking with 5% skim milk. Then 50  $\mu$ L/well of G8-Nluc fusion protein (0.5  $\mu$ g/mL) and an equal volume of serial concentrations of AFB<sub>1</sub> standard were added; the plate was mixed and incubated at 37 °C for 30 min. After washing, 100  $\mu$ L/well of 5  $\mu$ g/mL CZT-H substrate solution was added and the bioluminescent signal was recorded immediately. The standard curve was obtained by using the same formula as above for the ELISA. The BLEIA assay buffer has three critical parameters, including pH value, ionic strength, and methanol concentration, all of which were assessed in this study. The following BLEIA assay buffers were evaluated with varying methanol concentrations (0%, 5%, 10%, 20%, 40%, and 80%), ionic strength (0, 10, 20, and 50 mM), and pH (5, 6, 7, 8, and 9). BLEIA under different conditions was carried out using the same procedure, and their  $RLU_{max}$  and  $IC_{50}$  values were compared.

For cross-reactivity analysis, various concentrations of AFB<sub>1</sub>, FB<sub>1</sub>, OTA, ZEN, and DON standard solutions were prepared using optimized assay buffer for BLEIA, and a competitive inhibition standard curve was drawn to calculate  $IC_{50}$  and the cross-reactivities (CR) value using the following formula:

$$CR (\%) = [IC_{50}(AFB_1)/IC_{50}(AFB_1, FB_1, OTA, ZEN, DON)] \times 100\%$$

#### Sample Extraction and Analysis by BLEIA and LC-MS/MS.

For the spiking and recovery study, 5 g of crushed grain samples (corn, wheat), shown to be AFB<sub>1</sub> free by LC-MS/MS, were placed in three 50 mL centrifuge tubes and fortified with 5, 10, and 20  $\mu$ g/kg of AFB<sub>1</sub> standard, respectively. For the extraction, 15 mL of 80% methanol-PBS was added to the tube. After sonication for 15 min, the tube was centrifuged at 8000 rpm for 15 min and the supernatant was collected and filtered through a 0.22  $\mu$ m filter. The resulting extract was diluted with assay buffer and then subjected to BLEIA. Each sample was measured in triplicate. Experimental cereal samples purchased from the supermarket were treated in the same manner.

LC-MS/MS analysis of AFB<sub>1</sub> was performed on an Agilent SL liquid chromatograph connected to a 4000 Qtrap mass spectrometer. The separation was performed on a Kinetex C18 column (30 mm  $\times$  4.6 mm, 2.6  $\mu$ m). The column temperature was set at 50 °C. Water (solution A) and acetonitrile containing 0.1% (v/v) acetic acid (solution B) were used as the mobile phases with a flow rate of 0.6 mL/min. The sample injection volume was 5  $\mu$ L, and each sample had a running time of 3 min. The gradient is shown in Table S2a. The mass spectrometer was operated in negative ESI mode and multiple reaction monitoring mode. The optimized ion source parameters and MRM method are shown in Tables S2b and S2c, respectively. 12-(3-Cyclohexyl-ureido)-dodecanoic acid with a final concentration of 200 nmol/L was mixed with analytes as an internal standard to account for ionization suppression.

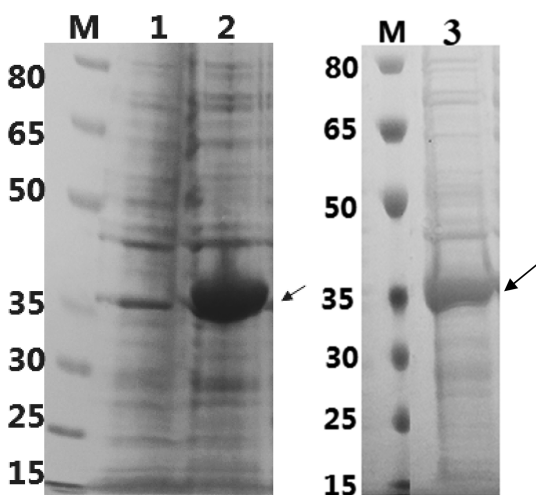
## RESULTS AND DISCUSSION

To evaluate the utility of nanoluciferase as a fusion partner for nanobodies, we selected a common competitive assay system for detection of AFB<sub>1</sub>, the major mycotoxin in cereals and the most harmful to humans as a model antigen. In our study, we focused on the following three scientific questions: (1) whether nanoluciferase fusion protein has the same binding and catalytic ability as its parents; (2) whether the bioluminescent immunoassay based on the fusion protein achieves higher sensitivity, with specific substrate and buffer conditions,



and (3) whether the novel one-step BLEIA is superior to the classical ELISA, for the detection of AFB<sub>1</sub> in cereals.

