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Gold nanoparticle-enhanced multiplexed imaging surface plasmon resonance (iSPR) detection of *Fusarium* mycotoxins in wheat

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ABSTRACT

A rapid, sensitive and multiplexed imaging surface plasmon resonance (iSPR) biosensor assay was developed and validated for three *Fusarium* toxins, deoxynivalenol (DON), zearalenone (ZEA) and T-2 toxin. The iSPR assay was based on a competitive inhibition format with secondary antibodies (Ab_2) conjugated to gold nanoparticles (AuNPs) used as a signal amplification tag. Signal was amplified nearly 25-fold for DON, 90-fold for ZEA and 12-fold for T-2 toxin assay using Ab_2-AuNPs. Analyses, including steps to regenerate the sensor, took 17.5 min. The antigen coated sensor chip was used for more than 46 cycles without affecting signal intensity (< 12%). Matrix matched calibration curves were used to determine *Fusarium* toxins in wheat. The mean recoveries ranged from 87% to 103% with relative standard deviations of repeatability of less than 5%. The limits of detection were 15 μg/kg for DON, 24 μg/kg for ZEA and 12 μg/kg for T-2 toxin. This provided sufficient sensitivity to monitor contamination of these mycotoxins in wheat in accordance with European Commission (EC) limits. Cut off levels for all three *Fusarium* toxins were validated using blank wheat and wheat spiked either at the EC regulated levels (100 μg/kg for ZEA and T-2 toxin) or at one third of the EC level (for DON: 400 μg/kg). The assay was successfully applied and further validated with naturally contaminated wheat samples. This is the first reported AuNP enhanced iSPR assay to detect and classify three agriculturally important *Fusarium* toxins in wheat.

1. Introduction

Mycotoxins are naturally occurring secondary metabolites produced by three major types of fungal genera, *Aspergillus*, *Penicillium* and *Fusarium*. Among these, *Fusarium* is the most widespread pathogen in cereals and is a heterogenous mycotoxin producer (Nathanail et al., 2015). Their widespread geographical distribution depends on several factors, including environmental conditions, crop production, storage, and processing and transportation methods (McNamee et al., 2017). The major toxins produced by *Fusarium* are the trichothecenes (deoxynivalenol, T-2 toxin and HT-2 toxin), fumonisins, and zearalenone (ZEA). After the aflatoxins, *Fusarium* toxins are the most reported mycotoxins in raw agricultural commodities (CAST, 2003). These toxins can enter into the food/feed chain and exert a wide range of adverse effects in humans and animals.

Among the trichothecenes, the most prevalent mycotoxin is deoxynivalenol (DON), produced by *F. graminearum* and *F. culmorum*. DON is frequently observed in U.S., Canadian and European wheat after invasion of the fungi, particularly during cool, wet growing and harvest seasons (CAST, 2003). In 1990s, DON was a severe problem in grain in the United States (U.S.). DON causes vomiting in animals and humans (D'Mello et al., 1999; Bhat et al., 1989). It is also affects the intestinal, hematopoietic, immune, endocrine, and nervous systems (Lin and Guo, 2016). The maximum level of DON permitted by the European Commission (EC, 2006) in unprocessed cereals, other than durum wheat, oats and maize is 1250 μg/kg.

Natural contamination with T-2 toxin (T-2) is less common in the U. S., but is observed more frequently in European grains, in particular oat and oat-based products (CAST, 2003; EFSA, 2011). T-2 is highly toxic, on the order of ten-fold more toxic than DON in mammals (Ueno, 1983). T-2 can penetrate skin and can cause non-specific symptoms such as weight loss, feed refusal, dermatitis, vomiting, diarrhea, hemorrhages and necrosis of the epithelium of stomach and intestinal, bone marrow, spleen, testis and ovary (EC, 2001). The EC has recommended a guidance level of 100 μg/kg in wheat for T-2 and HT-2 toxin (EC, 2013).

ZEA is an estrogenic mycotoxin, mainly produced by *F. graminearum*. In the U. S. and Canada, co-contamination of grain with ZEA

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and DON is frequently observed. The production of ZEA is favored by high humidity and low temperature conditions that are common in the Mid-western U.S. during autumn harvest (CAST, 2003). The commodities most commonly contaminated with ZEA are corn (maize), moldy hay, and pelleted commericial feed. ZEA and related congeners have estrogenic effects. These include edema of the vulva, prolapse of the vagina, enlargement of the uterus, atrophy of testicles, atrophy of ovaries, enlargement of mammary glands, and abortion (CAST, 2003). The maximum allowable levels for ZEA are 100 μg/kg in unprocessed cereals and 200 μg/kg in maize (EC, 2006).

Due to the public health concern and large economic losses caused by these toxins, a rapid, sensitive and economical analytical method is needed. Analytical methods for mycotoxins are generally divided into those that are confirmatory (quantitative analysis) or those that are used for rapid screening. Confirmatory methods such as high performance liquid chromatography, gas chromatography and mass spectrometry, are usually expensive, consume large volumes of solvent, and require skilled personnel. On the other hand, immunoassays such as enzyme linked immunosorbent assay (ELISA) and lateral flow devices ("dipsticks") are most commonly used to provide rapid, easy and sensitive detection. Most such assays offer only single toxin analysis. Therefore, rapid and sensitive multi-toxin screening assay is an attractive alternative.

