Simultaneous determination of Aflatoxin B1, Fumonisin B1 and Deoxynivalenol in beer samples with a label-free monolithically integrated optoelectronic biosensor

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Abstract The fast simultaneous determination of Aflatoxin B1 (AFB1), Fumonisin B1 (FB1) and Deoxynivalenol (DON) in beer samples employing an array of ten Mach-Zehnder interferometers (MZIs) monolithically integrated along with their respective light sources onto a Si chip is demonstrated. This is accomplished by functionalizing the sensing arms of individual sensors with mycotoxin-protein conjugates. For the assay, mixtures of calibrators or samples with monoclonal antibodies against the three mycotoxins were run over the chip, followed by reaction with an anti-mouse IgG antibody. Reactions are monitored by continuously recording the MZI output spectra, which are then subjected to discrete Fourier Transform to convert spectrum shifts to phase shifts. The detection limits achieved for AFB1, FB1 and DON were 0.8, 5.6 and 24 ng/ml, respectively, and the assay duration was 12 min. Recovery values ranging from 85 to 115% were determined in beer samples spiked with the three mycotoxins. Different beer types were analyzed with the biosensor developed and the results correlated well with those provided by LC-MS/MS and/or immunochemical methods. Moreover, the proposed immunosensor could be regenerated and re-used at least 20 times. These characteristics along with the small sensor size strengthen its potential for incorporation into a portable instrument for point-of-need applications.

Keywords: Aflatoxin B1, Fumonisin B1, Deoxynivalenol, Monolithically integrated Mach-Zehnder interferometers, Label-free biosensor

1. Introduction

Mycotoxins are low molecular weight compounds produced as secondary toxic metabolites by different types of fungi, belonging mainly to the Aspergillus, Penicillium and Fusarium species (Brase *et al*., 2009). Despite the high number of substances (~400) identified as mycotoxins, few of them are found as contaminants in food and animal feedstuff in hazardous quantities (Capcarova *et al*., 2016). In this category belong, amongst others, aflatoxins, fumonisins and trichothecenes. Aflatoxin B1 (AFB1) is considered the most toxic one, due to its teratogenic, mutagenic and carcinogenic effects (Murphy *et al*., 2006). Fumonisins are less toxic than aflatoxins; nevertheless fumonisin B1 (FB1) which accounts to about 80% of the fumonisins found in contaminated foods (Waskiewicz *et al*., 2012), has been classified as Group 2B carcinogen to humans by the International Agency for Research on Cancer (IARC). Trichothecenes, and especially their main representative deoxynivalenol (DON), are not carcinogenic to humans; nevertheless, acute exposure can cause gastroenteritis and chronic exposure was shown to impact immunotoxic effects in animal models (Pestka, 2010). The severe toxic effects on humans or animals by the acute or chronic exposure to mycotoxins motivated the European Commission to establish maximum allowable limits for the major mycotoxin contaminants in cereals and grains (Commission Regulation No 1881/2006). This has in turn motivated the development of methods for the detection and/or quantification of mycotoxins in food samples including both chromatographic or immunochemical techniques (Chauhan *et al*, 2016; Anfossi *et al*., 2016). Amongst instrumental chromatographic methods, HPLC coupled to mass spectrometry (LC-MS/MS) is currently the method more frequently used. The great advantage of this method is the ability to detect a large number of substances in complex matrices with high sensitivity (Zhang *et al*., 2016). On the other hand, they can be applied only in laboratory environment by highly skilled personnel, and the analysis cost is relatively high. Thus, immunochemical approaches have emerged as alternative methods (Chauhan *et al*, 2016; Anfossi *et al*., 2016) offering high detection sensitivity at lower analysis cost and increased potential for application at the Point-of-Need

(PoN) (Urusov *et al*., 2015). The main drawback of immunochemical versus the chromatographic techniques is their low level of multiplexity. To address this issue, multiplexed immunoassays based on appropriately-coded and antibody-functionalized beads have been developed and combined with compact and low cost read-outs (Peters *et al*., 2014; Xu *et al*., 2016). Despite these advancements, the only viable approach for small-size devices suitable for PoN application is the development of immunosensors. Thus, several immunosensors, based mainly on electrochemical or optical transduction principles, have been developed and applied for the detection of mycotoxins in various food matrices (Vidal *et al*., 2013; Puiua *et al*., 2014). Although they provided excellent analytical performance, they are capable for single-analyte determinations.

