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Analytical Methods

Development and optimization of a multiplex lateral flow immunoassay for the simultaneous determination of three mycotoxins in corn, rice and peanut

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ABSTRACT

A multiplex lateral flow immunoassay (LFA) is developed for the simultaneous on-site determination of three mycotoxins (aflatoxin B_1, zearalenone and ochratoxin A) in corn, rice and peanut. By systematically optimizing the preparation of antibody-gold nanoparticle conjugates, the size of gold nanoparticle and the position of capture antigen, the developed LFA can obtain a visual detection limit of 10 μg/kg for aflatoxin B_1, 50 μg/kg for zearalenone and 15 μg/kg for ochratoxin A. For quantitative analysis, the limits of detection were 0.10–0.13 μg/kg for aflatoxin B_1, 0.42–0.46 μg/kg for zearalenone, and 0.19–0.24 μg/kg for ochratoxin A, which were far below the regulatory limits set by the European Commission. At the spiked concentrations of 0.5–10.0 μg/kg, the mean recoveries of the three mycotoxins ranged from 86.2 to 114.5% with coefficients of variation less than 16.7%. These results demonstrated that the developed immunoassay can be used for routine monitoring of mycotoxin contamination.

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1. Introduction

Mycotoxin is a kind of toxic metabolites produced by fungi and it extensively exists in agricultural product (Anater et al., 2016; Brase, Encinas, Keck, & Nising, 2009). Because of the potential toxic effects such as carcinogenicity, mutagenicity, neurotoxicity, immunosuppressive and estrogenic effects (Brase et al., 2009; Rocha, Freire, Erlan, Izabel, & Rondina, 2014), mycotoxin can pose serious hazard to human and animal health. Surveillance survey indicated that mycotoxin contamination has been a global problem. It is estimated that approximately 25% of the world's crop production is contaminated with mycotoxin (Choudhary & Kumari, 2010), which can result in the reduction of livestock production, the increase of health care and regulatory costs, and thus will lead to tremendous economic losses. For protecting the health of consumers, maximum tolerable and guideline levels of mycotoxin have been established by many countries and areas (European Commission, 2006; Food and Drug Administration, US, 2011; Ministry of Health, PRC, 2011).

The analytical approaches for mycotoxin detection can be broadly divided into two categories: instrumental analysis and bioanalytical method (Anfossi, Baggiani, Giovannoli, D'Arco, & Giraudi, 2013; Turner, Subrahmanyam, & Piletsky, 2015). Instrumental methods mainly comprise liquid chromatography (Wang et al., 2008) and LC coupled with mass spectrometry (Hickert et al., 2015; Liao et al., 2015). These methods have high accuracy and precision, and are usually used for quantitative and qualitative analysis. However, they need expensive equipment and skilled personnel, and also require sophisticated sample preparation which often consumes hours and even days (Maragos & Busman, 2010). Thus, instrumental method is not suitable for the application in normal laboratory and field environment. The requirement for timely monitoring mycotoxin contamination in food industry has demanded more rapid and cost-effective methods, therefore, bioanalytical method especiallyimmunoassay has been developed for mycotoxin detection (Beloglazova et al., 2014; Li et al., 2013; Soleri et al., 2015; Song et al., 2014).

Lateral flow immunoassay (LFA) is one of the most widely used immunoassay because of its low-cost, easy-to-use and rapidity

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(Anfossi et al., 2013). However, routine LFA can only detect one target molecule at one time. By comparison, the multiplexing format of LFA can simultaneously detect several target chemicals, thus it can further reduce operating cost and improve detection efficiency (Li et al., 2013; Song et al., 2014). As aflatoxin B_1 (AFB_1), zearalenone (ZEA) and ochratoxin A (OTA) frequently occurred in foodstuff (Bennett & Klich, 2003), they were selected as target chemicals in the development of a multiplex LFA in this study. In previous report, Li et al. (2013) have developed a multi-component immunochromatographic assay for qualitative analysis of the three mycotoxins in agro-food. However, only qualitative analysis was achieved and their assay sensitivities needed further improvement. In this study, we presented both qualitative and quantitative analysis of the three mycotoxins in corn, rice and peanut with higher assay sensitivities.

For the development of a LFA method, the major challenge is to achieve high assay sensitivity while maintaining its rapidity. Hence, for acquiring satisfactory assay sensitivity, the optimization of immune-parameters is extremely important. Among the immune-parameters, the preparation of antibody-gold nanoparticle (Ab-GNP) conjugate is the most important factor since it directly relates with assay sensitivity. Flocculation test has been widely used to optimize the preparation of Ab-GNPs (Paek, Lee, Cho, & Kim, 2000; Xie et al., 2003; Ye et al., 2010), whereas it was originally designed to stabilize Ab-GNPs conjugate (Horrisberger, Rosset, & Bauer, 1975), not to pursue high assay sensitivity. Thus, if the flocculation test is suitable for optimizing assay sensitivity of competitive LFA still remained questionable. In addition, the effects of the size of gold nanoparticle and the test line position also remained unexplored. Therefore, we systematically investigated the effects of these three important immune-parameters on assay sensitivity of the developed LFA in this study.

