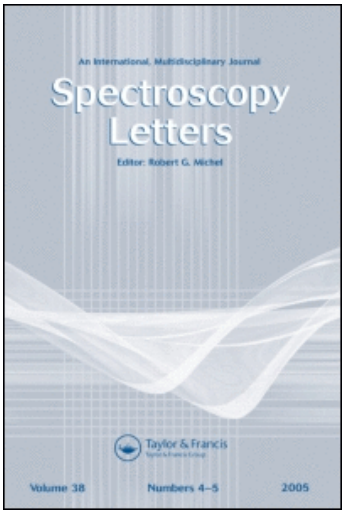

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Surface Plasmon Resonance-Based Immunoassay for the Detection of Aflatoxin B_1 Using Single-Chain Antibody Fragments

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Surface Plasmon Resonance-Based Immunoassay for the Detection of Aflatoxin B_1 Using Single-Chain Antibody Fragments

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Abstract: Aflatoxin B_1 (AFB_1) is a highly toxic secondary metabolite of the fungal species *Aspergillus flavus* and *Aspergillus parasiticus* produced under certain environmental conditions. The gene encoding an AFB_1-specific single-chain fragment variable (scFv) was isolated from a pre-immunized phage display library and used to express a monomeric and dimeric scFv, specific for AFB_1, in *Escherichia coli.* The monomeric and dimeric scFv were then applied to the development of surface plasmon resonance-based inhibition immunoassays for the detection of AFB_1. Regeneration of the sensor surface, which consisted of a CM5 chip immobilized with an AFB_1 derivative, was investigated and enabled at least 75 binding regeneration cycles. The inhibition

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assays developed had ranges of detection between 390 and 12,000 pg mL^{-1} (ppb) for the monomeric scFv and between 190 and 24,000 pg mL^{-1} (ppb) for the dimeric scFv, with coefficients of variation for the inter-day variability studies ranging from 1.9–4.18% and 3–11.53%, respectively.

Keywords: Aflatoxin B_1, surface plasmon resonance, immunoanalysis, food contamination

INTRODUCTION

Aflatoxins are a group of secondary fungal metabolites that are produced by *Aspergillus flavus* and *Aspergillus parasiticus* under certain conditions.[1] They were first discovered in 1960 after a toxin outbreak in England, which killed several thousand turkey poults after the consumption of contaminated Brazilian groundnut meal.[2] Contamination of crops such as maize, cottonseed, peanuts, and tree nuts occurs during growth, and to a greater extent, during storage. High humidity in tropical and subtropical climates favors fungal growth and therefore increases the levels and persistence of contamination. Aflatoxins are members of the coumarin family and are the most widely spread group of toxins from naturally occurring molds that result in contamination of food products. There are four main aflatoxins, B_1, B_2, G_1, and G_2, with AFB_1 being the most predominant and toxic. AFB_1 is linked to human hepatocellular carcinoma, and the International Agency for Research on Cancer regards it as a human carcinogen.[3] Hence, there is a requirement for a rapid and sensitive detection method for AFB_1 in food products.

Traditional methods of detection for AFB_1 include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas-liquid chromatography (GLC), and mass spectrometry.[4] These techniques have proved to be laborious, with increased sample preparation and cleanup, and lack sensitivity. Immunoanalytical techniques offer increased sensitivity and specificity for the detection of AFB_1. However, due to their low molecular mass (i.e., <1000 Da), aflatoxins do not stimulate an immune response and therefore must be covalently linked to a large carrier protein such as bovine serum albumin (BSA), which will elicit a strong immune response post immunization. The protein conjugate is required during the production, screening, and characterization of antibodies. Several polyclonal and monoclonal antibodies have been raised against aflatoxins and used in the development of immunoassays, with varying degrees of specificity and sensitivity.[3,5–10]

Recombinant antibody technology has provided an alternative source of antibodies with desirable affinity and specificity. Single-chain variable fragment (scFv) antibody fragments have been generated against small haptens such as morphine-3-glucuronide,[11] pesticides,[12–14] and the mycotoxins zearalenone[15] and aflatoxin B_1.[16,17]

A phage display system for the expression of scFv antibody fragments was described by Krebber et al.[18] This robust system offers vector stability and tight control of scFv expression fused to the wild-type geneIII coat protein of filamentous phage. It also offers a set of specific primers, for the polymerase chain reaction (PCR) amplification of variable region genes, a strategy for scFv assembly, and subsequent directional cloning using a single rare cutting restriction enzyme (SfiI) and compatible vector series.

The Krebber system has been used in the development of an AFB_1-specifc phage display library and used for the subsequent isolation of AFB_1-specifc scFvs.[17] The compatible vector series, described by Krebber et al., enables expression of the scFvs bound to phage particles (pAK100 and 200) or in soluble form (pAK300–600).