To this end, we present an immunosensor based on arrays of monolithically integrated onto silicon chip Mach-Zehnder interferometers (MZI) (Misiakos *et al*., 2014; Psarouli *et al*, 2015) for the fast label-free simultaneous determination of AB1, FB1 and DON in beer samples. All the parameters of the on-chip assays as well as the conditions for regeneration and reuse of chip have been optimized. The ability of the developed immunosensor to simultaneously detect the three mycotoxins was evaluated through analysis of beers of different types and origin and the results were compared with those received for the same samples by established mycotoxin analysis methods.

2.   Materials and Methods

2.1.   Materials and instruments

Mouse monoclonal antibodies against AFB1 and DON as well as mycotoxin-protein conjugates (AFB1-BSA, DON-OVA and FB1-OVA) were obtained from Aokin AG (Berlin, Germany). The mouse monoclonal antibody against FB1 as well as AFB1, FB1 and DON reference materials for the preparation of calibrators were kindly provided by RIKILT Wageningen UR (Wageningen, the Netherlands). 3-Aminopropyl-triethoxysilane (APTES) and highly pure methanol were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was purchased from Acros Organics (Geel, Belgium), goat anti-mouse IgG (affinity purified) and sodium dodecyl sulphate (SDS) were from Merck Millipore (Darmstadt, Germany), and absolute ethanol from Carlo Erba SpA (Milano, Italy). The water used throughout the study was doubly distilled.

Sensor chips accommodating an array of 10 integrated MZIs and their respective light sources were fabricated in the Nanotechnology & MEMS Laboratory (N-MEMS) of the Institute of Nanoscience and Nanotechnology of NCSR "Demokritos" (Athens, Greece), as it has been described in previous publications (Misiakos *et al*., 2014). The measuring apparatus involved a docking station that supported the fluidic and electrical connections. Aligned to the docking station was an optical fiber that provided for the collection of the transmission spectrum and its transfer to a miniaturized spectrometer (QE65000, Ocean Optics; Dunedin, FL) for the collection of the transmission spectra. The operation of the spectrometer was synchronized with a

dedicated electrical multiplexer that turned on/off sequentially the 10 light sources of a single chip every 1 s. In this way, one spectrum from each one BB-MZI of the chip was recorded every 10 s by the PC which controlled the measuring apparatus through a dedicated Labview application. The software allows for further signal processing with Discrete Fourier Transform to convert the shifts observed in the spectrum to phase shifts in both TE and TM polarization.

2.2   Preparation of calibrators and samples

Stock solutions with a concentration of 1 mg/mL were prepared for AFB1 and DON in 80% aqueous methanol solution and for FB1 in 10% aqueous methanol solution and stored in aliquots at -20 ºC. Calibrators were prepared in assay buffer (0.05 M Tris-HCl, pH 7.8, containing 9 g/L NaCl, 5 g/L BSA and 0.5 g/L NaN$_3$) containing 0.625% (v/v) absolute ethanol. Beer samples were degassed in an ultrasonic bath for 10 min (Elmasonic S30H; Elma Schmidbauer GmbH, Germany) and diluted 8 times with assay buffer prior to the analysis.

2.3.   Biochip preparation and assay performance

Chips accommodating 10 integrated BB-MZIs were chemically activated with APTES solution (Psarouli *et al*., 2015). The chips were then spotted with mycotoxins-protein conjugates (100 μg/ml in 0.05 M carbonate buffer, pH 9.2) using the BioOdyssey Calligrapher Mini Arrayer (Bio-Rad Laboratories, Inc.) following the spotting scheme, depicted in Figure 1. After spotting, the chips were incubated overnight at RT under controlled humidity conditions (75%). Then they were rinsed with washing buffer, blocked for 1 h through immersion in a 1% (w/v) BSA in 0.1 M NaHCO$_3$, pH 8.5, and then again rinsed with washing buffer (0.01 M Tris-HCl, pH 8.25, containing 0.9% NaCl) and distilled water and dried under a N$_2$ stream. Dried biochips were used either immediately or kept at 4 ºC in a desiccator until use.

Figure 1: Photograph of the chip depicting the areas functionalized with the different mycotoxin-protein (sensors 1-9) conjugates and the non-reactive protein (sensor 10).

For the assay, the fluidic module was assembled on top of the biochip and placed on the docking station. At first, the calibrators or the diluted beer samples were mixed at equal volumes with a solution containing the three monoclonal antibodies against mycotoxins in assay buffer. The biochip was equilibrated with assay buffer, and then the mixtures of the calibrators or the samples with the antibodies were run over the chip for 8 min at a flow rate of 40 μL/min.

