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A highly stable, sensitive, regenerable and rapid immunoassay for detecting aflatoxin B_1 in corn incorporating covalent AFB_1 immobilization and a recombinant Fab antibody

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ABSTRACT

A highly robust immunoassay applicable for the detection of aflatoxin B_1 (AFB_1) using a Fab antibody fragment was developed. A key factor was the use of covalently immobilized AFB_1 which allowed an almost three fold increase in sensitivity, reduced assay time and regeneration with retention of binding capacity. Various factors that might affect the sensitivity of the assay such as pH, organic solvents, storage stability and wash stringency were critically evaluated. It was also demonstrated that the assay was applicable for determination of AFB_1 in corn samples at concentration within the European union regulatory limits.

1. Introduction

Aflatoxins are toxic secondary metabolites produced by fungi of *Asperigillus flavus* and *A. parasiticus.* AFB_1 is the most potent carcinogen and is present in food products like maize, peanuts, pecans, almonds, hazelnuts, brazil nuts, cheese, corn, pistachio nuts, almonds, spices and walnuts [1]. Due to the adverse effects of this toxin on humans and livestock, European Union regulatory authorities have set the maximum limit to 20 ppb. Hence, there is a crucial requirement to develop highly sensitive immunoassays for its detection.

In recent years, a number of multi-toxin arrays were developed incorporating toxins linked to solid supports such as glass slides, gold surface and polystyrene microtiter plates through either covalent or non-covalent immobilization strategies [2–6]. Non-covalent approaches for coating hapten–protein conjugates onto solid surfaces have been used frequently [7]. However, a key issue is the sub-optimal orientation of hapten on the solid surface [8], leading to its non-availability for binding by the labeled antibody and, hence, weak signals may be obtained. More predictable orientation of hapten can be obtained with covalent immobilization to improve its accessibility to the antibody, thereby, resulting in a higher signal and associated improvement in sensitivity [9]. In general, the synthesis of hapten–protein conjugates with reproducible hapten density may be problematic. The efficiency of conjugation varies depending upon the experimental conditions and degree of degradation during storage [10]. In addition, the decreased sensitivity of immunoassays with hapten–protein conjugate-coated plates in the presence of organic solvents such as methanol [11,12] and pH changes [13] was reported. Therefore, the effective covalent immobilization of antigens onto solid supports is vital for the stability and sensitivity of the assay since the covalent bond is resistant to extremes of pH, ionic strength, substrates, solvent and temperature [14].

Critical requirement of a covalent immobilization strategy for the development of immunoassay involves a stable linkage between the solid support and hapten in its functionally active form. The stable assays developed on such platforms are particularly suitable for field analysis of mycotoxins. Usage of covalently-immobilized antigen on microtiter plates may also assist in performing biopanning techniques for specific recombinant antibody selection. Covalent attachment to solid supports requires available functional groups on the haptens that are not involved in specific antibody recognition. They commonly have hydroxyl, amine, carboxyl, carbonyl or sulfhydryl functional groups present either in the actual hapten structure or introduced by its derivatization with suitable linkers. Lack of suitable functional groups on the surfaces of microtiter plates was overcome by amine activation, which can be achieved by various methods. Most exploit the

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use of hydrophilic spacer arms such as 3-aminopropyl diisopropyl ethoxysilane, 3-glycidoxypropyl diisopropylethoxysilane or 3-aminopropyl triethoxysilane [15]. Chemical immobilization of haptens on microtiter plates has been successfully achieved using glutaraldehyde [16], amide [17–19], disulfide [20] and in particular, amide linkage. The latter is one of the most stable immobilization methods and hence, it was selected for the first demonstration of carboxylated AFB_1 covalent immobilization on amine-activated plates.

In the present study, we demonstrate the functionality of AFB_1 covalently immobilized onto microtiter plates by indirect and competitive ELISAs using a recombinant Fab antibody fragment that was generated in our lab [21]. The results show that the overall performance of the covalently-immobilized AFB_1 was significantly improved in comparison to a passively-adsorbed AFB_1–BSA conjugate and that the AFB_1 covalently-immobilized plates can readily be used for AFB_1 detection in corn samples.

2. Materials and methods

2.1. Reagents and apparatus

N-hydroxysuccinimide (NHS), N,N′ dicyclohexylcarbodiimide (DCC), H_2SO_4 (97% purity), H_2O_2, 1,4-dioxane, 3-aminopropyltriethoxy silane (APTES), bovine serum albumin (BSA), formaldehyde, methanol, urea, sodium dodecyl sulfate, aflatoxin B_1 (AFB_1), Tris–HCl, sodium hydroxide (NaOH), mercaptoethanol, sodium chloride, celite filter aid and tetrabutyl ammonium fluoride (TBAF) were obtained from Sigma aldrich (St. Louis, MO, U.S.A). Corn reference material (TR-A100) was obtained from Trilogy labs (Washington, MO, U.S.A). The recombinant anti-AFB_1 antibody Fab fragment was generated in Dublin City University [21]. The AFB_1–BSA, KLH, OVA conjugate was synthesized by the method of Chu et al. [22]. 1X PBS (0.15 M NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4 and 18 mM KH_2PO_4 in 1 L of distilled water with pH adjusted to 7.4) and 1X PBST (1X PBS with 0.5% (v/v) Tween 20) were used. Absorbance readings were measured on a TECAN Saffire II plate reader at 450 nm. MaxiSorb™ Nunc 96 well ELISA plates were used in all analyses. Mass spectroscopy was performed on a Bruker Daltonic Esquire-LC-ion trap instrument.

