Colloids and Surfaces B: Biointerfaces 174 (2019) 569–574

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Ochratoxin A detection in coffee by competitive inhibition assay using chitosan-based surface plasmon resonance compact system

Zainia Rehmat[a], Waleed S. Mohammed[b], Muhammad Bilal Sadiq[a], Manjunath Somarapalli[b], Anil Kumar Anal[a,*]

[a] Food Engineering and Bioprocess Technology, Department of Food, Agriculture and Bioresources, Asian Institute of Technology, Pathumthani 12120, Thailand
[b] Center of Research in Optoelectronics, Communication and Control Systems (BU-CROCCS), Bangkok University, Pathumthani 12120, Thailand

ARTICLE INFO

Keywords:
Ochratoxin A
SPR immunoassay
Chitosan
Carboxymethyl chitosan
Coffee

ABSTRACT

This study demonstrates the evaluation of ochratoxin A (OTA) in coffee on compact surface plasmon resonance (SPR) biosensors based on crosslinked chitosan and carboxymethyl chitosan nanomatrix substrates. Ochratoxin A is a toxic secondary metabolite widely produced by *Aspergillus* and *Penicillium* fungi and requires regular quantification and detection in food samples. The gold coated SPR chips were synthesized with chitosan and carboxymethyl chitosan through spin coating technique. The SPR nanomatrix chips were used for the immobilization of ochratoxin A-bovine serum albumin (OTA-BSA) conjugate to develop a competitive inhibition immunoassay. The monoclonal ochratoxin A antibodies (mAb-OTA) were used as biological receptors for the detection of OTA in buffer and coffee samples. The limit of detection (LOD) in coffee for chitosan (CS) and carboxymethyl chitosan (CMC) substrates was 5.7 ng/mL and 3.8 ng/mL, respectively. Compact surface plasmon resonance (SPR) system based on chitosan-based (CS-AU) nanomatrix substrates provides a platform for the detection of ochratoxin A with high sensitivity, accuracy, ease-of-use and cost-effectiveness. This compact SPR system can be used at farm and industrial levels for the detection of OTA in food matrices.

1. Introduction

Ochratoxin A (OTA) is highly toxic metabolite produced by molds of the *Aspergillus* and *Penicillium* families, which can grow on foods during harvesting and post-harvest stages and occurs naturally in numerous food products such as spices, coffee, cereals, beans and fruits, and in their processed products [1]. Moreover, OTA occurs in 25% of the world crops and poses a serious health risk due to its teratogenic, hepatotoxic and immunotoxic properties. Ochratoxin A has been categorized as a possible human carcinogenic (group 2B) by International Agency for Research on Cancer (IARC) [2–4]. Considering the public health importance, various chromatographic and immunoassay techniques have been developed for the detection and quantification of ochratoxin A in food commodities during last few decades. The most common techniques used for the detection of OTA are liquid chromatography–mass spectroscopy [5,6], high-performance liquid chromatography [7] and gas chromatography [8]. These all techniques are generally time-consuming, expensive and require trained personnel. It is thus, desirable to develop sensitive, low-cost, efficient and robust system for the detection of OTA in food commodities.

Coffee beverages are highly consumed worldwide and become the second largest traded food commodity. The intake of coffee has greatly been increased during the last two decades and it is estimated that more than 19 million tones will be consumed by 2019 [9]. The safe produce of processed coffee is highly desirable for the consumers as the filamentous fungi contaminate coffee during development, harvesting, transportation and, storage [10]. The OTA naturally occurs in green coffee beans in varying concentrations from 0.1 to 360 µg/kg [11–13]. The European Commission sets the highest maximum level of OTA in soluble and instant coffee at 10 ng/g and in roasted/ground coffee bean at 5 ng/g [14].