Then, a 10 μg/ml solution of goat-anti-mouse IgG in assay buffer was supplied for 4 min at the same flow rate. Regeneration of the chip was performed by passing a solution of 0.5% (w/v) SDS, pH 1.9, for 2 min followed by equilibration with assay buffer. After assay completion, the collected output spectra are subjected to Discrete Fourier Transform (Psarouli *et al.*, 2015) and the phase shift in both polarisations (TE & TM) were calculated. All the data presented in the present work refer to phase shifts in TE polarization. Thus, for the preparation of the calibration curves, the phase shift in radians corresponding to different calibrators (S_x) were expressed as percent ratios of the initial of the response obtained for the zero calibrator (S_0) and plotted versus each mycotoxin concentration in the calibrators in linear/log scale.

3. Results and Discussion

3.1. Optimization of the on-chip assays

All the assay parameters and especially the concentration of conjugates used for coating, the concentration of the antigen-specific antibodies and the duration of primary and secondary immunoreaction steps had to be optimized. The criteria used was the absolute signal obtained for each assay for the zero calibrator and the sensitivity of the assay as was defined by the % inhibition values obtained for certain calibrators. Thus, the selected concentration for all conjugates was 100 μg/mL whereas the specific antibody concentrations were: 6 μg/mL for anti-AFB1 Mab, 1.5 μg/mL for anti-FB1 Mab and 3.0 μg/mL for anti-DON Mab. The duration of the two immunoassay steps was also determined with respect to maximum plateau signal values. The conditions selected were 8 min for the primary immunoreaction and 4 min for the secondary, since under these conditions more that 85% of the maximum plateau signals was obtained.

Another parameter optimized was the composition of the regeneration solution. For this purpose the following solutions have been tested: a commercially available IgG elution buffer for immunoaffinity columns, a 0.1 M glycine-HCl buffer, pH 2.5, a 50 mM NaOH, a 100 mM HCl, and a 0.5% (w/v) SDS, pH 1.9. From preliminary experiments it was found that the maximum regeneration efficiency for all the above mentioned regeneration buffers was achieved when they were run over the chip for at least 2 min and thus this time was adopted in all cases. At first, the amount of analyte-specific Mab remaining on the surface after regeneration was assessed through incubation with secondary antibody and expression of the signal obtained as percent ratio of the zero calibrator signals. As shown in Figure 2, for the AFB1 and FB1 assay the higher regeneration efficiencies were received using either 100 mM HCl or 0.5% (w/v) SDS, pH 1.9, as regeneration solutions; while for DON 50 mM NaOH and 0.5% (w/v) SDS, pH 1.9, were the best performing regeneration solutions. In the frame of harmonization of the assays, 0.5% (w/v) SDS, pH 1.9, was selected as regeneration solution for further experimentation. The stability of the biochip towards regeneration was assessed through repetitive assay/regeneration cycles. A small signal drop was observed for the FB1 and DON assays after 15 assay/regeneration cycles which was however less than

10% of the zero calibrator signal obtained during the first assay run. These results demonstrate the stability of immobilized onto biochips molecules, i.e. the mycotoxin-protein conjugates.
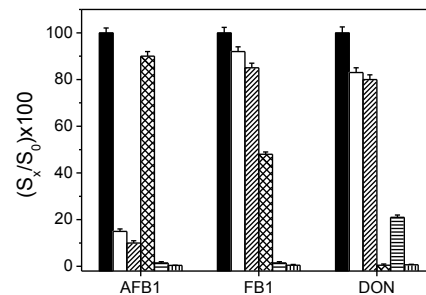

Figure 2: Percentage of signal corresponding to specific antibody remaining onto the chip after regeneration with IgG elution buffer (white columns), 0.1 M glycine-HCl buffer, pH 2.5 (striped columns), 50 mM NaOH (cross-stitched columns), 100 mM HCl (horizontally striped columns), or 0.5% (w/v) SDS, pH 1.9 (vertically striped columns) to the initial zero calibrator signal (black columns).