2.2. Covalent immobilization of AFB_1

2.2.1. Passivation and amine functionalization of microtiter plate surface

Individual wells of the microtiter plate were first cleaned three times with 250 μL of distilled water. The wells were incubated with 250 μL of freshly prepared piranha solution (1:3, H_2O_2:conc. H_2SO_4) for 1 h with constant shaking at 37 °C. The plate was then washed three times with distilled water (250 μL/well). Then 250 μL of 5% (v/v) APTES in distilled water was added to each well and incubated at 60 °C for 1 h. The amine-functionalized wells were washed three times with 250 μL of 30% (v/v) ethanol in water and once with 1X PBS and then with distilled water to remove excess APTES from the wells. The plates were dried at 80 °C for 1 h.

2.2.2. Synthesis of AFB1–CMO–NHS ester

AFB_1 (1 mg) was dissolved in 250 μL of ethanol and 50 μL of 1 M NaOH added. The reaction mixture was stirred for 15 min at room temperature after the addition of carboxylamine hydrochloride (10 mg). The mixture was refluxed in an oil bath for 3 h, with constant stirring, followed by overnight stirring at room temperature. The solvent was evaporated in a DNA 23050-A00 model Speed Vac apparatus (GeneVac Ltd., IP SWICH, England). The residue was then dissolved in 300 μL of water. The pH of the aqueous solution was adjusted to 9.1 using 0.5 M NaOH and then the unreacted AFB_1 was extracted with 500 μL of ethylacetate. The pH of aqueous layer was adjusted to 2.0 using 6 M HCl, followed by extraction with 500 μL of ethylacetate to obtain AFB_1–CMO (aflatoxin B_1–carboxymethyl oxime). The solvent was evaporated in a Speed Vac apparatus and the product was confirmed by TLC performed on silica gel plates with fluorescent indicator (254 nm) developed in methanol:dichloromethane (7:3). AFB_1–CMO (1 mg) was dissolved in 200 μL of dioxane, DCC (1.5 equivalents) and NHS (1.5 equivalents) were added and the reaction mixture was stirred overnight at room temperature. The precipitated dicyclohexyl urea was then removed by centrifugation at 14,000 rpm for 10 min. The presence of the NHS ester was confirmed by TLC using silica gel plates with fluorescent indicator (254 nm) developed in methanol:dichloromethane (7:3).

2.2.3. Immobilization of the AFB_1–CMO–NHS ester onto microtiter plates

The NHS ester in 1, 4-dioxane was dissolved in 1 M sodium hydrogen carbonate buffer (pH 9.4). To determine the optimal concentration of AFB_1–CMO–NHS ester for efficient signal generation, a checker board ELISA was performed by varying the NHS ester concentration (25, 50, 100 and 200 μg/mL). Optimization of the covalent attachment of the ester on amine-modified plates was performed by overnight incubation of the individual wells with 100 μL of ester at 4 °C, 37 °C and room temperature. A competitive assay was performed using plates incubated at three different temperatures. The IC_{50} value, which is half the maximal inhibitory value of the antibody in the presence of free antigen, was assessed.

2.2.4. Fluorescence assay to estimate loading value of AFB_1

To estimate the amount of covalently-immobilized AFB_1, individual wells of the microtiter plate were incubated with 150 μL of 1 M tetrabutylammonium fluoride (TBAF) in H_2O for 60 min at room temperature. A similar incubation procedure was performed with APTES-modified, unmodified and passively adsorbed (AFB1–BSA conjugate-coated) wells as negative controls. A set volume (100 μL) TBAF solution was removed from the wells and added to the individual wells of a fresh black MaxiSorb™ microtiter plate (Nunc). The fluorescence intensities were measured at 360/440 nm (excitation/emission) against standard AFB_1 concentrations (2.5–25 μg/mL of AFB_1). The unknown concentration of AFB_1 (nmol/cm^2) was calculated by subtracting the absorbance of APTES-treated wells from AFB_1 covalently-immobilized wells and then substituted in the slope equation $y=332.78x$ ($R^2=0.98$) of the calibration curve generated with fluorescence intensities on Y-axis against concentration of AFB_1 on X-axis.