Optical biosensing is an emerging technology alternative to traditional analytical techniques due to its high sensitivity, fast screening, the efficiency and the simple assay procedures for detection. Optical transducer in most of the developed sensors are based on the optical waveguide, fiber gratings, guided mode resonance, surface plasmon resonance and interferometer [15]. Among these, surface plasmon resonance (SPR) provides a rapid lab-free tool to study the biological interactions in real time and it is used for the quantification of toxins in complex food matrices [16,17]. Surface plasmon is excited by the

* Corresponding author at: Food Engineering and Bioprocess Technology, Asian Institute of Technology, PO Box 4 Klongluang, Pathumthani 12120, Thailand.
E-mail addresses: anilkumar@ait.ac.th, anil.anal@gmail.com (A. Kumar Anal).

https://doi.org/10.1016/j.colsurfb.2018.11.060
Received 19 September 2018; Received in revised form 17 November 2018; Accepted 24 November 2018
Available online 26 November 2018
0927-7765/

Kretschmann geometry when the polarized light at the interface of dielectric metal film undergoes total internal reflection. In general, a gold film is used as sensing substrate for the sensor chip because of its ability to excite the SPR response. The principle of molecular binding in SPR is basically similar to the other immunological techniques such as enzyme-linked immunosorbent assay (ELISA), immunochromatographic technique (ICT). Surface plasmon resonance technique has been used for the detection of OTA in wine, cereals and milk with the limit of detection (LOD) ranging from 0.155 ng/mL to 0.55 ng/mL [18,19].

The sensitivity of SPR depends on the volume, weight, and immobilization of biological molecules on the sensor chip, and causes the changes in resonance angle [20,21]. Therefore, detection of small molecules such as OTA (M_w = 403.8 Da) is difficult due to weak response from the sensor (insignificant change of the refractive index). Hence, selective polymers with gold (Au) nanoparticles, self-assembled monolayers, and molecular imprinted polymers are used for the signal amplification [22]. In this regard, chitosan is one of the most promising biopolymers for the immobilization of small biological molecules on the SPR chip surface because of its good film-forming ability, chemical stability, low-cost, biocompatible and eco-friendly nature [23,24]. Chitosan has been used as a matrix in SPR biosensors for the detection of heavy metal ions [25–27]. There is no report yet for the detection of OTA in coffee by SPR immunoassay on chitosan and carboxymethyl chitosan nanomatrix substrates.

Chitosan has the high binding capacity with biomolecules due to presence of protonated amino acids on its side chain and therefore, reduces the nonspecific binding of biological molecules on SPR chip surface during immobilization [28,29]. However, chitosan is not soluble at physiological pH due to its lower pka of nearly 6.5. In order to increase the hydrophilic properties and solubility, chemical modification, such as carboxymethylation of chitosan is generally conducted [30]. Carboxymethyl chitosan (CMC) is soluble in water and exhibits high hydrodynamic volume, high viscosity, low toxicity, biodegradable and biocompatible nature [31]. During the chitosan modification, hydroxyl (OH^-) groups are substituted by carboxymethyl groups (CH_2COOH). Therefore, presence of amino and carboxyl groups provides the chemical bases for the covalent attachment of biological molecules. Chemical modification on SPR sensor chip can be done through amine coupling, aldehyde coupling and thiol coupling [32].

In this study, in-house built compact SPR sensor based on chitosan and carboxymethyl chitosan nanomatrix was evaluated for detection of OTA in the buffer and coffee. Competitive inhibition immunoassay was developed for the detection of OTA in coffee as illustrated in Fig. 1. The limit of detection was identified on both chitosan and carboxymethyl chitosan nanomatrix substrates.

2. Methodology

2.1. Reagents and material

Ochratoxin A and mouse monoclonal to Ochratoxin A antibody (mAb) were purchased from Biomed Diagnostics (Thailand). Anhydrous magnesium sulfate and phosphate buffered saline were acquired from Fluka Co. Ltd (Germany). All other chemicals were of analytical grade and bought from Sigma-Aldrich Co. Ltd, USA. The gold (Au) sensor chip was provided by National Electronics and Computer Technology Center (NECTEC) NSTDA, Thailand. All other chemicals used were of analytical grade.