**Expression, Purification, and Identification of the G8-Nluc Fusion Protein.** The first step toward the creation of the fusion protein is construction of the recombinant plasmid. First, an oligo of the sequence of nanoluciferase was subcloned in the modified pET22b vector, and the nanoluciferase was expressed with high yield in *E. coli* as in the previous study. Though the gene of nanoluciferase is derived from deep sea shrimp, it was considered as the potential fusion partner because of its small size (19 kDa) and lack of glycosylation and disulfide bonds. The anti-AFB<sub>1</sub> specific nanobody G8 was chosen as the partner of nanoluciferase. Its gene was amplified from the original plasmid and subcloned into the modified pET22b vector containing the nanoluciferase gene. With use of the special primer (Table S1), G8 VHH was amplified with the upper hinge at the C-terminus and then fused with the N-terminus of nanoluciferase, resulting in a fusion protein with fusion partners being separated with the upper hinge as the spacer. The full sequence of the fusion gene is shown in Table S1. After transformation, the resulting recombinant plasmid was confirmed by DNA sequencing and then transformed into competent cells for expressing the soluble fusion protein. The crude fusion protein extracted by the B-PER reagent was purified by a Ni-NTA affinity column, and SDS-PAGE was used to determine the size and purity of the Nb-AP fusion protein (Figure 1). The gel shown has a band at approximately



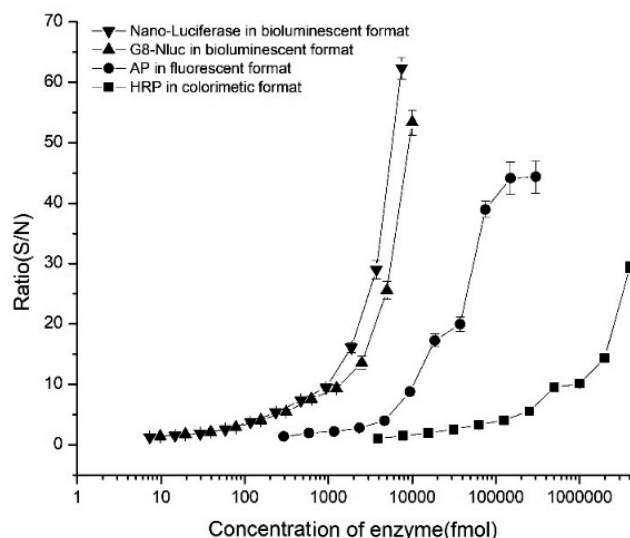
**Figure 1.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of expression of the fusion protein. Blots were stained with SYPRO Ruby protein gel stain. Key: lane M, PageRuler unstained protein ladder and spectrum multicolor broad-range protein ladder. Lane 1: Whole-cell extract under noninduced conditions. Lane 2: Whole-cell extract under induced conditions. Lane 3: G8-Nluc fusion protein.

35 kDa, as expected for the G8-Nluc fusion protein. Though the yield of the fusion protein is lower than the parent nanobody G8 and the parent nanoluciferase, we obtained almost 5 mg of purified fusion protein from 1 L of culture.

**Determination of Luminescence Activity of Fusion Protein with Different CTZ Substrates.** Coelenterazine (CTZ) is the natural substrate for luciferase, and over a dozen of CTZ analogs have been synthesized and are now available commercially. These CTZ analogues can function as substrates for *Renilla* and *Guassia* luciferase and have different bio-

luminescence properties.<sup>32</sup> CTZ-h (2-deoxy derivative of native coelenterazine) has a 2-fold higher initial light output than native CTZ from *Renilla* sp. CTZ-400a is a CTZ derivative that serves as a substrate for luciferase from *Renilla* sp. and generates an emission peak centered around 400 nm. Only a few studies screened the CTZ and its analogues as substrate of nanoluciferase; we intended to seek the best combination of CTZ analogues with nanoluciferase in this study. Three CTZ analogues, including CTZ-native, CTZ-400a, and CTZ-H, were chosen as substrates for nanoluciferase to generate a luminescence signal. The order of the initial luminescence intensity (RLU<sub>max</sub>) for the purified nanoluciferases was CTZ-H ( $2.3 \times 10^6$ ) > CTZ-400a ( $1.5 \times 10^6$ ) > CTZ-native ( $2.5 \times 10^5$ ) (Figure S2). The coelenterazine-H showed the strongest signal compared to two other analogues. These results indicated that 2-deoxy substituent on the phenyl group of coelenterazine significantly affects the catalytic function of nanoluciferase because there was a 10-fold improvement in signal intensity observed compared to CTZ-native. A similar order obtained with coelenterazine-1 for reactions with G8-Nluc showed that CTZ-H is a good substrate for both the nanoluciferase and its fused protein. In addition, the cost of CTZ-H is almost the same as the common CTZ-native, and significantly cheaper than the patented CTZ analogue fuzimazine from Promega.

Next, the luminescent signals for the serial dilutions of G8-Nluc with CTZ-H substrate were measured to determine the sensitivity of G8-Nluc detection. The limit of detection (LOD) for the enzyme catalytic activity in our study was defined as the lowest amount of enzyme (with a certain amount of substrate per well) that provided 2-fold higher signals than the blank (substrate background signal). As shown in Figure 2, the



**Figure 2.** Detection limit of fusion protein in bioluminescent format. S is the reading in the presence of enzyme and N is that in the absence of enzyme.

detection limit for G8-Nluc was as low as 50 fmol, at the same level as that for the nanoluciferase. This result indicated that G8-Nluc has similar catalytic activity to the original Nluc. For comparison, detection sensitivities for alkaline phosphatase (AP) and horseradish peroxidase (HRP) were measured in fluorescent and colorimetric formats, respectively. The

sensitivity of G8-Nluc detection in bioluminescent format was significantly higher compared to that for AP and HRP, the LODs of which were 1000 and 10 000 fmol, respectively. Thus, with use of well-matched CTZ-H substrate, G8-Nluc could be detected at femtomole level, which is 20- and 200-fold more sensitive than detection of AP with fluorescence readout and HRP with colorimetric readout, respectively. This result shows that Nluc is a very promising fusion partner for the development of Nluc-Ab fusion proteins that could be considered as ultrasensitive enzyme reagents for an immunoassay.