2.3. Confirmation of covalent attachment of AFB_1 on microtiter plate

2.3.1. Mass spectroscopy analysis

The cleavage of the AFB_1–CMO–NH–$(CH_2)_3$–Si–O-polystyrene from the wells of the microtiter plate that was covalently-immobilized with AFB_1 was performed by incubating the wells with 1 M TBAF in H_2O. The cleaved TBAF product (AFB_1–CMO–NH–$(CH_2)_3$–$Si(OH)_3$) was purified using a C18 reverse phase column. The sample was eluted using a mixture (1:1) of acetonitrile and water, dried in a Speed Vac apparatus and dissolved in LC/MS grade acetonitrile and water in a 1:1 ratio (v/v). The sample (100 μL) was injected into the ESI mass spectrometer and analyzed in positive mode with a range of interpreted molecular weights of AFB_1 and its derivates (300–600 Da).

2.3.2. Indirect ELISA

The titer of the recombinant antibody was determined by an indirect ELISA. The amine groups on the microtiter plate with covalently-immobilized AFB_1 were first blocked with 200 μL of 0.2 M formaldehyde in PBS for 2 h at 37 °C then washed three times with both PBST and PBS. Individual wells were incubated with 100 μL of serially diluted anti-AFB_1 antibody in 2% (w/v) milk Marvel PBST for 2 h at 37 °C followed by three washes with both PBST and PBS. The visualization was achieved by addition of 100 μL of chromogenic reporter antibody i.e. anti-HA-HRP in 2% (w/v) milk Marvel PBS (1:1000), to each well followed by incubation at 37 °C for 1 h and washing three times with both PBST and PBS. Finally, 100 μL of the substrate, TMB (3,3′,5,5′-tetramethylbenzidine dihydrochloride), solution was added to each well and incubated at room temperature for 15 min before the reaction was stopped with 50 μL of 1 M HCl. The resulting absorbance (450 nm) was read in a TECAN Saffire II plate reader at 450 nm. Similarly, the titer value of the recombinant antibody was determined on the microtiter plate that was coated with 5 μg/mL of AFB_1–BSA (passively-adsorbed AFB_1–BSA conjugate) with the single exception that 4% (w/v) milk Marvel in PBS was used as a blocking agent instead of 0.2 M formaldehyde. An anti-C reactive protein (anti-CRP) antibody was used as a control in the indirect ELISA performed on both the microtiter plates (with either covalently or passively adsorbed AFB_1).

2.3.3. Competitive indirect ELISA (CI-ELISA)

For competitive ELISAs microtiter plates with covalently-immobilized AFB_1 and passively-adsorbed AFB_1–BSA conjugate were blocked, as detailed above (Section 2.3.2). A 1:10,000 dilution of the anti-AFB_1 Fab antibody in 2% (w/v) milk Marvel PBST was incubated for 30 min at 37 °C with varying concentrations of free AFB_1 toxin (0.0048–2.5 μg/mL) in the ratio of 1:1. Samples were added to each well (in a total volume of 100 μL/well) and incubated for 2 h at 37 °C followed by three washes with both PBST and PBS. To each well 100 μL of secondary antibody (anti-HA-HRP in 2% (w/v) milk Marvel PBS (1:1,000 dilution)) was added and incubated for 1 h at 37 °C followed by three washes with both PBST and PBS. Finally, 100 μL of HRP substrate TMB (3,3′,5,5′-tetramethylbenzidine dihydrochloride) solution was added to each well and incubated at room temperature for 15 min and the reaction was stopped with 50 μL of 1 M HCl. The absorbance was read in a TECAN Saffire II plate reader at 450 nm. CI-ELISA was also performed on microtiter plates that were coated with 5 μg/mL of AFB_1–KLH and OVA. Due to the lack of variation in the antibody recognition characteristic when assays were performed using either KLH, BSA and OVA conjugates of AFB_1, further assays were performed using AFB1–BSA.

2.4. Cross reactivity studies

Cross reactivity studies were performed on both the covalently-immobilized and passively-adsorbed plates by CI-ELISA (Section 2.3.3) using 0.0048–2.5 μg/mL of AFB_2, G_1 and G_2 and 0.009–10 μg/mL Zearalenone, Fumonisin B_1 and T-2 toxins.

2.5. Evaluation of parameters and interfering agents affecting the assay sensitivity

The sensitivity of the CI-ELISA (Section 2.3.3) was evaluated by varying incubation time, washing, methanol concentration and acidic/basic conditions. The optimal period for antigen/antibody binding to get adequate response was determined by varying the incubation times of both primary and secondary antibody (20, 40, 60, 80, 100 and 120 min), whilst keeping substrate incubation time constant (15 min at room temperature). The influence of increased washing on the assay sensitivity was studied by varying the number of washes performed (3, 6, 9 and 12 times) with both PBST & PBS, with washes performed after each step of incubation during the ELISA. Assay performance in the presence of varying concentrations of methanol (0% (v/v), 10% (v/v), 20% (v/v), 30% (v/v) and 40% (v/v)) in the diluant used for diluting the primary antibody was also studied. The stability of the assay in acidic and basic conditions was tested by varying the pH of the primary antibody diluant to 6, 7.4 and 9.