2.2. Fabrication of SPR system

The compact and low-cost SPR system was designed based on edge coupling approach [33]. The sensor scheme is depicted in Fig. 1. The transverse magnetic polarized light was incident at the cross-section of the SPR chip therefore, avoiding the need for prism and index matching gel. The incident light underwent total internal reflection by striking the glass-metal interface at large to match SPR momentum. The output light was collected by a bare multimode optical fiber, which was further connected to a spectrometer. The SPR system was designed to have an SPR dip at 650 nm, that corresponds to SPR angle 0f ~73° and incident angle of ~26°. The bare fiber was also placed at an angle equals that of the incident angle. The mechanical alignment of the system was achieved using 3D printing (XYZ da vinci Jr 1.0). The test solution was introduced on the sensing surface using a micro fluidic flow channel fabricated using polydimethylsiloxane (PDMS) following the imprint method. The fluidic channel was placed on the top of SPR chip to help in limiting the area of interaction for the test solution on the sensing surface, that helps to improve the sensitivity of the system. The dimensions of the flow channel were approximately 10 mm × 1 mm × 100 µm by length, height and width respectively, with an inlet and outlet tube of 1 mm diameter.

2.3. Synthesis and immobilization of OTA-BSA conjugate on CS-AU surface

Surface plasmon resonance chips were coated with chitosan and carboxymethyl chitosan through spin coating technique. The SPR chips were coated with crosslinked chitosan and carboxymethyl chitosan for the binding of the ligand on the sensor surface. Carboxymethyl chitosan solution was prepared by dissolving 0.4 g in 50 mL of distilled water and crosslinked with 0.05 mL glutaraldehyde. Similarly, chitosan solution was prepared by dissolving 0.4 g in 50 mL acetic acid (1%, v/v) and crosslinked with 0.05 mL glutaraldehyde [34]. The mixture was stirred for 3 h thoroughly followed by vacuum filtration to remove any debris particles. The centrifugation (Sartorius, Göttingen, Germany) at 6000 rpm for 20 min, was conducted to settle down the suspended particles in the solution. Spin coating of the substrate was conducted on Donevcon Laurell spin-coater (6NPP/Lite) (Laurell Technologies) by spreading 0.55 mL of CS and CMC solution on rotating surface at the speed of 6000 rpm for 30 s. The chitosan-based substrate was fixed inside flow cell for the immobilization of OTA-BSA conjugate. The base line, the deionized water and PBS were injected subsequently into the flow cell. Immobilization of OTA-BSA conjugate was conducted on the fabricated microfluidic system directly inside the flow cell.

The surface was activated with 300 µl solution of EDC-NHC (1:1) by injecting the solution for 7 min followed by washing the surface with coupling buffer (maleic acid with NaOH at pH 5.5). Furthermore, the activated surfaces were immobilized with three different concentrations of OTA-BSA conjugates (50, 100 and 150 µg/mL in 10 mM sodium acetate pH 5.5). For this purpose, 300 µl solution of BSA-OTA conjugate was injected on the surface for 7 min at 25 C followed by regeneration of surface by 1 M NaCl. The surface was further rinsed by injecting deionized water and 300 µl ethanolamine (1 M) solution for 10 min. The purpose of ethanolamine was to block the reactive groups on the surface to reduce nonspecific binding on the sensor surface. During these, all steps of immobilization the SPR spectrum was recorded on the spectrometer (THORLABS CCS100).

2.4. Coffee samples preparation and analytical standard

The method for the preparation of coffee samples and analytical standards was adopted from Yuan et al. with slight modification [19]. The standard stock solution of OTA (1 mg/mL) was prepared in methanol (70%, w/v). Intermediate standard solutions (600, 300 and 100 µg/mL) were prepared by diluting the stock solution (1 mg/mL) in methanol. The HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20) was prepared according the method mentioned by Minoshim et al. [35] and buffer was used to prepare working standard solutions (0–50 ng/mL) by diluting the intermediate solutions in buffer and stored at −20 °C.