#### Optimization of the Luminescence Assay Buffer.

Luciferase is able to generate a glow-type luminescent signal by catalyzing the oxidation of specific substrates like luciferins and CTZ, with the concurrent emission of a photon. However, the signal half-life can decrease significantly, likely because of rapid depletion of substrate. Meanwhile, a substrate like CTZ is unstable in a weakly basic aqueous solution, which is the optimal pH value for many biological experiments. Thus, variation of the luminescence signal between the first sample and the last sample may become apparent when a large number of samples is processed sequentially. Many strategies were applied to address this problem, such as measurement of many samples simultaneously by using an injection-based reader to reduce the variation of luminescence.<sup>33</sup> Alternatively, some luciferase variants have been constructed whose luminescence half-life is stable over several minutes, although the specific activity of these variants is significantly lower.<sup>34</sup> In contrast to those complex solutions, a simple approach to achieve stable luminescent signal consists of adding detergent to lysis buffer when extracting the *Gaussia* luciferase from the cell.<sup>34</sup> Many commercial lysis buffers containing detergents have been developed for the detection of luminescence directly in mammalian cells, but the high cost of these buffers prohibits their use for immunoassay where large volumes of luminescence assay buffer is needed. In addition, suitability of these commercial luminescence assay buffers for nanoluciferase and CTZ-H combination is unknown. Therefore, we decided to develop an efficient luminescence assay buffer that enhances the half-life of the bioluminescence signal. Therefore, we prepared 10 mM PBS buffers containing various amounts of Triton X-100, Tergitol NP-10, BSA, and EDTA·Na<sub>2</sub> as additives. Next, a luminescence signal from reactions of G8-Nluc with CTZ-H substrate in each of these substrate buffers was recorded in 60 s intervals for more than 1200 s. When compared to blank PBS buffer (Figure 3), the surfactant additives like Triton X-100 and Tergitol NP-10 depressed the luminescence intensity slightly, but they both dramatically retarded the decay of luminescence. Surprisingly, BSA and EDTA·Na<sub>2</sub> increased the maximum intensity of the luminescence signal by 2- and 4-fold, respectively, while having no effect on the signal half-life. To improve the signal intensity and the half-life of luminescence, the concentrations of Triton X-100, BSA, and EDTA·Na<sub>2</sub> were optimized for the final buffer formulations. This luminescence assay buffer (pH 8.0, 10 mM PBS containing 1% Tergitol NP-10, 0.25 mg/mL BSA, and 8.8 mM EDTA·Na<sub>2</sub>) has great compatibility with the combination of nanoluciferase and CTX-H substrate and performs significantly better than commercial Glo-lysis and Passive lysis buffers, purchased from Promega (Figure 4). The present formulation of luminescence assay buffer shows great promise with comparable high luminescence intensity and more than 20 min half-life, which permits its application in high-

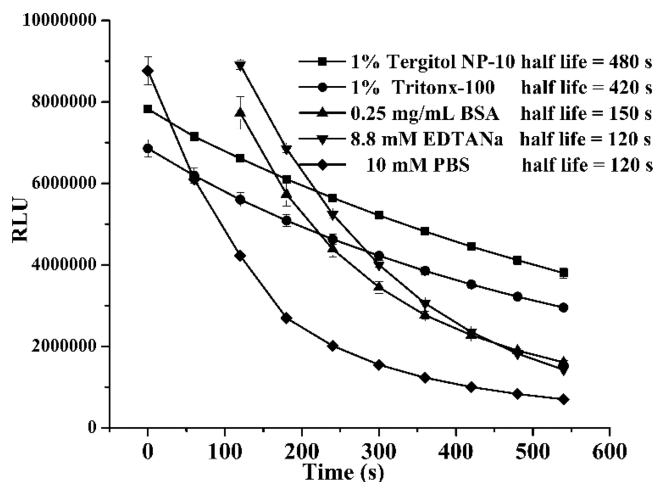


Figure 3. Kinetic analysis of substrate buffer mixed with different components.

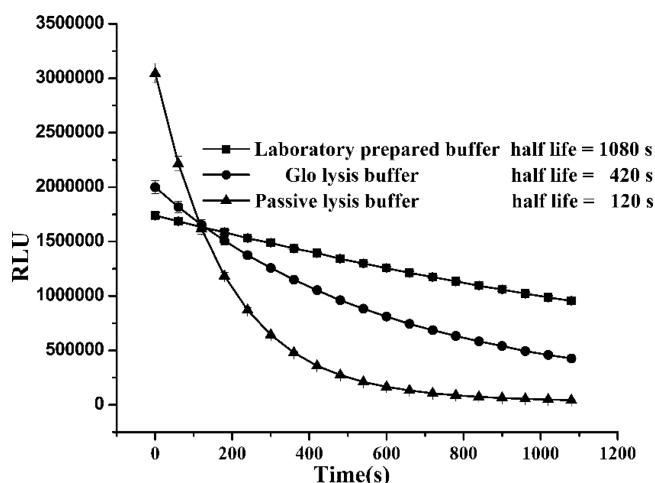


Figure 4. Comparison of the half-life of laboratory-prepared substrate buffer with commercial substrate buffers.

throughput immunoassays based on nanoluciferase and its fusions.