2. Material and methods

2.1. Reagents and equipments

Chemical standards of AFB_1, ZEA and OTA, bovine serum albumin (BSA), and chloroauric acid were purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-mouse IgG, Tween 20 and Triton X-100 were obtained from Thermo Fisher Scientific (West Palm Beach, FL). Purified water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA). Other reagents were purchased from Beijing Regent Corporation (Beijing, China). Nitrocellulose (NC) membrane HF 180 was purchased from Millipore Company (Millipore, Bedford, MA). Glass fiber membrane SB 08, absorbent pad CH 37, adhesive backing card were purchased from ShangHai GoldBio Co. Ltd. (Shanghai, China). The synthesis of AFB_1-BSA, ZEA-BSA and OTA-BSA conjugates and the preparation of respective anti-mycotoxin monoclonal antibodies (mAbs) were briefly described in Supplementary material. Strip reader (i-Check I type) was provided by Clover Technology Group (Beijing).

HM 3030 Dispensing Platform and ZQ 2000 Guillotine Cutting Module used for strip assembly were purchased from ShangHai GoldBio Co. Ltd. (Shanghai, China).

2.2. Synthesis of gold nanoparticles (GNPs)

GNPs were prepared as described by Frens (1973) with some modifications. Briefly, 100 mL of 0.01% (m/v) chloroauric acid in de-ionized water was heated to boiling, then an aliquot (1.0, 1.6 or 2.5 mL) of 1.0% tri-sodium citrate (w/v) was added under constant stirring. After reaction for 15 min, the solution was left to cool, and de-ionized water was complemented to the initial volume. Supplemented with 0.05% sodium azide, the obtained GNPs is stable at 4 °C for several months. Transmission electron microscopy (TEM, JEOL USA Inc., Peabody, MA) was used to determine the particle sizes of GNPs.

2.3. Flocculation test

To determine the optimal pH value and the minimum antibody amount for stabilizing Ab-GNPs conjugate, flocculation test was performed according to literature (Paek et al., 2000; Xie et al., 2003; Ye et al., 2010) with some modifications. First, the optimal pH value was measured. Briefly, the pH values of GNPs (32 nm) were adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 8.5 and 9.0 respectively by adding different amount of 0.1 M K_2CO_3, then each aliquot (1.0 mL) of these GNPs solution was mixed with 20 μg of respective anti-mycotoxin mAbs and incubated for 30 min. Subsequently, 100 μL of 10% NaCl (w/v) was added and mixed. After incubating for another 30 min, the absorbance of the solution was determined at 520 nm and 580 nm by UV–vis spectrophotometer (Varian Cary 50, Agilent Technologies Inc., Palo Alto, CA). Then the minimum antibody amounts were tested. The GNPs solutions were adjusted to the optimal pH values as determined above, then 0.16, 0.31, 0.63, 1.3, 2.5, 5.0, 10, 20, 40 and 80 μg of respective anti-mycotoxin mAbs in 100 μL of water were separately added into 1.0 mL of the GNPs solution and incubated for 30 min. Afterwards, 100 μL of 10% NaCl (w/v) was added and mixed. Following incubation for another 30 min, the absorbance of the solution was determined at 520 nm and 580 nm by UV–vis spectrophotometer. The differential absorbance ($A_{520\ nm}-A_{580\ nm}$) was used to evaluate the flocculation level. The optimal pH value and antibody amount to stabilize Ab-GNPs was the smallest pH value and antibody amount at which flocculation did not occur (Paek et al., 2000).

2.4. Checkerboard titration test

To measure the optimal pH value and antibody amount that can result in the best assay sensitivity, checkerboard titration test was performed. Briefly, aliquots (1.0 mL for each aliquot) of 32 nm GNPs was adjusted to pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 respectively by adding 0.1 M K_2CO_3, then 1.3, 2.5, 5.0, 10, 20 and 40 μg of respective anti-mycotoxin mAbs in 100 μL of water were separately added into GNPs with different pH values in the form of checkerboard, and the mixtures were incubated for 30 min. Then 100 μL of 1.0% BSA in 10 mM citrate buffer (pH 6.5) was added. After three centrifugation at 5000*g* for 10 min and re-suspended with 0.1% BSA in 10 mM citrate buffer (pH 6.5), the prepared Ab-GNPs conjugates were then applied to conjugate pad and subjected to LFA as described below. By adjusting dilution times with 0.1% BSA aqueous solution, the different Ab-GNPs conjugates were made to result in identical test line intensities. Then the cut-off value, which was defined as the lowest concentration to make test line invisible, was determined for each Ab-GNPs conjugate.