Roasted coffee beans were blended to prepare the fine texture powder. An aliquot of 5 g ground coffee was further spiked with OTA. From different concentrations of OTA (0, 0.3, 0.6, 1.25, 5, 10, 20 and

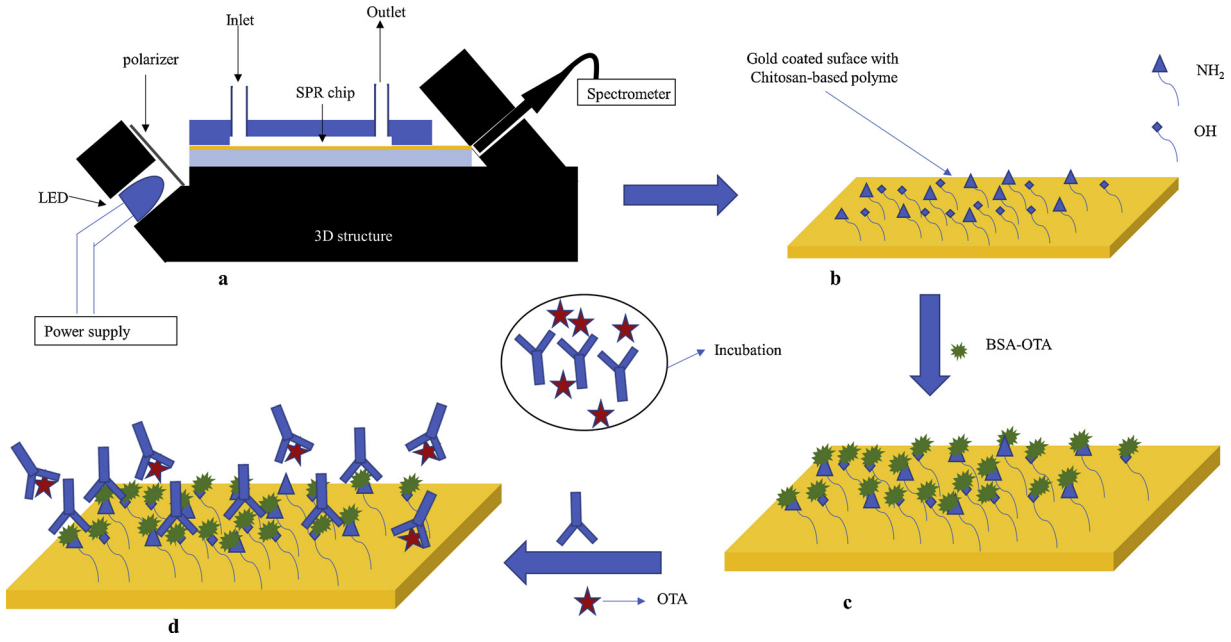

Fig. 1. Conceptual overview of the SPR immunoassay. Starting from 3D printed SPR biosensor (a), The chitosan and carboxymethyl chitosan nanomatrix on gold coated chip (b) The OTA- BSA conjugate is covalently immobilized on the surface by activated the carboxyl groups (c). The free OTA in solution and the immobilized OTA-BSA on the SPR sensor compete for the detection of OTA(d).

50 ng/mL), 0.5 mL of each concentration was spiked into each aliquot of coffee (5 g). The spiked samples were evaporated overnight in a laminar hood. Each spiked sample was extracted with 2 mL methanol (50%, v/v) first by overtax for 3 min followed by extraction in ultrasonic bath for 30 min. Later, the mixture was centrifuged at 6000 rpm at 4 ℃ for 15 min. The clear supernatants of aliquots were collected and diluted with HBS-EP buffer.

2.5. Indirect competitive inhibition assay of OTA in coffee samples on CS and CMC SPR sensor chip

The immunoanalytical methodology for indirect competitive inhibition assay was adopted from Estevez et al. [36] with slight modification. Ochratoxin A (OTA) immobilized surfaces CS and CMC were evaluated for the detection of OTA in coffee by SPR immunoassay. The OTA-BSA immobilized SPR chips were evaluated with pre- incubated mixtures of monoclonal ochratoxin A antibodies (mAb-OTA), OTA different concentrations in PBS and coffee samples. The calibration curves for both CS and CMC surfaces were established in PBS buffer and coffee samples separately. In the first step of SPR immunoassay, the HBS-EP buffer was run on the surface for 5 min to reach the stable baseline.