High-level expression of a monomeric scFv (Fig. 1A) is obtained using pAK400, with a strong Shine Dalgarno sequence (SDT7g10). The pAK400 also incorporates a C-terminal 6 × His tag for purification using immobilized metal affinity chromatography (IMAC) and detection using an anti-his tag antibody. Use of pAK500 results in the expression of a dimeric scFv (Fig. 1B), using a single-chain double helix (dHLX) for dimerization, followed by a 5 × His tag for purification and detection. Recently, scFvs have been applied to biosensor systems for the detection of small haptens including morphine-3-glucuronide[11,19] and the mycotoxins fumonisins[20] and aflatoxin B_1.[16,17]

The Biacore is a commercially available biosensor system based on the phenomenon of surface plasmon resonance (SPR).[21] This sensor enables biospecific interaction analysis (BIA) such as antigen–antibody binding in real-time. SPR is an optical technique that uses the principle of total

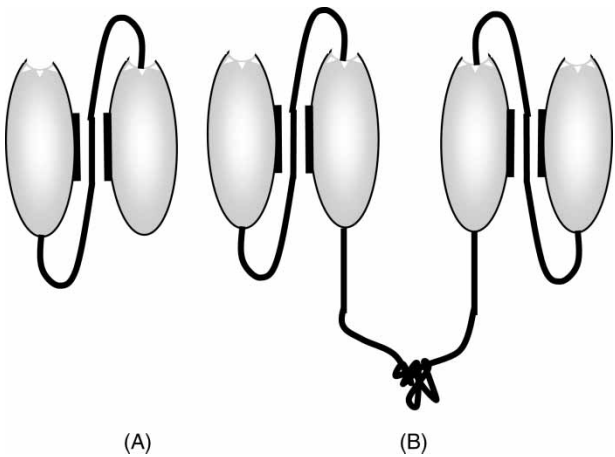

Figure 1. Schematic representation of the monomeric (A) and dimeric (B) scFvs. The monomeric scFv consists of a variable heavy and light chain domain stabilized with a serine-glycine linker; and the dimeric scFv comprises two scFv fragments dimerized via a double helix.

internal reflection (TIR). When a plane-polarized light beam propagates through a medium of higher refractive index (e.g., glass prism) and meets an interface with a medium of lower refractive index (e.g., sample solution), the light is totally internally reflected, above a certain critical angle. Under these conditions an evanescent wave, an electromagnetic field component of the light, penetrates into the low refractive medium to a magnitude of one wavelength. If the TIR interface is coated with a thin metal film, which is gold in the case of the Biacore, the evanescent wave propagates in the metal layer and causes the plasmons to resonate, resulting in a surface plasmon wave. At a particular angle of incidence, some of the energy of the reflected light causes excitation of the surface plasmons, causing a decrease in intensity of the reflected light. The specific angle at which SPR occurs is known as the SPR angle, and this can be used to monitor changes in refractive index of the medium adjacent to the metal layer. SPR can be used to monitor biological interaction on the metal film because changes in the refractive index of the media are directly proportional to changes in mass or concentration on the surface of the metal layer.

The Biacore biosensor was used in the development of inhibitive immunoassays for the sensitive detection of AFB_1 using a monomeric and a dimeric scFv. In this system, an AFB_1 derivative was immobilized onto the surface of a CM5 chip and antibody and free hapten standards were pre-incubated and then passed over the immobilized surface. The free hapten in solution prevents the antibody from binding to the immobilized hapten so that the amount of antibody binding the immobilized hapten is inversely proportional to the concentration of hapten free in solution.

MATERIALS AND METHODS

Caution

Aflatoxin B_1 is carcinogenic and should be handled with extreme care.

Reagents

All reagents and chemicals were of analytical grade and supplied by Sigma-Aldrich Co. (Poole, Dorset, UK), unless otherwise stated. Biacore 3000 was supplied by Biacore International AB (Uppsala, Sweden).

Aflatoxin B_1 scFv Phage Display Library

A pre-immunized phage display library was obtained from Dr. Stephen Daly (Dublin City University, Republic of Ireland).

Bacterial Strains and Plasmids

The suppressor *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA, USA) was used as a host strain for the initial cloning stages, and the nonsuppressor *E. coli* JM83 (DCU, Ireland) was used as a host strain for soluble scFv expression. The pAK400 and 500 expression vectors were kindly donated by Professor A. Plückthun (Universität Zürich, Switzerland).