Imaging surface plasmon resonance (iSPR) is an emerging semi-quantitative technique for the reliable, label free and sensitive detection of mycotoxins (Joshi et al., 2016). iSPR measures the changes in the refractive index due to biomolecular interactions at the sensor surface, the interface between a metal or dielectric media. Typically, SPR biosensors are suitable for medium and large compounds as the signal is proportional to the mass of the target molecules (Karczmarczyk et al., 2016). As mycotoxins are low molecular weight compounds, their binding with the antibodies is generally not adequate to produce sufficient SPR signal for practical use (Hu et al., 2014). A good alternative format uses the competition between free toxin and immobilized toxin (antigen) for limited amounts of primary (anti-toxin) antibodies added to the sample. The SPR signal can be further amplified by biofunctionalized nanomaterials such as gold nanoparticles (AuNPs), magnetic nanoparticles (MNPs), fluorophores or quantam dots (QDs) (Sendroiu et al., 2009; Yuan et al., 2011, Shabani and Tabrizian et al., 2013). AuNPs offer several advantages, including higher sensitivity, stability and selectivity, relatively easy and inexpensive synthesis, stability, unique optoelectronic properties, and high surface to volume ratio with excellent biocompatibility (Daniel and Astruc, 2004).

Although AuNPs have been successfully applied as amplification tags in the SPR-based detection of DNA, proteins and drug molecules, only a few mycotoxin studies have incorporated AuNPs. These include assays for ochratoxin A (OTA) (Karczmarczyk et al., 2016), and a combination of aflatoxins, OTA and ZEA (Hu et al., 2014). Karczmarczyk et al. (2016) reported a gold enhanced SPR assay for OTA in red wine using an indirect inhibition immunoassay format. Because multiplexing allows multiple toxins to be detected in a single run and because it reduces analysis time and the cost for reagents, multiplexing is becoming increasingly popular. Hut et al. (2014) described a multiplexed iSPR method for three mycotoxins in peanut samples. In both of these reports, however, the analysis times were long (~ 40 min or more), which is not suitable for practical purposes. Herein a rapid, reproducible, and sensitive iSPR assay is reported for three major *Fusarium* toxins in wheat. The method combines the multiplexing capability of iSPR with the strategy of using colloidal gold for amplification. Furthermore, the method was rapid and took only 17.5 min including the regeneration steps. To the best of our knowledge, no multiplex iSPR assay has been reported for the detection of DON, ZEA and T-2 in wheat that uses AuNPs for signal amplification.

2. Materials and methods

2.1. Chemicals and reagents

Gold (III) chloride trihydrate (HAuCl4. $3H_2O$) and mycotoxins (DON, ZEA and T-2) were purchased from Sigma Aldrich (MO, USA). PEG_3-OH was purchased from SensoPath Technologies (MT, USA) and PEG_6-COOH was purchased from Dojindo Molecular Technologies (MD, USA). 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and Sulfo-N-hydroxysucccinimide (Sulfo-NHS) were purchased from Thermo Scientific (USA). Goat anti-mouse IgG (secondary antibody: Ab_2) was purchased from Fisher Scientific (USA). The run buffer consisted of 0.1% w/v bovine serum albumin (BSA) in 10 mM sodium phosphate and 150 mM sodium chloride in water at pH 7.2. The water used in all experiments was purified by a nanopure water purification system (Fisher Scientific). Naturally contaminated wheat samples were purchased from Trilogy Analytical Laboratory (Missouri, USA). HPLC grade methanol was purchased from EMD Milipore (MA, USA). All other chemicals and reagents were purchased from Fisher Scientific.

2.2. Monoclonal antibodies

Mouse monoclonal antibodies (primary antibody: Ab_1) cross-reactive with DON, ZEA and T-2 toxin were produced at Envigo (Indianapolis, USA) using cell lines developed previously at the USDA-National Center for Agricultural Utilization Research (NCAUR, Peoria, IL). The monoclonal antibodies (Mab) used were Mab#1 for DON, Mab#22 for ZEA, and Mab#2–13 for T-2 toxin. The cross reactivities of the DON, ZEA and T-2 Mabs in several immunoassay formats were reported previously (Maragos et al., 2012, 2013; Maragos and Kim, 2006). For this manuscript the cross-reactivities of the antibodies were determined in the iSPR format. Calibration curves for the target mycotoxins and mycotoxin analogs were determined in blank wheat matrix, and the concentrations causing 50% inhibition (IC_{50}s) were calculated. The cross-reactivities were calculated as: (IC_{50} of the target mycotoxins/IC_{50} of the analog) × 100%. The analogs tested included 15-acetyl-deoxynivalenol (15-ADON), 3-acetyl-deoxynivalenol (3-ADON), nivalenol (NIV), α-zearalenol, β-zearalenol and HT-2 toxin (HT-2).

2.3. Preparation of mycotoxin-protein conjugates (antigens)

DON-β-lactoglobulin (DON-BLG) was synthesized using the carbodiimide technique described by Xiao et al. (1995) and Maragos and McCormick (2000). The protein concentration of the conjugate was determined with a commercial BCA assay (Pierce, Rockford, IL, USA). The conjugate was diluted to 1 mg/mL with 100 mM phosphate buffer saline (PBS), then distributed as 0.2 mL portions and lyophilized. To make the ZEA-BSA conjugate, the 6′-carboxymethyloxime of ZEA (ZEA-CMO) was first prepared according to the method of Thouvenot and Morfin (1983). The ZEA-CMO was then reacted with BSA as described by Maragos and Kim (2006). The T2-BSA protein conjugate was prepared according to the method of by Xiao et al. (1995) using 1, 1′-carbonyldiimidazole to link the 3-hydroxyl group of T-2 toxin to BSA. The lyophilized conjugates were reconstituted with water to achieve solutions of 1 mg/mL, which were then diluted as necessary with water during the immobilization procedure.