The ochratoxin A monoclonal antibodies and HBS-EP buffer (pH, 7.4) were mixed at ratio of 1:1, and poured over the chitosan-based substrate for 5 min. The resultant SPR spectra were continuously monitored. The regeneration solution with guanidine (5 M) in glycine (50 mM, pH 2.1) was passed after running each sample. Afterwards, the monoclonal ochratoxin A antibodies (10 µg/mL) and coffee samples containing series of standard OTA concentrations (0, 0.3, 0.6, 1.25, 5, 10, 20 and 50 ng/mL) were mixed with a ratio of 1:1, followed by incubation at 37 ˚C for 15 min. After incubation, 50 µl were flown over the substrate for 5 min with continuous flow of the sample and similar method discussed above was followed for the regeneration of substrate.

2.6. Data analysis

All the experiments were performed in triplicate and expressed as mean values ± standard deviation. Statistical analysis was conducted by using SPSS statistical software (SPSS, 23.0). ANOVA and Tukey's HSD test were carried out to determine the significant differences ($p < 0.05$) among the mean observations

The optical spectra as output were recorded using Thorlab spectrometer using Splicco software. Data were further processed with Origin lab 8. Logistic fit function was used for the calibration curve using Eq. (1) and LOD was calculated by using Eq. (2) [37].

$$Y = A2 + \frac{(A1 - A2)}{1 + (\frac{x}{X0})\hat{}p} \quad (1)$$

where A_1: SPR signals at zero analyte concentration,
A_2: SPR signals for infinite analyte concentration
X: Desired concentration
X_0: Inflection point
p: Sigmoidal curve slope (steepness of the curve), and
Y: SPR shift.

$$LODX = X0 \times \sqrt[p]{\frac{A1 - A2}{(LODY - A2) - A1}} \quad (2)$$

The established bioassay LOD was calculated by using Eq. (2)

Where, LODY = Y_0 - 3 - S_0, with Y_0 is the average response for 0 ng/mL OTA and S_0 is the mean standard deviation for Y_0.

3. Results and discussion

Effective immobilization of ligand on the sensor surface is required towards the detection of OTA in coffee and to increase the sensitivity of SPR immunosensor. Chitosan (CS) and carboxymethyl chitosan (CMC) coated substrates were immobilized with OTA-BSA conjugate and evaluated, through indirect competitive inhibition assay for the analysis of OTA in buffer and coffee samples. All the experiments were performed in ambient conditions.

3.1. Characterization of SPR setup

The developed SPR system was tested with solutions of different refractive index i.e. water (W = 1.331), and acetone solutions (A1 = 1.334, A2 = 1.339 A3 = 1.342, A4 = 1.345). The SPR system

Table 1
SPR response for solutions of different refractive index on different sensing surfaces gold + chitosan (AU + CS) and gold + carboxymethyl chitosan (AU + CMC).

Solutions	Refractive Index (RI)	Au	Au + CMC	Au + CS
water	1.331	620	633	638
A_1	1.334	626	639	645
A_2	1.339	634	647	653
A_3	1.342	640	653	659
A_4	1.345	645	658	664

A_1 = 10%, A_2 = 20%, A_3 = 30%, A_4 = 40% acetone (v/v).

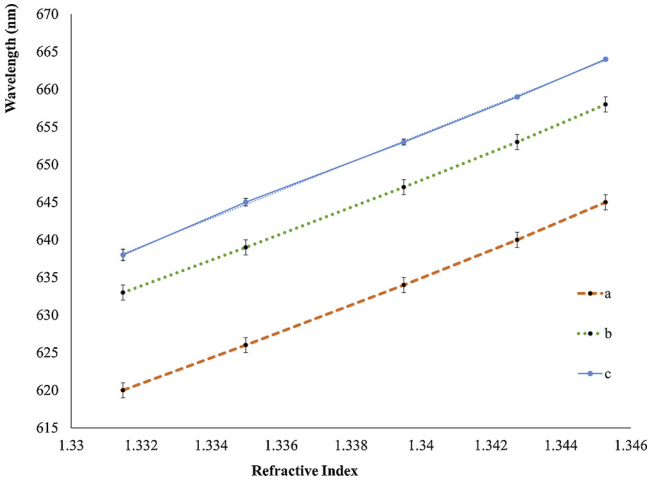

Fig. 2. The average of resonance wavelength shift on three different SPR chips coated with polymer. SPR chip based on Gold (a); SPR chip based on CMC (b); and SPR chip based on CS (c).

was also tested with two surface functionalization: a CMC polymer and CS polymer. The system response for the different solutions SPR sensing surfaces are shown in Table 1. An additional layer of polymer (CMC/CS) on top of the SPR chip showed an obvious shift due to refractive index of the polymer layer [38].

