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Surface plasmon resonance biosensor for the detection of ochratoxin A in cereals and beverages

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ABSTRACT

Ochratoxins are a group of mycotoxins produced as secondary metabolites by fungi which contaminate a large variety of food and feed commodities. Due to their teratogenic and carcinogenic properties, ochratoxins present a serious hazard to human and animal health. There is an increasing need to establish a simple sensitive method to detect these toxins. Here we report a rapid and highly sensitive surface plasmon resonance (SPR) assay of ochratoxin A (OTA) using Au nanoparticles for signal enhancement on a mixed self-assembled monolayer (mSAM) surface. A competitive immunoassay format was used for the development of the OTA immunoassay, which is based on the immobilization of target OTA through its ovalbumin (OVA) conjugate with a polyethylene glycol (PEG) linker. The new OTA conjugate (OTA-PEG-OVA) showed remarkably enhanced performance characteristics compared with those based on the immobilization of a commercial bovine serum albumin BSA-OTA conjugate without a PEG linker. Although OTA concentrations as low as 1.5 ng mL^{-1} could be directly detected on this surface, the limit of detection (LOD) can be dramatically improved to 0.042 ng mL^{-1} for OTA by applying large gold nanoparticles (40 nm) for signal enhancement. Various chemical conditions to minimize the influence of the food matrix on assay performance were also investigated. Grain samples were simply extracted with 50% methanol and liquid samples treated with poly(vinylpyrrolidone) (PVP) (3 or 5%), without any sample clean-up or pre-concentration step prior to analysis. The LODs for OTA in oats and corn were 0.3 and 0.5 ng g^{-1}, respectively, while in wine and other beverages, LODs ranged from 0.058 to 0.4 ng mL^{-1}. No cross-reactivity was observed with three other common mycotoxins. In addition, the mSAM/OTA-PEG-OVA surface exhibited high stability with over 600 binding/regeneration cycles. This approach with simple sample preparation provides a powerful tool for the rapid and sensitive quantitative determination of OTA in food matrices.

1. Introduction

The ochratoxins are a group of highly toxic fungal secondary metabolites that are produced by several species of the genera *Aspergillus* and *Penicillium* [1,2]. Ochratoxin-producing species are found throughout the world, and may contaminate agricultural products pre- and post-harvest. Ochratoxin A (OTA) is the most toxic and prevalent among these toxins, and is receiving increasing attention because of the hazard imposed on both human and animal health. OTA has been shown to be nephrotoxic, carcinogenic, immunotoxic and teratogenic to all animal species tested, and implicated as a factor in human disease called "Balkan Endemic Nephropathy" [3]. OTA has also been associated with an increased incidence of tumors of the upper urinary tract in humans. As a result, the International Agency of Research on Cancer (IARC) has classified OTA in group 2B as a possible human carcinogen [4].

The worldwide occurrence of OTA contamination in a variety of food and feed products has been amply documented [5]. It has been found in a wide range of commodities such as cereal grains, oil seeds, dried fruits, coffee and cocoa beans and has also been detected in beverages such as beer, wine and grape juice [5–7]. OTA contaminant in raw agricultural products used as feed can pass unchanged through the food chain to be present in meat and meat products [8,9]. Because of the persistence of OTA in the food chain, it poses a serious threat to human health. The European Food Safety Authority (EFSA) has conducted detailed risk assessments for OTA and established maximum allowable levels presented in food and feed products and in raw materials (EC No 123/2005) (10 μg kg^{-1} in dried vine fruits and instant coffee, 5 μg kg^{-1} in raw cereal grains and roasted coffee, 3 μg kg^{-1} in processed cereal foods and 2 μg kg^{-1} in grape juice and all types of wine) [6]. According to

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this, several countries have proposed individual regulations with a wide variability in the maximum tolerated OTA levels, which, in turn, requires the development of validated official analytical methods for rapid and cost-effective monitoring on a large scale [10].

Many different analytical methods are now available for the determination of OTA in a variety of matrices [11]. Chromatographic methods such as thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC) with immunoaffinity columns and fluorescence detection [12–16] provide sensitive and specific techniques, but generally require multiple steps prior to detection. These multiple steps include extraction with organic solvents from complex matrices, extensive sample clean-up, and pre-concentration and sometimes analyte derivatization. Such sample treatment not only makes the analysis time-consuming and costly but also requires skilled personnel. Immunological methods such as enzyme-linked immunosorbent assays (ELISA) have been developed to overcome these limitations, and now are widely used for screening purposes [17–19]. Conventional ELISA methods are less demanding for sample clean-up and allow parallel analysis of multiple samples, but they still require several hours to obtain results. Rapid ELISA kits for OTA detection are commercially available, for example Veratox (Neogen, Lansing, MI), and require only 20–30 min after sample extraction, but their sensitivity is limited. Recently, some new immunosensing techniques have been emerged for rapid OTA screening [20–23]. In particular, Biacore biosensors which exploit the phenomenon of surface plasmon resonance (SPR) to monitor interaction between molecules in real time have proven to be extremely useful for this purpose. For example, van der Gaag et al. [24] have developed an SPR method for the simultaneous detection of multiple mycotoxins within a time frame of 25 min, with detection limits for aflatoxin B1, zearalenone, ochratoxin A, fumonisin B1 and deoxynivalenol reported at 0.2, 0.01, 0.1, 5.0, and 0.5 ppb, respectively. Yu and Lai [25] have combined an SPR device and a molecularly imprinted polymer (MIP) technique to study the interaction of OTA. In their study, the molecularly imprinted polypyrrole (MIPPy) film was synthesized on the Spreeta sensor surface [25]. The SPR angle increased when binding of OTA molecules occurred, within a linear correlation range from 0.05 to 0.5 ppm.

The ability of OTA to persist through the food chain and the observation of both acute and chronic effects in animals and humans have highlighted the need to develop reliable, easy and highly sensitive techniques for its rapid and cost-effective monitoring in food products. Here, we report on the development of a simple and highly sensitive SPR assay for fast (<10 min) screening of OTA in a variety of food and beverage samples. The method is based on our previous ultrasensitive SPR assay format which controls the proximity of the large anti-mouse IgG-gold nanoparticles (40 nm) to the mixed self-assembled monolayers (mSAM) sensor surface through protein conjugation with an optimal PEG linker for maximum signal enhancement [26].

