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Multiplex chemiluminescent immunoassay for screening of mycotoxins using photonic crystal microsphere suspension array†

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A novel multiplex chemiluminescent mycotoxin immunoassay suspension array system was developed by combining the silica photonic crystal microspheres (SPCMs) encoding technique and a chemiluminescent immunoassay (CLIA) method. The SPCMs were used as a carrier of the suspension array and encoded by their reflectance peak positions, which overcome fluorescence photobleaching, and the potential interference between the encoding fluorescence and detection fluorescence. Aflatoxin B1 (AFB1), fumonisin B1 (FB1) and ochratoxin A (OTA) artificial antigens were immobilized on the surfaces of SPCMs by using 3-glycidoxypropyltrimethoxysilane as a linker. Horseradish peroxidase (HRP) was used as a labeling enzyme for the secondary antibody in the enzyme-catalyze H_2O_2–luminol chemiluminescence system. The CLIA detection system was easily integrated with a multifunctional microplate reader and displayed a two to three orders of magnitude dynamic linear detection range from 0.001 to 1, 0.001 to 1, and 0.01 to 1 ng mL^{-1} for AFB1, FB1 and OTA with 50% inhibitory concentrations (IC_{50}) of 0.01, 0.036, and 0.04 ng mL^{-1}, respectively. The recovery rates are in the range of 63.5 to 121.6% for the three mycotoxins in three kinds of spiked cereal samples. The results of detection in 12 naturally contaminated cereal samples were consistent with that of the classic enzyme-linked immunosorbent assay (ELISA) method. This proposed system is simple, rapid, low cost and high throughput for multiplex mycotoxin assay.

1. Introduction

Mycotoxins are low-molecular weight secondary metabolic fungal products, which are toxic to both animals and humans because they cause a variety of acute and chronic diseases.[1] Fungi species are usually parasitic in the grain, feedstuff and food. The most common types of fungi which produce toxins and cause major health risks belong to the genera *Aspergillus fusarium* and *Penicillium*.[2,3] Due to their very high chemical and physical resistance, some mycotoxins have the tendency to remain in the human food chain in the form of the original toxins or their metabolites.[2] In addition, the co-occurrence of different mycotoxins in one matrix implies a potential risk for additional or even synergistic toxic effects.[4] These properties cause great potential safety risks to human health once these mycotoxins contaminate grains or feedstuffs. Therefore, it is of great significance to develop a multimycotoxin detection method for correct assessment of the degree of mycotoxin contamination of grains, feedstuffs and food.

Generally, mycotoxins are present in trace amounts in matrix samples, which make it still a challenge to develop sensitive, rapid, efficient and low cost assay techniques for multi-mycotoxins. At present, liquid chromatography combined with mass spectrometry (LC-MS/MS) is the most popular method for multimycotoxin analysis because it is highly sensitive, selective, accurate and has good repeatability. For example, multi-mycotoxin LC-MS/MS methods have been used to simultaneously analyze 25, 26 and even 186 mycotoxins in grains and feedstuffs, respectively.[4–6] The chromatographic methods not only require expensive instruments and skilled operators but also complicated sample pretreatment (such as immunoaffinity or solid phase extraction columns), which limit the application of the method, especially in developing countries. For this reason, all kinds of biosensor techniques based on immunoassays have been developed for multimycotoxin assay. The typical commercialized technique involves a protein microarray chip, which is a high throughput determination method for parallel multimycotoxin assay and requires only nanolitre (nL) analysis reagents.[7] However, it requires an expensive robot for spotting probes and a confocal fluorescence scanner for obtaining the detection signals, which make its application very difficult.