2.6. Stability and re-usability studies

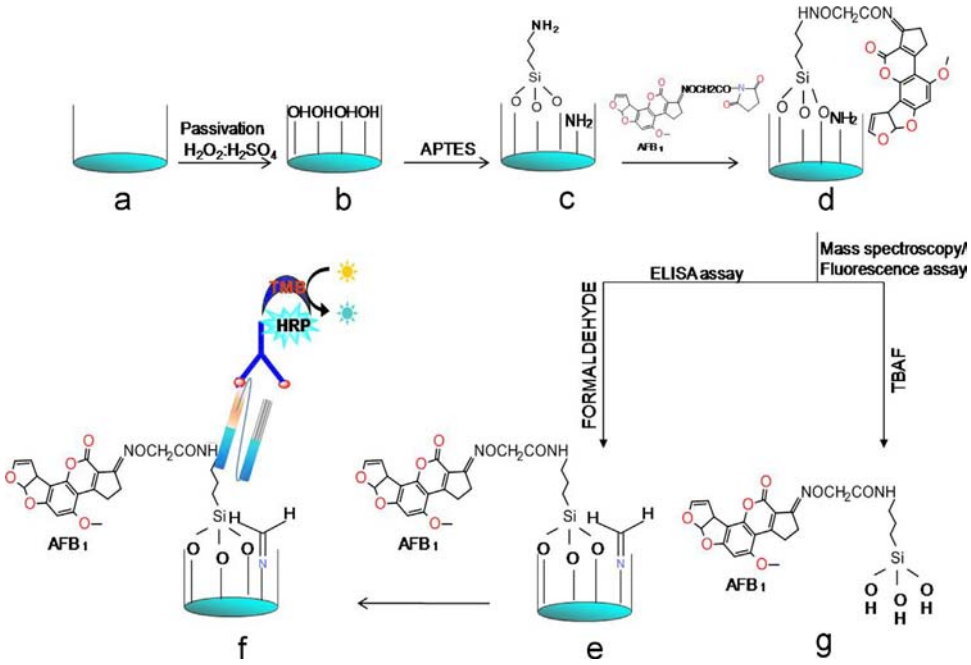
The time-dependent stability of both covalently and passively adsorbed microtiter plates was investigated for 14 days by performing CI-ELISA (Section 2.3) using the microtiter plates that were stored in a vacuum desiccator at room temperature. Regeneration of the surface of microtiter plates was studied using three different dissociation solutions, with chaotropic agents for antibody removal, i.e., glycine-HCl [23], 2 M NaOH and a mixture of 8 M urea, 2% (w/v) sodium dodecyl sulfate with 2% (v/v) mercaptoethanol. The individual wells of the plates were treated with 150 μL of the dissociation solution for 30 min on an orbital IKA MTS 2/4 digital microtiter shaker at 300 rpm at room temperature after performing CI-ELISA, as described in Section 2.3. The plates treated with 8 M urea mixture were washed once with a solution containing 20 mM Tris–HCl (pH 7.5), 0.1% (v/v) Tween 20 and 500 mM NaCl [24]. The plates, treated with glycine-HCl and 2 M NaOH, were washed once with PBS. The competitive immunoassay was performed after incubating the plates for 30 min at 4 °C. In order to ensure complete removal of the immuno-complex after the chaotropic treatment, control experiments were performed. These included the addition of substrate only, secondary enzyme-labeled antibody with substrate, non-specific primary antibody with secondary enzyme-labeled antibody and substrate to the chaotrope-treated microtiter plates. The absence of signal with the negative controls signifies complete removal of the immuno-complex.

2.7. Extraction of AFB_1 from corn and its utilization for validating immunoassay developed on AFB_1 covalently-immobilized plates

Reference corn material (TR-A100) (10 g) containing 3.5 μg/kg of aflatoxin B_1 and 0.2 μg/kg of aflatoxin B_2 was extracted for 30 min with 40 mL of 80% (v/v) methanol in the presence of 500 mg of celite filter aid and 500 mg of sodium chloride [25]. The extract was centrifuged at 4,000 rpm for 30 min and filtered using Whatman Grade 1 filter paper. The solvent was evaporated in a rotary evaporator and the residue was diluted in 40% (v/v) methanol in water. The concentration of AFB_1 in the crude extract was quantified by fluorescence estimation (Section 2.2.4) at 360/440 nm (Excitation/Emission) using standard calibration curve (slope equation $y=361.7x$ ($R^2=0.99$)) with known amounts of AFB_1 (10–320 ng/mL) diluted in 40% (v/v) methanol in water. The efficiency as well as the repeatability of extraction procedure was evaluated through performing 10 similar extractions using 10 g of corn samples. The corn extract was used to perform CI-ELISA on AFB_1 covalently immobilized microtiter plates (Section 2.3.3). The limit of blank (LOB) was determined through recording the absorbance value of twenty blank samples. The limit of detection (LOD) was calculated using the formula LOB+3 × S.D. of LOB.

