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ORIGINAL ARTICLE

Development of an immunoassay for the detection of mycotoxins using xMAP technology and its evaluation in black tea samples

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Abstract Mycotoxins, a natural food contaminant, are secondary metabolites of fungi. Aflatoxin B1 (AFB1) and ochratoxin A (OTA) are two major mycotoxins found in various food commodities. These mycotoxins are hepatotoxic, nephrotoxic, cytotoxic, mutagenic and carcinogenic, thus they are a public health concern and their monitoring in food commodities is necessary. There are several conventional techniques available for mycotoxin detection, such as HPLC, LCMS, and ELISA. However, extensive nature and huge cost allowances make it challenging to deploy these techniques for monitoring of mycotoxins in the large sample size. Therefore, a robust, responsive and high-throughput technique is required. Here, we aimed to develop a multiplexed Luminex suspension assay based on multi analyte profiling (xMAP) technology for the simultaneous detection of AFB1 and OTA in the black tea, which is found to be contaminated with these mycotoxins during the cultivation or processing steps. Limit of detection for AFB1 and OTA, was 0.06 ng/ml and 0.49 ng/ml, respectively without any cross-reactivity with other mycotoxins and this assay is suitable for simultaneous detection of AFB1 and OTA in the same sample. Collectively, based on the results, we suggest that the developed Luminex suspension assay is sensitive, accurate, rapid and suitable for high-throughput screening of multiple mycotoxins.

Keywords Mycotoxins · Aflatoxin B1 · Ochratoxin A · xMAP technology · Black tea

Abbreviations

AFB1	Aflatoxin B1
AFG2	Aflatoxin G2
AFM1	Aflatoxin M1
BSA	Bovine serum albumin
CSV	Comma-separated values
DMSO	Dimethyl sulfoxide
DON	Deoxynivalenol
EDC	*N*-(3-dimethylaminopropyl)-*N*′-ethyl carbodiimide hydrochloride
FB2	Fumonisin B2
IARC	International Agency for Research on Cancer
LOD	Limit of detection
LOQ	Limit of quantification
mAbs	Monoclonal antibodies
MFI	Mean fluorescence intensity
NHS	*N*-hydroxysuccinimide
o-CMA	*o*-carboxymethyl (hydroxylamine) hemihydrochloride
OTA	Ochratoxin A
OTα	Ochratoxin α
QuEChERS	Quick, easy, cheap, effective, rugged and safe
STG	Sterigmatocystin

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TNBS	2,4,6-Trinitrobenzene sulphonic acid
UPLC	Ultra-performance liquid chromatography
xMAP	(x = number of analyte) Multi analyte profiling
ZEN	Zearalenone

Introduction

Mycotoxins are secondary metabolite products of various fungi. Out of four hundred mycotoxins, 30 are known to be potentially toxic (Juraschek et al. 2022). Mycotoxin contamination of food and feed is a worldwide concern. However, the degree of mycotoxin contamination is strongly influenced by physical and chemical factors such as temperature, humidity, water activity, pH, relative humidity and oxygen levels. Because, these inter-related factors collectively contribute to the favorable conditions for the production and accumulation of mycotoxins in food and raw materials (Pleadin et al. 2019). Additionally, thermostability and the ability to grow at very low temperatures make mycotoxins, a unique natural food contaminant, which can contaminate food and feed in the pre- and post-harvest stages. Once these mycotoxins enter the food chain, it is very difficult to get rid of them. (Pleadin et al. 2019; Stadler et al. 2020; Schrenk et al. 2020a, b).

Although a number of mycotoxins were identified and chemically characterized, in-depth investigation was limited to about fifty mycotoxins. Among them, AFB1 and OTA are particularly important because of their wide spread and potential health risks (Schrenk et al. 2020a). AFB1 is one of the major mycotoxins in contaminated food samples. It is primarily produced by *Aspergillus flavus* and *Aspergillus parasiticus* fungi. It is a natural contaminant in a variety of foods, including cereals, grains, coffee, nuts, and legumes etc. It also contaminates animal feed, which may cause contamination of animal origin products like milk and meat (Schrenk et al. 2020a). After ingestion, AFB1 is absorbed in to the small intestine and distributed to the liver, where it initially undergoes metabolism and forms epoxides, which can covalently bind with the proteins and cause various harmful effects in humans and animals (Rushing and Selim 2019). IARC has classified AFB1 as a Group 1 carcinogen, however, addition to carcinogenic effects, AFB1 may also have other harmful effects such as immune system suppression, hepatitis and liver cirrhosis (Ostry et al. 2017; Rushing and Selim 2019; Hua et al. 2021). Another important mycotoxin is OTA, which can coexist with AFB1 and are produced primarily by *Aspergillus ochraceus, Aspergillus carbonarius, and Penicillium verrucosum.* It is also a naturally occurring contaminant in a variety of food commodities, including cereals, grains, tea, coffee, cocoa beans and wine (Schrenk et al. 2020b). OTA is found to be a major culprit linked to kidney damage, however, it may have some other adverse effects, including genotoxicity, neurotoxicity, and immunotoxicity (Schrenk et al. 2020b). Moreover, on the basis of carcinogenic efficacy in cell-based assays and animal studies, OTA is classified as Group 2B carcinogen by IARC.

Furthermore, considering the adverse effects of these mycotoxins (AFB1 and OTA) on animals and humans, surveillance and analytical determination of these toxins in several food commodities have been widely investigated in the past several years.

In most cases, these mycotoxins have been identified by thin layer chromatography (TLC), high-performance liquid chromatography coupled mass spectrometry (HPLC–MS/MS), gas chromatography-mass spectrometry (GC–MS) and oligonucleotide aptamer-based graphene oxide systems etc. (González-Jartín et al. 2019; Klarić et al. 2009; Amelin et al. 2013; Puntscher et al. 2018; Kim et al. 2019; McMaster et al. 2019; Nasaruddin et al. 2022; Ye et al. 2023). Besides the chromatographic methods, various immune-based assays such as ELISA, later flow immunoassay (Klarić et al. 2009; Omar et al. 2020; Suo et al. 2023) and biosensors based on aptamers or molecular imprinting polymers have also been used for the quick detection of mycotoxins (Kadota et al. 2010).