2.5. Preparation of detection reagents

The purified anti-AFB_1, anti-ZEA and anti-OTA mAbs were dialyzed against phosphate buffer saline (PBS, 10 mM, containing 0.15 M sodium chloride, pH 7.4) at 4 °C for 24 h with constant stirring. Before conjugation between antibody and GNPs, the optimal pH value and antibody concentration were determined by checkerboard titration test as described above. With gentle stirring, 10.0 mL of GNPs solution was adjusted to the optimal pH values (pH 6.5 for AFB_1, pH 7.5 for ZEA, pH 6.5 for OTA) with 0.1 M K_2CO_3, then 1.0 mL of purified mAb (0.025 mg/mLanti-AFB_1, 0.050 mg/mL anti-ZEA, and 0.025 mg/mL anti-OTA) was added. After incubation at room temperature for 60 min, 1.0 mL of 1.0%

BSA in 2 mM citrate buffer (pH 6.5) was added and stirring was continued for another 15 min. The mixture was centrifuged at 5000*g* for 10 min, and the precipitate of Ab-GNPs was re-suspended in 10.0 mL of 0.10% BSA in 2 mM citrate buffer (pH 6.5). The centrifugation and re-suspension steps were repeated twice and finally the precipitate was re-suspended in 1.0 mL of 4.0% sucrose in 2 mM citrate buffer (pH 6.5). The three Ab-GNPs conjugates were then mixed at a ratio of 2:1:3 (AFB_1:ZEA:OTA) and dispensed onto a conjugate pad (1 μL per mm^2; glass fiber membrane, SB 08, GoldBio Co. Ltd.). The conjugate pad was then dried rapidly at 37 °C for 2 h and stored under dry condition at room temperature.

2.6. Immobilization of capture reagents

The goat anti-mouse IgG (2.0 mg/mL) in 50 mM phosphate buffer (PB, pH 7.4) was applied to NC membrane as control line, while AFB_1-BSA, ZEA-BSA and OTA-BSA (0.25 mg/mL in 50 mM carbonate buffer, pH 9.5; from right to left, as shown in Fig. 1) were applied as the three test lines, respectively. The dispensed volumes were both 1 μL per cm line. After dispensing, the NC membrane was dried at 37 °C for 2 h and stored under dry conditions at room temperature until use.

2.7. Assembly of strips

As shown in Fig. 1, the multiplex lateral flow strip is composed of five components: sample pad, conjugation pad, NC membrane, absorbent pad and adhesive backing card. The strip assembly procedure was similar to that described by our group (Chen et al., 2008). Briefly, the NC membrane (HF180) lined with capture reagents (AFB_1-BSA, ZEA-BSA and OTA-BSA) was pasted on the center of plastic backing card, then the conjugate pad dispensed with detection reagent was attached with one end overlapping (2 mm) the NC membrane. After that, the sample pad (SB 08) was pasted with one end partly covering (2 mm) the conjugation pad. Next, the absorbent pad (CH 37) was pasted on the other side of the backing card and it also laid-over (2 mm) the NC membrane. Finally, the assembled plate was cut to appropriate size (4 mm width) for each strip, which was then mounted in a plastic housing and ready for use.

2.8. Test procedure

At room temperature, a standard or sample solution (150 μL) was added onto sample pad and then the solution migrated toward the absorbent pad. When the solution flew through the conjugation pad, it will dissolve the detection reagent (Ab-GNPs conjugate). When one mycotoxin (AFB_1, ZEA or OTA) was absent in the sample, the respective detection reagent would be trapped by the capture antigen immobilized on NC membrane to form a visible test line. When AFB_1, ZEA or OTA was present in the sample, they would compete with the immobilized capture antigen for the limited amount of detection reagent. If sufficient AFB_1, ZEA or OTA molecules existed, they would then hinder detection reagents from combining with the capture antigen, thus a positive sample gave no respective visible test line on the NC membrane. When the test procedure was properly performed, the control line was always visible. After 10 min, the result can be judged by naked eyes for qualitative analysis or by a strip reader for quantitative analysis. For qualitative analysis, the cut-off value was defined as the lowest concentration that can cause invisible test line. For quantitative analysis, calibration curves were constructed by plotting the ratios between optical intensity of spiked and blank sample (B/B_0) against the logarithm concentrations of mycotoxins. The optical intensity of unknown sample was then compared to respective calibration curve to determine analyte concentration.

2.9. Sample preparation and analysis

Corn, rice and peanut samples were minced and then divided into aliquots. An aliquot of minced sample (5.0 g) was weighed and transferred to a 50 mL polypropylene centrifuge tube. Ten milliliter of 70% (v/v) methanol aqueous solution was added and vortexed for 1 min for sufficient extraction. After the solution was left stand for 5 min, 1.0 mL of the supernatant was mixed with 4.0 mL of PBS (0.01 M, containing 0.15 M sodium chloride, pH 7.4) and then Tween 20 was added to a final concentration of 0.5% (v/v). Following a vortex step, 150 μL of the mixture was subjected to LFA.