2.4. iSPR biosensor

An iSPR biosensor (iSPRimager®II) system was purchased from GWC technologies (Wisconsin, USA). The system used chips (slides) with 17 gold spots affixed in a grid pattern, with each spot surrounded by a hydrophobic polymer layer (SpotReadyTM chips, GWC Technologies). The chips were affixed to the optics of the instrument with a refractive index-matching fluid. The optical system (CCD

Camera, Horizon SPRimager) and software (V^{++} Precision Digital Imaging, version 5) captured data from all of the regions of interest (spots) on the array. To facilitate introduction of defined volumes of samples (generally 0.5 mL), a manual injection system consisting of a model 7125 valve (Rheodyne, Cotai, CA, USA) was added between the peristaltic pump and the flow cell.

2.5. Preparation of sensor surface

The gold surface of the sensor chip was modified with two hetero-bifunctional monothiolalkanes with polyethylene glycol (PEG) tethers to yield a surface with carboxylate groups (for immobilization of ligands) and hydroxyl groups (to minimize non-specific binding). Sensor chips were immersed in a mixture of 0.9 mM PEG3-OH and 0.2 mM PEG6-COOH in ethanol (99.95%) and held for a minimum of 3 days at ambient temperature in the dark in order to allow sufficient coupling to take place. The free carboxylates on the surface were then activated with a mixture of 100 mM Sulfo-NHS and 300 mM EDC in water for 1 h. After washing with water and drying with nitrogen gas, stock solutions of the DON-BLG, ZEA –BSA and T2-BSA conjugates were diluted with 50 mM phosphate buffer at pH 7.0 and spotted (0.1 μg) onto the gold surface. A BSA-immobilized gold spot was used as a control. The spotted chips were held in a humid environment at 4 °C overnight to avoid drying of the spots. Following this incubation remaining free carboxylates on the surface were reacted with 1 M ethanolamine, pH 8.5 for 30 min at ambient temperature. After washing with water and drying under nitrogen, the antigen coated sensor chips were stored dry at 4 °C until use.

2.6. Preparation of gold AuNPs and Ab_2-AuNPs conjugation

Gold nanoparticles (AuNPs) were prepared based on the citrate reduction method described by Ye et al. (2014). The remaining volume of the colloidal gold solution was adjusted to pH 8.5–9.0 with 100 mM potassium carbonate. Ab_2 was non-covalently attached to colloidal gold as described by Maragos (2012). The product was washed several times with 0.1% BSA in 2 mM $Na_2B_4O_7$ at pH 7.4, and then stored in a buffer consisting of 0.5% BSA in 0.05% of sodium azide with 2 mm $Na_2B_4O_7$ at pH 7.4. The product, Ab_2 conjugated with gold nanoparticles (Ab_2-AuNPs), was stored at 4 °C until further use.

2.7. iSPR assay conditions

The stock Ab_1 solutions were diluted 10,000-fold (for DON and ZEA) or 5,000-fold (for T-2) with 0.1% PBS-BSA (run buffer). This corresponded to antibody concentrations of 0.68 μg/mL for anti-DON, 0.42 μg/mL for anti-ZEA and 1.34 μg/mL for anti-T-2 toxin. To begin an assay, the sensor chip was equilibrated with the run buffer for 150 s. The sample was mixed with an equal volume of Ab_1, and introduced into the system at a flow rate of 300 μL/min. At 400 s, the labeled Ab_2-AuNPs was added. At 650 s, a series of regeneration steps were begun. The regeneration solutions (RS) consisted of the following: 0.05% Tween 20 in water (RS1); 50 mM sodium dodecyl sulfate and 20 mM NaOH in water at pH 12.0 (RS2); and 100 mM Glycine-HCl in deionized water at pH 3.0 and dimethylformamide, 9:1(v/v, RS3). The regeneration solutions were introduced in the following order: RS1 at 650 s, RS2 at 700 s, RS3 at 850 s and RS1 again at 1000 s. Then the sensor was re-equilibrated with run buffer to make it ready for the next cycle. The entire cycle, including regeneration, took 17.5 min.

2.8. Preparation of wheat samples and validation parameters

The extraction method for wheat was modified from the method of Joshi et al. (2016). Briefly, 2.5 g of ground wheat was weighed into a 50 mL centrifuge tube and mixed with 10 mL of methanol (80%). The sample was mixed vigorously for 10s, and shaken for 30 min on a wrist-action shaker. The samples were kept at 4 °C for 30 min, then centrifuged at 4000 rpm for 10 min. A 1 mL aliquot of the supernatant was diluted 5-fold with run buffer. The solution was then centrifuged again at 12,000 rpm for 10 min. Before being introduced into the instrument, the sample supernatant was mixed with an equal volume of Ab_1 solution. Therefore the mixture of injected sample solution contained 8% of methanol. To evaluate matrix effects, calibration curves were prepared in run buffer (buffer calibration) and in extracts from blank wheat samples (matrix matched calibration: MMC). The concentrations tested were 0, 0.5, 1, 5, 10, 50 and 100 ng/mL. For measuring toxins in spiked or naturally contaminated wheat samples, the MMC curves were constructed over a smaller range for DON and T-2 (0–50 ng/mL) at six concentrations, and ZEA (0–20 ng/mL) at five concentrations with three replicates. To determine recoveries, mycotoxins (DON, ZEA and T-2 toxin) were spiked into blank wheat in triplicate at 50, 100 and 500 μg/kg. The limit of detection (LOD) of the assay was defined as the concentration causing 10% inhibition of the binding (IC_{10}).