These methods are considered reliable and widely used; however, they do have certain limitations. Particularly among these methods, ELISA is highly temperature sensitive and requires optimization of several parameters, such as timing of the reaction, selection of suitable substrate and enzymes for colour reaction etc. In GC–MS and LC–MS methods electric source is essential for the functioning of the spectroscopic instruments. Moreover, maintenance of proper vacuum pressure is essential for these instruments. The other problem associated with these methods is the specific calibration nature of the instruments. Lastly, the involvement of costly oligonucleotide aptamer increases the expenses of the protocol and also caution should be taken as the stability of these aptamers are extremely dependent on the pH and ionic strength of the medium.

Keeping in view of these challenges, a reproducible, reliable, sensitive, cost-effective and high-throughput method is needed. Hence, a sensitive multiplexed Luminex suspension assay that utilizes xMAP technology was developed. Specificity and cross-reactivity of developed Luminex assay was checked for other mycotoxins. Finally, developed Luminex multiplexed assay was evaluated for simultaneous determination of AFB1 and OTA in black tea samples. Black tea samples were chosen for detection of AFB1 and OTA because it is one of the most consumed non-alcoholic drinks globally and contribute potential health benefits (Hazra et al. 2019). Despite of these, black tea found to be contaminated with various mycotoxins

during the growing or processing steps, which cause risks to human health (Ye et al. 2023). In addition, mycotoxins contamination causes huge economic losses due rejection of contaminated black tea samples by the consumers and regulatory authorities. Thus, our developed Luminex suspension assay would be an accurate, sensitive, high-throughput, and reproducible detection of AFB1 and OTA in black tea samples.

Material and methods

Chemicals

AFB1, OTA, AFM1, AFG1, OTB, OTα, STG, FB2, DON, ZEN, citrinin, o-CMA, EDC, NHS, BSA and TNBS were procured from Sigma Aldrich (St. Louis, Missouri, USA). Protein marker was purchased from Puregene (Etzmatt, Zeiningen, Switzerland). Paramagnetic microsphere bead set (Magpax®-C 064 and 026) and the xMAP antibody coupling kit was purchased from Luminex Corporation (Austin, Texas, USA). Anti-AFB1 and anti-OTA mAbs were purchased from Abcam (Cambridge, United Kingdom). Phycoerythrin labelled anti-mouse antibody was purchased from Invitrogen (Waltham, Massachusetts, USA). DMSO and HPLC grade Methanol was purchased from Merck (Darmstadt, Germany).

Synthesis of mycotoxin-BSA conjugates

The conjugation of mycotoxins with BSA involves the use of BSA and amino or carboxylic group of mycotoxins. OTA has a carboxylic group in its chemical structure; however, this group is not present in AFB1. Hence, a reaction of AFB1 and o-CMA in pyridine solution was conducted to add a carboxylic group into AFB1 (Lee et al. 2004). After an overnight reaction, the solution was evaporated, which resulted in AFB1-oxime, that react with BSA. Next, AFB1/OTA-BSA conjugates were synthesized using the EDC-NHS method (Venkataramana et al. 2015). Briefly, AFB1-oxime/OTA (1 mg) was dissolved in 200 µl of DMSO, which contains 1.5 mg NHS and 1.2 mg EDC. This solution mixture was incubated 2 h at RT, followed by overnight at 4℃. The next day, 1.5 ml sodium carbonate buffer (pH 9.6) containing 2 mg BSA was added and incubated on magnetic stirrer for 2 h at RT. Subsequently, conjugated AFB1/OTA-BSA reaction mixture was transferred to dialysis tubing and dialyzed against 500 ml of PBS (pH 7.4) for 24 h at 4°C with the exchange of PBS thrice. At the end, AFB1/OTA-BSA was lyophilized and stored at − 20°C for further use.

Characterization of mycotoxin-BSA conjugates

SDS–Polyacrylamide gel electrophoresis

To visualization of bands of mycotoxin-BSA conjugates, 5 µg of each sample (BSA, OTA-BSA and AFB1-BSA) in SDS-PAGE loading dye were separated on 10% SDS-PAGE with 1.5 mm gel thickness. Standard protocol for the stacking and resolving gel was followed using Mini-PROTEAN system casting stand, Bio-rad, California, USA. Electrophoresis was carried out at 80 V for 2 h. After electrophoresis, gels were stained with coomassie blue dye and later destained for visualization of protein bands.

Quantification of the molar ratio of mycotoxin-BSA conjugates by TNBS assay

The amount of free lysine in BSA before and after mycotoxin conjugation was measured using the TNBS assay to estimate the molar ratio of mycotoxin-BSA conjugate. With some modifications, the assay was carried out as stated in the method (Habeeb 1966). In brief, 125 µL of TNBS (0.5%; w/v) was incubated with 250 µl of diluted BSA standards (12.5–800 µg/ml) and mycotoxin-BSA conjugates (50 µg/ml) separately for 2 h at 37°C. Then, 125 µl of 10% SDS was added to the reaction mixture to solubilize it. Finally, 62.5 µl IN HCl was added and absorbance was recorded at 335 nm.

Percent substitution and the molar ratio of mycotoxin-BSA conjugates were calculated using the following formula:

$$\%\text{Substitution} = \frac{\text{Abs of carrier protein} - \text{Abs of conjugate}}{\text{Abs of carrier protein}} \times 100$$

$$\text{No. of moles attached to protein} = \frac{\%\text{Substitution}}{100} \times \text{lysine residues } \textit{in} \text{ the carrier protein}$$

Spectrophotometric measurements

Spectrophotometric measurements (200–400 nm) were used to examine the spectral peak shifts of mycotoxin-BSA conjugate in comparison with AFB1/OTA and BSA alone). Mycotoxin-BSA (100 µg/ml), BSA (100 µg/ml), AFB1 (5 µg/ml) and OTA (5 µg/ml) was diluted in PBS and spectra were acquired using dual beam Shimadzu spectrophotometer (UV-2600 240 V EN, Shimadzu Corporation Kyoto, Japan).