Fig. 2 illustrates the SPR response for different solutions at different surfaces. The response was observed linear and the sensitivity was measured to be $2.59*10^{-4}$, $4.05*10^{-4}$, and $5.4*10^{-4}$ for Au, Au + CMC, and Au + CS respectively. The testing of the system was performed in ambient environment conditions, That might influence the properties of the polymer upon exposure and might consequently affects the sensitivity of the system [33]. The CS and CMC polymer solutions were further cross-linked with glutaraldehyde to detect OTA in buffer and coffee samples. Spin coating technique was applied to develop homogenous nanomatrix of CS and CMC on gold coated SPR chips. The homogenous nanomatrix surfaces considerably reduce nonspecific binding due to its extreme chain flexibility and availability of polymer hydroxyl group on its side chain [38]. The nanomatrix increases the protein adsorption with surface and the nanomatrix coating is stable under the physiological flow rate [39].

3.2. Competitive inhibition immunoassay based on CS and CMC nanomatrix substrates

As SPR sensitivity depends on the deposition of molecular mass on the sensor surface, competitive inhibition assay is required for the detection of small molecules on SPR sensors due to the lack of signals from the transducer surface [36]. For the detection of OTA (M_w = 403.8 Da), direct immunoassay is less appropriate as the analyte directly flows on the antibody- immobilized surface. As shown in Fig. 1, fixed concentration of antibodies was incubated with different concentrations of

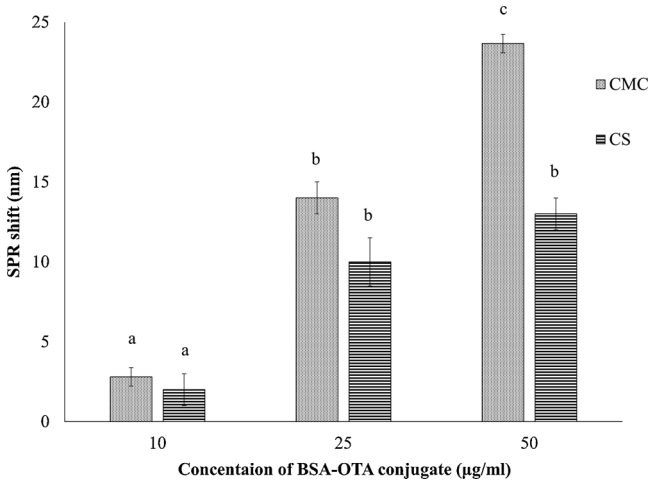

Fig. 3. Different concentrations of BSA-OTA conjugate (10, 25 and 50 µg/mL) immobilized on chitosan (CS) and carboxymethyl chitosan (CMC) coated surface. Different letters (a–c) above the bars indicate significant ($p < 0.05$) difference among mean observations for each treatment.

OTA. There was competition between immobilized OTA-BSA on the sensor substrates and free OTA in solution. Therefore, SPR response was high for the lower amount of toxin in solution as more free antibodies in solution interacted with the immobilized conjugates. The specificity towards mAb-OTA towards OTA was confirmed after pouring the mAb-OTA on the BSA immobilized surface without OTA. There was no significant SPR shift observed.

The composite nanomatrix sensor surface contains abundant aldehyde groups for adsorption and immobilization of bioactive compounds without dissolution. Chitosan and carboxymethyl chitosan SPR chips were immobilized with OTA-BSA conjugate through amine coupling kit. To optimize the OTA-BSA conjugate concentration for competitive inhibition assay on CS and CMC nanomatrix three different concentrations (10, 25 and 50 µg/mL) of OTA-BSA conjugate were immobilized on CS and CMC based substrates. The immobilization with 50 µg/mL OTA-BSA concentration resulted in significantly higher ($p < 0.05$) SPR shift with coefficient variance below 4% as shown in Fig. 3. Sensogram was developed for immobilization of ligand on chitosan-based substrates as shown in Fig. 4. The analyte was run on the bare sensor surface (without OTA-BSA conjugate) to evaluate the nonspecific binding and the SPR wavelength shift was negligible as compared to BSA-OTA conjugate.