3. Results and discussion

The covalent immobilization strategy for AFB_1 involves a three step process on an amine-activated polystyrene surface of the



Scheme 1. Schematic representation of AFB_1 immobilization and assay format development. The wells of the microtiter plate were activated for the generation of hydroxyl functional groups (b) and addition of 3-aminopropyltriethoxy silane (APTES) led to the formation of amine groups (c). The modified AFB1–CMO-ester was attached to the surface through amide bonding (d). Two alternative procedures were performed on these plates. One set of plates was first reacted with 0.2 M formaldehyde to block the unreacted amine groups (e). Anti-AFB_1 antibody, its chromogenic reporter anti-HA-HRP and substrate 3,3′, 5,5′-tetramethylbenzidine dihydrochloride (TMB) solution were added (f). Another set of plates were used for estimation of the levels of AFB_1 immobilized on the surface, through cleaving Si–O-bond from the surface of the modified microtiter plate using 1 M tetrabutyl ammonium fluoride (TBAF). The cleaved product (g) was used for quantification of the covalently immobilized AFB_1 by fluorescence assay and the molecular weight of the cleaved compound was determined using ESI mass spectroscopy.

microtiter plates as depicted in Scheme 1. Firstly, hydroxyl functional groups were generated through treating individual wells of the microtiter plates with piranha solution. Secondly, NH_2 groups were incorporated by surface modification with APTES (3-aminopropyl-triethoxy silane). To avoid multi-layers of APTES on the surface, the plates were washed thoroughly with 30% (v/v) ethanol and PBS. The plates were then incubated at 80 °C to achieve stable cross-linked attachment of APTES to the surface. The final step in the immobilization process was the covalent linkage of AFB_1 onto the modified surfaces of the microtiter plates. This was performed through the addition of AFB_1–CMO ester, which was synthesized according to the procedure described in Section 2.2.2. The stability of AFB_1–CMO–NHS was verified by TLC every week up to 8 weeks, and it was found to be stable at 4 °C. The stable amide bond formation, which governs the covalent linkage of AFB_1 to the modified surfaces of the microtiter plate, was achieved through overnight incubation of the plates with AFB_1–CMO ester at 4 °C.

The covalent attachment of AFB_1 on the microtiter plate was optimized through varying the ester concentration and temperature. The efficiency of AFB_1–CMO–NHS ester coupling on microtiter plates was evaluated by varying the coupling temperature (4 °C, 37 °C and room temperature). The efficiency of covalent linkage was reduced at 37 °C and room temperature, which may be due to the increase of hydrolysis of NHS ester at higher temperatures that leads to reduced amine reactivity. Checker-board ELISA was performed to determine the optimal amount of AFB_1–CMO–NHS ester for covalent immobilization. For this varying concentrations of ester were immobilized and competitive inhibition assays performed. It was found that 50 μg/mL of ester was more effective in producing an acceptable absorbance response, with minimal undesirable background and better 'signal-to-noise' ratios (*S*/*N* ratio). Thus, for the generation of an ideal AFB_1 covalently-immobilized plate, usage of 50 μg/mL of ester at 4 °C incubation was optimal.

The degree of covalent linkage was determined by quantifying the amount of covalently-immobilized AFB_1 released following cleavage with 1 M TBAF [26], using a fluorescence-based assay. The initial loading value of AFB_1 onto the surface of covalently-immobilized microtiter plates was 0.733 nmol/cm^2, as determined by fluorescence assay. The molecular weight of the cleaved product was determined by ESI mass spectroscopy. The chromatogram of the cleaved product from the mass spectroscopy data showed a major peak at 503.6 (Fig. 1), which corresponds to AFB_1–CMO–NH–$(CH_2)_3$–$Si(OH)_3$ (calculated: 504 Da, observed: 503.6 Da). From this it can be concluded that neither free AFB_1 (M.Wt. 312.27) nor the ester of AFB_1–CMO-COOH (M.Wt. 385) was present on the surface of the covalently-immobilized plate.