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Recently, a suspension array system based on multiplexed fluorescent encoding microspheres was found to be a powerful platform for multimycotoxin assay because of its high flexibility, fast reaction, and good repeatability for detection.[1,8–11] The Multi Analyte Profiling (xMAP) technology system from Luminex Corporation (Austin, Texas, USA) is the most prominent suspension microarray commercially available for simultaneously detecting a large array of analytes.[12,13] The microspheres in this system can be coupled with a different biological probe and are detected by a specialized flow cytometer.[9] Czeh *et al.* used this technology to simultaneously analyze six mycotoxins and the sensitivity range increased by between 13% and 100% compared to commercial ELISA.[1] Anderson *et al.* reported that a competitive suspension immunoassay with the Luminex100 system showed 50% inhibition values of 300 pg mL^{-1} and 30 ng mL^{-1} for fumonisin B and ochratoxin A in buffer, respectively.[10] Wang *et al.* used this method to simultaneously detect four different mycotoxins in grains and showed limits of detection at the pg level and recovery rates of 80.16% to 117.65% in real samples.[14] Peters *et al.* observed that this method showed good sensitivity and no cross-interactions between the assays in buffer for six mycotoxins.[9] These results show that the xMAP technology system has a great application potential for multimycotoxins in the field of food safety. Obviously, the key technology of the xMAP system lies in the encoding of microspheres with organic dyes (on which microsphere is the molecular probe immobilized) and decoding of microspheres by flow cytometry (how many target molecules are on each microsphere). However, the current technologies including the encoding and decoding in the xMAP system show several disadvantages. For example, fluorescent dyes tend to be quenched or bleached,[15,16] which causes the fluorescence intensity levels to vary, and the dual-laser flow cytometer causes the light path system to be much more complicated and expensive. In addition, the spectral features of fluorescence dyes or quantum dots show a wide spectral band range, and using many emission wavelengths for encoding may cause interference with the fluorescence signal from the labeling reporters.[17–19]

Silica photonic crystal microspheres (SPCMs) encoding technique is a physical encoding technique, which is based on the unique reflection spectra of microspheres rather than the emission spectra of fluorescent dyes.[11,17,20,21] The unique reflection spectra of microspheres or structure colors are very stable during the whole detection, and overcome the disadvantage of encoding with fluorescent dyes. The chemiluminescence immunoassay (CLIA) method has high immunological specificity and can improve the sensitivity of immunoassays by at least two to three orders of magnitude compared with conventional colorimetric detection.[22] In this work, we combined an SPCMs decoding method and CLIA to detect three mycotoxins in cereal samples. In the new CLIA system, horseradish peroxidase (HRP) was used as a labeling enzyme for the secondary antibody for the enzyme-catalyzed H_2O_2–luminol–4-iodophenol (PIP) chemiluminescence system. As the most common major mycotoxins in cereal samples, aflatoxin B1 (AFB1), fumonisin B1 (FB1) and ochratoxin A (OTA) were detected by the developed technique. The low signal intensities of detection could be markedly improved and fluorescence photobleaching could be avoided. We demonstrated that the CLIA detection system was easily integrated with a multifunctional microplate reader and the multimycotoxin assay system was furthermore simplified.

2. Experimental section

2.1. Materials

AFB1, FB1 standard substances, AFB1-BSA, Tween-20, 3-glycidoxypropyl-trimethoxysilane (GPTMS), bovine serum albumin (BSA) and tetraethoxysilane (TEOS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The mouse monoclonal anti-AFB1 and mouse monoclonal anti-FB1 antibodies (Ab) were purchased from Abcam (UK). OTA standard substance, OTA-BSA, FB1-BSA, AFB1, FB1 and OTA ELISA kits were bought from Huaan Magnech Bio-Tech Co., Ltd (Beijing, China). Horseradish peroxidase-labeled goat anti-mouse antibody (HRP-IgG) was purchased from Nanjing Shengxing Bio-Tech Co., Ltd (Nanjing, China). Luminol was bought from Sangon Biotech Co., Ltd (Shanghai, China). 4-Iodophenol (PIP) and dimethylsulfoxide (DMSO) were bought from Sinopharm Chemical Reagent Co., Ltd., China. 30% H_2O_2, ammonia and absolute ethanol were from Nanjing Chemicals Ltd., China. Luminol stock solution (50 mM) was prepared in 0.05 M pH 10.3 carbonate buffer solution (CBS). PIP stock solution (4 mM) was prepared in 100 mL of DMSO. 0.1 M H_2O_2 stock solution was prepared in triple distilled water with 30% H_2O_2. Prior to use, luminol, PIP and H_2O_2 stock solutions were mixed and diluted using 0.05 M pH 8.2 Tris–HCl EDTA buffer solution to 0.5 mM, 0.4 mM and 4 mM for luminol, PIP and H_2O_2, respectively. Methylsilicone oil was bought from Yunuo Chemicals Ltd., China. Monodisperse silicon nanoparticles with diameters of 210 ± 11 nm, 223 ± 15 nm and 252 ± 23 nm were synthesized by the Stöber method in our lab.[23] Deionized water was produced with a Millipore purification water system. Samples were purchased from a local supermarket in Nanjing (China). All other chemicals were of analytical grade.