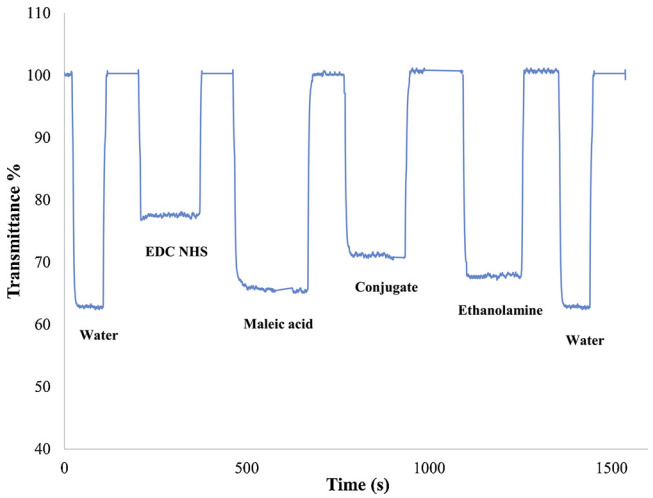

Fig. 4. Sensogram for immobolization of OTA-BSA conjugate on CS coated SPR chip.

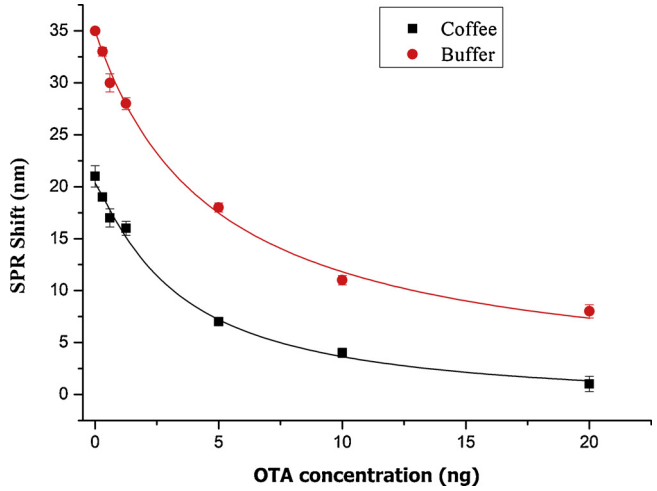

Fig. 5. logistic fit calibration curve (based on Eq. (1)) are plotted as a function of the OTA concentrations on chitosan surface based on SPR shift (nm) in buffer and coffee.

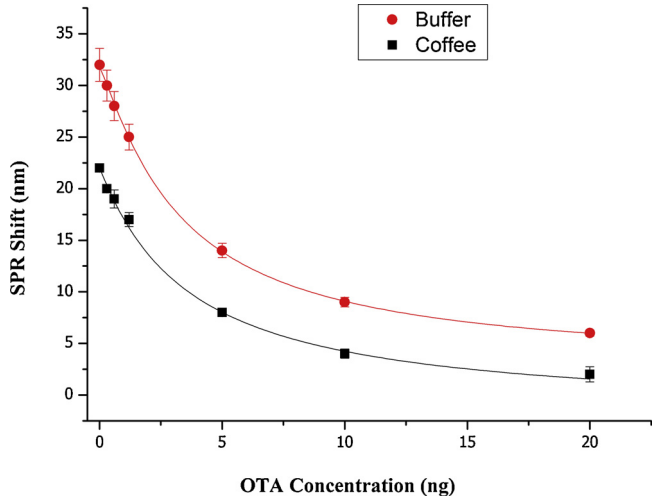

Fig. 6. Logistic fit calibration curves (based on Eq. (1)) are plotted as a function of the OTA concentrations on carboxymethyl chitosan surface based on SPR shift (nm) in buffer and coffee.