**Binding Capacity of Fusion Protein Compared to the Parent Nanobody.** The G8-Nluc fusion protein could serve as a dual functional reagent in an immunoassay, not only for antigen binding but also the reporter reagent for signal amplification. Thus, the binding properties of the fusion protein is a key feature to guarantee the sensitivity of a bioluminescent ELISA. To evaluate the sensitivity of the G8-Nluc and the parent G8 nanobody, classic two-step ELISAs were performed. Anti-6X His tag HRP-conjugated antibody was used as a secondary antibody to recognize the His tag at the C-terminus of both recombinant proteins. As shown in Figure 5, the binding between G8 or G8-Nluc with the coating antigen could be inhibited by AFB<sub>1</sub>. The IC<sub>50</sub> for both ELISAs are comparable, with 8.14 ng/mL for G8 and 10.43 ng/mL for G8-Nluc, respectively. This result indicated that the developed G8-Nluc fusion protein maintains the binding properties of the parent G8 nanobody. In this study, the spacer between the nanobody and nanoluciferase was the flexible linker from the upper hinge of the nanobody's C terminus (Table S1). This result indicated that the developed G8-Nluc fusion was designed properly, where the two parts of the fusion protein

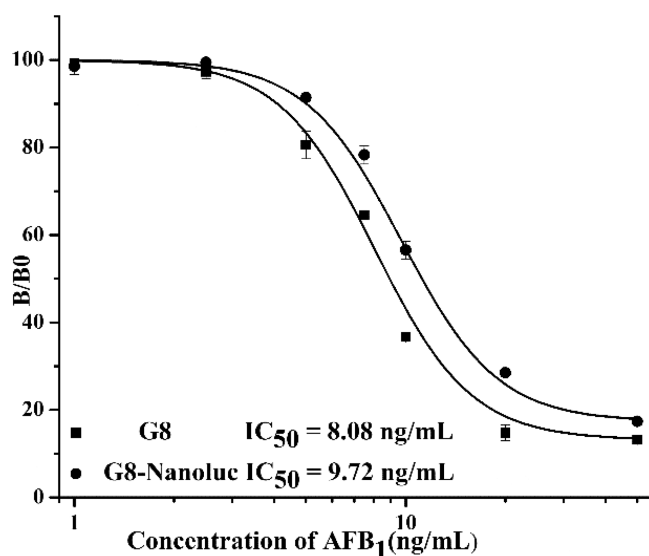


Figure 5. Standard curve of fusion protein and parent nanobody in two-step ELISA.

were separated by a spacer and their natural protein conformations were maintained.

**Performance of BLEIA Using the Fusion Protein in One-Step Format.** As shown above, the G8-Nluc is a dual functional reagent, which contains a recognition domain that can bind the antigen and a reporter domain which generates the luminescent signal. We next evaluated the usefulness of this dual functional reagent for the detection of AFB<sub>1</sub>. Therefore, a one-step direct-competitive bioluminescent enzyme immunoassay (BLEIA) was developed. Optimal concentrations of G8-Nluc and the coating antigen were determined by using checkerboard titration. Because of the high intensity of our luminescent system (the combination of G8-Nluc with CTZ-H in the luminescent assay buffer generated in house), the concentration of immunoreagents could be dramatically reduced compared to that of a classical two-step ELISA. G8-Nluc at 0.5  $\mu\text{g/mL}$  and AFB<sub>1</sub>-BSA at 1  $\mu\text{g/mL}$  were optimal based on the two performance parameters: the  $\text{IC}_{50}$  and the maximum relative bioluminescence intensity ( $\text{RLU}_{\text{max}}$ ). Because other assay parameters such as pH, ionic strength, and organic solvent could also influence immunoreactions, these parameters were optimized to increase the sensitivity of the assay. The BLEIA for detection of AFB<sub>1</sub> was carried out in different conditions, including five different pH values of PBS, four different ionic strengths, and six different concentrations of methanol (Figure S6). The lowest  $\text{IC}_{50}$  and the highest  $\text{RLU}_{\text{max}}$  were obtained at 50 mM PBS, pH 6.0, and 10% methanol. Under the optimized conditions, a standard curve was established for one-step BLEIA (Figure 6). The standard curve had a good correlation coefficient of 0.997 and a limit of detection of 0.05 ng/mL. The assay had an  $\text{IC}_{50}$  of 0.41 ng/mL with a linear range of 0.10–1.64 ng/mL. The  $\text{IC}_{50}$  of the BLEIA based on the G8-Nluc fusion protein was almost 20 times lower than that of the classical two-step ELISA based on that of the parent nanobody ( $\text{IC}_{50} = 8.14$  ng/mL), the performance of BLEIA was also better than that of our previous AFB<sub>1</sub> assay based on G8-Alkaline phosphatase fusion.<sup>12</sup> The specificity of the G8-Nluc fusion protein was studied with five mycotoxin analogues (Table 1). The cross-reactivity was calculated using the following formula:  $[(\text{IC}_{50} \text{ of AFB}_1)/(\text{IC}_{50} \text{ of tested mycotoxin})] \times 100\%$ . As shown in Table 2, no cross-reactivity was observed for any of the