Cloning into pAK400/500

Following third round panning on the pre-immunized phage display library, an AFB_1-specific clone was isolated. The plasmid DNA was purified using the Wizard Plus Miniprep kit (Promega, Southampton, UK), and the gene encoding the scFv of interest was restricted from pAK100 using the restriction enzyme SfiI (New England Biolabs, Hertfordshire, UK). The scFv gene was then gel-purified using silica mesh 325 glass beads (Dr. Paul Clarke, DCU, Ireland) and ligated into pAK400/500, previously restricted using SfiI. The vectors, containing the cloned gene of interest, were then transformed into $CaCl_2$-competent JM83 *E. coli.*

Expression of Soluble scFv Fragments

An overnight culture of JM83 *E. coli*, harboring the pAK vector containing the scFv gene of interest, was used to inoculate 200 mL of 2 × tryptone-yeast extract (TY) containing 25 $\mu g\,mL^{-1}$ chloramphenicol. The culture was incubated at 37°C, with vigorous shaking, until the OD_{550nm} reached 0.5–0.6. The culture was then induced using 1 mM isopropylthiogalactopyranoside (IPTG) and incubated for a further 4 hr for pAK400 (or 16 hr for pAK500), at 26°C, with vigorous shaking, and then centrifuged at 4000 rpm for 20 min. The bacterial pellet was resuspended in 10 mL of TES (100 mM Tris-HCl, pH 8, 0.5 M sucrose, 0.5 mM EDTA) and incubated on ice for 1 hr. Cellular debris was then removed after centrifugation at 4000 rpm for 20 min and the supernatant (crude periplasmic lysate) dialyzed against PBS (pH 7.4) overnight at 4°C and used in the various immunological techniques described below.

AFB_1 Sensor Chip

A Biacore CM5 chip immobilized with an AFB_1 derivative was kindly donated by XenoSense Ltd. (c/o N.I. Science Park, Queens Island, Belfast, UK).

Surface Regeneration

Regeneration of the AFB_1 sensor surface was carried out using 10 mM NaOH, for the monomeric scFv and 25 mM NaOH for the dimeric scFv. The regeneration solution (5 μL) was passed over the sensor surface at a flow rate of $10\,\mu L\,min^{-1}$.

Sample Preparation for Use in Inhibitive Assay

A $1\,mg\,mL^{-1}$ of free AFB_1 was prepared in 100% methanol. Standards, ranging in concentrations from 190 to 24,000 $pg\,mL^{-1}$, were prepared in PBS containing 5% (v/v) methanol. Each AFB_1 standard was then pre-incubated with an equal volume of a 1/4 dilution (approximately $10\,\mu g\,mL^{-1}$) for the monomeric scFv and a dilution of 1/35 (approximately $1.5\,\mu g\,mL^{-1}$) for the dimeric scFv for 30 min. Twenty microliters of each concentration was then passed over the sensor surface (at a flow rate of $10\,\mu L\,min^{-1}$) three times.

Measurement of Cross-Reactivity

Both the monomeric and dimeric scFv were assayed with a range of structurally related aflatoxin molecules, which included aflatoxin B_2, M_1, M_2, G_1, and G_2, in order to determine potential cross-reactivity. Stock solutions of each aflatoxin were prepared in methanol and diluted in PBS-5% (v/v) methanol to a range of concentrations from 780 to 6250 $pg\,mL^{-1}$. Biacore inhibition assays were carried out against each aflatoxin, with the monomeric and dimeric scFvs, as described above. The results were normalized and plotted.

RESULTS AND DISCUSSION

Preliminary Steps for the Development of an scFv-Based Assay Using the Biacore

This paper focuses on the development of Biacore inhibition assays for the detection of AFB_1. However, previous studies on the development of Biacore assays for AFB_1 have encountered several difficulties. Problems have been encountered when trying to immobilize antibodies onto a sensor surface, either directly or indirectly.[22] When directly immobilizing the antibodies onto the sensor surface, the coupling chemistry affected the antibodies binding capacity, and indirectly immobilizing the antibodies, using either protein A or species-specific antibodies, resulted in no binding between the captured antibody and the protein conjugate. Daly et al. also encountered difficulties developing a Biacore assay for the detection of AFB_1 using polyclonal

antibodies.[10] In this case, a sensor surface immobilized with an AFB_1–BSA conjugate was used in the development of an inhibition assay format, but difficulties were encountered when trying to regenerate the sensor surface.

Therefore, it was decided to use a CM5 chip immobilized with an AFB_1 derivative for the development of inhibition assays, incorporating the monomeric and dimeric scFvs. The gene encoding an AFB_1-specific scFv was isolated from a phage display vector, pAK100, and subcloned into pAK400 for the soluble expression of a monomeric scFv and into pAK500 for the soluble expression of a dimeric scFv fusion protein. The monomeric and dimeric scFv were then applied to the development of inhibition assay for the detection of AFB_1 using the Biacore.

For the successful development of an inhibition assay for the detection of AFB_1, the optimization of a number of parameters was required. These included antibody dilutions, removal of nonspecific interactions and surface regeneration conditions.

Several scFv dilutions were passed over the AFB_1 surface, and the dilution resulting in the binding of approximately 300–400 response units (RU) was selected as optimal. A 1/8 dilution of the monomeric and a 1/70 dilution of the dimeric scFv were found to produce binding responses of approximately 350 and 250 RU, respectively.