Coupling of mycotoxin-BSA conjugates with microsphere beads

AFB1-BSA and OTA-BSA was coupled to paramagnetic color-coded microsphere beads region 064 and 026 using xMAP antibody coupling kit (Luminex Corporation, Austin Texas, USA). Microsphere beads have unique spectral signature based on the concentration of internal dye with magnetic properties. The carboxyl groups on the surface of microsphere beads were used for covalent coupling with AFB1/OTA-BSA as per instructions given in the kit. Briefly, 2.5×10^6 microsphere beads of each set (Magpax®-C 064 and 026) were activated with the activation buffer and 10 µl of Sulfo-NHS and EDC reagent was added. Reaction mixture was incubated for 20 min at RT to facilitate the coupling of microspheres with mycotoxin-BSA conjugates. After incubation, the microsphere beads were washed on a magnetic separator and again incubated with AFB1/OTA-BSA (10 µg) for 2 h at RT. Coupled microsphere beads were further washed and stored in the wash buffer at 4°C in the dark. Next day, coupling was confirmed using PE-labeled anti-mouse antibody. Working concentration of coupled microsphere beads were prepared by diluting the stock solution up to50 beads/µL. Fifty µL of diluted microspheres (50 beads/µL) were added to a clear black plate with a flat bottom. Subsequently, 50 µl of anti-AFB1/OTA mAbs (0.5 µg/ml) were added to each well except blank and plate was incubated on a shaker for 30 min at RT in dark. After incubation, plate was washed threetimes on a magnetic separator using wash buffer (PBS with 0.05% Tween-20) and varying concentration of PE-labeled anti-mouse antibodies (ranging from 0.06 to 4 µg/ml) were added to the wells. The plate was then washed and incubated for 5 min at RT and fluorescence was measured using a Luminex analyzer (Luminex Corporation, Austin, Texas, USA).

Development of Luminex suspension assay for the detection of mycotoxins

Optimization of the number of microspheres and antibody concentration

The ideal number of microspheres and antibody concentration was established using the checkerboard titration method in order to develop the Luminex suspension assay for the detection of mycotoxins. The final working concentration of microspheres and mAbs was decided after a number of tests. Using the variable number of microspheres (500 beads per well, 1000 beads per well, and 2000 beads per well for AFB1 and 1250 beads per well, 2500 beads per well, and 5000 beads per well for OTA), the number of microspheres was optimized.

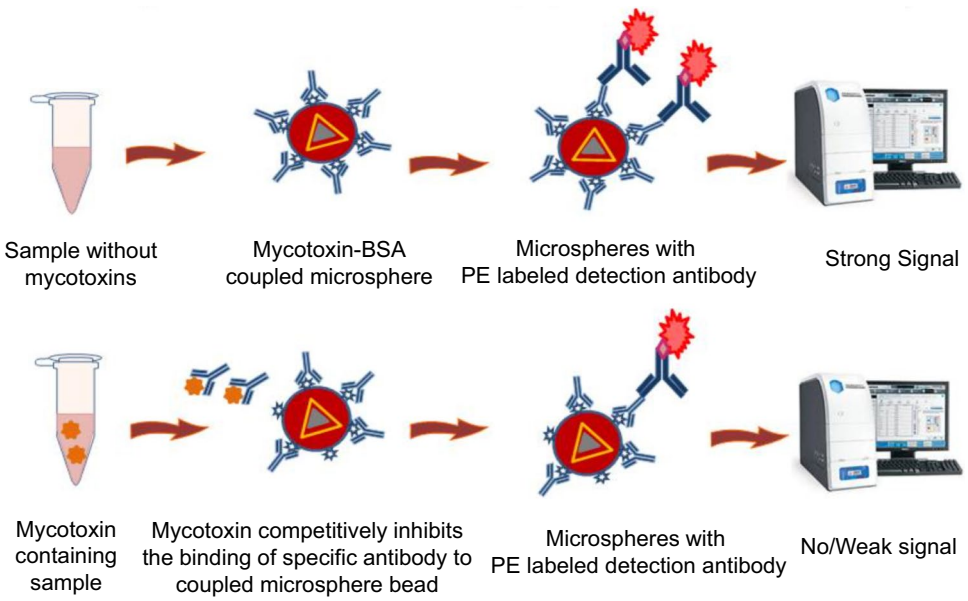

Fig. 1 Working principle of Luminex suspension assay: based on a competitive immunoassay format

Briefly, 50 µl of each bead set in triplicate was added to a clear black plate with a flat bottom and diluted mAbs (0.06–0.5 µg/ml) were subsequently added to all the wells except the blank ones. Plate was incubated on a shaker for 30 min at RT and washed three times using magnetic separator. Subsequently, PE-labeled anti-mouse antibody (4 µg/ml) was added, incubated as before, and plate was washed again. Finally, plate was incubated on a shaker for 5 min at RT and fluorescence was measured with a Luminex analyzer.

Development of Luminex suspension assay

The competitive inhibition approach was used to develop the Luminex suspension assay (Fig. 1). Immune complex was formed by incubating the diluted AFB1 (0.06–31.25 ng/ml) and OTA (0.49-250 ng/ml) standards with their respective mAbs (0.06 µg/ml for AFB1 and 0.5 µg/ml for OTA) for 30 min at RT. Subsequently, coupled microspheres were diluted to working concentrations (500 beads/well for AFB1 and 1250 beads/well for OTA) and dispensed into a clear black plate with a flat bottom. Further, prepared immune complexes (50 µl/well) were added to every well containing microsphere beads except blank, and the plate was shaken for 30 min at RT. Following washing step, 4 µg/ml of PE-labeled anti-mouse antibodies were added to each well, and the plate was further incubated for 30 min at RT. After incubation, fluorescence was measured, and standard curves were plotted by comparing the average MFI value to the concentration of mycotoxins.