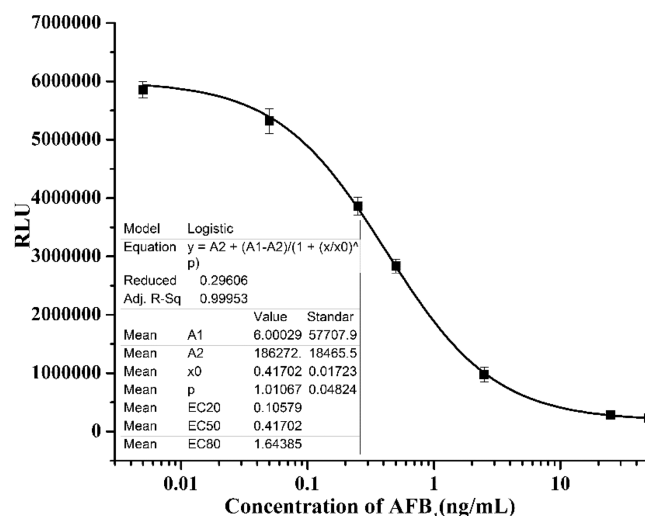
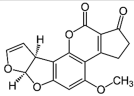
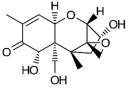
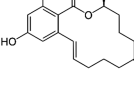
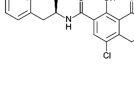
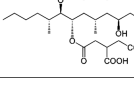


Figure 6. Standard curve of one-step BLEIA for detection of AflatoxinB<sub>1</sub>. Each point was tested in triplicate.

Table 1. Cross-Reactivity of G8-Nluc to Aflatoxin-Related Mycotoxins in One-Step BLEIA

| Analyte                  | Chemical structure  | Cross-reactivity (%) |
|--------------------------|---|----------------------|
| Aflatoxin B <sub>1</sub> |   | 100                  |
| Deoxynivalenol           |  | 0.31                 |
| Zearalenone              |  | 0.38                 |
| Ochratoxin A             |  | 0.33                 |
| Fumonisin B <sub>1</sub> |  | 0.37                 |

As shown in Table 2, no cross-reactivity was observed for any of the

Table 2. Detection of the Cereal Spiked with Aflatoxin and the Real Sample<sup>a</sup>

| sample matrix | AFB <sub>1</sub> spiked | mean $\pm$ SD ( $\mu\text{g/kg}$ ) | recovery (%) | CV (%) |
|---------------|-------------------------|------------------------------------|--------------|--------|
| corn          | 5                       | 5.70 $\pm$ 0.54                    | 113          | 9.47   |
|               | 10                      | 9.61 $\pm$ 0.37                    | 106          | 3.85   |
|               | 20                      | 19.51 $\pm$ 0.50                   | 98           | 2.56   |
| wheat         | 5                       | 5.32 $\pm$ 0.38                    | 106          | 7.14   |
|               | 10                      | 8.73 $\pm$ 0.72                    | 107          | 8.25   |
|               | 20                      | 18.19 $\pm$ 0.81                   | 91           | 4.00   |

<sup>a</sup>Each assay was performed in triplicate.



mycotoxins tested, consistent with the cross-reactivity pattern for the parent nanobody.<sup>12</sup> This result indicated that the G8-Nluc fusion preserved the recognition features of the parent nanobody. Negligible cross-reactivity with other mycotoxins demonstrated that the BLEIA based on the novel fusion protein had good selectivity and could effectively and reliably detect AFB<sub>1</sub>.

**Matrix Effect and Validation.** Matrix effect is a crucial factor to be considered in an immunoassay because it can interfere with the reaction between the antigen and antibody by quenching or enhancing readouts. To reduce the matrix effect, the extraction solution and method can be optimized. To apply the one-step BLEIA for sample analysis, the matrix effect was evaluated with wheat and corn in representative cereal samples. Aflatoxin B<sub>1</sub> is insoluble in water and is easily soluble in oil, fatty acid esters, and various organic solvents including chloroform, methanol, ethanol, and similar organic reagents. Water is miscible with methanol and is the most common extraction solvent used in aflatoxin analysis. To maximize the recovery rate, we extracted the cereal sample using 80% methanol in water. To reduce the solvent effect against the immune reaction, dilution of the resulting sample solution was necessary before loading the sample in the plate. Additionally, fat and protein are added to the methanol–water extraction solution, which can affect the immune reaction. Dilution of the sample with BLEIA assay buffer is the most common method to reduce or eliminate the matrix effect. To apply the one-step BLEIA for sample analysis, the standard inhibition curves generated in BLEIA assay buffer (10% methanol, 1× PBS, MeOH–PBS) were compared to those using diluted AFB<sub>1</sub>-free grain (corn and oat) extracts. As the sample extract was serially diluted, the OD<sub>max</sub> and IC<sub>50</sub> of the curve were varied as shown in Figures S7 and S8. When the extracts of corn and wheat were diluted eight times, it produced a sufficient standard curve and the matrix effect was minimized. The developed BLEIA had an LOD of 0.84 and 2.64 μg/kg in wheat and corn and a linear detection range of 1.68–89.28 and 1.93–41.32 μg/kg in wheat and corn for AFB<sub>1</sub>, respectively. These results meet EU regulatory requirements for AFB<sub>1</sub> in cereals (5 μg/kg). This result indicated that the sample extract could be analyzed directly by diluting 8-fold without losing sensitivity.