Nonspecific binding analysis was carried out on the monomeric [Fig. 2A(ii)] and dimeric [Fig. 2B(ii)] scFvs by passing each over an unactivated carboxyl-methylated (CM) dextran surface. Negligible binding was observed with each scFv to the dextran, and as a result there was no need to incorporate dextran into the diluent buffer.

The regeneration of the sensor surface is a major factor affecting the development of Biacore assays. The regeneration conditions for the removal of the monomeric and dimeric scFv from the AFB_1 sensor surface were optimized. A 1 min pulse of 10 mM NaOH and 25 mM NaOH enabled complete removal of the monomeric and dimeric scFvs, respectively. Figure 3 shows a typical sensogram for the binding and regeneration of the monomeric (1) and dimeric (2) scFvs on the AFB_1 sensor surface. Previous studies have encountered problems regenerating the sensor surface immobilized with hapten–protein conjugates after injection of specific polyclonal antibodies. The need for stringent regeneration conditions were required, including 1 M ethanolamine, pH 13.6 for the regeneration of a morphine-3-glucuronide–ovalbumin (M3G–OVA) surface[23] and 1 M ethanolamine with 20% (v/v) acetronitrile, pH 12.0, for the regeneration of an AFB_1–BSA surface.[10] In the case of this study, the use of 10 and 25 mM NaOH for the monomeric and dimeric scFvs, respectively, enabled the complete regeneration of the AFB_1 surface. The need for a higher NaOH concentration with the dimeric scFv suggests that the two binding sites increase the avidity of scFvs for AFB_1. Recent publications on the development of Biacore assays with monomeric scFvs have also reported the need for less stringent regeneration conditions in comparison with the use of polyclonal antibodies.[11,17,19]

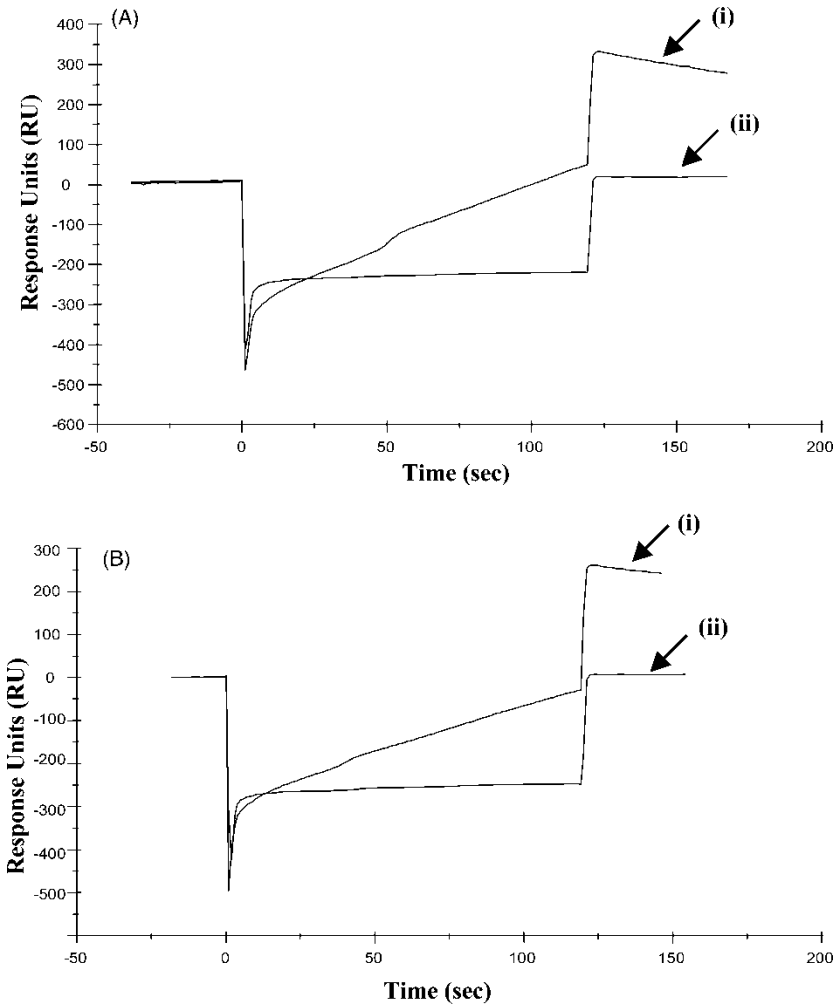

Figure 2. Overlay plot demonstrating binding of the monomeric (A) and dimeric (B) scFvs to the AFB_1 surface (i) and an unactivated dextran surface (ii). Negligible binding of the monomeric scFv or dimeric scFv to the unactivated dextran was observed. However, approximately 350 and 250 response units of the monomeric and dimeric scFv, respectively, bound to the AFB_1 surface. This indicates the specificity of the monomeric and dimeric scFvs toward AFB_1.