After successful covalent attachment of AFB_1 onto the microtiter plates surface, the next step was to demonstrate a functional immunoassay. In the process of assay development, first the unreacted NH_2 groups were blocked using 0.2 M formaldehyde, which leads to the formation of a Schiff's base. The blockage of the plates was followed by the determination of functionality of the microtiter plate using indirect and CI-ELISAs with the anti-AFB_1 Fab antibody fragment. An anti-C-reactive protein (anti-CRP) recombinant antibody, which should not bind to the hapten or its conjugate was used as a control. The absence of signal without anti-AFB_1 Fab antibody fragment in the assay, indicated integrity of the immobilized AFB_1. The negative control experiments were performed by coating the microtiter plate with milk Marvel and BSA in the case of the AFB1–BSA passively-adsorbed plate and formaldehyde and PBS in the case of the AFB_1 covalently-immobilized plates. CI-ELISAs performed on AFB_1 covalently-immobilized and AFB_1–BSA passively-adsorbed microtiter in buffer has an IC_{50} of 27 ± 2.7 ng/mL (mean ± S.D.; n=3) and 77 ± 2.6 ng/mL (mean ± S.D.; n=3) (Fig. 2), respectively. Thus the assay with chemically immobilized AFB_1 has a 2.7 fold higher detection sensitivity when compared to the passively-adsorbed AFB_1–BSA with 1.67 nmol/cm^2 of AFB_1 (calculation done

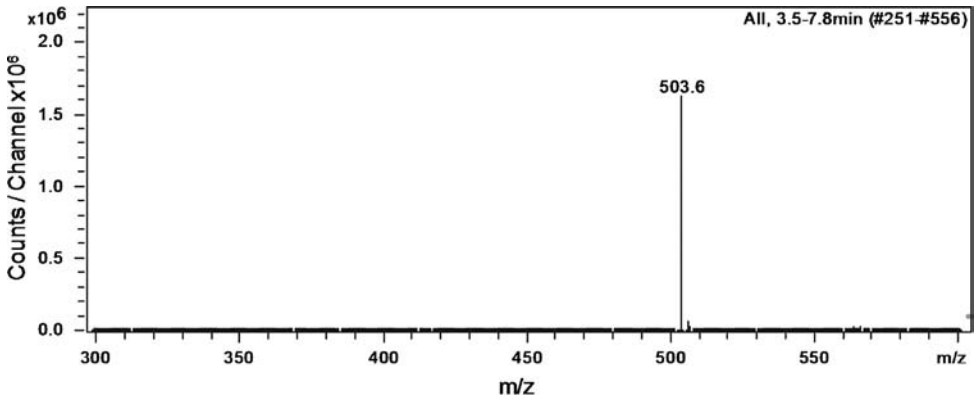


Fig. 1. Analysis of the cleaved AFB_1 that was covalently immobilized on the surface of the microtiter plate. The covalently linked AFB_1 was cleaved from the surface using 1 M tetrabutyl ammonium fluoride (TBAF). ESI mass spectrum in positive mode showing the products cleaved form AFB1–CMO–NH–(CH2)3–Si–O-polystyrene (wells) using 1M TBAF. The cleaved product was purified on C18 column and eluted with acetonitrile and water (1:1 (v/v)). The molecular weight of the cleaved product corresponds to 503.6 Da.

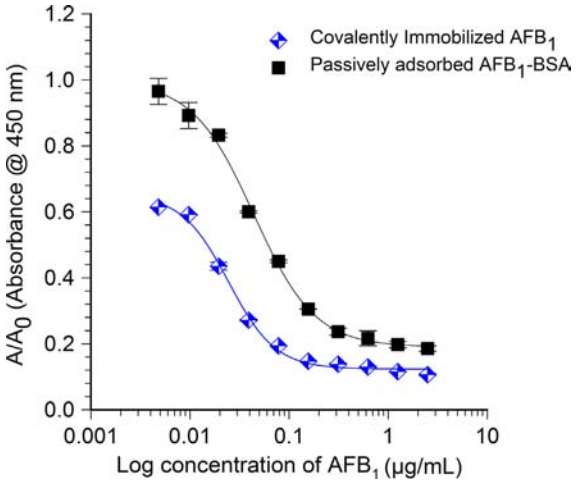


Fig. 2. Representative standard curve for CI-ELISA. The CI-ELISA was performed on the microtiter plates with covalently- (◆) immobilized AFB_1 and passively- (■) adsorbed AFB_1–BSA conjugate using a 1:10,000 dilution of the anti-AFB_1 Fab antibody inhibited with varying concentrations of AFB_1 (0.0048–2.5 µg/mL). A/A_0 was calculated where A is the absorbance of antibody in presence of varying AFB_1 concentration and A_0 is the absorbance of antibody in the absence of free AFB_1. The error bars represents the standard deviation of three independent determinations.

based on MALDI-TOF analysis, which indicates the presence of 30 mol of AFB_1/mole of BSA).