The effect of beer presence on the zero calibrator signals was assessed for each one of the three mycotoxins in order to define also a matrix for the preparation of calibrators. Although for all assays, an 8-times dilution seemed to minimize the effect of beer matrix, the zero calibrator values still differed from those of buffer. Thus, assay buffer containing 0.625% (v/v) ethanol was also tested, which corresponded to a mean ethanol content of a typical beer diluted 8-times with assay buffer. Using this buffer, the calibration curves were identical to those obtained using calibrators prepared in beer diluted 8 times with assay buffer. This was true for different types of beers (lager, dark, ale, etc.), leading to adoption of assay buffer containing 0.625% (v/v) ethanol as the matrix for calibrators' preparation

3.2. Evaluation of assays specificity

The possible cross-reaction of the anti-mycotoxin-specific antibodies with the different immobilized mycotoxin-protein conjugates was tested using chips modified as indicated in Figure 1 by running over the chip separately each one of the monoclonal anti-mycotoxin antibody followed by reaction with secondary antibody and regeneration. Response distinguishable from that of the blank sensor (spotted with OVA) was obtained by each one of the three sensor groups only when the specific Mab was run over them. In addition, the responses obtained from a chip also modified with the three mycotoxin-protein conjugates upon running a mixture of the three anti-mycotoxin specific Mabs followed by secondary antibody are identical to those provided from the respective sensors when probed only with the respective Mab further confirming the specificity of the assays.

3.3. Analytical characteristics of the assays

Typical calibration curves for AFB1, FB1, and DON are provided in Figure 3.
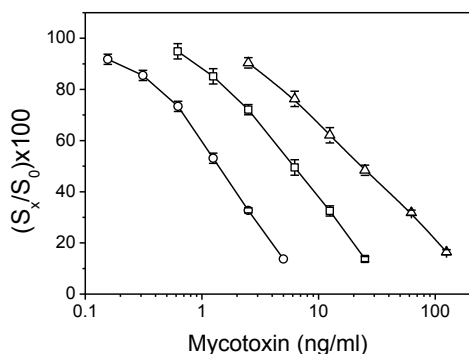

Figure 3: Typical calibration curves of AFB1 (□), FB1 (○), and DON (△) obtained with the developed immunosensor. Each point is the mean of 3 sensors (3 replicates each) ± SD.

The assays limit of detection (LOD) was calculated as the concentration corresponding to mean value -3 standard deviations (SD) of 21 replicate measurements of zero calibrator and was 0.1, 0.7 and 2.5 ng/mL, for AFB1, FB1, and DON, respectively, in assay buffer which corresponded to 0.8, 5.6 and 20 ng/mL, respectively, in undiluted beer taking into account the 8-times dilution. The linear response dynamic range reached up to 2.5, 25 and 125 ng/mL, respectively, in buffer or 20, 200, and 1000 ng/mL in undiluted beer for AFB1, FB1, and DON, respectively. The assay repeatability was determined by analyzing three control samples prepared in a lager beer and spiked so as the three mycotoxins to be present at different concentration levels that covered the whole range of the calibrators. The intra-assay coefficient of variation (CV) was determined by running 3 replicates of each control in the same day while the inter-assay CV was determined by duplicate measurements performed in 10 consecutive days and their values for the three controls were less than 12% and 16%, respectively, for the three assays. The assay accuracy was also evaluated through recovery experiments. For this purpose, six beers corresponding to different types, i.e., lager, pilsner, ale, dark, etc., were fortified with 3 different concentrations of each one of the three mycotoxins (5.0, 7.5 and 10 ng/mL for AFB1, 20, 50 and 100 ng/mL for FB1, and 150, 300, and 750 ng/ml for DON). All beers used were free of OTA as was determined by an LC-MS/MS analysis of these samples and confirmed by the sensor developed. The recovery values determined for the three mycotoxins and the 6 different beer samples ranged between 85.0 and 115%, indicating the good accuracy of the developed immunosensor.

Finally 30 beers of different types purchased from the Greek market have been analysed with the developed immunosensor. AFB1 and FB1 were not found in detectable amounts in any of the beers analysed. DON was detected in a few beers at concentrations close to detection limit. The results were verified for AFB1 and FB1 through a validated LC-MS/MS, whereas in case of DON

comparison was done with Luminex bead-based immunoassays, further supporting the accuracy of the results obtained with the immunosensor developed.

4.   Conclusions

The development of an immunosensor, based on monolithically integrated arrays of Broad-Band Mach-Zenhder interferometers, for the simultaneous label-free determination of mycotoxins AFB1, FM1 and DON in beer samples was demonstrated. The assays were characterized by high repeatability and accuracy, while the values determined in beer samples were in good agreement with those provided for the same samples by already established laboratory methods, supporting further the accuracy of the measurements performed by the developed immunosensor. These characteristics along with the small sensor size strengthen its potential for incorporation into a portable instrument for point-of-need applications.

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