Efficiency of Regeneration

Regeneration of the sensor surface is essential in the development of an assay in order to enable the analysis of multiple samples, making the biosensor a more cost-effective method of detection. Multiple binding–regeneration cycles were carried out on the sensor surface to determine

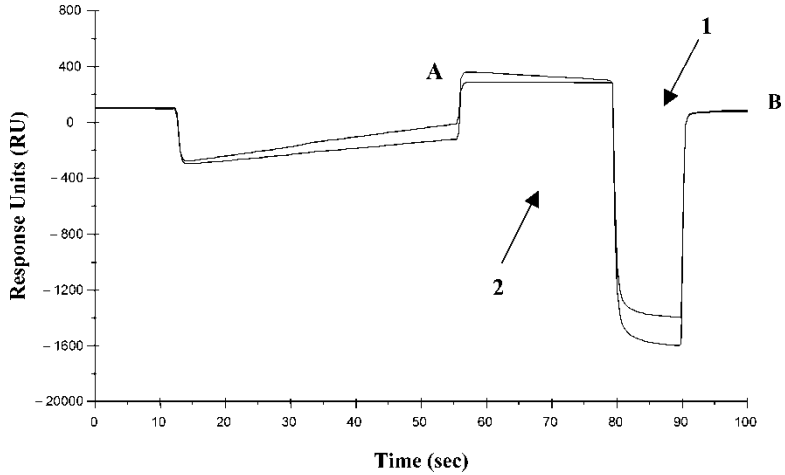

Figure 3. Typical sensorgram showing the binding and regeneration of the monomeric scFv (1)and the dimeric scFv (2) on the AFB_1 surface. A $1/8$ and a $1/70$ dilution of the monomeric and dimeric scFvs, respectively, was passed over the sensor surface at $10\,\mu L/min$ for 4 min with approximately 400 response units of scFv binding (A). The surface was then completely regenerated using a 1-min pulse of 10 and 25 mM NaOH for the monomeric and dimeric scFvs, respectively (B).

the binding–regeneration cycles, the binding capacity of the scFv to AFB_1 should not decrease by more than 20%.[24] After optimization of the regeneration solution for use with the monomeric and dimeric scFv, regeneration studies were conducted, which involved repeatedly injecting the scFv over the AFB_1 sensor surface and regenerating it with the appropriate regeneration solution. It was possible to regenerate the sensor surface at least 75 times using the monomeric scFv, before a decrease of 12% in the ligand binding capacity was observed (Fig. 4A). The sensor surface could be regenerated at least 75 times using the dimeric scFv, with a decrease in the ligand binding capacity of 10% being observed (Fig. 4B). It should be noted at this point that the majority of work reported in this paper was performed using only one sensor surface, making it possible to carry out at least 530 regenerations on the CM5 surface immobilized with the AFB_1 derivative.

Development of a Biacore Inhibition Assay for AFB_1

After optimization of the various assay parameters, inhibition assays, incorporating the monomeric or dimeric scFvs, were developed for the detection of AFB_1 using the CM5 chip immobilized with an AFB_1 derivative. Free AFB_1 standards, ranging in concentration from 375 to 12,000 $pg\,mL^{-1}$ for the