2.9. Determination of cut-off level for DON, ZEA and T-2 toxin

To evaluate the cut-off level for each toxin, two sets of 10 replicate samples were prepared. One set were blank samples of wheat, containing no added mycotoxins. The second set were blank wheat samples spiked at the target concentrations of 400 μg/kg (for DON) or 100 μg/kg (for ZEA and T-2 toxin). Spiked samples were held overnight at 4 °C before testing. To determine the cut-off level, the guidelines from the EC were used (EC, 2014). For a screening assay, the cut-off level was determined by the following equation:

$$\text{Cut} - \text{off level} = R_{STC} + t - \text{value } 0.05^{*}SD_{STC}$$

where, R_{STC} is the mean response of the positive (spiked) samples at the target concentration ($_{STC}$), the t-value is the one tailed t-value for a rate of false negative results of 5%, and SD_{STC} is the standard deviation observed with the spiked samples. When the relative response (B/Bo) is equal to the cut-off level or below, the sample is considered as “screen positive” sample. The threshold (T) level was also determined using the equation described by Joshi et al. (2016) as follows:

$$T = B - t\text{–value } 0.05^{*}SD_b$$

where B is the average mean response of the blank samples and SD_b is the standard deviation of these blank samples. The iSPR assay was further applied to the naturally contaminated wheat samples (n = 8) with known levels of mycotoxin contamination to validate either positive or negative based on cut-off levels.

2.10. Data analysis

Raw data from the iSPR biosensor were obtained and analyzed using Microsoft Excel 2007. LOD (IC_{10}) and non-linear regression analysis were determined using the four-parameter logistic dose-response model from automated curve fitting software, TableCurve 2D (version 5.01).

3. Results and discussion

3.1. Design principle and differential image of iSPR assay

A schematic diagram of the iSPR assay for *Fusarium* mycotoxins is shown in Fig. 1. The scheme is based on a competitive immunoassay format using the toxins immobilized in the form of toxin-protein conjugates (antigens). Antigens were covalently immobilized onto the gold surface after activation of the surface with EDC and sulfo-NHS. When the sample mixture (sample mixed with Ab_1) was introduced, the immobilized antigens competed with toxins present in the sample for binding to Ab_1. Signal from the surface bound Ab_1 was then amplified using a secondary antibody conjugated to gold nanoparticles (Ab_2-AuNPs). The sensogram in Fig. 2(A) shows one full cycle of assay, which includes binding of the Ab_1 (association), binding of the Ab_2-AuNPs

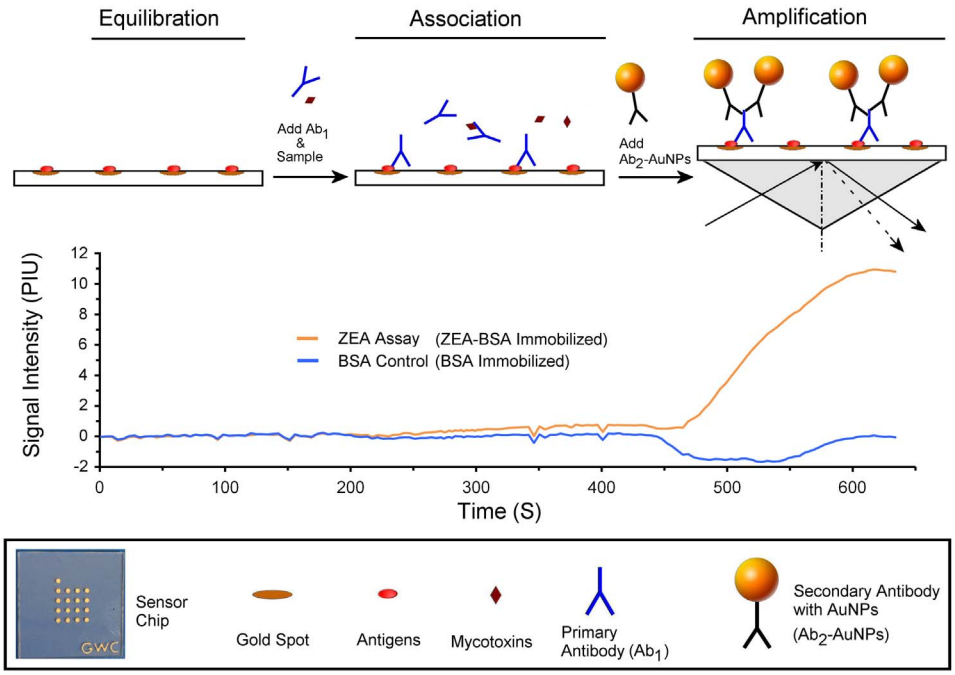


Fig. 1. Schematic illustration of the iSPR assay for the detection of *Fusarium* mycotoxins with signal amplification using a label of secondary antibody-gold nanoparticles (Ab_2-AuNPs). Toxin-protein conjugates (antigens) are immobilized onto the surface of the gold spots on the sensor chip. The antigen competes with free toxin contained in the sample for a limited amount of primary antibody (Ab_1). Attachment of the Ab_1 is quantified following amplification with Ab_2-AuNPs. The sensorgrams depict the responses that result with the application of a ZEA antibody and corresponding amplification with Ab_2-AuNPs when either BSA is immobilized (control, blue line) or when ZEA-BSA is immobilized (orange line). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

(amplification), and multiple regeneration steps (RS1, RS2, and RS3). Differential images obtained at various stages of a full iSPR assay cycle in real time are shown in Supplementary Fig. 1.