2. Experimental

2.1. Reagents and materials

Mouse anti-ochratoxin A monoclonal antibody (mAb) and ochratoxin A were purchased from Biodesign. IgG/nanogold (40 nm) was obtained from British Biocell International (BBI). Ochratoxin A-BSA conjugate (OTA-BSA), succinic anhydride, ovalbumin (OVA), poly(vinylpyrrolidone) (PVP); 11-mercaptoundecanol (11-MUOH), 16-mercaptohexadecanoic acid (16-MHA), triethylamine, and trioxatridecanediamine were supplied by Sigma (St. Louis, MO); *N,N′*-dicyclohexyl-carbodiimide (DCC), was from Lancaster (Morecambe, England). *N*-Hydroxysuccinimide (NHS) was from ICN (Aurora, OH). Polyethylene glycol (PEG)-400 and ethyl glycol were from Prolabo (Founteray sous bois, France). Anhydrous magnesium sulphate, guanidine, phosphate buffered saline with 0.05% Tween 20 (PBS/T) were from Fluka (Buchs, Germany). The amine coupling kit (0.1 M NHS, 0.4 M EDC, and 1 M ethanolamine, pH 8.5), HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20), SIA kit Au were from GE Healthcare (Uppsala, Sweden).

2.2. Instrumentation

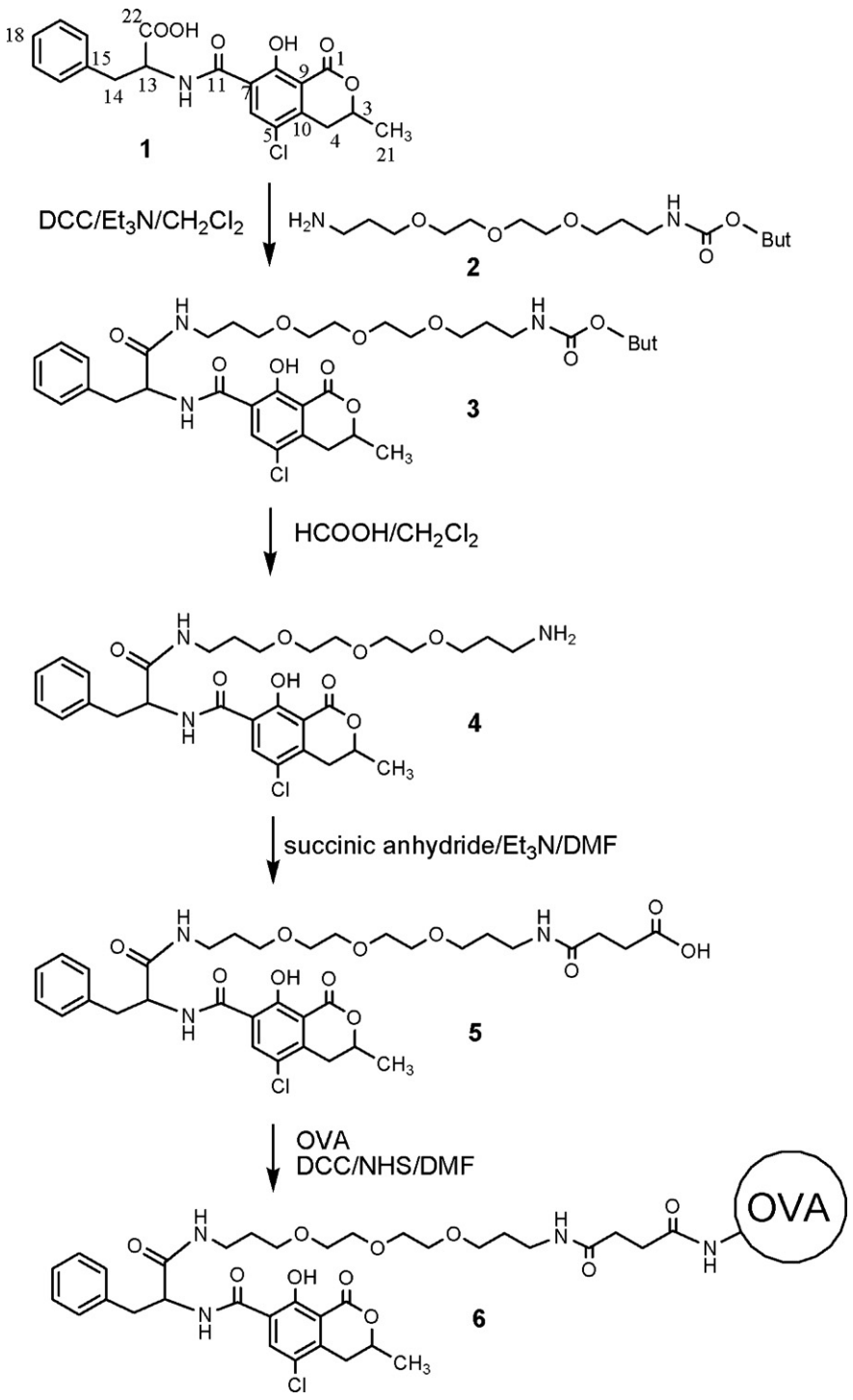
SPR-based measurements were performed using an Optical SPR Biosensor System (Biacore Q) equipped with BIA control and evaluation software (GE Healthcare, Uppsala, Sweden). The NMR spectra were produced on a 400 MHz Bruker Avance DRX400 NMR (Rheinstetten, Germany). Electrospray mass spectra (ES-MS) were generated using a VG Platform II instrument (Beverley). Matrix-assisted laser desorption/ionization mass spectrometry with time-of-flight (MALDI-TOF) spectra were recorded on a Bruker Daltonics Autoflex spectrometer.

2.3. Procedures

2.3.1. Chemical synthesis of OTA-PEG-CO_2H (Scheme 1)

OTA-PEG-CO_2H (**5**) was synthesized according to reported methods with modifications [26,27]. Briefly, a mixture of ochratoxin A (**1**) (25 mg, 0.06 mmol), *N*-(*t*-butoxycarbonyl)-trioxatridecanediamine (**2**) (25 mg, 0.08 mmol), DCC (16 mg, 0.08 mmol) and triethylamine (1 mL) in dichloromethane (15 mL) was stirred at room temperature under nitrogen for 20 h. The solution was filtered and concentrated under vacuum at 40 °C. The residue was flash chromatographed on silica gel (5 g) using dichloromethane–methanol (9:1) to yield OTA-PEG-NHBoc (**3**) (42 mg, 96%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 12.77 (s, 1H, H-8), 8.61 (d, 1H, H-12), 8.40 (s, 1H, H-6), 7.26 (m, 6H, H-phenyls), 4.98 (m, 1H, H-13), 4.78 (m, 1H, H-3), 3.62–3.39 (m, 12H, CH_2–O, PEG), 3.28 (m, 1H, H-14a), 3.20 (m, 1H, H-4a), 3.12 (m, 1H, H-14b), 2.85 (m, 1H, H-4b), 1.93 (m, 2H, CH_2–N, PEG), 1.70 (m, 2H, CH_2–N, PEG), 1.59 (d, 3H, H-21), 1.43 (s, 9H, CH_3, But-*t*-O), 1.33 (m, 2H, OCH_2-CH_2-CH_2N), 1.11 (m, 2H, OCH_2–CH_2–CH_2N); ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm): 170.4 (C-22), 169.7 (C-1), 162.5 (C-11), 159.0 (C-8), 156.1 (N–CO–O), 140.7 (C-5), 138.9 (C-6), 136.9 (C-15), 129.4 (C-16,20), 128.9 (C-17,19), 127.0 (C-18), 123.1 (C-10), 120.9 (C-7), 110.1 (C-9), 77.3 (O-C, *t*-But), 75.9 (C-3), 70.5 ($2CH_2$–O, PEG), 70.3 (CH_2–O, PEG), 70.1 (CH_2–O, PEG), 69.6 (2 CH_2–O, PEG), 55.7 (C-13), 38.6 (NH–CH_2), 37.7 (C-14), 32.3 (C-3), 29.7 (NCH_2–CH_2–CH_2O), 28.9 (NCH_2–CH_2–CH_2O), 28.5 ($3CH_3$, *t*-But), 20.7 (C-21). ESI-MS (negative mode, *m*/*z*) of **3**: 704.3 $[M-H]^-$.