The accuracy and reliability of the developed one-step BLEIA for detecting AFB<sub>1</sub> were evaluated by analysis of spiked wheat and corn samples, confirmed to be free of aflatoxin by LC-MS. Wheat and corn were spiked with three different concentrations of Aflatoxin B<sub>1</sub> (5, 10, and 20 ng/g). After extraction with methanol and subsequent dilution, the samples were analyzed by one-step BLEIA. As shown in Table 2, the average recovery rate for AFB<sub>1</sub> measured by the one-step BLEIA ranged from 91% to 113%. In addition, five wheat and five corn samples collected from the local supermarket were analyzed with one-step BLEIA and LC-MS/MS. As shown in Table 3, 10 samples were positive and the concentration of aflatoxin in these cereal samples ranged from 3 to 8 μg/kg. AFB<sub>1</sub> was undetectable in most wheat samples, but one sample was contaminated at a level exceeding MRL (5 μg/kg). However, most of the corn samples contained AFB<sub>1</sub>, though the contamination levels in three of them were less than the MRL. The results obtained from one-step BLEIA and LC-MS were in good agreement with each other, indicating the accuracy and reliability of the developed one-step BLEIA.

**Table 3. Detection of the Cereal Purchased from the Supermarket**

| samples | no. | one-step BLEIA ( <i>n</i> = 3) <sup>a</sup><br>(μg/kg) | HPLC-MS/MS ( <i>n</i> = 3) <sup>a</sup><br>(μg/kg) |
|---------|-----|--|--|
| corn    | C1  | 5.94 ± 0.29  | 6.50 ± 0.34  |
|         | C2  | ND <sup>b</sup>  | ND   |
|         | C3  | 3.11 ± 0.05  | 3.50 ± 0.46  |
|         | C4  | 3.93 ± 0.19  | 4.60 ± 0.34  |
|         | C5  | 3.51 ± 0.94  | 3.80 ± 0.35  |
| wheat   | W1  | 6.15 ± 0.53  | 7.17 ± 0.30  |
|         | W2  | ND   | ND   |
|         | W3  | ND   | ND   |
|         | W4  | ND   | ND   |
|         | W5  | ND   | ND   |

<sup>a</sup>Each assay was carried out in triplicate on the same day. <sup>b</sup>Not detectable.

In this study, a sensitive and reliable dc-BLEIA based on a novel G8-Nluc fusion protein for detecting Aflatoxin B<sub>1</sub> in cereal samples was successfully developed. The nanoluciferase was an excellent fusion partner compared to alkaline phosphatase and GFP; the fusion protein remained small in size and easily expressed in *E. coli*. In our study, G8-Nluc fusion had both nanobody binding capacity and luciferase catalytic activity. This dual-functional reagent was used to develop a one-step ultrasensitive BLEIA successfully. The high sensitivity due to bioluminescent format compensated for the loss of sensitivity during the dilution of samples after extraction. In addition, the one-step procedure shortened the assay time within 2 h from sample dilution to data analysis. These results indicated that the nanobody/nanoluciferase fusion can be considered as an attractive and powerful reagent for an immunoassay, and the one-step BLEIA can be a simple and rapid analytical tool for quantification of the pollutants in commercial foods.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b00688.

The sequence for G8-Nluc fusion protein, the assay condition for best performance in BLEIA, LC-MS/MS condition, the structure mycotoxins, and optimization of substrate and substrate buffer matrix effect on the performance of BLEIA (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Authors

\*Tel.: +86-791-88329479. Fax: +86-791-88333708. E-mail: xuyang@ncu.edu.cn (Y.X.).

\*Tel.: 530-752-0492. Fax: 530-752-1537. E-mail: bdhammock@ucdavis.edu (B.D.H.).

### ORCID

Bruce D. Hammock: 0000-0003-1408-8317

### Funding

This work was financially supported by the National Key R&D Program of China (Grants 2018YFC1602203), the National Natural Science Foundation of China (No. 31471648, 31860260, 31301479, and 31671924), the National Institute of Environmental Health Science Superfund Research Program (P42ES004699), Major Program of Natural Science Foundation of Jiangxi, China (20152ACB20005), the Jiangxi Province

Key Technology R & D Program (20171BBG70072), Jiangxi Province Science Fund for Distinguished Young Scholar (Grant 20171BCB23023), Natural Science Foundation of Jiangxi Province, China (Grant 20181BAB204019), and Open Project Program of State Key Laboratory of Food Science and Technology, Nanchang University (SKLF-KF-201825).

## Notes

The authors declare no competing financial interest.