3.2. Signal enhancement by AuNPs

Because of their small size, it is difficult to use iSPR to directly detect the binding of mycotoxins to immobilized antibodies. This is because the binding causes only small changes in refractive index. However, the binding of Ab_1 with immobilized antigen can produce a significant iSPR signal. This is the reason why most SPR assays for small molecules indirectly measure the binding of a larger molecule (antibody) to immobilized antigens. However, higher signals can require binding of significant amounts of Ab_1. Since the sensitivity depends upon a competition between the immobilized antigen and the free toxin, for a limiting amount of antibody, high concentration of antibody can limit the performance of the technique (Dorokhin et al., 2011). AuNPs have been widely use as a signal amplifier (Nguyen et al., 2015). In this work a secondary antibody labeled with AuNPs (Ab_2-AuNPs) was used to increase the total mass bound. This greater mass helps to produce the higher refractory index changes on the sensor surface. Because of the signal enhancement, less Ab_1 is needed, which promotes improved sensitivity and reduces reagent costs. Fig. 2 (B) shows the signal amplification with Ab_1 and Ab_2-AuNPs obtained with the DON, ZEA and T-2 toxin assays. It was observed that the iSPR signal increased almost 25-fold for DON, 90-fold for ZEA and 12-fold for T-2 toxin assay when Ab_2-AuNPs were used instead of Ab_1 alone. The binding affinity between functionalized AuNPs and the gold surface of the sensor chip, and therefore the extent of the amplification, can be influenced by several factors such as coulombic interactions, steric effects, and stability of the complex with an analyte (Springer et al., 2014).

3.3. Specificity of antibodies (Mab) on coated antigens

Covalent attachment of the conjugates (antigens) to the sensor surface was crucial for the stability and reusability of the sensor chip. Non-specific binding of antibodies at the sensor chip surface with immobilized antigens can lead to inaccurate estimation of mycotoxins in the sample. To explore this fact, the specificity of Ab_1, and Ab_2-AuNPs were tested for each antigen. Supplementary Fig. 2(A) shows the

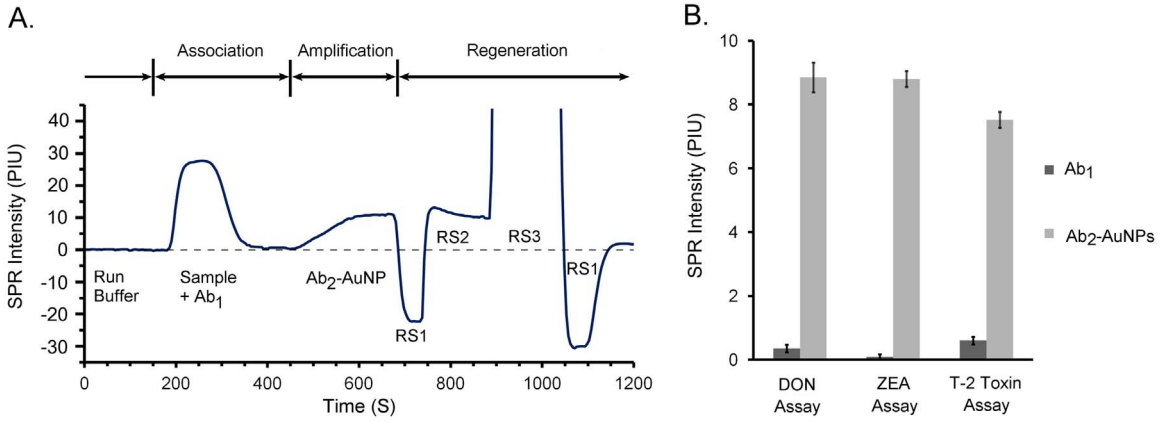


Fig. 2. (A): A complete iSPR cycle. The sensorgram shows binding of the primary antibody (Ab_1) in the association step, binding of the labeled secondary antibody (Ab_2-AuNPs) in the amplification step, and dissociation of the bound complex in the regeneration step. RS1, RS2, and RS3 refer to regeneration solutions described in the text. (B): Signals obtained without amplification (i.e. Ab_1 alone) and signals obtained with amplification using Ab_2-AuNPs for three toxin assays.

response of DON, ZEA and T-2 antibodies (i.e. three Ab_1) with their respective antigens after the signal was amplified by Ab_2-AuNPs. The assay showed excellent specificity as no significant response was observed that would have indicated binding of either Ab_1 or Ab_2-AuNPs with BSA-immobilized control (Supplementary Fig. 2A). In between the cycles, no carryover/binding of Ab_1 was observed when Ab_2-AuNPs were introduced alone as shown in Supplementary Fig. 2(B).

3.4. Cross reactivity of antibodies with mycotoxin analogs

The cross-reactivity of antibodies against DON, ZEA and T-2 with commonly found mycotoxin analogs in food and feed is described in Supplementary Table 1. The DON antibody was cross-reactive for 15-ADON (150%) but showed negligible cross reactivity for 3-ADON (< 1%) and NIV (< 2%). A similar trend was observed with the same antibody in an ELISA format (Maragos et al., 2012). The antibody against ZEA was cross-reactive with α-Zearalenol (104%) followed by β-Zearalenol (56%). Both of the ZEA analogs showed higher cross-reactivity in the iSPR format compared to a fluorescence polarization immunoassay format (Maragos and Kim, 2006). The T-2 antibody showed no significant cross-reactivity with HT-2 or DON, similar to what was observed in an earlier report (Maragos et al., 2013).