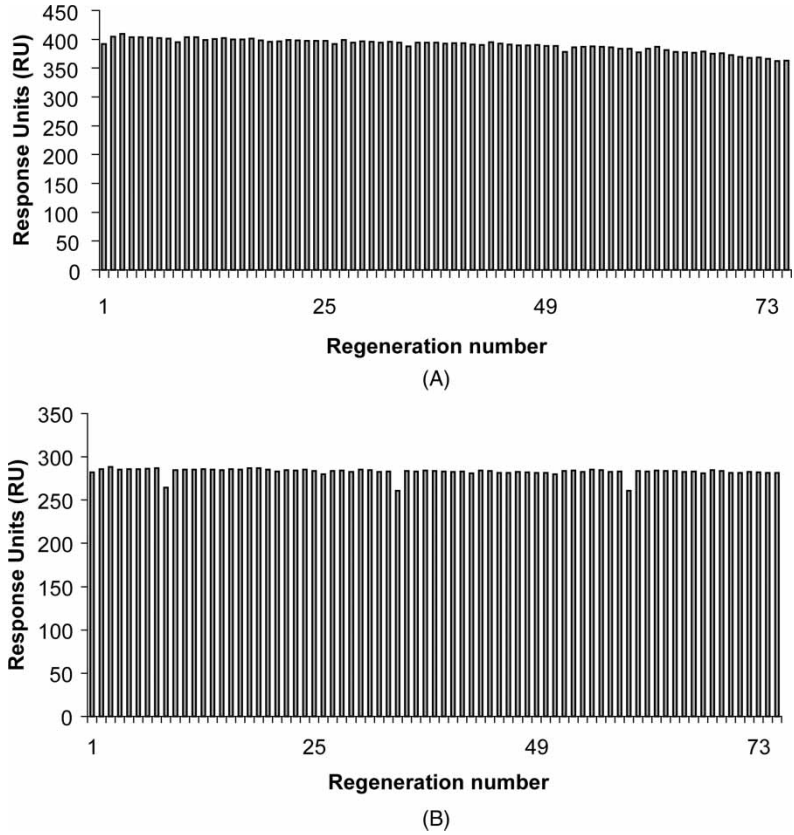

Figure 4. Graph showing the regeneration profile for 75 consecutive regeneration cycles of the monomeric (A) and dimeric (B) scFvs on the AFB_1 sensor chip. A 2-min pulse of the monomeric or dimeric scFv diluted to 1/8 and 1/70, respectively, was followed by a 0.5-min injection of 10 mM NaOH for the monomeric scFv and 0.5-min injection of 25 mM NaOH for the dimeric scFv as the regeneration solutions. The regeneration solution enabled the complete removal of all bound scFv after each binding cycle. This results in highly reproducible binding cycles, as shown, with no significant decrease in the binding response measured over the course of the regeneration studies.

monomeric scFv and 190 to 24,000 pg mL^{-1} for the dimeric scFv, were prepared in PBS containing 5% (v/v) methanol. Each free AFB_1 concentration was incubated with an equal volume of either the monomeric scFv diluted to 1/4 (to ensure a final dilution of 1/8) or a 1/35 dilution (to ensure a final dilution of 1/70) of the dimeric scFv and allowed to equilibrate for 30 min at 37°C. The equilibrated samples were then passed over the sensor surface, in random order, followed by regeneration of the AFB_1 sensor surface using the

appropriate regeneration solution. This was carried out in triplicate for each concentration. The change in response for each AFB_1 standard was then plotted against the concentration of free AFB_1. Studies on the intra-day variability showed that the monomeric scFv had a range of detection for free AFB_1 from 375 to 12,000 pg mL^{-1} with coefficients of variation (CVs) remaining below 0.61%. The intra-day variability assay with the dimeric scFv had a range of detection between 190 and 24,000 pg mL^{-1} and the CVs remained below 3.37%. Inter-day variability studies were also carried out in order to determine the reproducibility of the assay over 3 days. Figures 5A and 5B show the inter-day assay curves for the monomeric and dimeric scFvs, respectively, where the range of detection of free AFB_1 was 375–12,000 pg mL^{-1} for

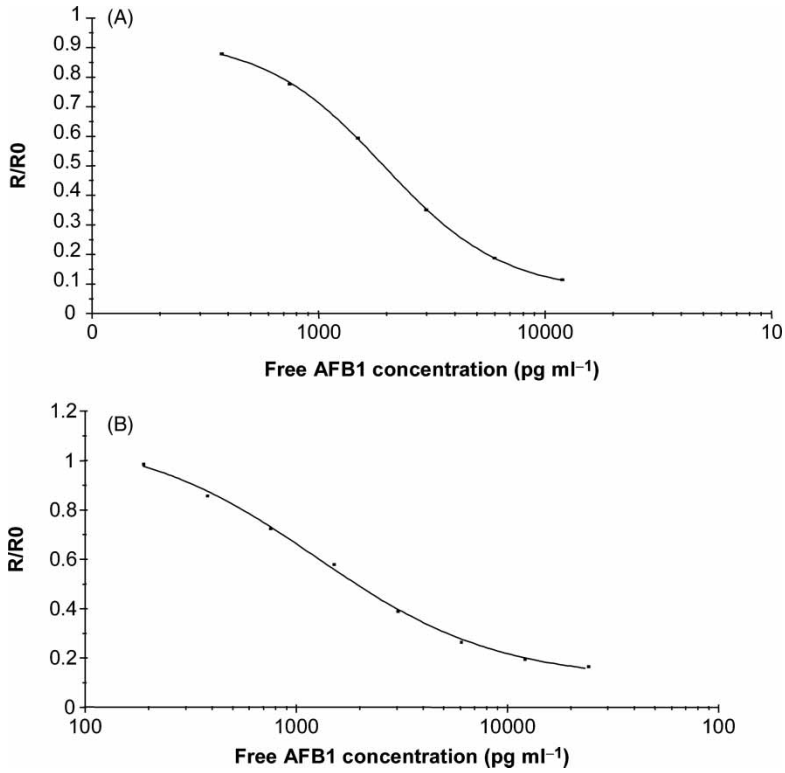

Figure 5. Inter-day Biacore inhibition assay for the detection of AFB_1 using the monomeric scFv (A) and the dimeric scFv (B) on the chip immobilized with the AFB_1 derivative. The results displayed show the average of three replicate results, and the range of detection for AFB_1 was found to be between 375 and 12,000 pg mL^{-1} using the monomeric scFv and between 190 and 24,000 pg mL^{-1} with the dimeric scFv. The mean binding response (R) at each concentration was divided by the antibody binding response obtained in the presence of zero AFB_1 (R0) to give a normalized binding response (R/R0).