2.2. Fabrication of SPCMs

The fabrication procedures of SPCMs were described in a previous study from our lab.[11] The characteristics of SPCMs were shown in Fig. 1S, ESI.†

2.3. Immobilization of mycotoxin artificial antigens (Ags)

The surfaces of SPCMs were hydroxylated with piranha solution (30% hydrogen peroxide and 70% sulfuric acid) for 6 h. After the SPCMs were washed with water and dried by a nitrogen flow, their surfaces were epoxidized with 0.5 mL of 5% GPTMS toluene solution at 60 °C for 6 h. Then, these modified microspheres were washed with toluene and absolute ethanol solution three times. Then, the SPCMs were reacted with 10 μL of AFB1-BSA, FB1-BSA and OTA-BSA in 0.05 M, pH 9.6 CBS at 37 °C for 1 h. After washing with phosphate buffer solution with Tween-20 (0.5% v/v) (PBST) and 0.01 M pH 7.4 phosphate buffer

solution (PBS) 3 times, unbound active sites were blocked with 100 μL of 1% BSA CBS at 37 °C for 1 h. After washing with PBST 3 times, these SPCMs were stored at 4 °C for detection.

2.4. Multiplex competitive immunoassays

Three kinds of SPCMs (4–5 microspheres of each kind for a set of repeated data) respectively modified with AFB1, FB1, and OTA Ags and control PBS were used for multiplex CLIA. Three kinds of SPCMs were mixed together and incubated with mycotoxin standard solutions (10 μL) at a range of concentrations from 0.001 pg mL^{-1} to 100 ng mL^{-1} in the centrifuge tube. Then, 10 μL of anti-mycotoxin Abs diluted to certain concentrations in PBS were added into the centrifuge tube and incubated at 37 °C for 1 h in the dark, and then the reaction solution was removed by pipette and washed with PBST and PBS buffer 3 times. Then 10 μL of HRP-goat anti-mouse antibody diluted 1 : 6000 was added and incubated for 1 h at 37 °C. The samples were washed with PBST solution 3 times. These SPCMs were classified by their different structure colors under a metallographic microscope equipped with a CCD camera or the reflective peak positions and put into the wells of a 384 microplate. 20 μL of chemiluminescence substrate was added into each well. The mixture was incubated for 4 min at room temperature in the dark, then the emitted photons were measured with a multifunctional microplate reader (expressed as relative light unit, RLU) (TECN, Infinite 200, Switzerland). The whole procedure is shown in Scheme 1. The standard curves were obtained by plotting chemiluminescent intensities against the logarithm of analyte concentration.

2.5. Sample preparation and assays

Corn, wheat and rice were pretreated according to previous procedures.[11] The grain samples were confirmed to contain no toxins by HPLC. The mycotoxin standard solutions were prepared in 2 mL of 100% methanol. Two grams of homogenized sample was spiked with standard solutions at a series of concentrations of AFB1, FB1, and OTA. The samples were mixed and then placed in a fume hood overnight.

Sample extractions were performed with 10 mL of methanol–water (6 : 4 v/v). The samples were firstly shaken for 1 h at 150 rpm and then the extracts were centrifuged at 4000 rpm for 10 min. The supernatant solution diluted with 0.01 M, pH 7.4 PBS solution at a ratio of 1 : 10 was used for analysis. The control samples were extracted under the same conditions. The samples were measured by the above method (see Section 2.4).

The traditional ELISA for validation assays was carried out in according to ELISA kit instructions and the extract supernatant solutions were not diluted.

2.6. Specificity

The specificities of this method were investigated by adding 0.1 ng mL^{-1} of one kind of toxin standard substance into the centrifuge tubes which contained the above SCPMs modified with the three kinds of mycotoxin Ags. The respective corresponding mycotoxin Abs were then added to the system for investigating cross-talk with each other. The flowing procedures were the same as for multiplex competitive immunoassays.