Determination of specificity

Using the method described in Sect. "Development of Luminex suspension assay", the cross-reactivity of developed Luminex suspension assay with various additional mycotoxins was examined. Briefly, the mAbs cocktail (anti-AFB1, 0.06 g/ml and anti-OTA, 0.5 g/ml) was incubated for 30 min at RT with 100 ng/ml of each mycotoxin standard (AFB1, AFG1, AFM1, OTA, OTB, STG, FB2, ZEN, AOH, DON and citrinin).Prepared immune complexes were added into a clear black plate with a flat bottom that contained coupled microsphere beads, and incubated for 30 min at RT followed by three washes on magnetic separator. After that, 50 µl of PE-labeled anti-mouse antibodies were added to each well and after 30 min incubation at RT, plate was washed three times on a magnetic separator. Finally, fluorescence was measured on a Luminex analyzer and cross-reactivity was calculated using following formula:

$$\text{Cross - reactivity} = \frac{\text{MFI of control sample} - \text{MFI of other mycotoxin}}{\text{MFI of control sample}} \times 100$$

Evaluation of developed luminex suspension assay in black tea samples

For the evaluation of developed Luminex suspension assay, 25 µl of mycotoxin negative control (Wash buffer), mycotoxin standards (10 ng/ml) and three different infused tea samples, were incubated with the 25 µl mAbs cocktail (anti-AFB1, 0.06 µg/ml and anti-OTA, 0.5 µg/ml) for 30 min at RT. Then the prepared immune complex was incubated with the mixture of coupled microsphere beads in a black plate for 30 min at RT on a shaker followed by three washes on the magnetic separator. Subsequently, 50 µl of PE-labeled anti-mouse antibodies were added into each well and incubated on a shaker for 30 min at RT. The plate was washed three times on a magnetic separator with 100 µL of wash buffer. Plate was then further incubated at RT for 5 min and the results were read using a Luminex analyzer.

Tea sample preparation for UPLC

Black tea samples were procured from local market of Lucknow, Uttar Pradesh, India and prepared using a modified version of Ye et al. 2023. Briefly, 2 gm of black tea sample was brewed in 100 ml of water (95–100°C) for 5 min and filtered. Five ml of filtrate was combined with a solution of acidified acetonitrile (2 ml), ethyl acetate (500 µl) and NaCl (2 gm), vortexed, and then incubated for 30 min at RT on a shaker. After incubation, the solutions were centrifuged at 5000 rpm for 10 min at −4°C to separate the organic and aqueous layers. After collecting the top organic layer, QuEChERS salt (NaCl & $MgSO_4$) was added, and the mixture was spun down by centrifuging at 5000 rpm for 5 min. After centrifugation, supernatant was collected and evaporated by a rotary evaporator. The obtained residue was reconstituted in 500 µl of ACN:Water (60:40) and stored at −20°C for further use.

Chromatographic conditions for analysis of AFB1 and OTA

For analysis of AFB1 and OTA in black tea samples, Nexera X2 UPLC systems equipped with quaternary pump LC-30AD, auto sampler Sil 30AC, column oven CTO 20 AC, prominence fluorescence detector RF-20A and control bus module CBM-20A was used. The chromatographic separations of both mycotoxins were performed using ACQUITY UPLC BEH C18 column having 100 mm × 2.1 mm

dimensions with 1.7 µm particle size guard column. The mobile phases A & B (A-0.1% formic acid in water; B-0.1% formic acid in methanol) were used. The flow-rate of mobile phase was set at 0.5 ml/min. The excitation and emission wave lengths were fixed 360 nm and 430 nm for AFB1, 335 nm and 460 nm for OTA, respectively. Using an auto-injector, filtered samples (5 µl) were injected, and chromatograms were then recorded and analyzed with Lab Solutions software (Shimadzu Corporation Kyoto, Japan).

Data analysis of Luminex suspension assay

The data analysis was performed using xPONENT 4.2 software (Luminex Corporation, Austin, Texas, USA). The dilution factor was set, and the wells for blank, standard and samples were identified for data acquisition. An analyzed report was generated as output CSV files.

Statistical analysis

The obtained data are presented as mean ± SE. The statistical significance of the data was determined by one-way ANOVA. The p-value of < 0.05 was considered statistically significant.

Results and discussion

The detection of multiple mycotoxins in the same sample has gained attention due to their co-occurrence and valuable step towards gaining a clearer picture of mycotoxins contamination pattern in food and feed samples. Thus, efforts are being made at international level to detect multiple mycotoxins at a time. While LC–MS/MS can identify numerous mycotoxin metabolites at a time (Streit et al. 2013), it is not suitable for a rapid and high-throughput analysis, and it requires skilled personnel and is time-consuming. ELISA technique is, however, fast and suitable for high-throughput screening but cannot detect multiple mycotoxins simultaneously (Suo et al. 2023). Some studies have used suspension array technology and immunochip methods to quantify multiple mycotoxins, but they are complex and costly (Wang et al. 2013; Liu et al. 2021).

Keeping these points in view, an attempt has been taken to develop a rapid, easy and high throughput screening technique for simultaneous detection of two mycotoxins using xMAP technology. This technology utilizes a suspension array system, where different types of microspheres are internally labeled with unique fluorescent dyes or other detection moieties. Each microsphere can be coupled with specific mycotoxin-BSA conjugate to detect different target mycotoxins. Because of these capabilities, running independent assays takes less time and can identify up to 50 different microsphere beads conjugated with different analytes.