the monomeric and 190–24,000 pg mL^{-1} for the dimeric scFv. The CVs obtained for the inter-day variability studies on the monomeric scFv ranged between 1.9% and 4.18% (Table 1) and between 3% and 11.53% for the dimeric scFv (Table 2), indicating that both assays were reproducible over the 3 days. The degree of accuracy was also estimated for each assay by calculating the percentage recovery. This concept, described by Findlay, is used to express the closeness of agreement between a measured test result and its theoretical true value.[25] The back-calculated values were determined using the four-parameter fit of the inter-day calibration curve. The degree of accuracy was then calculated by expressing the observed (back-calculated) concentration as percentage of the theoretical concentration value. Tables 1 and 2 display the percentage recovery for the monomeric and dimeric assays, respectively. Recovery levels of between 97.28% and 102.36% and 83.06% and 110.08% were observed with the assays incorporating the monomeric and dimeric scFvs, respectively. The results show that both the monomeric and dimeric scFvs offer excellent sensitivity, specificity, reproducibility, and accuracy for a model assay system for AFB_1. The dimeric scFv offers improved sensitivity over the monomeric scFv, which is due to the fact that the sensitivity of an SPR-based detection method is dependent on the molecular weight of the surface-binding antigen, which in the case of this study is an scFv. However, this statement may imply that conventional monoclonal and polyclonal antibodies, with molecular weights of approximately 150 kDa, would offer increased sensitivity over the smaller scFv antibody fragment (approximately 32 kDa), which has not proved to be the case. The panning process used during the selection of scFvs enables the isolation of antibody fragments with improved sensitivities.

The limits of detection for AFB_1 demonstrated in this paper (375 and 190 pg mL^{-1} for the monomeric and dimeric scFvs, respectively) compare favorably with published literature on the detection of AFB_1 using a Biacore-based assay. Biacore-based inhibition assays for the detection of AFB_1 have previously been developed. Van der Gaag et al. developed an assay in spiked grain samples using a monoclonal antibody with similar detection limits of

Table 1. Inter-day assay coefficients of variation and recovery levels obtained for the Biacore inhibition assay for the detection of AFB_1 using the monomeric scFv

AFB_1 concentration (pg mL^{-1})	Calculated mean ± SD	Coefficient of variation (%)	Recovery (%)
12,000	0.11 ± 0.004	3.75	98.56
6,000	0.19 ± 0.006	3.16	100.49
3,000	0.35 ± 0.015	4.18	100.54
1,500	0.59 ± 0.020	3.40	98.80
750	0.77 ± 0.012	1.58	102.36
375	0.88 ± 0.017	1.90	97.28

Table 2. Inter-day assay coefficients of variation and recovery levels obtained for the Biacore inhibition assay for the detection of AFB_1 using the dimeric scFv

AFB_1 concentration ($pg\,mL^{-1}$)	Calculated mean ± SD	Coefficient of variation (%)	Recovery (%)
24,000	0.16 ± 0.013	7.95	83.06
12,000	0.19 ± 0.017	8.89	104.87
6,000	0.26 ± 0.030	11.53	108.02
3,000	0.39 ± 0.020	5.27	103.08
1,500	0.58 ± 0.048	8.28	91.40
750	0.72 ± 0.055	7.66	101.93
375	0.85 ± 0.054	6.36	110.08
190	0.98 ± 0.029	3.00	90.25

0.2 ppb (0.2 $ng\,mL^{-1}$).[26] Van der Gaag and associates focused their research on the comparison between HPLC and Biacore for the detection of mycotoxins in feed. However, information was not provided on regeneration studies or on the reproducibility of their Biacore assay. Daly and collaborators developed a Biacore-based assay using a polyclonal antibody in PBS with a limit of detection of 3 $ng\,mL^{-1}$[10] and an scFv-based Biacore assay with a limit of detection of 3 $ng\,mL^{-1}$ in PBS and 0.75 $ng\,mL^{-1}$ in spiked grain.[17]

The limits of detection described in this paper also compare favorably with several other immunoassay formats including a fluorescence polarization assay for aflatoxins with a range of detection between 5 and 200 ppb,[27] a dipstick assay with a limit of detection of 2 $ng\,mL^{-1}$,[28] and ELISA formats described by Candlish et al.,[29] Aldao et al.,[30] and Daly et al.[10,17] with limits of detection at 0.2 $ng\,mL^{-1}$, 0.25 $\mu g\,kg^{-1}$, 3 $ng\,mL^{-1}$, and 98 $ng\,mL^{-1}$, respectively. Analytical techniques, including the HPLC detection system described by Kussak et al.[31] have offered greater sensitivity with limits of detection for aflatoxins B_1, B_2, G_1, and G_2 at 6.8 $pg\,mL^{-1}$ in urine, a sol-particle lateral flow immunoassay with limits of detection of 0.1 ppb in buffer and 10 ppb in grain samples,[32] and an immunoaffinity fluorometric biosensor with a lower limit of detection, at 0.1 ppb.[33] Although some assay formats may offer lower limits of detection, it must be noted that the assay described is a relatively rapid and cost-effective assay that occurs in real-time and one that is capable of detecting AFB_1 levels well below the EU maximum residue levels, which are set between 2 and 8 ppb ($ng\,mL^{-1}$).