For spiked experiment, blank samples (corn, rice and peanut) were spiked with mycotoxin standard solutions to produce a series of spiked concentrations. After treated as described above, the obtained sample solution was applied to the multiplex LFA. For real sample analysis, 19 real samples collected by our lab were simultaneously measured by the multiplex LFA and LC analysis (Wang et al., 2008). The results obtained by both methods were compared.

3. Results and discussion

3.1. Optimization of Ab-GNPs preparation

According to DLVO (Derjaguin-Landau-Verwey-Overbeek) theory, citrate-capped GNPs can be stabilized in solution by a balance between electrostatic repulsion and London-van der Waals

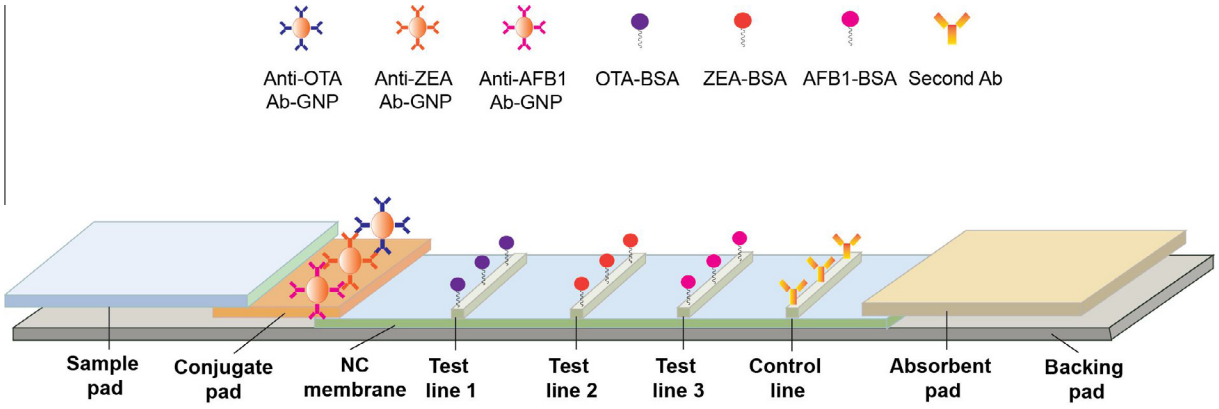


Fig. 1. Schematic illustration of multiplex lateral flow strip.

attraction among the particles (Yang, Schatz, & Van Duyne, 1995). However, on addition of ionic substance, electrostatic repulsion is shielded, London attractive force would then induce GNPs to approach each other and form aggregation, which can result in significant red-shifting in surface plasmon resonance band of GNPs, accompanying a color change from red (520 nm) to blue (580 nm) (Paek et al., 2000). This instability can be prevented by coating GNPs surface with protein molecules because of the introduction of steric hindrance. As the passive adsorption of protein on GNPs can be easily affected by solution environment and protein concentration, flocculation test has been widely employed to optimize pH value and antibody amount for preparing Ab-GNPs conjugate (Paek et al., 2000; Xie et al., 2003; Ye et al., 2010), and it was also used at first in this study.

It was found that at low pH value, the formed Ab-GNPs are very susceptible to aggregation, which was indicated by the low A_{520}–A_{580} value. When the solution pH was increased to be higher than a critical value, the value of A_{520}–A_{580} maintained a plateau, demonstrating that the Ab-GNPs conjugate became tolerable to salt solution (Fig. S1). The pH values for stable conjugate formation were determined to be 7.0, 8.0 and 7.5 for anti-AFB_1, anti-ZEA and anti-OTA mAbs, respectively (Table 1). In addition, although the adsorption of protein on gold surface is reported to be mainly mediated by London-van der Waals force and hydrophobic interaction (Paek et al., 2000), the dramatic effect of pH value on the stability of Ab-GNPs implies electrostatic interaction plays an important role on the formation of Ab-GNPs.

The results also indicated that at these optimal pH values, with the increased antibody amount, the stability of Ab-GNPs conjugate was also increased and finally maintained little change. The lowest antibody amounts to stabilize Ab-GNPs were found to be 10, 40 and 20 μg per mL GNPs for anti-AFB_1, anti-ZEA, and anti-OTA mAbs, respectively (Fig. S1). It is assumed that these minimal amounts of antibodies would have saturated GNPs surface and introduced a large steric hindrance around GNPs, thus the particles have no chance to interact with each other to form aggregation. In summary, based on this flocculation test, the optimal pH and antibody amount to stabilize 1.0 mL of 32 nm GNPs were pH 7.0 and 10 μg of anti-AFB_1 mAb, pH 8.0 and 40 μg of anti-ZEA mAb, pH 7.5 and 20 μg of anti-OTA mAb, respectively (Table 1).