2.7. Data analysis

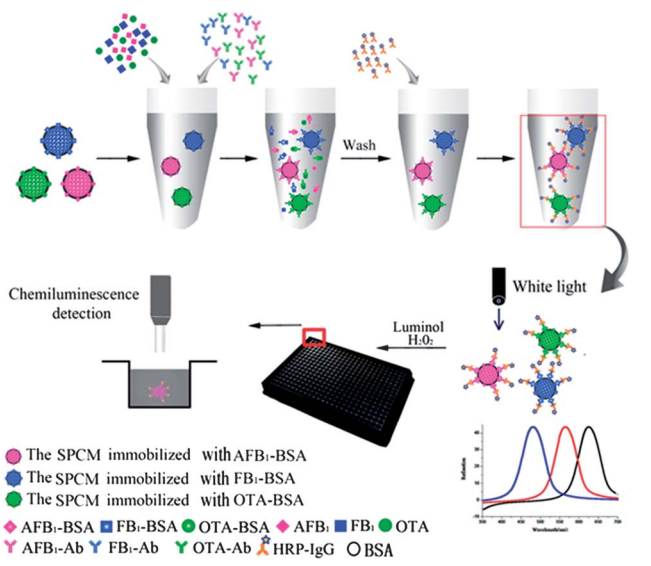
These CLIA data were analyzed with Origin8.5 (OriginLab Corp. USA). The average value and standard deviation for each sample was calculated from no less than 4 samples.

3. Results and discussion

3.1. The scheme of multiplex chemiluminescence immunoassay for mycotoxins

The whole procedure of multiplex chemiluminescence immunoassay for mycotoxins is shown in Scheme 1. The artificial antigens of mycotoxins were immobilized on the surfaces of three kinds of SPCMs by covalent bonding. The unbound activated sites were blocked with 1% BSA. The mycotoxin standards or samples competed to bind to their corresponding antibodies with the artificial antigens. After the SPCMs were incubated and washed, HRP-labeled secondary antibody was added into the reaction system and incubated and washed. Then these SPCMs were classified by their different structure colors or reflectance peak positions and each SPCM was transferred into one well of a 384 microplate. After this, the SPCMs were washed and 10 μL of chemiluminescence substrate was added into each well and incubated in the dark. The CLIA signals were collected by the multifunctional microplate reader.

Currently, it is common practice to first screen a large number of samples for possible contamination and then subject suspected samples to further confirmation, since food safety and environmental monitoring has become a more and more challenging task.[13] The suspension-array-based technologies could provide a powerful platform for this need. Spectral encoding technology for these microspheres is one of the key technologies. The encoding method using the structure colors



Scheme 1 The scheme of the multiplex chemiluminescence immunoassay for mycotoxins.

or reflectance peak positions of SPCMs is significantly superior to that of fluorescent dyes encoding microspheres. The advantages of SPCMs lie in the fact that they could overcome fluorescence photobleaching and the potential interference between the encoding fluorescence and detection fluorescence. More importantly, compared with the fluorescence labeled system our group has previously reported,[11] the CLIA detection system was easily integrated with a multifunctional microplate reader and furthermore simplified.

Planar-array technologies have also been used for multiplex mycotoxin analysis. The main planar-array technologies already applied to food and environmental toxin analysis include the protein microarray,[7] the surface plasmon resonance (SPR)-based biosensor,[24,25] the Naval Research Laboratory (NRL) array biosensor[26,27] and other biosensors.[2,28,29] These encoding methods of planar-arrays mainly depend on the different positions of the different probe molecules on the planar chips. This results in increase of the reaction time and reduction of the detection signal because the rates of hybridization and binding on planar arrays are limited by diffusion to the surface.[19,30] Furthermore, protein microarray technology for multiplex mycotoxin detection requires an expensive robot for spotting probes, a fluorescence scanner for signals and the labeled reporters.[11] Compared with previous reports of microplate and lateral flow CLIA for mycotoxin detection,[22,31–33] the CLIA based on SPCMs system has these advantages: (1) high throughput parallel detection, (2) smaller volume of detection reagents, (3) rapid reaction rate.