3.5. Durability of the sensor chip

One of the advantages of iSPR over other immunoassay assay platforms is the ability to use the sensor multiple times, which reduces the cost per assay. However, it is a challenging task to determine the optimal conditions for binding and regeneration when multiple antigens, and antibodies are involved, such as with a multiplexed assay. The regeneration of the sensor surface is a crucial factor for the effective utilization of iSPR. For this reason, it was important to determine how many times a sensor chip could be used without decreasing the signal intensity. The ability of immobilized DON-BLG, ZEA-BSA and T2-BSA antigens to withstand repeated cycles of use and regeneration was determined. As depicted in Fig. 2(A), multiple regeneration solutions were used to facilitate disassociation of Ab_1 from the sensor, followed by equilibration with run buffer in preparation for the next cycle. No significant changes in the baseline and antibody binding capacity were observed after regeneration with Tween 20 (RS1), SDS: NaOH (RS2) and Glycine: DMF (RS3) and re-equilibration with run buffer. Joshi et al. (2016) reported that OVA conjugates were sensitive to regeneration and the conjugates were partially lost from the surface as manifested by a reduced signal. In this study, conjugates of DON, ZEA and T-2 on a single sensor chip showed excellent stability over 46 cycles without significant loss of signal, as shown in Fig. 3. Fig. 3(A) shows the variation in signal response among 4 replicate spots (spot to spot variation) for each DON, ZEA and T-2 toxin assay on the sensor chip. The spot to spot variation (n = 4) of cycle 1, 24, 36 and 46 were less than 12% for DON, 16% for ZEA and 11% for T-2 toxin assay. For individual spots the signal variations between cycles (cycle 1, 24, 36 and 46) were less than 12% (< 7% for DON assay, < 12% for ZEA assay and < 5% for T-2 toxin assay) as shown in Fig. 3(B). It can be concluded from the results that variations (spot to spot variation and cycle to cycle variation) are not prominent and will not interfere with the determination of the *Fusarium* toxins in wheat.

Table 1
Performance parameters of the multiplexed iSPR assay, with standards in either buffer, or wheat matrix (n = 3).

Toxins	MLs[a] (μg/kg)	Buffer calibration			Matrix-matched calibration		
		LOD	IC_{50}	Working range (μg/kg)	LOD	IC_{50}	Working range (μg/kg)
DON	1250	38	366	83–2652	15	362	48–2827
ZEA	100	57	289	102–835	24	212	54–790
T-2	100[b]	24	154	46–571	12	310	42–1836

[a] Maximum limits (MLs) set by European Commission.
[b] Recommended level for sum of T-2 and HT-2.

3.6. Matrix effects and assay validation

To evaluate the effects of matrix, buffer calibration and MMC curves were constructed for DON, ZEA and T-2 toxin. The coefficient of determination ($r^2 > 0.99$) was higher with the matrix matched calibration than with the buffer calibration ($r^2 > 0.96$). MMC curves appeared more sensitive relative to buffer calibration (Table 1). Fig. 4 shows the MMC curve for DON, ZEA and T-2 toxin at six points. The performance data for buffer and MMC calibration, including LOD, IC_{50} and working range are described in greater detail in Table 1. For quantification purpose, MMC curves were constructed in a smaller range equivalent to 0–2000 μg/kg for DON and T-2 toxin, and 0–800 μg/kg for ZEA in wheat for spiking/recovery and naturally contaminated wheat studies. The coefficients of determination (r^2) of the smaller range MMC curves were higher than 0.99. Recoveries at individual spiking concentrations (50, 100 and 500 μg/kg) ranged from 73% to 103% for DON, 85–103% for ZEA, and 95–100% for T-2 toxin with RSDrs less than 7%. The mean recoveries were 87% for DON, 94% for ZEA and 103% for T-2 toxin with RSDrs of less than 5%. The mean recoveries and RSDr for all three *Fusarium* toxins were within the range of EC guidelines (DON: recovery: 60–110%; ZEA: recovery 70–120%; T-2: recovery 60–130%) (EC, 2006). The LODs for the three toxins were 15 μg/kg for DON, 24 μg/kg

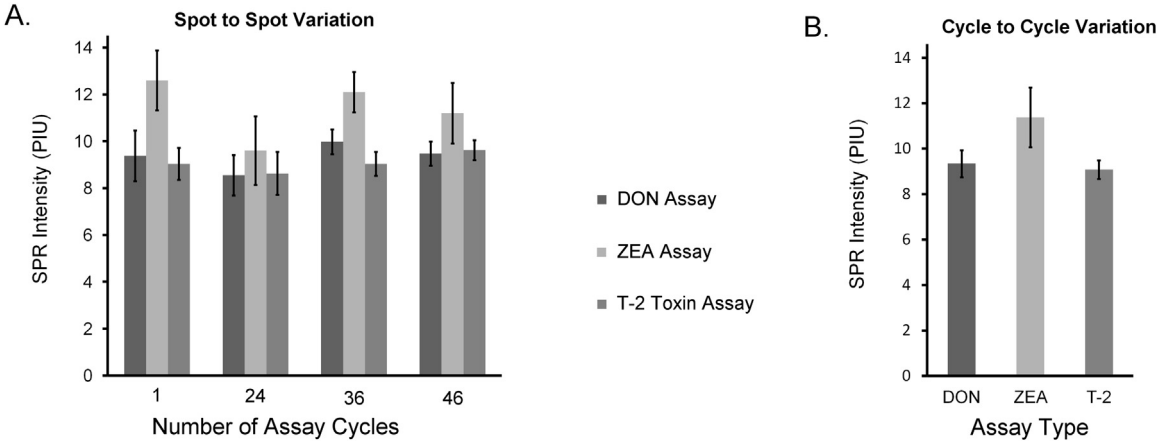


Fig. 3. Durability of a sensor chip over 46 cycles; A) Spot to spot variation. Shown are the mean ± one standard deviation of 4 spots at each of the indicated cycles. B) Cycle to cycle variation. Shown are the mean ± one standard deviation of the average of four spots over 4 cycles (cycles 1, 24, 36, and 46).