Flocculation test was originally designed to stabilize Ab-GNPs conjugate (Horrisberger et al., 1975) and has been widely used to optimize the pH value and antibody amount for the preparation of Ab-GNPs in LFA (Paek et al., 2000; Xie et al., 2003; Ye et al., 2010). However, the prepared Ab-GNPs by this method can only result in LODs of 3.0, 25 and 8 ng/mL for AFB_1, ZEA and OTA, respectively in this study (Table 1), which cannot meet the requirements for monitoring mycotoxin contamination and prompts us to further optimize the preparation of Ab-GNPs conjugate. As shown in Fig. 1, Ab-GNPs conjugate can be considered a multivalent antibody. Theoretically, when interacting with multivalent antigen, the apparent affinity (avidity) constant of multivalent antibody (Ka) can be expressed as: $Ka = (K_1)^n$, where n is the number of binding sites, K_1 is the intrinsic affinity constant of individual antibody. Therefore, the avidity of Ab-GNPs can be much larger than the intrinsic affinity of adsorbed antibody, and the increase of avidity would be dependent on the number of antibody adsorbed on GNPs (Tobita, Oda, & Azuma, 2004). As the increase of added antibody amount will increase the surface antibody density, while the increase of pH value will decrease the surface antibody density on GNPs (Geoghegan, 1988), thus the pH value and added antibody for the preparation of Ab-GNPs would have important impact on the avidity of the produced Ab-GNPs, which in turn will affect the assay sensitivity. For non-competitive LFA, high avidity of Ab-GNPs is beneficial for the recognition of antigen in sample, and thus can lead to higher assay sensitivity. However, for competitive LFA as developed in this study, the increased antibody density on Ab-GNPs would require more free molecules to saturate these antibodies and prevent their combination with coating antigen to form a visible test line. That means higher LODs are expected to be obtained when Ab-GNPs with higher avidity was used. Consequently, we supposed that there should be an optimal avidity of Ab-GNPs, which can produce high test line intensity for negative sample, while generating invisible test line at low concentration of target molecule in sample. This assumption prompted us to perform a checkerboard titration test to determine the optimal pH value and antibody amount which can lead to the best assay sensitivity.

Table 1
Assay sensitivities of Ab-GNPs prepared according to flocculation test and checkboard titration test.

Mycotoxin	Flocculation test			Checkerboard titration test		
	Antibody amount (μg/mL)	pH value	Cut-off value (ng/mL)	Antibody amount (μg/mL)	pH value	Cut-off value (ng/mL)
AFB_1	10	7.0	3.0	2.5	6.5	0.5
ZEA	40	8.0	25	5	7.5	5.0
OTA	20	7.5	8.0	2.5	6.5	1.0

As a result of titration test, it was observed that with the increase of added antibody amount in a certain range, the prepared Ab-GNPs will produce higher intensity of test line. In contrast, with the increase of pH value, the test line intensity was decreased. Thus, the Ab-GNPs prepared at different pH value and antibody amount were diluted or concentrated to obtain equivalent line intensity. At similar test line intensity, the optimal pH value and antibody amount that gave rise to the lowest LODs (0.50 ng/mL of AFB_1, 5.0 ng/mL of ZEA and 1.0 ng/mL of OTA) were measured to be pH 6.5 and 2.5 μg of anti-AFB_1 mAb, pH 7.5 and 5.0 μg of anti-ZEA mAb, pH 6.5 and 2.5 μg of anti-OTA mAb (Table 1). Comparing the result of flocculation test and titration test, we can find that the optimal pH value obtained by titration test were 0.5–1.0 unit lower than that determined by flocculation test, while the optimal antibody amounts by titration test were only one-fourth to one-eighth of that measured by flocculation test. Most importantly, the assay sensitivities generated by titration test were significantly improved by 5–8 times as compared to that by flocculation test.

3.2. Effect of particle size

To investigate the effect of particle size on the sensitivity of LFA, three sizes of GNPs were synthesized by adjusting the added amount of sodium citrate to gold solution (Frens, 1973). TEM images of the three GNPs are shown in Fig. 2A–C, and their sizes were determined to be 32, 18 and 12 nm, respectively. After optimizing the pH value and antibody amount by checkerboard titration test as described above, the prepared Ab-GNPs were applied to LFA. As shown in Fig. 2D, at the optimal conditions, when the sizes of GNPs were increased from 12 nm to 32 nm, the cut-off values were lowered from 2.0 ng/mL to 0.5 ng/mL for AFB_1, from 12 ng/mL to 5.0 ng/mL for ZEA, and from 3.0 ng/mL to 1.0 ng/mL for OTA. These results are consistent with that reported by Liu, Tsao, Wang, and Yu (2008), which demonstrated that 40 nm GNPs could lead to better assay sensitivity than 10 nm GNPs in their developed LFA for OTA. Accordingly, 32 nm GNPs was finally used for Ab-GNPs preparation in this study.