3.2. Kinetics of chemiluminescence reaction

After HRP-goat anti-mouse antibody bound to mycotoxin primary antibody, 20 μL of chemiluminescence substrate was added into the well and the chemiluminescence signal intensity was recorded with a multifunctional microplate reader for 15 min at room temperature. The kinetics of chemiluminescence reaction with time was shown in Fig. 1. The chemiluminescence signal intensity reached a maximum value within 4 min and then slowly decreased. Compared with CLIA on the planar surface of a 96 well microplate, the porous SPCMs result in faster mass transfer of enzyme substrates and significantly shorten the chemiluminescence reaction time (30 min for the planar 96 well microplate 30).[31] The 3D nanoporous structure of the SPCMs and suspension array are helpful for accelerating both mass transport of the immunoreagents and immunoreaction of antigen and antibody, which shortens the chemiluminescence development time. The decrease of signal intensity was ascribed to the inhibition of HRP activity by H_2O_2 after a long exposure.[34]

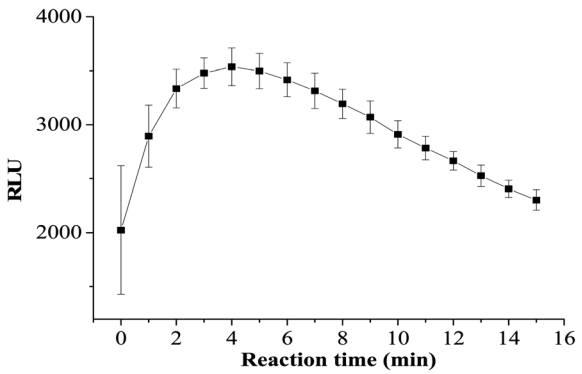


Fig. 1 Kinetics of chemiluminescence reaction on the surface of SPCM.

3.3. Optimization of the multiplex CLIA procedure

3.3.1. The influence of chemiluminescence substrates on the RLU. The chemiluminescence substrates including the concentrations of luminol, PIP and H_2O_2 and buffer solutions affect the RLU. Under a fixed concentration of luminol, PIP and H_2O_2, we found the different buffer dilution solutions of chemiluminescence substrates significantly affect the stability of the RLU of SPCMs. The chemiluminescence emission in 0.05 M pH 8.2 Tris–HCl EDTA buffer is more stable than in 0.05 M pH 9.6 CBS and 0.01 M pH 7.4 PBS solutions (Fig. 2S, ESI†).

3.3.2. Immobilization of artificial antigens on the surfaces of SPCMs. The pH of the buffer solution for the immobilization medium of artificial antigens on the surfaces of SPCMs is one of the important factors influencing the sensitivity of CLIA.[31] The buffer solutions of different pH values including 0.05 M pH 4.8 citric acid, 0.01 M pH 7.4 PBS, 0.05 M pH 8.0 Tris–HCl, and 0.05 M pH 9.6 CBS were used to investigate the influence of buffer solutions of different pH on the CLIA signal. Fig. 3S, ESI† indicates that the RLU is affected by the different pH values of the buffer solutions and the highest RLU is obtained in pH 9.6 CBS. This was due to the most efficient ring opening of the epoxide group which leads to many more Ags becoming immobilized on the surface of SPCMs in this immobilization medium.[35]

3.3.3. The influence of different concentrations of mycotoxin artificial Ags, Abs and HRP-labeled secondary antibody on the RLU. The immobilization concentrations of mycotoxin artificial Ags in the range of 50 to 3200 ng mL^{-1} were investigated to enable a high RLU intensity. Fig. 4S, ESI† indicated that the RLU reached a maximum value when the immobilization concentrations of AFB1-BSA, FB1-BSA and OTA-BSA were 1200, 1200 and 400 ng mL^{-1}, respectively. Therefore, these concentrations were selected for further experiments.

The working concentrations of AFB1-Ab, FB1-Ab and OTA-Ab were optimized by a series of respective dilution ratios. Fig. 5S, ESI† showed that the dilution ratios of the primary antibodies affect the RLU signal, and the optimum concentrations of AFB1-Ab, FB1-Ab and OTA-Ab were 1 : 10 000, 1 : 10 000 and 1 : 30 000 dilution ratios, respectively.