3.3. Detection of OTA in buffer and coffee

Coffee samples were spiked with various concentrations of OTA extracted in methanol (50% v/v). The spiked coffee and buffer samples were analyzed on compact SPR system. The coffee extracts were further diluted with HBS-EP buffer. The final concentration of methanol remained only 10%, MAb-OTA (15 µg/mL) was further mixed and incubated with different concentrations of OTA in the coffee sample at a 1:1 ratio. The samples were poured over the BSA-OTA immobilized surface. The higher IC_{50} value (4.7 ± 2.5 ng/mL) was obtained using HBS-EP buffer as compared to the coffee (3.2 ± 2.5 ng/mL).

Fig. 5 illustrates the logistic curve (based on Eq. (1)), fitted on the measurements for chitosan-based SPR substrate for analysis of OTA in coffee and buffer. Buffer (R^2 = 0.99, n = 4) resulted in LOD values of 3.2 ng/mL with coefficient of variance (CVs) of 8%. Similarly, OTA analysis in coffee (R^2 = 0.99, n = 3) resulted in LOD values of 5.2 ng/mL and CVs 16%. The LOD value for buffer was lower as compared to the coffee samples. The variation in values could be explained due to the fact that methanol concentration interfered with the sensitivity of the SPR immunoassay by inhibiting the interaction between the antibody and OTA [19]. Furthermore, the coffee residues were attached to chitosan coated SPR nanomatrix substrate, resulted in low system signals.

Similarly, for carboxymethyl chitosan SPR nanomatrix substrate, the different concentrations of OTA solution were mixed with mAb at a 1:1 ratio and poured the sample over the CMC based SPR chip. The IC_{50} value for the buffer (3.39 ± 0.5 ng/mL) obtained on carboxymethyl chitosan surface was similar to coffee (3.39 ± 2.5 ng/mL). The logistic curve (Eq. (1), Fig. 6) was fitted on the measurements for CMC based substrate for analysis of OTA in buffer (R^2 0.99, n = 3) resulted in LOD value of 2.5 ng/mL with CVs of 5%. While OTA analysis in coffee (R^2 0.99, n = 3) resulted in LOD value of 3.8 ng/mL with CVs of 10%. The LOD value for the buffer was lower as compared to the coffee samples because coffee is comprised of complex chemical structures including different amino acids, carbohydrates, and lipids [40]. Consequently, it may result in the nonspecific binding of the sensor substrate. The LOD was less for chitosan as compare to the carboxymethyl chitosan due to high adoption capability of CMC. Carboxymethyl chitosan showed high adsorption capacity than chitosan due to the presence of more carboxyl (−COOH) groups on the surface to bind with ligand [41]. Previous studies reported about the analysis of ochratoxin A in wine, cereals, wheat and peanut oil [18,42–44], however, these immunosensors have not extensively explored the analysis of coffee samples, which is a highly contaminated with OTA [45]. Herein, we fabricated a low-cost, small, portable and compact one piece system with the involvement of three dimensional (3D) printing technology for the development of system.

4. Conclusion

A rapid and sensitive competitive inhibition SPR immunoassay was developed on 3D printed compact SPR system. SPR chips were coated with chitosan and carboxymethyl chitosan through spin coating technique for the detection of OTA in buffer and coffee. Various analytical conditions were optimized such as regeneration of the surface and immobilization of conjugate on substrate. Buffer and coffee samples spiked with OTA were used in the concentration range between 0–50 ng/mL to stablish a calibration curve. The LOD of detection for carboxymethyl chitosan was higher as compared to chitosan both in buffer and coffee samples. On chitosan, coated surface the LOD for buffer and coffee was found to be 3.2 and 5.2 ng/mL respectively, while for carboxymethyl chitosan it was 2.5 and 3.8 ng/mL. The developed compact SPR system with biopolymer chitosan and carboxymethyl chitosan resulted in highly sensitive and cost-effective immunoassay for the analysis of OTA in a complex coffee matrix. This system can be extended for the detection of OTA in other food commodities.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare no conflict of interest.

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