## REFERENCES

- (1) Shephard, G. S. Current Status of Mycotoxin Analysis: A Critical Review. *J. AOAC Int.* **2016**, *99*, 842–848.
- (2) Rushing, B. R.; Selim, M. I. Aflatoxin B<sub>1</sub>: A review on metabolism, toxicity, occurrence in food, occupational exposure, and detoxification methods. *Food Chem. Toxicol.* **2019**, *124*, 81–100.
- (3) Stettler, P. M.; Sengstag, C. Liver carcinogen aflatoxin B<sub>1</sub> as an inducer of mitotic recombination in a human cell line. *Mol. Carcinog.* **2001**, *31*, 125–138.
- (4) Van de Perre, E.; Jaxsens, L.; Lachat, C.; El Tahan, F.; De Meulenaer, B. Impact of maximum levels in European legislation on exposure of mycotoxins in dried products: case of aflatoxin B<sub>1</sub> and ochratoxin A in nuts and dried fruits. *Food Chem. Toxicol.* **2015**, *75*, 112–117.
- (5) Zhang, Q.; Ran, C. C.; Chen, D.; Li, J. M.; Jiang, Y. Determination of aflatoxin B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub> in Ben Lame granules by HPLC-FLD after multi-pretreatment clean-up. *Zhongguo Zhong Yao Za Zhi* **2015**, *40*, 3780–3785.
- (6) Di Nardo, F.; Alladio, E.; Baggiani, C.; Cavallera, S.; Giovannoli, C.; Spano, G.; Anfossi, L. Colour-encoded lateral flow immunoassay for the simultaneous detection of aflatoxin B<sub>1</sub> and type-B fumonisins in a single Test line. *Talanta* **2019**, *192*, 288–294.
- (7) Pan, D.; Li, G.; Hu, H.; Xue, H.; Zhang, M.; Zhu, M.; Gong, X.; Zhang, Y.; Wan, Y.; Shen, Y. Direct Immunoassay for Facile and Sensitive Detection of Small Molecule Aflatoxin B<sub>1</sub> based on Nanobody. *Chem. - Eur. J.* **2018**, *24*, 9869–9876.
- (8) Yamasaki, T.; Miyake, S.; Sato, N.; Hirakawa, Y.; Iwasa, S.; Narita, H.; Watanabe, T. Development of Enzyme-Linked Immunosorbent Assay for Analysis of Total Aflatoxins Based on Monoclonal Antibody Reactive with Aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>. *Shokuhin Eiseigaku Zasshi* **2018**, *59*, 200–205.
- (9) Edupuganti, S. R.; Edupuganti, O. P.; Hearty, S.; O'Kennedy, R. A highly stable, sensitive, regenerable and rapid immunoassay for detecting aflatoxin B<sub>1</sub> in corn incorporating covalent AFB<sub>1</sub> immobilization and a recombinant Fab antibody. *Talanta* **2013**, *115*, 329–335.
- (10) Liu, A.; Ye, Y.; Chen, W.; Wang, X.; Chen, F. Expression of V(H)-linker-V(L) orientation-dependent single-chain Fv antibody fragment derived from hybridoma 2E6 against aflatoxin B<sub>1</sub> in *Escherichia coli*. *J. Ind. Microbiol. Biotechnol.* **2015**, *42*, 255–262.
- (11) Bever, C. S.; Dong, J. X.; Vasylieva, N.; Barnych, B.; Cui, Y.; Xu, Z. L.; Hammock, B. D.; Gee, S. J. VHH antibodies: emerging reagents for the analysis of environmental chemicals. *Anal. Bioanal. Chem.* **2016**, *408*, 5985–6002.
- (12) Cao, D. M.; Xu, Y.; Tu, Z.; Li, Y. P.; Xu, L.; Fu, J. H. One-step enzyme linked immunosorbent assay for detection of aflatoxin B<sub>1</sub> using a nanobody alkaline phosphatase fusion protein. *Chinese Journal of Analytical Chemistry* **2011**, *44*, 1085–1091.
- (13) Xiong, Y.; Tu, Z.; Huang, X.; Xie, B.; Xiong, Y.; Xu, Y. Magnetic beads carrying poly (acrylic acid) brushes as “nanobody containers” for immunoaffinity purification of aflatoxin B<sub>1</sub> from corn samples. *RSC Adv.* **2015**, *5*, 77380–77387.
- (14) Wang, S. H.; Zhang, J. B.; Zhang, Z. P.; Zhou, Y. F.; Yang, R. F.; Chen, J.; Guo, Y. C.; You, F.; Zhang, X. E. Construction of single chain variable fragment (ScFv) and BiscFv-alkaline phosphatase fusion protein for detection of *Bacillus anthracis*. *Anal. Chem.* **2006**, *78*, 997–1004.
- (15) Jiacomini, I.; Silva, S. K.; Aubrey, N.; Muzard, J.; Chavez-Olortegui, C.; De Moura, J.; Billiald, P.; Alvarenga, L. M. Immunodetection of the “brown” spider (*Loxosceles intermedia*) dermonecrotin with an scFv-alkaline phosphatase fusion protein. *Immunol. Lett.* **2016**, *173*, 1–6.
- (16) Xu, Z. L.; Dong, J. X.; Wang, H.; Li, Z. F.; Beier, R. C.; Jiang, Y. M.; Lei, H. T.; Shen, Y. D.; Yang, J. Y.; Sun, Y. M. Production and Characterization of a Single-Chain Variable Fragment Linked Alkaline Phosphatase Fusion Protein for Detection of O,O-Diethyl Organophosphorus Pesticides in a One-Step Enzyme-Linked Immunosorbent Assay. *J. Agric. Food Chem.* **2012**, *60*, 5076–5083.