The concentration of HRP-labeled secondary antibody was also optimized for the improvement of CLIA efficiency. The optimal dilution of HRP-labeled secondary antibody was established by incubating it at different dilution ratios under the above optimized CLIA detection conditions. Fig. 6S, ESI† demonstrated that the RLU decreased with increasing dilution ratios, and appeared stable between 1 : 4000 and 1 : 6000 dilution ratio. In order to minimize the use of reagents, a 1 : 6000 dilution of HRP-labeled secondary antibody was chosen for these experiments.

3.4. Sensitivity and calibration curves of CLIA for multiplex mycotoxins

A series of concentrations of mycotoxin standard solutions in the range from 0.01 pg mL^{-1} to 100 ng mL^{-1} were added into the system to establish their calibration curves. Fig. 2 shows the dose–response relationships between the inhibition ratio and the concentrations of AFB1, FB1 and OTA. The inhibition ratios decrease with increasing concentrations of three kinds of mycotoxin. The calibration inhibition curves of CLIA for the three mycotoxins were typical sigmoidal curves with half maximal inhibitory concentrations (IC_{50}) of 0.01, 0.036, and 0.04 ng mL^{-1} for AFB1, FB1 and OTA, respectively. The IC_{50} values were one order of magnitude lower than those obtained by a Luminex system.[9,10] This developed method displays dynamic linear detection ranges over two to three orders of magnitude. The linear ranges between RLU and the concentrations of mycotoxins varied from 0.001 to 1 ng mL^{-1}, 0.001 ng mL^{-1} to 1, and 0.01 to 1 ng mL^{-1} with high coefficients of determination, $R^2 = 0.994$, 0.992 and 0.995 for AFB1, FB1 and OTA, respectively. The linear detection ranges were far wider than those reported by Peters[9] and similar to those detected by Czeh using the Luminex system.[1] The limits of detection (LODs) were calculated at a signal/noise ratio of 3 to be 1.19, 0.60, and 0.73 pg mL^{-1} for AFB1, FB1 and OTA, respectively, which are much lower than those reported by other immunoassays[22,29,31] and the Luminex system.[1,9,10]

Compared with the Luminex suspension array, the 3D nanoporous structure of the SPCMs has a large surface area which could bind many more probe molecules and allow more opportunities for probe molecules to bind their targets.[11] These result in the detection being more sensitive, shorter in time, and more highly effective than other immunoassay methods.

3.5. Specificity evaluation

The cross-reactivity may mainly arise from the nonspecific adsorption of nonspecific primary antibodies on the surfaces of SPCMs and lead to false signals. In addition, this may occur between the mycotoxins and the nonspecific primary antibodies, which depend on the specificity of antibodies. Therefore, the specificity of CLIA for multiple mycotoxins was evaluated among the three mycotoxins. Fig. 3 shows the detection signal is positive only between the matched Ags and Abs, and nonspecific adsorption on each surface of SPCMs can be ignored. This result showed that the cross-reactivity was avoided and multiplex mycotoxin CLIA could be performed using this system.

3.6. Multiplex mycotoxin CLIA performance

To study the recovery rates of different mycotoxins in the system, three concentrations, 10, 100 and 1000 pg mL^{-1} of AFB1, FB1 and OTA were spiked in rice, corn and wheat samples. These artificially contaminated cereal samples were analyzed by the developed method. As shown in Fig. 4, the recovery rates ranged from 72.9 to 113.9% for AFB1, 63.5 to 115.6% for FB1, 77.7 to 121.6% for OTA in the three kinds of cereal samples, respectively. Several recovery rates were less than 80% and most recovery rates are in range of 80–122%. These low recovery rates may arise from the matrix effect during the extraction of mycotoxins, which still needs further research.

The precision of the suspension array is investigated by intra- and interassay variation coefficients with different concentrations of AFB1, FB1 and OTA (Table 1S, ESI†). The intraassay variation coefficients (generated on the same day) varied from 3.0 to 8.7% for AFB1, 1.8 to 7.9% for FB1 and 2.1 to 8.0% for OTA. The interassay variation coefficients (generated on different days) were lowe than 14.4% for AFB1, 14.5% for FB1 and 13.8% for OTA. The suspension array is stored in pH 7.4 PBS solution at 4 °C when not in use. There are no obvious changes in their shape and bioactivity after storing for 3 months.