3.3. Effect of test line position

LFA is a non-equilibrium immunoassay. In this assay format, free mycotoxin in sample firstly interacts with Ab-GNPs on

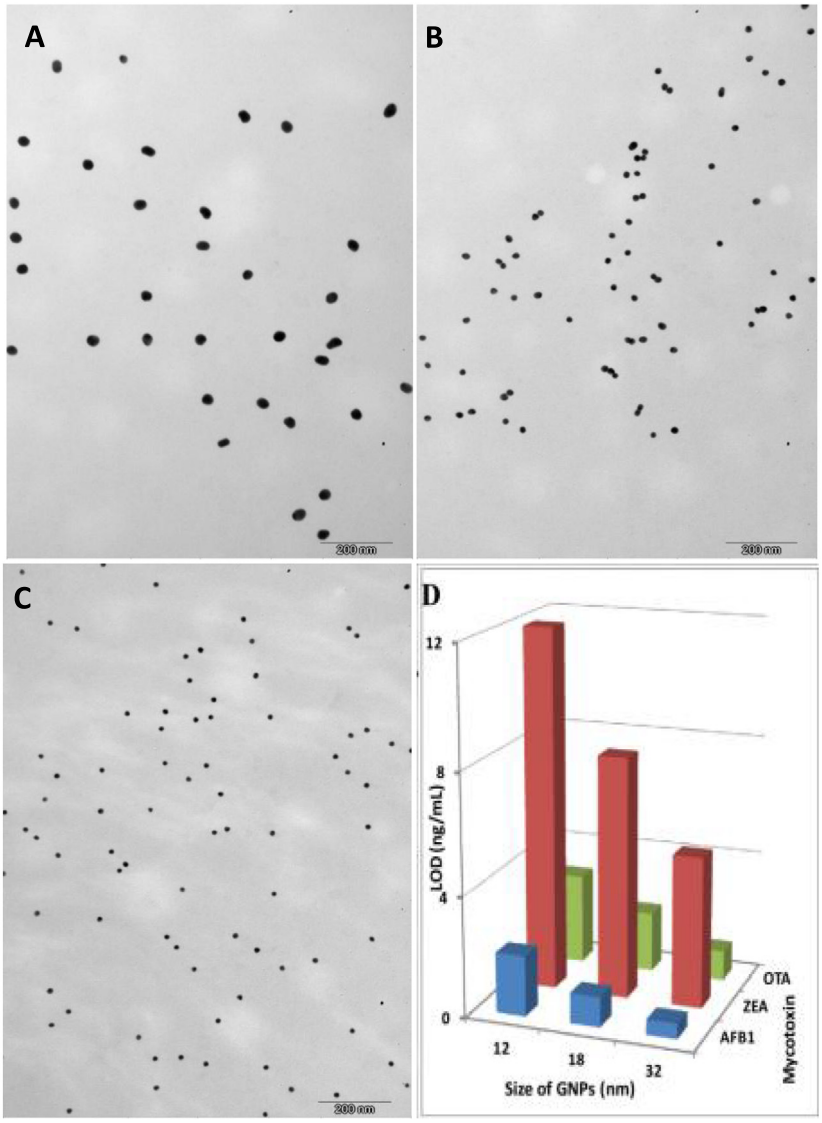


Fig. 2. TEM images of GNPs with different sizes, and the effects of GNP sizes on assay sensitivities. The determined particle diameters were 32 nm (A), 18 nm (B) and 12 nm (C), respectively.

conjugate pad to form an immune-complex, and then compete with capture antigen on test line for the limited amount of Ab-GNPs. The interaction time for free mycotoxin and Ab-GNPs would determine the amount of the formed immune-complex and thus will affect the assay sensitivity. Since this interaction time was mainly determined by the distance between conjugate pad and test line, we assumed that the test line positon of each mycotoxin antigen would have influence on assay sensitivity. To investigate these effects, AFB_1-BSA, ZEA-BSA and OTA-BSA were applied onto three different positions (the proximal end, the middle and the distal end, as shown in Fig. 1) of NC membrane and then subjected to LFA. The results indicated that for all the three mycotoxin, the respective antigens on the distal positions can produced the best assay sensitivities, while the antigens on the proximal positions produced the lowest assay sensitivities (Fig. S2, Supplementary material). This means the farther the test line position was away from conjugation pad, the better assay sensitivities can be obtained for all the three antigens. However, it was observed that the extent of this effect is varied. OTA-BSA is the least influenced while AFB_1-BSA is the most influenced coating antigen, which may be attributed to the different associate rate constants of their respective antibodies. Therefore, in order to maintain high assay sensitivities for all the three mycotoxin, the test lines from the proximal to distal positions were coated with OTA-BSA, ZEA-BSA and AFB_1-BSA, respectively (Fig. 1).