- (17) Liu, X.; Xu, Y.; Wan, D. B.; Xiong, Y. H.; He, Z. Y.; Wang, X. X.; Gee, S. J.; Ryu, D.; Hammock, B. D. Development of a nanobody-alkaline phosphatase fusion protein and its application in a highly sensitive direct competitive fluorescence enzyme immunoassay for detection of ochratoxin A in cereal. *Anal. Chem.* **2015**, *87*, 1387–1394.
- (18) Wang, J.; Majkova, Z.; Bever, C. R.; Yang, J.; Gee, S. J.; Li, J.; Xu, T.; Hammock, B. D. One-step immunoassay for tetrabromobisphenol A using a camelid single domain antibody-alkaline phosphatase fusion protein. *Anal. Chem.* **2015**, *87*, 4741–4748.
- (19) Zhang, Y. Q.; Xu, Z. L.; Wang, F.; Cai, J.; Dong, J. X.; Zhang, J. R.; Si, R.; Wang, C. L.; Wang, Y.; Shen, Y. D.; Sun, Y.; Wang, H. Isolation of Bactrian Camel Single Domain Antibody for Parathion and Development of One-Step dc-PEIA Method Using VHH-Alkaline Phosphatase Fusion Protein. *Anal. Chem.* **2018**, *90*, 12886–12892.
- (20) Faulin, T. E. S.; Guilherme, D. F.; Silva, A. S.; Abdalla, D. S.; Hering, V. R.; Politi, M. J.; Maranhao, A. Q. GFP-SCFV: expression and possible applications as a tool for experimental investigations of atherosclerosis. *Biotechnol. Prog.* **2014**, *30*, 1206–1213.
- (21) Rinaldi, A. S.; Freund, G.; Desplancq, D.; Sibling, A. P.; Baltzinger, M.; Rochel, N.; Mely, Y.; Didier, P.; Weiss, E. The use of fluorescent intrabodies to detect endogenous gankyrin in living cancer cells. *Exp. Cell Res.* **2013**, *319*, 838–849.
- (22) Wongso, D.; Dong, J.; Ueda, H.; Kitaguchi, T. Flashbody: A Next Generation Fluobody with Fluorescence Intensity Enhanced by Antigen Binding. *Anal. Chem.* **2017**, *89*, 6719–6725.
- (23) Li, Y.; Li, Y.; Zhao, J.; Zheng, X.; Mao, Q.; Xia, H. Development of a Sensitive Luciferase-Based Sandwich ELISA System for the Detection of Human Extracellular Matrix 1 Protein. *Monoclonal Antibodies Immunodiagn. Immunother.* **2016**, *35*, 273–279.
- (24) Shifera, A. S.; Hardin, J. A. Factors modulating expression of Renilla luciferase from control plasmids used in luciferase reporter gene assays. *Anal. Biochem.* **2010**, *396*, 167–172.
- (25) Wu, N.; Rathnayaka, T.; Kuroda, Y. Bacterial expression and re-engineering of *Gaussia princeps* luciferase and its use as a reporter protein. *Biochim. Biophys. Acta, Proteins Proteomics* **2015**, *1854*, 1392–1399.
- (26) Patel, K. G.; Ng, P. P.; Kuo, C. C.; Levy, S.; Levy, R.; Swartz, J. R. Cell-free production of *Gaussia princeps* luciferase-antibody fragment bioconjugates for ex vivo detection of tumor cells. *Biochem. Biophys. Res. Commun.* **2009**, *390*, 971–976.
- (27) Venisnik, K. M.; Olafsen, T.; Gambhir, S. S.; Wu, A. M. Fusion of *Gaussia* luciferase to an engineered anti-carcinoembryonic antigen (CEA) antibody for in vivo optical imaging. *Mol. Imaging Biol.* **2007**, *9*, 267–277.
- (28) England, C. G.; Ehlerding, E. B.; Cai, W. B. NanoLuc: A Small Luciferase Is Brightening Up the Field of Bioluminescence. *Bioconjugate Chem.* **2016**, *27*, 1175–1187.
- (29) Ding, Y.; Hua, X.; Chen, H.; Liu, F.; Gonzalez-Sapien, G.; Wang, M. Recombinant Peptidomimetic-Nano Luciferase Tracers for Sensitive Single-Step Immunodetection of Small Molecules. *Anal. Chem.* **2018**, *90*, 2230–2237.
- (30) He, S. X.; Song, G.; Shi, J. P.; Guo, Y. Q.; Guo, Z. Y. Nanoluciferase as a novel quantitative protein fusion tag: Application for overexpression and bioluminescent receptor-binding assays of human leukemia inhibitory factor. *Biochimie* **2014**, *106*, 140–148.
- (31) Ling, Y.; Jiang, P.; Li, N.; Yan, Q.; Wang, X. A luciferase immunoprecipitation assay for the detection of proinsulin/insulin autoantibodies. *Clin. Biochem.* **2018**, *54*, 51–55.



(32) Zhao, H.; Doyle, T. C.; Wong, R. J.; Cao, Y.; Stevenson, D. K.; Piwnicka-Worms, D.; Contag, C. H. Characterization of coelenterazine analogs for measurements of Renilla luciferase activity in live cells and living animals. *Mol. Imaging* **2004**, *3*, 43–54.

(33) Degeling, M. H.; Bovenberg, M. S.; Lewandrowski, G. K.; de Gooijer, M. C.; Vleggeert-Lankamp, C. L.; Tannous, M.; Maguire, C. A.; Tannous, B. A. Directed molecular evolution reveals Gaussia luciferase variants with enhanced light output stability. *Anal. Chem.* **2013**, *85*, 3006–3012.

(34) Maguire, C. A.; Deliolanis, N. C.; Pike, L.; Niers, J. M.; Tjon-Kon-Fat, L. A.; Sena-Esteves, M.; Tannous, B. A. Gaussia luciferase variant for high-throughput functional screening applications. *Anal. Chem.* **2009**, *81*, 7102–7106.