The OTA-PEG-NHBoc (**3**) (41 mg, 0.06 mmol) was dissolved in dichloromethane (2 mL) and formic acid (2 mL) added. The mixture was stirred at room temperature under nitrogen for 8 h. The solvents were removed under nitrogen stream to give OTA-PEG-NH_2 (**4**) (35 mg, 99%). ESI-MS (negative mode, *m*/*z*) of **4**: 604.7 $[M-H]^-$. OTA-PEG-NH_2 (**4**) (35 mg, 0.058 mmol) was dissolved in DMF (15 mL). To the vigorously stirred solution, triethylamine (0.1 mL) was added, and then succinic anhydride (6 mg, 0.06 mmol) in DMF (3 mL) was added dropwise in 20 min. The solution was stirred at room temperature under nitrogen for 22 h. The solvents were removed under nitrogen stream. The residue was flash chromatographed on silica gel (10 g) eluting with dichloromethane–methanol–acetic acid (85:10:5) to yield OTA-PEG-CO_2H (**5**) (35 mg, 85%). ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 8.31 (s, 1H, H-12), 8.16 (s, 1H, H-6), 7.27 (m, 6H, H-phenyls), 5.01 (t, 1H, H-13), 4.79 (m, 1H, H-3),



Scheme 1. The schematic processes for the synthesis of ochratoxin A–ovalbumin conjugate (OTA-PEG-OVA).

3.65–3.51 (m, 12H, CH_2–O), 3.34 (m, 1H, H-14a), 3.26 (m, 1H, H-4a), 3.14 (m, 1H, H-14b), 2.85 (m, 1H, H-4b), 2.59 (t, 2H, CO–C*H*$_2$–CH_2–CO), 2.47 (t, 2H, CO–CH_2–C*H*$_2$–CO), 1.77 (m, 4H, CH_2–N), 1.68 (m, 4H, OCH_2–C*H*$_2$–CH_2N), 1.54 (d, 3H, H-21); ^{13}C NMR (400 MHz, CD_3OD) δ (ppm): 177.4 (NH–CO–CH_2–CH_2–*C*OOH), 177.3 (NH–*C*O–CH_2–CH_2–CO), 175.4 (C-22), 174.0 (C-1), 171.6 (C-11), 159.0 (C-8), 144.3 (C-5), 139.3 (C-6), 138.9 (C-15), 131.4 (C-16,20), 130.4 (C-17,19), 128.8 (C-18), 122.7 (C-10), 120.9 (C-7), 110.1 (C-9), 77.7 (C-3), 72.4 (CH_2–O, PEG), 72.3 (CH_2–O, PEG), 72.1 (CH_2–O, PEG), 72.0 (CH_2–O, PEG), 70.7 (CH_2–O, PEG), 70.6 (CH_2–O, PEG), 57.7 (C-13), 38.7 (2CH_2–N), 34.2 (C-14), 32.6 (CO–CH_2–*C*H_2–CO), 31.4 (C-4), 31.2 (CO–*C*H_2–CH_2–CO), 31.1 (OCH_2–*C*H_2–CH_2N), 31.0 (OCH_2–*C*H_2–CH_2N), 21.7 (C-21). ESI-MS (negative mode, *m*/*z*) of **5**: 704.5 $[M-H]^-$.

2.3.2. Preparation of OTA-PEG-OVA conjugate (Scheme 1)

OTA-PEG-CO_2H (**5**) (0.0255 mmol) was dissolved in a solution of DCC (0.1625 mmol) and NHS (0.1625 mmol) in dry DMF (0.2 mL), and the reaction was stirred at room temperature for 3 h until a white precipitate (ureas) was formed. The solution was then added to OVA (0.51 μmol) in phosphate buffer (2 mL, 0.2 M, pH 7.4) without separation, and stirred at 4 °C overnight. The conjugate was

dialyzed against deionized water for 2 days (3 changes per day) and PBS/T for 8 h (2 changes) at 4 °C. Finally, the conjugate (2.5 mL) was further purified by passing it through a PD-10 column (Amersham Pharmacia Biotech, Sweden) using PBS/T as eluent and the purified conjugate (3.5 mL) was collected. The protein concentration of OTA-PEG-OVA (**6**) was determined by a bicinchoninic acid assay.

2.3.3. Preparation of analytical standards and food samples

A stock standard solution (1 mg mL^{-1}) was prepared by dissolving OTA in methanol. Intermediate standard solutions of 100, 10, and 1 μg mL^{-1} were prepared in methanol by diluting the stock. These solutions were stored at −20 °C. The working standard solutions were prepared by diluting the intermediate solutions in HBS-EP buffer.

For a spiking study of cereals, several 0.5 g replicates of both finely ground corn and oats were weighed out. Solutions (0.5 mL) of OTA diluted in methanol at various concentrations were spiked into individual 0.5 g replicates of each cereal, and then allowed to evaporate overnight in a fume-hood. The spiked-samples were mixed with 2 mL of methanol/water (50:50, v/v) and extracted for 30 min in a ultrasonic bath, after which the mixture was centrifuged at 5000 rpm at 4 °C for 10 min. Aliquots of the clear supernatants were removed, diluted with HBS-EP, and used directly for analysis without clean-up.

For a spiking study of beverages, aliquots of grape juice, apple juice, red wine (2006, 12.5% alcohol) and white wine (2007, 12% alcohol) were spiked with the stock solution of OTA to obtain a final concentration of 10 μg mL^{-1} OTA. Then, using the unspiked-samples as diluent, several dilutions from each spiked sample were made to obtain different concentration samples containing OTA in the range 0–5 μg mL^{-1}. Different treatment protocols were studied to minimize matrix effects, including PVP and sample dilution [28]. The optimum treatment of beverages was based on the addition of 3% PVP in fruit juice and 5% PVP in wine samples. The mixtures were then shaken for 5 min at room temperature, and the pH of each aliquot was then adjusted to 7.2 with 3 M NaOH prior to analysis.