3.4. Analysis of spiked and real samples

Blank corn, rice and peanut samples spiked with different concentrations of AFB_1, ZEA and OTA were extracted and measured by the multiplex LFA. The typical photo images for the spiked tests are shown in Fig. 3. For qualitative analysis, the cut-off values of the three mycotoxins were measured to be 10 μg/kg for AFB_1, 50 μg/kg for ZEA, and 15 μg/kg for OTA respectively (Fig. 3). For quantitative analysis, calibration curves were constructed by plotting the ratios between optical intensities of spiked and blank sample (B/B_0) against the logarithm concentrations of analytes (Fig. 4). The shapes of calibration curves for various sample types were slightly different from each other, thus respective calibration curves were constructed for each sample type. The sensitivity of this LFA was evaluated by the limit of detection (LOD), which was defined as the analyte concentration that produce 10% decrease in line

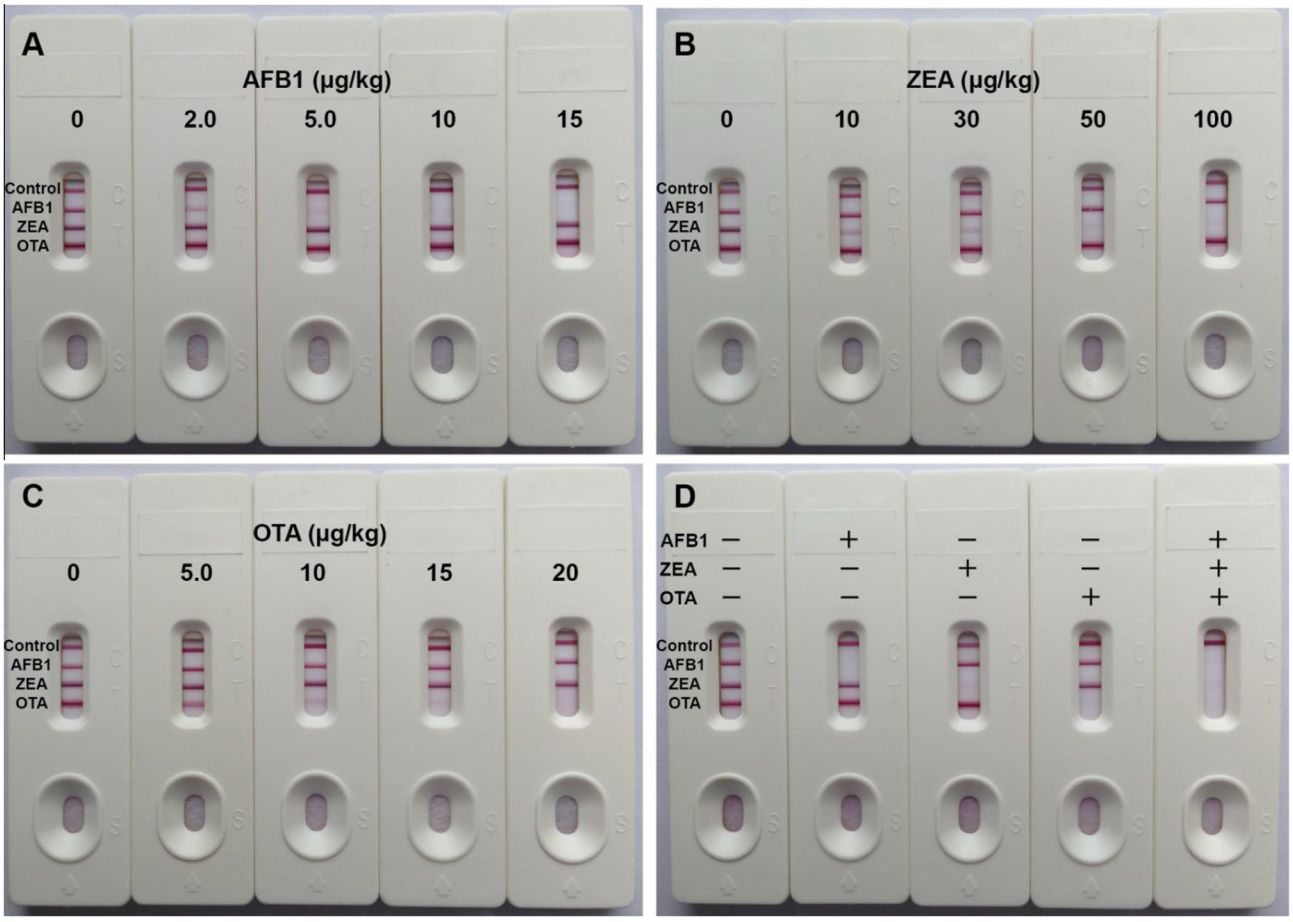


Fig. 3. Typical photo image of multiplex LFA for mycotoxins in spiked corn samples. A: AFB_1 at spiked concentrations from 0 to 15 μg/kg; B: ZEA at spiked concentrations from 0 to 100 μg/kg; C: OTA at spiked concentrations from 0 to 20 μg/kg; D: Positive results for single and multiple mycotoxins.