The specificity of the assay developed on AFB_1 covalently immobilized plates and AFB_1–BSA passively adsorbed plates was determined against aflatoxin congeners (AFB_1, B_2, G_1 and G_2) and other mycotoxins that might co-occur with aflatoxins like Zearalenone, Fumonisin B_1 and T-2 toxin at concentrations of 10 µg/mL. It is evident from the results that the antibody is specific to AFB_1 in comparison to other aflatoxin variants, as indicated by the percentage cross reactivity data presented in Table 1. The lack of cross reactivity to other mycotoxins such as Zearalenone, Fumonisin B_1 and T-2 toxin (data not shown) indicates the specificity of the antibody to aflatoxins. Upon comparing the percent cross reactivity profile data obtained from assay performed on AFB_1 covalently-immobilized and AFB_1–BSA passively-adsorbed microtiter plates, it was noticed that there was a variation in the percentage cross reactivity profile exhibited. Lower percentage cross reactivity towards all the aflatoxin variants was found when the assay was performed on covalently-immobilized plates.

The effect of incubation times of the primary and secondary antibody on the assay sensitivity was investigated. The sensitivity

Table 1
Cross reactivity profile of the antibody when assayed on AFB_1-covalently immobilized and AFB_1–BSA passively adsorbed microtiter plates.

Mycotoxin	AFB_1-covalently immobilized plate		AFB_1–BSA passively adsorbed plate	
	IC_{50} value (ng/mL ± S.D.)[a]	% Cross reactivity[b]	IC_{50} value (ng/mL ± S.D.)[a]	% Cross reactivity[b]
AFB_1	20 ± 1	100	72 ± 1	100
AFB_2	1000 ± 1	2	1600 ± 1	4
AFG_1	110 ± 1	18	240 ± 1	30
AFG_2	1500 ± 1	1	1700 ± 1	4

[a] IC_{50} is a measure of the amount of AFB_1 required to inhibit the anti-AFB_1 Fab antibody by half the value of maximum inhibition value.
[b] is calculated using the formula IC_{50} value of AFB_1/IC_{50} value of test mycotoxin × 100.

of the assay performed on microtiter plate with covalently-immobilized AFB_1 remains unaltered, with the increase of incubation time from 20–120 min. The usage of the microtiter plate with covalently-immobilized AFB_1 eliminates longer incubation steps, thus reducing the assay time to 75 min resulting in a more rapid assay. Contrastingly, decreasing the incubation time to 20 min, led to a decreased assay sensitivity from 79 ± 1.34 ng/mL (mean ± S.D.; $n=3$) to 100 ± 0.98 ng/mL (mean ± S.D.; $n=3$) for the assays performed on passively-adsorbed AFB1–BSA conjugate microtiter plates. From the experimentation it was found that 60–80 min (for AFB1–BSA passively adsorption) and 20 min (for AFB_1 covalent immobilization) of incubation times are required for attaining the desired sensitivity levels. Hence, it is clearly evident that assay time does not affect the relative antibody binding to covalently-immobilized plates, which will affect the sensitivity of the assay.

The robustness of the assay was evaluated through investigating various physiochemical factors that might affect the assay sensitivity. Increasing the stringency of washes (3–12 times with both PBST and PBS) did not have any significant effect on the sensitivity of the assay performed (Table 2). This suggests that the level of haptens availability on the surface for antibody binding is not effected through stringent washes performed on the plates that were covalently-immobilized with AFB_1 or passively-adsorbed AFB_1–BSA conjugate.

In many analytical sample preparation regimes, AFB_1 will be extracted from food matrices by methanol, so the effect of methanol concentration (Table 2) on the sensitivity of assay was deemed an important parameter to evaluate. From the results obtained, a decrease in the sensitivity of assay was determined

Table 2
Evaluation of the factors altering the assay performance.

Factor	IC_{50} value[a] (ng/mL ± S.D.)	
	Covalently immobilized AFB_1	Passively adsorbed AFB1–BSA
Number of washes with both PBST & PBS		
3	17 ± 1	72 ± 1
6	25 ± 0.5	80 ± 1
9	25 ± 0.6	75 ± 1
12	22 ± 1	77 ± 1
Methanol (v/v)		
0%	22 ± 2	75 ± 3
10%	32 ± 1	110 ± 1
20%	35 ± 1	112 ± 2
30%	25 ± 2	115 ± 2
40%	33 ± 2	125 ± 2
pH		
6	22 ± 1	79 ± 1
7.4	28 ± 1	80 ± 0.5
9	26 ± 1	74 ± 1.5

[a] IC_{50} is a measure of the amount of AFB_1 required to inhibit the anti-AFB_1 Fab antibody by half the maximum inhibition value. The units are ng/mL ± S.D. of the value obtained from three independent determinations. The IC_{50} values of both the covalently-immobilized AFB_1 and passively-adsorbed AFB_1–BSA conjugate were compared under varying experimental conditions.

with the increasing methanol concentration from 0% to 40% (v/v) i.e. from 75 ± 2.5 ng/mL (mean ± S.D.; $n=3$) to 125 ± 2 ng/mL (mean ± S.D.; $n=3$) in the case of passively-adsorbed AFB_1–BSA conjugate. The sensitivity of the assay performed on the microtiter plate with covalently-immobilized AFB_1 remains unaltered upto 40% (v/v) methanol, after which the assay sensitivity was reduced drastically.