2.3.4. Biacore assay

2.3.4.1. Immobilization of OTA-PEG-OVA and OTA-BSA on mSAM surface. The preparation of the mSAM and the immobilization procedures were performed as described previously [29]. Briefly, the mSAM surface was formed by deposition of a mixture of 11-MUOH and 16-MHA (10 mM, 9:1) on a bare gold chip (SIA Kit Au). Then, the OTA-BSA or OTA-PEG-OVA conjugates at protein concentration of 0.5 mg mL^{-1} in 10 mM sodium acetate pH 4.0 buffer were immobilized on the surface, using the amine coupling kit supplied by the manufacturer. A similar level of immobilization of 2500 RU was achieved for both ligands. A reference flow cell was constructed by immobilization of chloramphenicol-PEG-OVA [26].

2.3.4.2. Evaluation of sensor surfaces. Two OTA immobilized surfaces were evaluated for the development of the OTA SPR immunoassay. Indirect competitive assays were performed by mixing mAb-OTA (final concentration of 20 μg mL^{-1} for OTA-BSA surface and of 5 μg mL^{-1} for OTA-PEG-OVA surface) with a series of standard OTA solutions in HBS-PE buffer (0–1 μg mL^{-1}). After incubation, 75 μL mixtures were then injected onto the immobilized surface at a flow rate of 25 μL min^{-1}, followed by surface regeneration with 5 M guanidine in 50 mM glycine (pH 2.0, 25 μL min^{-1}). Five replicates were performed using a Biacore wizard program.

2.3.4.3. Binding signal enhancement and competitive assays of OTA in food samples. Signal enhancement by IgG/nanogold particles was performed by injecting a fixed amount of mAb solution (0.5 μg mL^{-1}, 75 μL, 25 μL min^{-1}) over the OTA-PEG-OVA surface, followed by sequential injection of IgG/nanogold (40 nm, 40 μL, 10 μL min^{-1}) at various dilution ratios (0, 1:5, 1:10, 1:15 and 1:20 in HBS-EP containing 1% (v/v) PEG-400, 2 M ethylene glycol and 0.5 M NaCl) from a commercial stock solution (Optical Density = 10.2 at 520 nm).

A binding curve of SPR responses versus the ratio of IgG/nanogold was plotted, giving a maximum signal enhancement and minimum non-specific binding, which was used for the antibody binding enhancement study. The antibody binding with IgG/nanogold enhancement curve was established by varying the concentration of mAb in HBS-EP ranging from 0 to 2 μg mL^{-1} but keeping the Au nanoparticles at a 1:15 dilution ratio. Regeneration was achieved using the same procedure as above.

Determination of OTA in buffer and various food samples was carried out using a competitive inhibition assay format. Assays were performed by mixing mAb (0.5 μg mL^{-1}) with a series of standard OTA solutions in HBS-EP buffer or sample extracts ranging from 0 to 1 μg mL^{-1} at 1 to 1 ratio (v/v). After incubation, the mixtures were injected over the surface (75 μL, 25 μL min^{-1}), followed immediately by injection of IgG/nanogold (40 nm, 40 μL, 25 μL min^{-1}) and then regeneration for the next binding cycle. The results were analyzed statistically using Sigma Plot version 8.0 (SPSS, Chicago, IL). All assay standard curves were fitted to a four-parameter logistic plot.

2.3.4.4. Cross-reactivity determination. To determine the specificity of the antibody in the presence of other common mycotoxins, the cross-reactivity of the anti-OTA antibody with aflatoxin M1, deoxynivalenol, and zearalenone was evaluated in buffer using the same assay conditions as described in the competitive assay of OTA. Cross-reactivity was determined by comparing the concentration of standard OTA inhibiting 50% of antibody binding (IC_{50}) with the concentration of other compounds inhibiting 50% of antibody binding according to the following equation.

$$\text{Cross-reactivity}\,(\%) = \frac{IC_{50}\ \text{for OTA}}{IC_{50}\ \text{for other compounds}} \times 100\%$$

3. Results and discussion

The effective immobilization of OTA is a critical step for developing a sensitive SPR immunosensor. As recognition by the antibodies is easier when the antigen is more exposed, surface immobilization by OTA conjugates (OTA-PEG-OVA) containing a PEG linker was evaluated and compared with the surface coated by an OTA-BSA conjugate without a PEG linker. Then, indirect competition between the immobilized OTA and free OTA in the sample for binding the antibody in solution was carried out to compare the performance of the two sensor surfaces. Finally, IgG/nanogold (40 nm) solution was flowed over the surface to enhance the antibody binding signals and therefore improve the assay sensitivities.

3.1. Competitive immunoassay based on mSAM/OTA-BSA surface

The commercial OTA-BSA conjugate was covalently immobilized onto a mixed SAM surface consisting of 10% 16-MHA and 90% 11-MUOH using an amine coupling kit, achieving an immobilized level of 2500 RU. This corresponds to 2.5 ng of OTA-BSA per mm^2 on the sensor surface, calculated according to the relationship 1 RU corresponds to a change of 1 pg per mm^2 of surface protein concentration [30]. The optimum Biacore conditions were found to be 20 μg mL^{-1} antibody, mixed with OTA standards at a 1:1 ratio, a flow rate of 25 μL min^{-1} coupled to a contact time of 3 min, followed by a 1-min pulse of regeneration solution (50 M guanidine in 50 mM glycine, pH 2.0).