Cross-Reactivity Studies on the Monomeric and Dimeric scFvs in a Biacore Inhibition Assay Format

Cross-reactivity studies were then carried out on each scFv in an inhibition assay format on the Biacore. Cross-reactivity potential of the scFvs

were determined against five structurally related aflatoxins, B_2, G_1, G_2, M_1, and M_2. Comparisons of the least detectable dose (LDD) and inhibition concentration (IC_{50}) values were used to accurately estimate levels of cross-reactivity.[34] The IC_{50} value is defined as the analyte concentration that results in 50% inhibition and the LDD as the analyte concentration that results in 90% inhibition or as the smallest concentration of analyte that produces a response that can be significantly distinguished from zero.[34] Levels of cross-reactivity were estimated at the LDD (CR_{90}) and at the IC_{50} (CR_{50}) as 100-fold the ratio between the LDD and IC_{50} values of the antigen and of the cross-reactant, respectively. The monomeric scFv (Table 3) displayed the highest level of cross-reactivity with aflatoxin M_1 and G_1 at the LDD (12.5%) and aflatoxin G_1 at the CR_{50} (13%). Minimal cross-reactivity with the monomeric scFv was observed with aflatoxins B_2, G_2, M_1, and M_2 at the IC_{50} (i.e., $\leq$5%) and with aflatoxins B_2, G_2, and M_2 at the LDD (i.e., $\leq$3%). The dimeric scFv (Table 4) displayed minimal cross-reactivity with aflatoxins B_2, G_2, M_1, and M_2 at the IC_{50} and LDD (i.e., $\leq$5%). Slightly higher levels of cross-reactivity were observed with aflatoxin G_1 at the IC_{50} (10%) and LDD (20%). This suggests that both the monomeric and dimeric scFvs appear to specifically bind to AFB_1 with minimal levels of cross-reactivity (i.e., $\leq$20%) observed at the IC_{50} and LDD.

CONCLUSIONS

A gene encoding an AFB_1-specific scFv was isolated from a phage display library and cloned into a series of compatible vectors for the expression of a monomeric and dimeric scFv in *E. coli.* The two scFvs were then applied to a Biacore inhibition assay format for the detection of AFB_1. Several assay parameters including scFv dilution, nonspecific binding interactions, and regeneration conditions were optimized. Two inhibition assays were then developed

Table 3. Cross reactivity studies on the monomeric scFv with aflatoxins

Aflatoxin	LDD[a] (pg mL^{-1})	IC_{50}[b] (pg mL^{-1})	%CR_{50}[c]	%CR_{90}[d]
B_1	375	2,000	100	100
B_2	12,500	200,000	1	3
M_1	3,000	40,000	5	12.5
M_2	31,250	>250,000	<1	1.2
G_1	3,000	15,000	13	12.5
G_2	12,500	200,000	1	3

[a]Least detectable dose calculated at 90% A/A0.
[b]50% inhibition concentration (50% A/A0).
[c]Percentage cross-reactivity determined at IC_{50}.
[d]Percentage cross-reactivity determined at LDD.

Table 4. Cross reactivity studies on the dimeric scFv with aflatoxins

Aflatoxin	LDD[a] ($pg\,mL^{-1}$)	IC_{50}[b] ($pg\,mL^{-1}$)	$\%CR_{50}$[c]	$\%CR_{90}$[d]
B_1	300	2,000	100	100
B_2	25,000	300,000	<1	1.2
M_1	20,000	200,000	1	1.5
M_2	125,000	>250,000	<1	<1
G_1	1,500	20,000	10	20
G_2	25,000	250,000	<1	1.2

[a]Least detectable dose calculated at 90% A/A0.
[b]50% inhibition concentration (50% A/A0).
[c]Percentage cross-reactivity determined at IC_{50}.
[d]Percentage cross-reactivity determined at LDD.

with the monomeric and dimeric scFvs and had ranges of detection between 390 and 12,000 $pg\,mL^{-1}$ and 190 and 24,000 $pg\,mL^{-1}$, respectively. Each assay was capable of detecting AFB_1 at levels well below the EU maximum residue limits, which are currently set between 2 and 8 ppb ($ng\,mL^{-1}$). Cross-reactivity studies indicated that each scFv had a high level of specificity for AFB_1. The monomeric and dimeric scFvs provided excellent sensitivity and specificity for AFB_1 and enabled the development of highly accurate and reproducible Biacore inhibition assays for the detection of AFB_1.

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