In the present investigation, AFB1/OTA-BSA conjugate was synthesized and characterized using SDS-PAGE, TNBS assay and UV spectrophotometry. Characterized AFB1/OTA-BSA conjugates were coupled with two different microsphere beads (064 and 026) that have a distinct spectral signature and optimum number of microspheres and antibody concentration was determined. Using the optimized parameters, Luminex suspension assay was developed and evaluated for detection of AFB1 and OTA in black tea samples. The following sections provide more specific results and discussion:

SDS-PAGE analysis confirmed the conjugation of BSA with AFB1 and OTA

BSA and AFB1/ OTA-BSA conjugates were subjected to SDS-PAGE to check the binding of mycotoxin with BSA. BSA only showed a sharp band on 66 kDa, however, a notable difference in electrophoretic pattern was observed in AFB1/OTA-BSA conjugates. Both the conjugates showed sharp bands on 90 kDa as shown in the Fig. 2a. Densitometric analysis of AFB1-BSA and OTA-BSA respect to BSA alone showed 1.4 and 1.21 fold change respectively. AFB1/OTA-BSA conjugates migrated slowly as compared to BSA alone due to their higher molecular mass (Charlermroj et al. 2021). These results clearly suggest the conjugation of AFB1/OTA with BSA.

Molar ratio of AFB1/OTA and BSA in conjugates was determined using TNBS assay

Electrophoresis results indicated the increment in molecular weight of BSA upon conjugation, but could not give the definite information about how many moles of AFB1/OTA was attached to one mole of BSA. Hence, TNBS assay was performed to analyze the molar ratio of AFB1/OTA and BSA. As reported earlier, TNBS reacts with primary amines and produces highly chromogenic product N trinitrophenyl protein derivatives (TNP-protein) that can be measured spectrophotometrically at 335 nm (Habeeb 1966). The intensity of the color is directly proportional to the number of primary amines present in the sample. As shown in the Fig. S2, a standard curve was plotted, and moles of AFB1/OTA conjugated with per mole of BSA was calculated. Although, it has been reported that approximately 30–35 primary amines per BSA molecule are available for conjugation with mycotoxins (Singh et al. 2004), we found that 8 mol of AFB1 and 7 mol of OTA were coupled to a single mole of BSA.

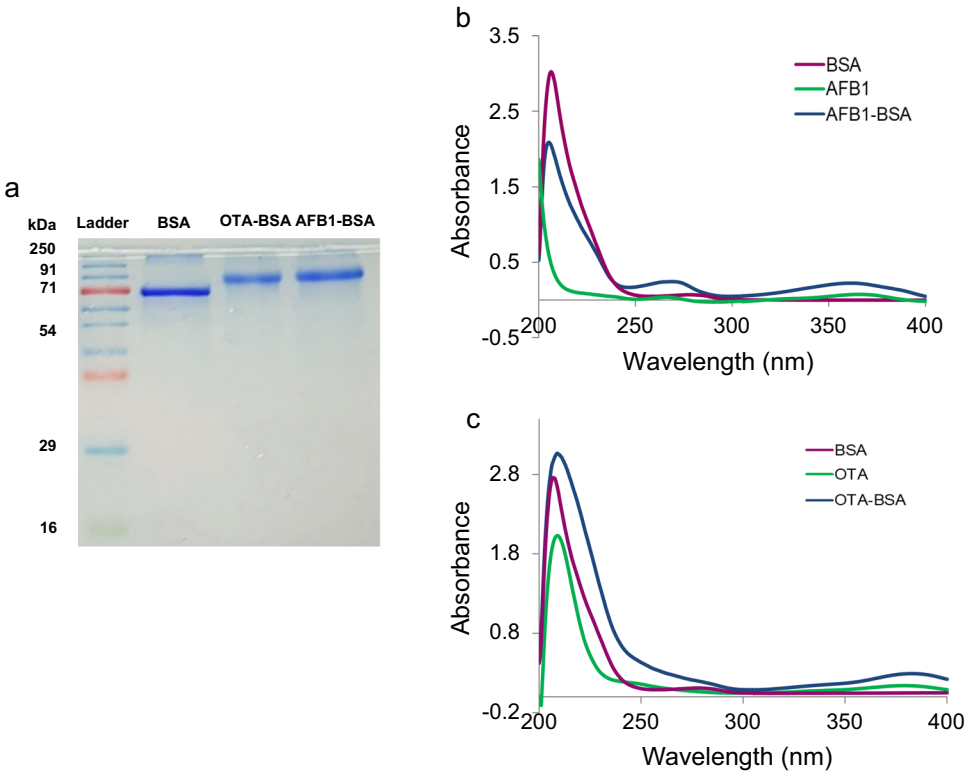

Fig. 2 Characterization of AFB1/OTA-BSA conjugates. **a** SDS-PAGE of mycotoxin-BSA conjugates. **b** UV spectrophotometry of AFB1-BSA conjugates. **c** UV spectrophotometry of OTA-BSA conjugates

UV spectrophotometry showed structural alterations in mycotoxin-BSA

After determining the molar ratio of AFB1/OTA and BSA in the conjugates, the structural alterations in the AFB1/OTA-BSA conjugates were assessed using UV/visible spectroscopy. The absorption peak at 280 nm, attributed to chromophoric amino acids like tryptophan and tyrosine, was observed for BSA only. However, an increase in absorbance was noted in the mycotoxin-BSA conjugates (Fig. 2b, c), indicating the unfolding of BSA structure upon conjugation (Khan et al. 2016). This suggests that the mycotoxins are masking the surface of BSA. Specifically, AFB1-BSA exhibited an absorbance of 0.151 at 280 nm, whereas BSA alone had an absorbance of 0.069. Similarly, OTA-BSA displayed an absorbance of 0.189, compared to 0.11 for BSA alone. Notably, AFB1-BSA showed a 54% hyperchromacity, while OTA-BSA exhibited a 41% hyperchromacity, relative to BSA alone. These findings provide valuable insights into the structural changes occurring upon conjugation of mycotoxins with BSA.