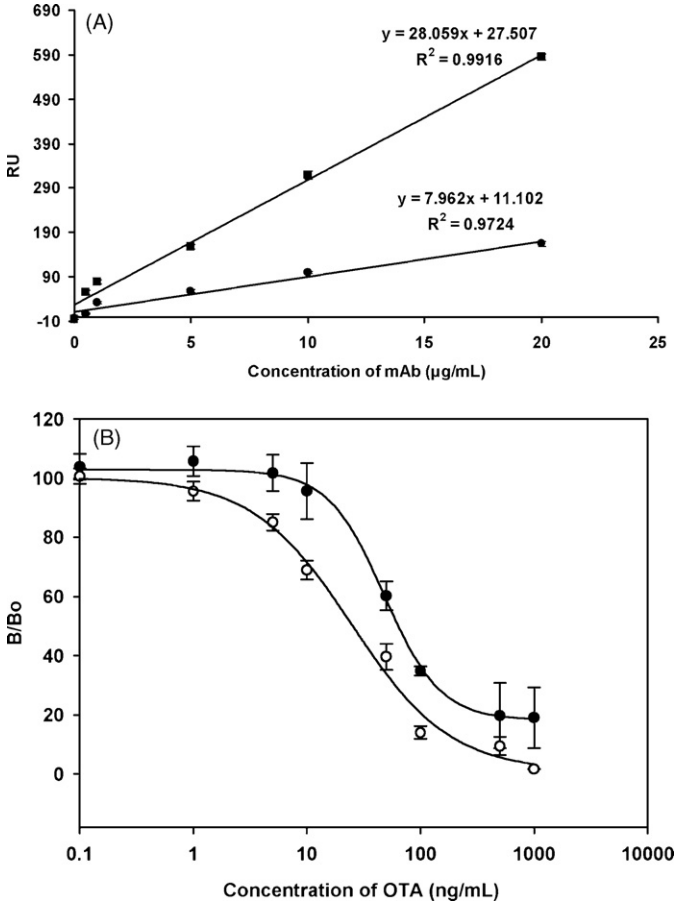


Fig. 1. (A) Binding responses in HBS-EP buffer for mAb on OTA-BSA surface (●) and OTA-PEG-OVA surface (■). Some error bars were too small to be seen (based on five measurements for each mAb concentration). (B) Comparison of two assay standard curves of OTA in HBS-EP buffer on OTA-BSA/mSAM surface (OTA standard solutions containing 0.3 M NaCl) (●) and OTA-PEG-OVA/mSAM surface (○). Error bars are standard error of the mean with $n = 5$.

The non-specific binding of OTA on the OTA-BSA surface was about 19%. It has been reported that OTA can bind strongly to plasma proteins such as BSA [31]. This non-specific binding can seriously affect the performance of the assay, as free OTA used in the solution can be adsorbed onto the surface BSA. It is well known that increasing the salt concentration in the assay buffer can reduce the non-specific binding [32]. Therefore, the competition assay was performed with 0.3 M NaCl in the OTA working solutions (Fig. 1B), and gave an IC_{50} value of 54.1 ± 4.9 ng mL^{-1} with an LOD of 16.4 ± 2.59 ng mL^{-1}.

3.2. Competitive immunoassay based on mSAM/OTA-PEG-OVA surface

Preparation of appropriate hapten-carrier protein conjugates for surface immobilization is a critical step for developing an effective SPR immunoassay of small molecules. Our previous work on the design of protein conjugates has demonstrated that insertion of a water-soluble, 23-atom long PEG linker between the small molecule and the protein can significantly increase the degree of hapten density in the protein conjugate, thereby increasing the density of surface immobilization and the assay sensitivity [26,29]. In the present work, an OTA-PEG-OVA conjugate (**6**) was prepared by a similar procedure to that described previously [26,29] and the number of hapten molecules conjugated to the protein was determined by MALDI-TOF. A centering m/z value (MW 51816) from the conjugate in the MALDI-TOF spectrum was taken as an average molecular weight of the OTA-PEG-OVA conjugate. Based on this result and a pure OVA sample (MW 44343) as a reference, the calculated mass due to the OTA derivative (OTA-PEG-CO_2H, MW 705) in the OTA-PEG-OVA conjugate (**6**) was 7473. Therefore, the number of OTA constituents was calculated to be 10.6 or around 11 molecules of the OTA derivative per protein molecule. This is similar to the number of hapten molecules attached to protein in our previous work [26,29]. There are several advantages of using a PEG chain as an intermediate linker in hapten-carrier conjugates. It can increase water solubility through the multiple ether linkages and thus improve the protein conjugation in aqueous solution [33]. This significantly increases the surface density of hapten. In addition, high surface densities and a long PEG chain can effectively prevent non-specific binding by resisting biomolecule adsorption on the sensor surface [34]. Furthermore, the PEG linker can effectively project the antigen, such as OTA, out from the sensor surface to allow maximum antibody binding and more efficient surface regeneration.

A checkerboard assay was performed in order to determine the optimal working conditions. An immobilization level of 2.5 ng per mm^2 on the mSAM surface was achieved using OTA-PEG-OVA at 0.5 μg mL^{-1}. This is similar to that achieved for the OTA-BSA surface. The antibody binding curve showed that the optimal concentration of mAb required for competition assays was 5 μg mL^{-1}, which is a 4-fold reduction compared with that used on the OTA-BSA surface (Fig. 1A). The non-specific binding of OTA on the OTA-PEG-OVA surface was negligible at only 1.7%, compared with large non-specific binding (19%) of OTA on the OTA-BSA surface, indicating that PEG has effectively minimized surface non-specific bindings of OTA. A 1:1 antibody: antigen volume ratio was applied in a competition assay for aqueous OTA (Fig. 1B). Comparison of SPR assays on the two SPR surfaces based on their LOD values of 1.5 ± 0.3 ng mL^{-1} for OTA-PEG-OVA and 16.4 ± 2.59 ng mL^{-1} for OTA-BSA is shown in Table 1. The lower values achieved using OTA-PEG-OVA, resulted from the higher density of OTA on protein OVA (11 to 1 ratio) compared with OTA-BSA (4 to 1 ratio), and the long PEG spacer, which minimized the steric effects thus allowing much easier regeneration and creating a stable surface. The results showed that the OTA-PEG-OVA coated mSAM chip underwent over 600 binding/regeneration cycles, whereas the OTA-BSA surface performed only 250 cycles. Consequently, the OTA-PEG-OVA surface was used in all further tests to evaluate the combination with Au nanoparticle enhancement of the SPR signals to further improve the assay sensitivities.

3.3. Au nanoparticle enhanced the sensitivity of SPR immunoassay

Recently, we developed an ultrasensitive SPR assay for small molecules by using Au nanoparticles as external labels for signal amplification on mSAM/analyte-PEG-OVA surfaces [26,29]. The assay achieved an LOD of 4.9 pg mL^{-1} for progesterone with small Au nanoparticles (10 nm) or an LOD of 0.74 fg mL^{-1} for chloramphenicol using large Au nanoparticles (40 nm). Based on these results, 40 nm Au nanoparticles were chosen to improve the sensitivity and lower the detection limit of the competitive assay on the mSAM/OTA-PEG-OVA surface.

The two major challenges to successful application of Au nanoparticles for SPR signal enhancement are the minimizing of non-specific binding of Au nanoparticles on the sensor surface and the retention of efficiency of regeneration. Use of BSA protein and/or PEG has been widely reported as a useful technique for blocking non-specific binding on the sensor surfaces [35]. Throughout these experiments, non-specific binding of IgG/gold on the sensor surface was efficiently minimized by the use of a special

Table 1
Summary of OTA SPR assay parameters including IC_{50} sensitivity, and ratios of signal enhancement and LOD improvement.