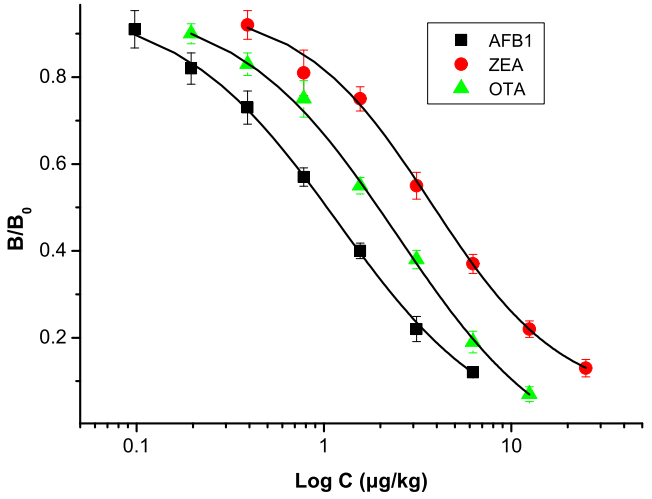


Fig. 4. Typical calibration curves of AFB_1, ZEA and OTA in corn sample. Each B/B_0 represents the average value of three measurements by three strips and the error bar represents the standard deviation of the three measurements.

intensity as compared to blank sample. The LOD values and detection ranges were as shown in Table 2. The calculated LOD values were 0.10–0.13 μg/kg for AFB_1, 0.42–0.46 μg/kg for ZEA, and 0.19–0.24 μg/kg for OTA, which were far below the regulatory limits established by authorities (European Commission, 2006; Food and Drug Administration, USA, 2011; Ministry of Health, PRC, 2011). The assay sensitivity of this LFA is comparable to or better than that of other LFAs for the detection of mycotoxin (Table S1). Li et al. (2013) has developed a multi-component immunochromatographic assay for the detection of the same three mycotoxins in agro-food, but their assay can only provide qualitative results. As compared to this method, the multiplex LFA we developed can provide both qualitative and quantitative result. Furthermore, the assay sensitivities were better than their method.

Table 2
The LODs and detection range of AFB_1, ZEA and OTA in corn, rice and peanut.

Analyte	Corn		Rice		Peanut	
	LOD (μg/kg)	Detection range (μg/kg)	LOD (μg/kg)	Detection range (μg/kg)	LOD (μg/kg)	Detection range (μg/kg)
AFB_1	0.10	0.10–7.0	0.12	0.12–7.2	0.13	0.13–7.2
ZEA	0.42	0.42–35.5	0.43	0.43–35.5	0.46	0.46–35.7
OTA	0.19	0.19–10.1	0.21	0.21–10.2	0.24	0.24–10.3

The accuracy and precision of this assay were also evaluated by the spiked recovery experiment. Blank samples were spiked with different concentrations of mycotoxins and then measured by the developed LFA. As shown in Table S2, at the spiked concentrations of 0.5–10.0 μg/kg, the mean recoveries of the three mycotoxins (AFB_1, ZEA and OTA) ranged from 86.2 to 114.5% with the coefficient of variation (CV) values less than 16.7% (n = 4). In order to further validate the developed method, 19 real samples collected by our lab were then detected by the developed multiplex LFA and LC method, respectively. The results are as shown in Table S3.

Twelves samples were determined as negative by the developed LFA, and the same 12 samples were confirmed as negative by LC method. This indicated that no false negative result was obtained by the developed LFA. Seven samples were determined to contain at least one mycotoxin, and the detection values obtained by the developed LFA were in good agreement with that of LC method (Table S3). These results demonstrated that the developed LFA could be applied for quantitative analysis of real samples. Among the seven positive samples, one corn sample and one peanut sample were determined to be co-contaminated with AFB_1 and ZEA, AFB_1 and OTA, respectively. In these cases, the developed multiplex LFA is advantageous to simplex immunoassay since it can further reduce detection cost and increase detection efficiency. Therefore, this multiplex LFA can be a useful and cost-effective tool for the simultaneous detection of the three mycotoxins in corn, rice and peanut.

4. Conclusions

In this study, the development and optimization of a multiplex LFA for three mycotoxins (AFB_1, ZEA and OTA) were presented in this study. As compared to instrumental methods and other immunoassays, the multiplex LFA allowed the simultaneous detection of the three mycotoxins within 15 min, thus it can be employed for rapid on-site screening of mycotoxin contamination, especially in the case of mycotoxin co-contamination in the sample matrixes. Meanwhile, the checkerboard titration test proposed in this study proved to be a feasible way for optimizing Ab-GNPs preparation in competitive LFA since it significantly improved assay sensitivity and meanwhile reduced antibody consumption. The size of GNPs and the test line position also posed significant effects on the assay sensitivities of the developed immunoassay. Thus, clarifying the effects of these immune-parameters will help us to obtain better assay sensitivity in the development of a competitive LFA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.06.116.

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