3.4 Development of multiplexed immunoassay using Luminex xMAP technology

Luminex suspension assay, based on xMAP technology, is a multiplexed bead-based immunoassay for the simultaneous detection and quantification of multiple analytes in a single sample. In order to develop the Luminex suspension assay, AFB1/OTA-BSA conjugates were coupled with microsphere beads of two distinct spectral region using EDC-NHS. Coupling of conjugates with microsphere beads is one of the most important requirements of the Luminex suspension assay. AFB1/OTA-BSA conjugates bind to carboxylated surface of microsphere beads (Reslova et al. 2017).Coupling of microsphere beads to mycotoxin-BSA was confirmed with two antibodies. The reaction mixture was initially probed with anti-AFB1/OTA mAbs for 30 min, and then it was probed again with variable concentrations of PE-labeled anti-mouse antibodies. These coupled microspheres were then examined using a Luminex analyzer. Interestingly, there was a increase in the average MFI was observed with the increase of PE-labeled anti-mouse antibodies (Fig. 3a, b), whereas the MFI of the background wells was very low (less than 25) it suggests that the AFB1/OTA-BSA were successfully coupled with microsphere beads.

Additionally, to optimize the Luminex suspension assay, we employed the chessboard titration method to determine the appropriate number of coupled microspheres and the concentration of anti-AFB1/OTA mAbs. By testing various combinations, we recorded the MFI of each well. Our optimization finding suggested that 500 coupled microspheres per well, along with a concentration of 0.06 µg/mL of anti-AFB1 mAbs, were sufficient for the establishment of the AFB1 assay. Similarly, for the OTA assay, 1250 coupled

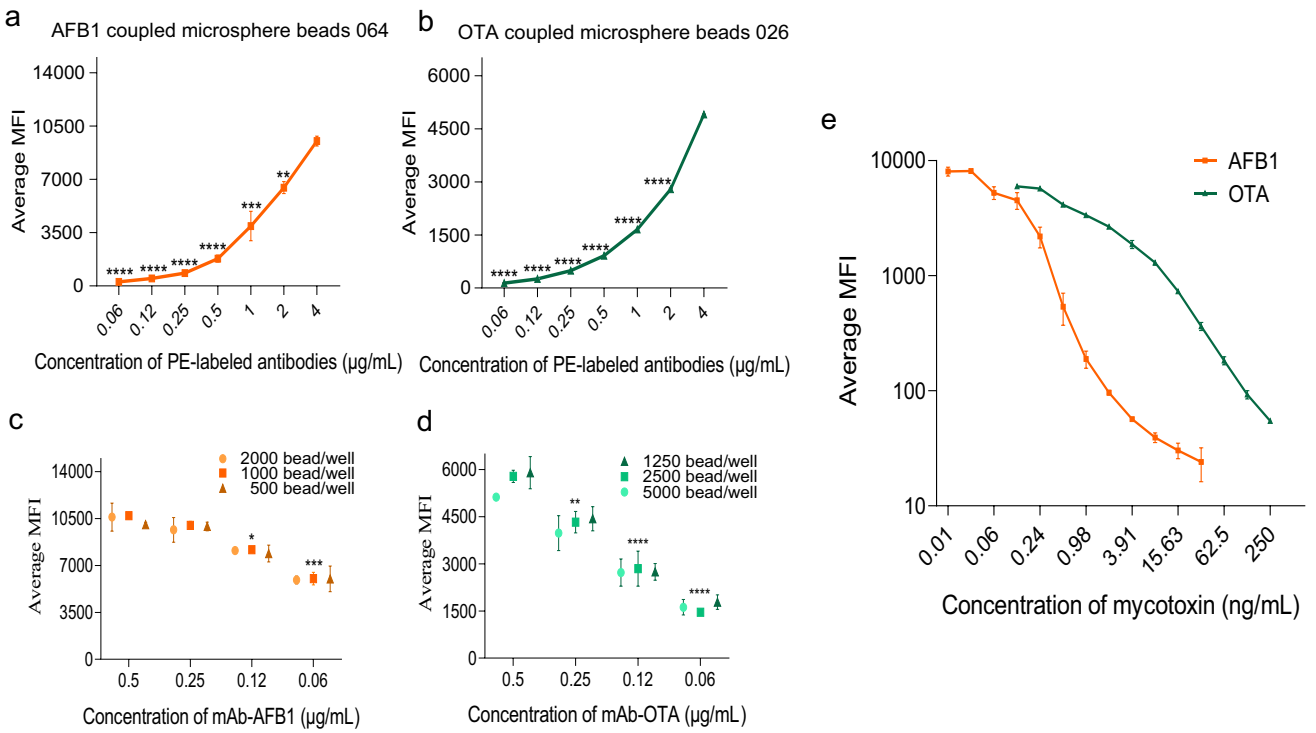

Fig. 3 Development of Luminex suspension assay. **a** Coupling confirmation of AFB1-BSA with microsphere beads region 064 using different concentration of PE-labeled anti-mouse antibodies. **b** Coupling confirmation of OTA-BSA with microsphere beads 026 using different concentration of PE-labeled anti-mouse. **c** Optimization of the number of microsphere beads and anti-AFB1 mAbs concentration. **d** Optimization of the number of microsphere beads and anti-OTA mAbs concentration. **e** Standard curves for the simultaneous detection of AFB1 and OTA in antibody dilution buffer. Each data point represents the mean of three replicates (n = 3). One-way ANOVA was performed and significance is indicated as $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$

microspheres per well and a concentration of 0.5 µg/ml of anti-OTA mAbs were determined to be optimal (Fig. 3c, d).

Subsequently, a competitive inhibition approach was employed to establish the detection range of the Luminex suspension assays. In this approach, the mycotoxin of interest (AFB1/OTA) present in the sample competes with the mycotoxin-BSA conjugate for a limited number of binding sites on the anti-AFB1/OTA-mAb. The availability of binding sites on the mAbs for the mycotoxin-BSA conjugate is inversely related to the concentration of targeted mycotoxin in the samples. At lower concentrations of mycotoxin, there is a higher abundance of binding sites on the mAbs, allowing the mycotoxin-BSA conjugates to effectively bind and increase the average MFI value. Conversely, as the concentration of mycotoxin increases, there is an increased competition between the mycotoxin-BSA conjugate and the mycotoxin in the sample for binding sites on the mAb. This competition reduces the binding of the mycotoxin-BSA conjugate to the mAb, leading to a decrease in the mean MFI values.