Assay format	mAb ($\mu g\,mL^{-1}$)	LOD ($ng\,mL^{-1}$)	IC_{50} ($ng\,mL^{-1}$)	Inter assay CV%[a]	Signal enhancement ratio	LOD improvement ratio
OTA-BSA surface	20	16.4 ± 2.59	54.1 ± 4.9	14.4		
OTA-PEG-OVA surface	5	1.5 ± 0.3	24.9 ± 2.5	8.2	3.5	2.2
IgG/gold (40 nm) enhancement	0.5	0.042 ± 0.004	3.4 ± 0.26	8.1	17.2	35.7
OTA in HBS-EP/MeOH (10%)	0.5	0.068 ± 0.009	5.4 ± 0.6	5.3		
OTA in HBS/MeOH (25%)	0.5	0.47 ± 0.078	20.5 ± 2.2	7.8		
OTA in oat spiked-samples	0.5	0.33 ± 0.034[b]	47.2 ± 5.3[b]	9.5		
OTA in corn spiked-samples	0.5	0.5 ± 0.022[b]	56.5 ± 3.9[b]	6.3		
OTA spiked in white wine (0.5% PVP)	0.5	0.74 ± 0.089	49.5 ± 3.5	4.9		
OTA spiked in white wine (1% PVP)	0.5	0.65 ± 0.051	45.1 ± 4.5	2.8		
OTA spiked in white wine (3% PVP)	0.5	0.56 ± 0.1	44.4 ± 2.8	6.8		
OTA spiked in white wine (5% PVP)	0.5	0.41 ± 0.043	13.2 ± 1.2	7.8		
OTA spiked in red wine (5% PVP)	0.5	0.33 ± 0.028	10.7 ± 0.7	5.6		
OTA in grape juice spiked-samples	0.5	0.058 ± 0.005	7.7 ± 0.6	8.5		
OTA in apple juice spiked-samples	0.5	0.084 ± 0.007	8.5 ± 0.3	5.1		

[a] Inter-assay CV% values are based on five replicates.
[b] Unit for LOD or IC_{50} for oat and corn samples is $ng\,g^{-1}$.

gold dilution buffer consisting of 1% (v/v) PEG-400, 2 M ethylene glycol, and 0.5 M NaCl into HBS-EP. This cocktail combined with polymer stabilizers and low ionic strength significantly prevented aggregation and non-specific adsorption of Au nanoparticles and also significantly facilitated the surface regeneration.

To estimate the binding capacity of the OTA-PEG-OVA sensor chip, different dilutions of IgG/gold (40 nm) (1:5, 1:10, 1:15 and 1:20 in optimum dilution buffer) from the original stock solution were applied after injection of a fixed amount of mAb ($0.5\,\mu g\,mL^{-1}$) as described in Section 2. Binding signals increased in an almost linear fashion up to 1349 RU with decreasing dilution of the Au particle complexes. In contrast, the non-specific binding decreased from 14.8% (1:5 IgG/gold applied) to 8.1% (1:10 IgG/gold applied) and to

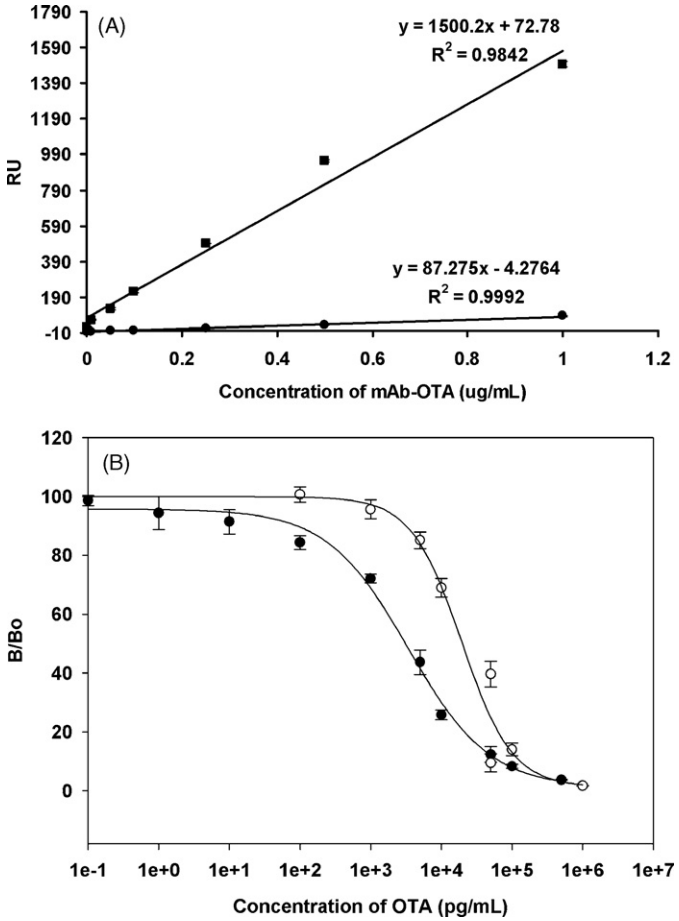


Fig. 2. On the OTA-PEG-OVA/mSAM surface: (A) Binding responses in HBS-EP buffer for mAb only (●) and IgG/nanogold (40 nm) enhancement (■). (B) Comparison of two assay standard curves with mAb only (○), and IgG/nanogold (40 nm) enhancement (●). Error bars are standard error of the mean with $n = 5$.

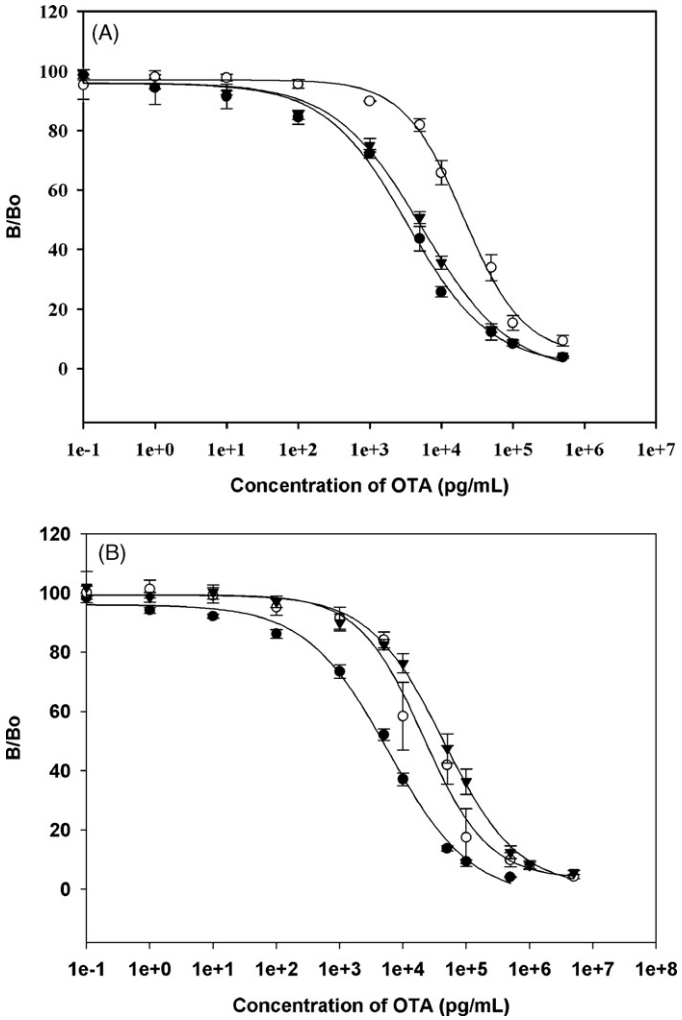


Fig. 3. (A) Comparison of three SPR assays of OTA in HBS-EP buffer only (●), HBS-EP containing 10% MeOH (▼) and HBS-EP containing 25% MeOH (○). (B) Comparison of three assay standard curves of OTA in HBS-EP buffer (●), and for spiked oat samples (○) or spiked corn samples (▼). Error bars are standard error of the mean with $n = 5$.