The proposed CLIA for multiple mycotoxins was applied to evaluate AFB1, FB1 and OTA in 12 naturally contaminated cereal samples including rice, corn and wheat. Meanwhile, we have compared the results of our system to those of commercial ELISA kits (Fig. 7S, ESI†). The results showed that there were good correlations between the proposed method and classic ELISA. This furthermore indicated the proposed CLIA method could be applied in real agricultural products.

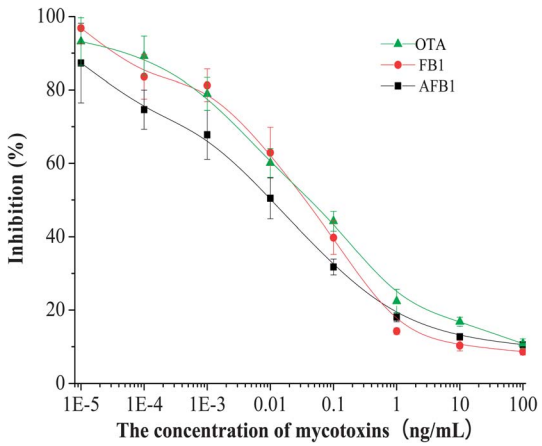


Fig. 2 The CLIA calibration curves for multiple mycotoxins.

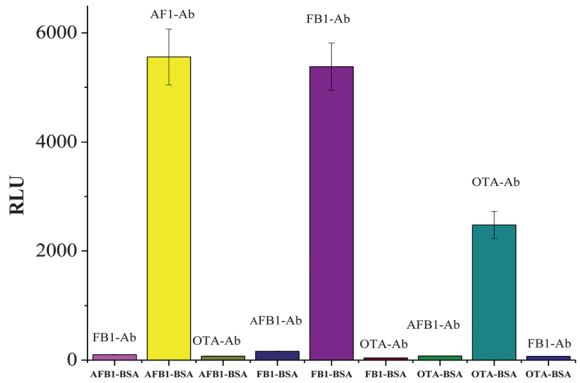


Fig. 3 The specificity test for multiple mycotoxin detection among AFB1, FB1 and OTA.

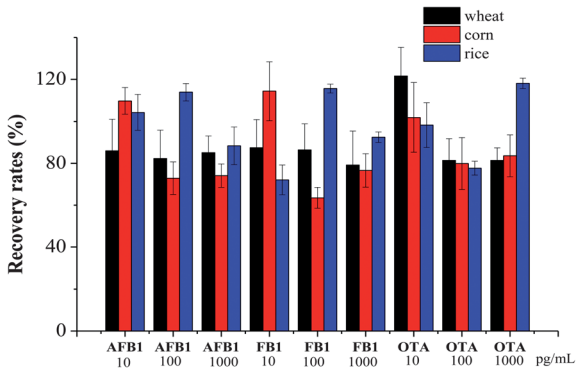


Fig. 4 The recovery rates of AFB1, FB1 and OTA from artificially contaminated cereal samples determined by the developed method ($n = 4$).

Apart from immunoassay methods, LC-MS/MS techniques have also been developed for multimycotoxin assays. Pamel *et al.* reported that the LOD and quantitation ranges for this technique were 5–348 and 11–695 ng g^{-1} for 26 mycotoxins with recovery ratios between 61 and 116%.[4] Sulyok *et al.* used this technique to detect 39 mycotoxins in cereal samples and the LODs ranged from 0.03 to 220 ng g^{-1} with recovery ratios between 20 and 110%.[36] Though LC-MS/MS is a sensitive, selective and high resolution method for multimycotoxin assay, it requires expensive instruments, skilled operators and complicated sample pretreatment, which is not suitable for the screening of large numbers of samples for multimycotoxin assays. On the contrary, the developed CLIA technique is simple, cost-effective, and has great potential for practical application.

4. Conclusions

This work described a new multiplex mycotoxin CLIA system based on SPCMs suspension array for agricultural products. The unique optical porous microspheres provide good biocompatibility, high capacity for loading of protein and fast mass transport of analytes. The new CLIA system on the surfaces of SPCMs for multiple mycotoxins is more sensitive, rapid, cost-effective, flexible and simple compared with classic ELISA methods. The multiplex mycotoxin CLIA system has been successfully applied to detect AFB1, FB1 and OTA in practical samples. Although more work is required to improve recovery rates and validation with HPLC, the established method shows a great application potential in the screening of large numbers of samples.

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