Based on this concept, the standard curves were plotted which reflect the competitive inhibition relationship (Fig. 3e). At low AFB1/OTA concentrations, the mycotoxin-BSA dominates the binding to the anti-AFB1/OTA mAbs, leading to higher MFI values. However, below 0.06 ng/ml and 0.49 ng/ml (AFB1 and OTA respectively), the MFI values reach a plateau, indicating that the binding sites on the mAbs was fully accessible to the mycotoxin-BSA, with minimal or no competition from the AFB1/OTA present in the sample. As the mycotoxin concentration increases, the competition between the mycotoxin in the sample and the mycotoxin-BSA reduces the binding of the mycotoxin-BSA to the mAbs, resulting in a decrease in the MFI values. We observed, beyond 31.25 ng/ml and 250 ng/ml (AFB1 and OTA respectively), there was a plateau on MFI values was observed as all the binding sites on mAbs become fully occupied by the mycotoxin present in the sample.

The saturation observed in the standard curves indicates the importance of working within the linear range of the assay where the MFI values are inversely proportional to the AFB1/OTA concentration. Based on the standard curves, the detection range for AFB1 was determined to be 0.06–31.25 ng/ml, while for OTA; it was 0.49–250 ng/ml. Furthermore, LOD for AFB1 and OTA, calculated to be 0.06 ng/ml and 0.49 ng/ml, respectively, represents the lowest concentration of mycotoxin that can be reliably detected above the background signal. Overall, these

Table 1 Per cent cross reactivity of other mycotoxins and metabolites in multiplexed Luminex suspension assay

Mycotoxin (100 ng/mL)	Per cent cross reactivity	
	AFB1 (mAbs)	OTA (mAbs)
AFB1	99.7	< 1
OTA	< 1	96.3
AFM1	94	< 1
AFG1	99.9	< 1
STG	< 1	< 1
FB2	< 1	< 1
DON	1.8	< 1
ZEN	< 1	< 1
Citrinin	< 1	< 1
OTB	< 1	< 1
OTα	–	3.7

results demonstrate the detection range and sensitivity of the Luminex suspension assay for AFB1 and OTA, providing valuable information for its application in mycotoxin analysis and monitoring in various food and feed samples.

Specificity of newly developed Luminex suspension assay

The developed Luminex suspension assay was tested for specificity against the other mycotoxins and their metabolites. AFB1 does not have cross reactive interactions with other mycotoxins (OTA, STG, FB2, ZEA, citrinin, and OTB), while there was high cross reactivity with AFG1 and AFM1. One of plausible reason would be that all aflatoxins share a similar structural make-up, it is conceivable that the employed antibody detected the same epitope in all of them. However, in the case of OTA, there was no cross-reactivity with other mycotoxins (AFB1, STG, FB2, ZEA, Citrinin and OTB), only negligible cross-reactivity with OTα was observed. The results of this experiment are summarized in Table 1.