about 3.5% for both the 1:15 and 1:20 IgG/gold dilutions. However, the efficiency of regeneration using 5 M guanidine in 50 mM glycine (pH 2.0) increased from 87% to 92% and to 100% with increasing dilution of the Au particle complexes. Taking into consideration the four most important parameters, i.e. consumption of IgG/gold conjugates, the degree of signal enhancement, the level of non-specific binding and efficiency of regeneration, a 1:15 dilution of IgG/gold was chosen for further experiments.

A sequential injection assay was performed to determine the amount of signal enhancement upon antibody binding achieved by injecting various concentrations of mAb followed immediately by injecting a fixed amount of IgG/gold (40 nm, 1:15 dilution). The results showed that 40 nm Au nanoparticle/IgG greatly increased the signal of antibody binding 17.2-fold (Fig. 2A). This signal enhancement led to a huge reduction of mAb concentration required in the competitive assay of OTA from 5 to 0.1 $\mu g\,mL^{-1}$. The competition assays were evaluated by mixing mAb with a series of OTA standard solutions in HBS-EP buffer and followed a sequential injection procedure. A wide detection range (0–1 $\mu g\,mL^{-1}$) of OTA was achieved with an LOD of $42.3 \pm 4.3\,pg\,mL^{-1}$ (Fig. 2B). The sensitivity was increased 100-fold following nanogold enhancement. However, the LOD achieved in this assay was much higher than our previous LOD of $0.75\,fg\,mL^{-1}$ for chloramphenicol [26] which is probably because of the lower affinity of the anti-OTA antibody for its antigen. To further characterize the binding properties of this antibody, four different concentrations of mAb (0.1, 0.5, 0.75 and 1 $\mu g\,mL^{-1}$) were used in separate nanogold enhanced competition assays for OTA. The results showed that the LOD values achieved with the four mAb concentrations were similar (data not shown), suggesting the lowest LOD is reached at relatively higher monoclonal anti-OTA antibody concentration compared with our previous results with other small molecule immunoassays [26].

3.4. Detection of OTA in cereals

Methanol is commonly used to extract OTA from food matrices, with various solvent combinations yielding different results [36]. To test the effects of methanol on the binding performance of the anti-OTA antibody, competitive assays were performed in pure HBS-EP buffer as well as HBS-EP containing 10% or 25% methanol (Fig. 3A). A much higher IC_{50} value ($20.5 \pm 2.2\,ng\,mL^{-1}$) was obtained when using 25% methanol. This clearly shows that the high methanol concentration interfered with the sensitivity of the SPR immunoassay by inhibiting the interaction between the OTA and the antibody [36]. However, the IC_{50} value ($5.4 \pm 0.6\,ng\,mL^{-1}$) obtained with 10% methanol was similar to the value obtained using HBS-EP buffer ($3.4 \pm 0.26\,ng\,mL^{-1}$), indicating that an extract containing 10% methanol can be safely assayed without any detrimental effects on the tracer antibody (Table 1).

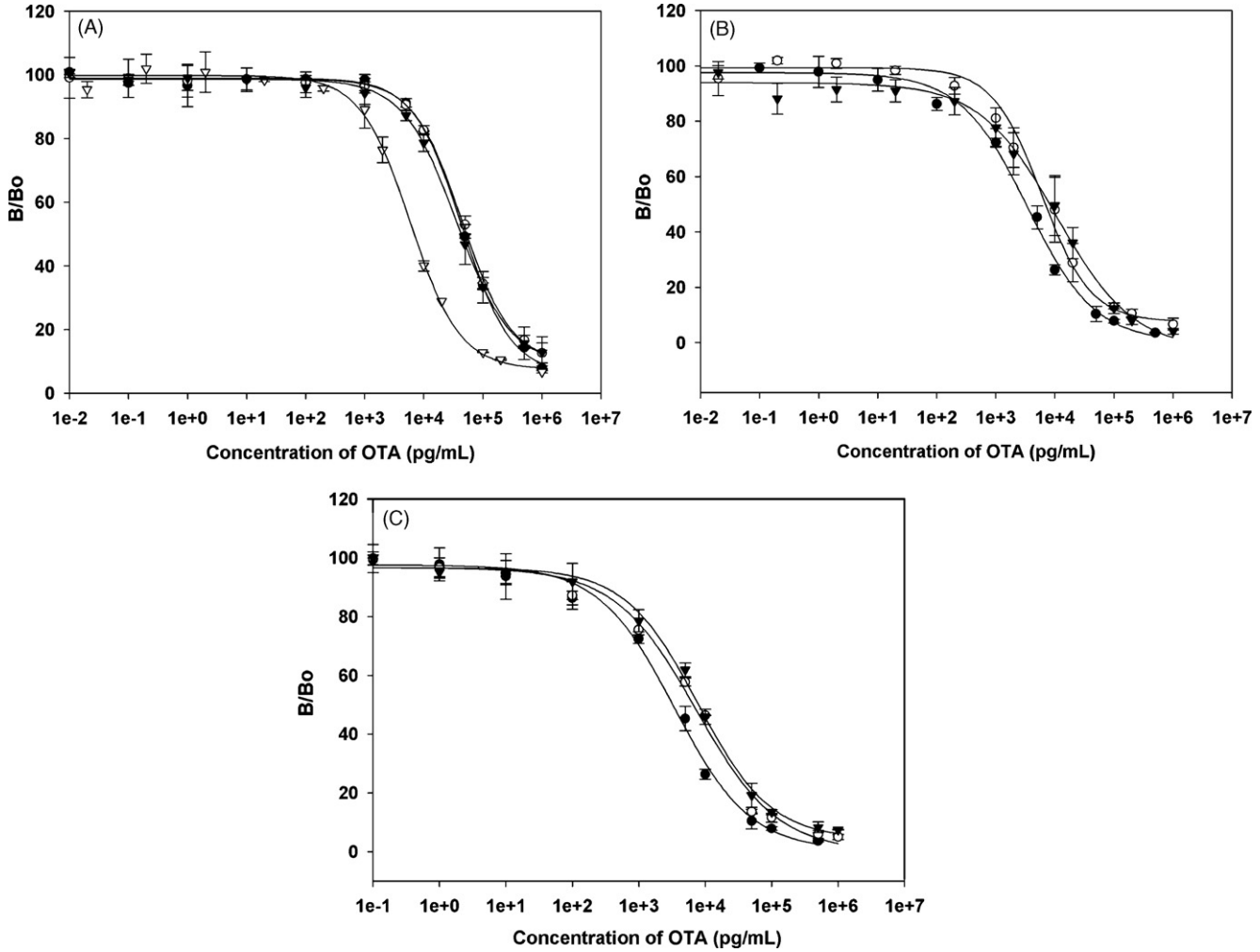


Fig. 4. (A) Comparison of four assay standard curves of OTA for spiked white wine samples containing PVP of 0.5% (●), 1% (○), 3% (▼), or 5% (△). (B) Comparison of three assay standard curves of OTA for white wine (○), red wine (▼) or HBS-EP buffer (●) spiked-samples. (C) Comparison of three standard curves of OTA for grape juice (○), apple juice (▼) or HBS-EP buffer (●) spiked-samples. Error bars are standard error of the mean with $n = 5$.