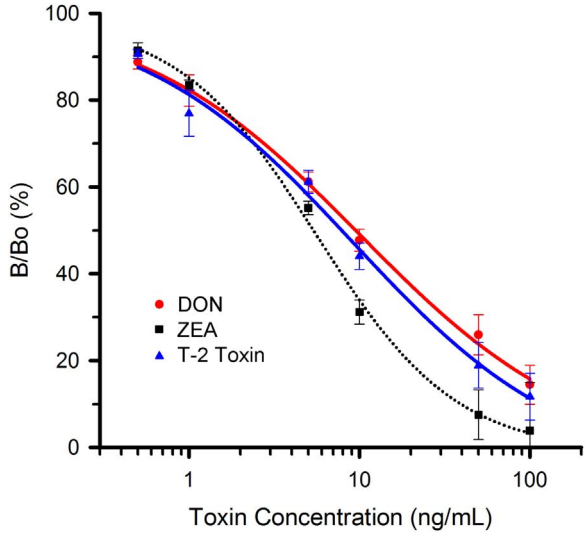


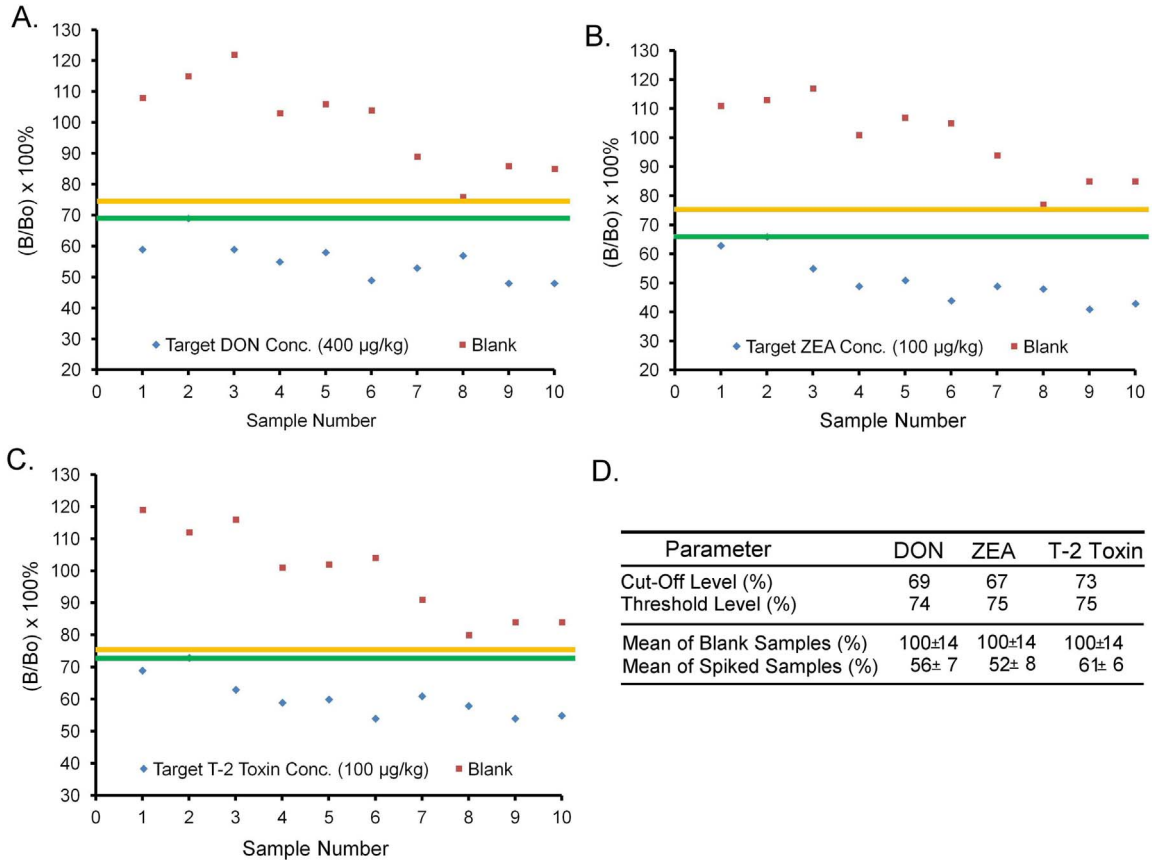
Fig. 4. Matrix matched calibration of the iSPR assay for DON (•), ZEA (■) and T-2 toxin (▲) in wheat.

for ZEA, and 12 μg/kg for T-2 toxin. The concentrations at which 50% inhibition of binding occurred (IC_{50}s) were 362 μg/kg (DON), 212 μg/kg (ZEA), and 310 μg/kg (T-2 toxin) in wheat. The LOD of this multiplex assay was more sensitive than a recently reported 6-plex nanostructured portable iSPR assay (Joshi et al., 2016). To the best of our knowledge, there are no reports in the literature where AuNPs have been used to enhance a multiplex iSPR assay for DON, ZEA and T-2 toxin in wheat. Previous studies with non-imaging SPR have multiplexed either DON and ZEA (Dorokhin et al., 2011) or DON and T-2/HT-2 (Meneely et al., 2012). Considering previous immunoassay and multiplex SPR assays, the iSPR method developed here is more sensitive and could detect all three *Fusarium* toxins at sufficiently low levels in wheat.