Alterations in pH of the diluants demonstrated no significant impact on the CI-ELISA sensitivity performed on both the microtiter plates with covalently- immobilized AFB_1 and passively-adsorbed AFB_1–BSA conjugate. It was clearly evident that both covalently-immobilized AFB_1 and the passively-adsorbed AFB_1–BSA conjugate are stable with regard to CI-ELISA sensitivity over the range of pH 6–9 (Table 2).

Storage stability of microtiter plates (covalently-immobilized and passively-adsorbed) were determined by placing the plates in a vacuum dessicator at room temperature. CI-ELISAs were performed on the stored microtiter plates. The covalently-immobilized AFB_1 and passively-adsorbed AFB_1–BSA conjugate microtiter plates were found to be stable up to 14 and 10 days, respectively. The re-usability of the microtiter plates was tested by treating the plates with a chaotropic mixture of 8 M urea, sodium dodecyl sulfate and mercaptoethanol. The sensitivity of the assay performed on the non-chaotropic-treated microtiter plate was considered as 100%. The percentage variation in the sensitivity of the assay performed on both microtiter plates with covalently-immobilized AFB_1 and passively-adsorbed AFB_1–BSA conjugate, was analyzed by using the formula (obtained value/initial value) × 100. Upon successive usages, after treatment with the dissociation buffer, the percentage binding retention when compared to the initial 100% were 96%, 96%, 84%, 80% and 60% in the case of covalently-immobilized microtiter plate, whereas it was 75%, 73%, 50% and inconsistent values from the fourth usage onward, in the case of passively-adsorbed plates. In short, the microtiter plates that were covalently-immobilized with AFB_1 could be reused for four times with an average agreement of 89% for four successive re-uses, whereas the passively adsorbed AFB_1–BSA conjugate plates could be reused twice with an average agreement of 74% with the initial usage.

Microtiter plates with covalently-immobilized AFB_1 were successfully used to detect AFB_1 in corn reference samples. Based on the findings of Lee et al. [27] that methanol concentration below 80% (v/v) will result in lowered extraction efficiency from food samples, 80% (v/v) methanol was used to extract AFB_1 from corn samples. After extraction the final methanol concentration was set to 40% (v/v). The amount of AFB_1 recovered was assessed by fluorescence estimation at Ex/Em=365/455 nm [28], whose values were in agreement with the values obtained by the Triology standard protocol. The certified level of aflatoxins in the corn sample (TR-A100) was 3.7 μg/kg and the obtained levels (fluorescence estimation) were 3.5 ± 0.9 μg/kg (mean ± S.D.; $n=10$).

The IC_{50} value of the CI-ELISA performed on AFB_1-covalently immobilized plate was 35 ± 1.1 ng/mL (matrix) and 27 ± 2.7 ng/mL (buffer) (mean ± S.D.; $n=3$) with LODs 12 ± 2.3 ng/mL (mean ± S.D.; $n=3$) (matrix) and 9 ± 1.2 ng/mL (mean ± S.D.; $n=3$) (buffer). The discrepancies of LODs are comparable with the other immunoassays for the corn matrix [26]. The trueness of the assay was evaluated through performing intra- ($n=3$) and inter-day ($n=5$) assay using the corn extract. The percent CVs obtained in both intra- and inter-day assays were less than 5. It is apparent from the results that the corn matrix has not significantly affected the robustness and sensitivity of the CI-ELISA performed on covalently-immobilized AFB_1 microtiter plates. Despite of the matrix effect, the sensitivity of assay developed on AFB_1-covalently immobilized microtiter plate are within the FDA action levels of 20 ppb (ng/g) and this assay can readily be used for the detection of aflatoxin B_1 in corn samples and might be used for the detection of AFB_1 in other food matrices.

4. Conclusion

In conclusion we have demonstrated an immunoassay for detection of AFB_1 using AFB_1 covalently linked to microtiter plate. This covalent immobilization strategy can be applied for various haptens with carboxyl or hydroxyl functional groups. The CI-ELISA developed on the microtiter plate with covalently immobilized AFB_1 was 2.7 fold more sensitive and also the assay time was reduced to 75 mins. The stability of these modified plates to organic solvents, which are quite often used for dissolving haptens was confirmed. The retention of the activity of covalently-immobilized AFB_1 during storage was demonstrated. The re-usability of the microtiter plates that were covalently-immobilized with AFB_1 is a major advantage. Further studies will be undertaken for the development of toxin arrays using different haptens immobilized on polystyrene plates. The re-usability, storage stability and assay rapidity make the usage of these AFB_1 covalently-immobilized microtiter plates a more robust and versatile platform facilitating the detection of aflatoxins in corn samples with good precision.

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