Based on these results, grain samples spiked with various concentrations of OTA were extracted using 50% methanol in water (v/v). The extracts were then diluted with HBS-EP to yield an OTA solution with a final concentration of 10% methanol. After mixing the OTA solution with mAb at a 1:1 ratio and flowing the sample over the OTA surface, Au nanoparticles were then injected onto the surface to enhance the sensitivity of SPR immunoassays. Fig. 3B shows the standard curves of OTA in oats and corn sample extracts. Compared with the curve for OTA in a solution of HBS-EP/methanol (10%), the assay curves for both oats and corn have shifted slightly to a higher concentration, suggesting some inhibition of binding was caused by the cereal matrix alone. However, the limits of detection for OTA spiked in oat or corn were measured as 0.33 ± 0.034 and $0.5 \pm 0.022\ \text{ng mL}^{-1}$, respectively (Table 1). These are lower than those reported using other biosensor measurements [21], and similar to the values produced by ELISA/HPLC but achieved much more rapidly and more simply [11].

3.5. Detection of OTA in beverages (wine and fluid juice)

The percentage of alcohol in wine (around 12%) is not high enough to influence the binding interaction between the antibody and antigen [21]. However, the additional content of polyphenols in wine could cause antibody inactivation and therefore interfere with the immunoassay performance. To address this, a simple pre-treatment of the wine sample with the binding agent poly(vinylpyrrolidone) (PVP) was used to remove polyphenols, followed by pH neutralization [21,28]. The spiked-samples were then directly analyzed without further dilution. Fig. 4A shows the assay curves produced by different pre-treatments with PVP in samples spiked with white wine in a competitive assay format with nanoparticle enhancement. The results clearly demonstrate that higher assay sensitivity, or a lower IC_{50} value, was achieved for OTA analysis in the wine sample with the addition of 5% PVP treatment, while there was no significant difference in IC_{50} values in the samples with the other three PVP treatments (0.5, 1 and 3%) (Table 1). It was also found that the amount of PVP in the HBS-EP buffer had almost no influence on the binding activity of the OTA antibody (data not shown); therefore a higher concentration of PVP can be used to stabilize the antigen–antibody binding in wine. This approach was also applied successfully to the red wine-spiked sample. Fig. 4B shows the standard curves of OTA in both white and red wine sample against HBS-EP buffer all with addition of 5% PVP. Compared with OTA in HBS-EP buffer, the IC_{50} value for OTA increased about 2-fold in white wine and 3-fold in red wine, probably because of the influence of other components such as anthocyanins on the antibody–antigen interaction. In this study, the LODs for OTA in white and red wine were determined to be 0.41 ± 0.043 and $0.33 \pm 0.028\ \text{ng mL}^{-1}$, respectively, with the IC_{50} values of 13.2 ± 1.2 and $10.7 \pm 0.7\ \text{ng mL}^{-1}$, respectively. These LOD values were lower than values in previous reports [9,13,31]. Recently, Flajs et al. reported lower detection limits ($5\ \text{ng L}^{-1}$) for OTA in wine using HPLC and modified ELISA but both of the methods used required complex sample clean-up and pre-concentration steps prior to analysis [37].

Addition of PVP to fruit juice samples followed by pH neutralization can also effectively remove the influence of endogenous polyphenols on competitive assay performance. Fig. 4C presents the assay curves for OTA analysis in HBS-EP, grape juice and apple juice with the addition of 3% PVP treatment. The standard curve for OTA in HBS-EP/PVP buffer was not significantly different from those obtained for OTA spiked in grape or apple juices, indicating that the matrix interference was not significant. The LOD obtained for OTA was as low as $0.058 \pm 0.005\ \text{ng mL}^{-1}$ with an IC_{50} value of $7.7 \pm 0.6\ \text{ng mL}^{-1}$ in grape juice and $0.084 \pm 0.007\ \text{ng mL}^{-1}$ (LOD) with an IC_{50} of $8.5 \pm 0.3\ \text{ng mL}^{-1}$ in apple juice.

3.6. Cross-reactivity of anti-OTA antibody

The cross-reactivity of monoclonal anti-OTA antibody with other common mycotoxins (aflatoxin M1, deoxynivalenol, and zearalenone) was examined. No cross-reaction was observed with any of these mycotoxin compounds (<0.01%) which may coexist in food, suggesting that the antibody is specific for the OTA on the surface.

4. Conclusion

A rapid and sensitive competitive SPR immunosensor with Au nanoparticle enhancement has been developed to detect OTA in various foodstuffs. The different analytical conditions were optimized including surface immobilization, non-specific binding, and regeneration. Our new OTA-PEG-OVA surface showed higher antibody affinity compared with the commercial OTA-BSA immobilized surface due to a higher density or accessibility of the protein-conjugated OTA and a long PEG linker which significantly increased the antibody binding response and therefore improved the performance of the SPR assay performance [26,29]. The use of Au nanoparticles for the enhancement of the OTA assay sensitivity demonstrated a 17-fold signal enhancement and an improvement in LOD of two orders of magnitude. These results represent a significant improvement in assay sensitivity over existing OTA biosensor assays. A simple one-step extraction of grain samples with aqueous methanol for grain samples, or for liquid samples pre-treatment with PVP to remove interference by polyphenols, and pH adjustment provides simple and rapid detection of OTA in situ. The detection limit for OTA in both cereals and beverages was less than $0.5\ \text{ng mL}^{-1}$ and the total time for sample analysis was less than 10 min (after extraction). This is competitive with or even superior to ELISAs and other analytical methods currently carried out with foodstuffs. Furthermore, our technique uses inexpensive reagents (IgG/nanogold) to significantly reduce the required concentration of the more expensive mAb reagent used in the assay. It is also based on a highly stable sensor surface, capable of performing over 600 binding/regeneration cycles with a single chip. Overall the described method provides a powerful tool for the rapid and cost-effective screening for OTA in food matrices.

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