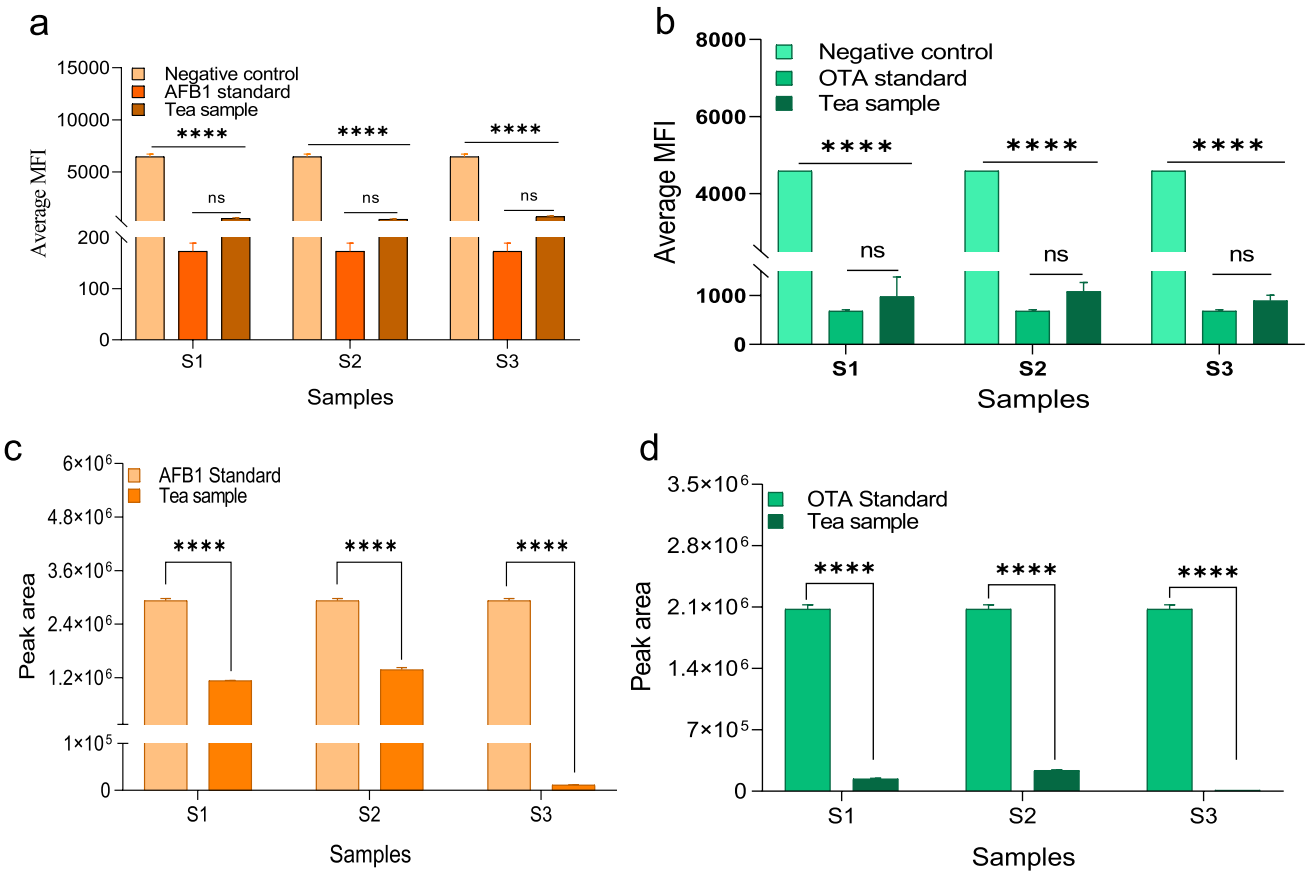

Fig. 4 Evaluation of Luminex suspension assay with tea samples. **a** Multiplexed detection of AFB1using Luminex suspension assay. **b** Multiplexed detection of OTA using Luminex suspension assay. **c** Detection of AFB1 using UPLC. **d** Detection of OTA using UPLC. Each data point represents the mean of three replicates (n = 3). One way ANOVA was performed and significance is indicated as $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$

Table 2 Comparison of the results obtained using UPLC-FLD and Luminex suspension assay

Spiked sample	UPLC-FLD (ng/mL)		Luminex suspension assay (ng/mL)	
	AFB1	OTA	AFB1	OTA
LOD	0.43	0.47	0.06	0.49
LOQ	1.42	1.57	0.12	0.98

Determination of AFB1 and OTA in black tea sample using newly developed Luminex suspension assay

The goal of developed assay was to analyze mycotoxins in food commodities; hence, there is a need to evaluate the developed assay in real sample. Here, working performance was evaluated using black tea samples. This experiment revealed that the negative control for mycotoxins exhibited a higher MFI compared to mycotoxin standards and tea samples. The reason behind this observation is that the negative control, being a buffer solution, does not contain any mycotoxin (AFB1/OTA) that could compete with the AFB1/OTA-BSA-coupled microspheres in terms of binding and detection. On the other hand, the tea samples displayed a lower MFI compared to the mycotoxin negative control, indicating the presence of mycotoxins (AFB1/OTA) in the tea samples. However, the MFI of the tea samples was higher than that of the mycotoxin standards, suggesting that the mycotoxins are present in the tea samples, albeit at lower concentrations than the mycotoxin standards (Fig. 4a, b).

Further, accuracy in mycotoxin quantification of the developed Luminex suspension assay was checked by UPLC. For this purpose, calibration curves were plotted using five different concentrations (0.5–10 ng/ml and 0.25–5 ng/ml for AFB1 and OTA respectively) (Fig. S1). Then, same tested tea samples were analyzed and the higher peak area was observed in mycotoxins standards compared to tea samples. It revealed that the tea samples contained lower concentration of mycotoxins than the mycotoxins standards (Fig. 4c, d). Obtained results are similar to the results obtained using developed luminex suspension assay.

Moreover, the LOD for AFB1 and OTA was determined to be 0.43 ng/ml and 0.47 ng/ml, respectively, using UPLC-FLD. In contrast, the Luminex suspension assay exhibited LOD values of 0.06 ng/ml for AFB1 and 0.49 ng/ml for OTA.

Interestingly, the sensitivity of the developed Luminex suspension assay was significantly higher for AFB1 and OTA (0.12 ng/ml and 0.98 ng/ml, respectively) compared to the UPLC method (1.42 ng/ml and 1.57 ng/ml) (Table 2). This increased sensitivity enables the detection of lower levels of AFB1 and OTA in black tea samples. To ensure the specific interactions, monoclonal antibodies were used and no cross-reactivity was observed, confirming the specificity of these antibodies. This specificity was crucial in establishing a reliable multiplex assay system. Moreover, the Luminex suspension assay required less sample volume of the tea samples and could analyze the concentration of AFB1/OTA within 2.5 h. Developed Luminex suspension assay is less time taking as it is faster than UPLC due to reduced sample preparation time, simultaneous analysis of multiple analytes, and rapid assay development. This increased efficiency and reduced sample volume compared to UPLC methods were achieved by enabling the simultaneous detection of multiple mycotoxins.

The developed Luminex suspension assay is more sensitive compared to UPLC and less time taking as it is faster than UPLC due to reduced sample preparation time, and it is useful forsimultaneous analysis of multiple analytes in a single sample. The use of monoclonal antibodies and the multiplex assay system in conjunction with the Luminex platform allowed for accurate and simultaneous detection of AFB1 and OTA in food samples.

In conclusion, traditional detection methods are limited in terms of throughput and simultaneous detection of multiple mycotoxins. The developed Luminex suspension assay using x-MAP technology offers a sensitive, scalable, and time-efficient solution for multiplexed mycotoxin detection. Moreover, developed Luminex suspension assay is able to detect AFB1 and OTA simultaneously in tea samples. In future research, there is potential to expand the method to encompass the testing of additional mycotoxins and improve multiplexed analysis beyond the current simultaneous detection of two mycotoxins. Ultimately, this assay offers a valuable tool for high-throughput screening of multiple mycotoxins, thereby, enhancing capabilities for food safety analysis.

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Author contributions IJS conceived, carried out the experiments and wrote the manuscript; MK carried out the experiments, KM carried out the experiments; PM analyzed and interpreted the data; VS supervised the Luminex suspension array experiments; KMA conceived, designed the experiments; corrected the manuscript.

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Availability of data and material Data is available from the corresponding author upon reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest (include appropriate disclosures) Not applicable.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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