3.7. Cut-off and threshold levels

A successful screening assay must be able to distinguish between positive (spiked) samples at the target concentration level and negative (blank) samples. To determine whether the iSPR method can be used as a qualitative screening assay, and to establish a cut-off level for such an assay, we analyzed blank wheat samples (n = 10) and samples spiked (n = 10) at the target levels of 400 μg/kg (DON), and 100 μg/kg, (ZEA and T-2 toxin). After obtaining responses for blank and spiked samples and their corresponding t-value, the cut-off and threshold levels were calculated. The experiment was conducted simultaneously for three pairs of antigens and antibodies (DON-BLG with Mab 1; ZEA-BSA with Mab 22; T2-BSA with Mab 2–13) (Fig. 5). The results obtained for the cut off and threshold levels are shown in Fig. 5D. It was demonstrated in Fig. 5A-C that samples spiked at the target mycotoxin concentrations were clearly separated at the calculated cut off level from blank samples. In this cut off level study, no false positive or negative samples were observed. The acceptable estimated rate of false positives should be lower than 5% of the blank samples.

After successful validation of the cut-off levels, the performance of the method on several naturally contaminated wheat samples with known toxin levels was evaluated (Table 2). Three samples containing DON were tested. Of these, two (1600 and 500 μg/kg) were considered as a positive (above the cut-off level). The third sample, with DON at



D.

Parameter	DON	ZEA	T-2 Toxin
Cut-Off Level (%)	69	67	73
Threshold Level (%)	74	75	75
Mean of Blank Samples (%)	100±14	100±14	100±14
Mean of Spiked Samples (%)	56± 7	52± 8	61± 6

Fig. 5. Validation of cut-off levels for DON (A), ZEA (B) and T-2 toxin (C). Shown are results from blank samples (■) and spiked samples (♦). Spiked samples contained toxin at the indicated target levels (100 μg/kg for ZEA and T-2; 400 μg/kg for DON). Green lines indicate cut off levels and yellow lines indicate the threshold levels for each toxin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Table 2
Evaluation of the cut-off levels in samples of naturally contaminated wheat.

Sample No	DON		ZEA		T-2 toxin	
	+/-ve[a]	Concentration (μg/kg)[b]	+/-ve	Concentration (μg/kg)	+/-ve	Concentration (μg/kg)
Wheat 1	–	–	–	–	–	45
Wheat 2	–	–	–	–	–	–
Wheat 3	–	–	+	1085	–	–
Wheat 4	–	–	+	410	–	–
Wheat 5	–	–	–	–	–	–
Wheat 6	+	1600	–	–	–	–
Wheat 7	+	500	–	–	–	–
Wheat 8	–	100	–	–	–	5

100 μg/kg, appeared as negative (below the cut-off level of 400 μg/kg). For ZEA, two of the wheat samples contained amounts above the cut-off level, and both samples (containing 1085 and 410 μg/kg) were considered positive. Only two of the wheat samples tested contained T-2 toxin, and the level of contamination for both was very low (45 μg/kg and 5 μg/kg). As a result, none of the 8 samples tested by the iSPR assay yielded a positive result for T-2 toxin (cut-off level, 100 μg/kg). It can be concluded from the results that the cut-off level allowed the effective separation of naturally contaminated wheat from uncontaminated wheat. This finding affirms the applicability of this cut-off level for differentiating samples at the EU target concentration for monitoring of *Fusarium* toxins.

4. Conclusions

A rapid and sensitive iSPR assay was developed for *Fusarium* toxins in wheat. This is the first method to use a secondary antibody with AuNPs as an amplification tag to determine three *Fusarium* toxins in wheat. The signals from the iSPR biosensor were amplified as much as 12–90-fold by using Ab_2 labeled with AuNPs. After optimization of pairs of antigens and antibodies and development of an effective regeneration protocol, the responses from sensors were consistent over time. Sensor chips could be re-used for over 46 cycles without significant signal loss (< 12%). In spiking studies with wheat, the mean recoveries ranged from 87% to 103%, with an RSDr of less than 5%. The cross-reactivity of the antibodies against DON and T-2 with mycotoxin analogs in the iSPR format was similar to previous reports with the same antibodies in ELISA formats. However, cross-reactivity of the ZEA antibody against two analogs was different in the iSPR format than seen previously in a fluorescence polarization immunoassay format. The multiplexed iSPR assay was sensitive enough to be used to monitor all three *Fusarium* toxins in wheat at low levels. This reported multiplex iSPR assay was more sensitive than previously reported immunoassay and multiplexed SPR assays. Finally, cut-off levels were successfully validated to differentiate blank samples from the target concentrations regulated by the European Commission for all three *Fusarium* mycotoxins. This promising multiplex iSPR assay based on AuNP signal amplification can be extended into a wide range of agriculturally important mycotoxins produced by *Aspergillus* and *Penicillium* species.

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Conflict of interest

None

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2017.10.033.

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