DETECTION OF OCHRATOXIN A IN BEER SAMPLES BY ELISA AND A LABEL-FREE OPTOELECTRONIC BIOSENSOR

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ABSTRACT

Ochratoxin A (OTA), a mycotoxin produced as secondary metabolite by several fungi of Aspergillus and Penicillium species, in often detected in cereals and associated products. OTA is a potent teratogen, immunosuppressant, nephrotoxic and carcinogenic substance for animals and was classified as a possible carcinogen for humans. Thus, analytical methods that can detect OTA at concentrations lower than those established by the European Commission (5 μg/kg in unprocessed cereals) are highly valued. In this work three different competitive enzyme-immunoassay configurations were developed for the detection of ochratoxin A in beer, so as to select the most sensitive one to be transferred on a label-free optoelectronic biosensor based on an array of monolithically integrated Mach-Zehnder Interferometers (MZI) on a single silicon chip. The assay configuration finally selected was that involving immobilization of an anti-mouse IgG antibody on microtitration wells and pre-incubation of OTA standard solutions (8-times diluted beer) with a mouse monoclonal anti-OTA antibody followed by addition of biotinylated OTA–OVA conjugate. Detection was achieved using a streptavidin-peroxidase conjugate in combination with chromogenic substrate (H_2O_2/ABTS). The assay had a detection limit of 0.2 ng/mL in beer and a dynamic range up to 16 ng/mL. This assay format was also applied for the detection of OTA in beer using the MZI sensor. In this case, detection was performed in label-free format by continuously recording the output spectra of each MZI which were then subjected to Fourier Transform to convert the observed spectral shifts to phase shifts. The detection limit achieved in this case was 5 ng/mL in beer and the dynamic range extended up to 50 ng/mL. The assay was precise (intra-assay CVs <10%) and accurate (%recovery 86-118%) and was completed in 20 min. Given the analytical performance of the sensor and its small size (4.2x8 mm^2), that could facilitate the development of a portable device; OTA could be detected in beer samples as well as in other foodstuffs at the point-of-need.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced as secondary metabolite by several fungi of Aspergillus and Penicillium species and can be found in cereals and associated products such as wheat, maize, barley, coffee, cacao, beer and vine (Duarte *et al.*, 2011). OTA is a potent teratogen, immune suppressant, nephrotoxic and carcinogen to animals (Castegnaro *et al.*, 2006), and was classified by the International Agency for Research on Cancer (IARC) as a possible carcinogen to humans (IARC monographs, 1993). Thus, analytical technics that can detect OTA at concentrations lower than those established as maximum allowable levels by the European Commission (5 μg/kg in unprocessed cereals) are needed (EC regulation No 1881/2006).

Chromatographic techniques such as liquid chromatography (Beláková *et al.*, 2011) and immunochemical methods such as enzyme-linked immunosorbent assays (ELISA) (Zhang *et al.*, 2011) are being used frequently to determine OTA in food. The instrumental techniques offer sensitivity and specificity, but require advanced equipment and qualified personnel. On the other

hand, ELISAs offer simplicity, specificity and easy sample preparation. Nevertheless, during the last years biosensors have emerged as tools for detection of food contaminants due to their ability for miniaturization, multi-analyte determination and label-free detection that can lead to the development of portable devices for point-of-need determinations.

In the present study, a novel label-free optoelectronic biosensor based on an array of monolithically integrated on a single silicon chip Mach-Zehnder Interferometers (MZI) (Misiakos *et al.,* 2014) is evaluated for the immunochemical detection of ochratoxin A in beer samples. To select the assay format for ochratoxin A detection with the biosensor, three different competitive ELISA formats have been developed in microtitration plates and compared with respect to their analytical performance, i.e., in terms of sensitivity, repeatability and accuracy. The most sensitive one was selected for transfer on the label-free optoelectronic biosensor and the analytical performance of the biosensor was evaluated.

2. Experimental

2.1. Materials and Instruments

A mouse monoclonal antibody against ochratoxin A was purchased from Soft Flow Hungary Ltd. (Hungary). Ochratoxin A and ochratoxin A-ovalbumin conjugate (OTA-OVA) were from Aokin AG (Berlin, Germany). Streptavidin-Peroxidase conjugate (streptavidin-HRP), goat anti-mouse IgG antibody, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride (EDC), methanol, ovalbumin (OVA) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was from Acros Organics (Geel, Belgium), absolute ethanol from Carlo Erba SpA (Milano, Italy) and 96-well polystyrene plates from Greiner Bio-One GmbH (Frickenhausen, Germany). The water used was doubly distilled. Optical density of the microtitration wells was measured at 405 nm using a Victor3 1420 Multilabel Counter (Perkin Elmer; Milano, Italy).

2.2. Buffers and Solutions

Coating buffer was 0.05 M carbonate buffer, pH 9.3; blocking buffer consisted of 1% (w/v) BSA in 0.1 M $NaHCO_3$, pH 8.5; washing buffer was 0.01 M Tris-HCl, pH 8.25, containing 0.9% (w/v) NaCl and 0.05% (v/v) Tween 20; assay buffer was 0.05 M Tris-HCl, pH 7.8, containing 0.9% (w/v) NaCl and 0.5% (w/v) BSA; HRP-labeled secondary antibody dilution buffer was 0.15 M Tris-HCl, pH 8.25, containing 0.5% (w/v) BSA; HRP-labeled streptavidin dilution buffer was 50 mM phosphate-buffered saline (PBS), pH 7.4, containing 1% BSA (w/v). The HRP chromogenic substrate solution contained 0.03% (v/v) H_2O_2 and 1.9 mM ABTS in 0.1 M citrate–phosphate buffer, pH 4.5.

2.3. Sample preparation

A 1 mg/mL ochratoxin A stock solution was prepared in methanol and stored at -20º C. Calibrators with concentration ranging from 0.2 to 16 ng/mL were prepared in assay buffer containing 5% (v/v) ethanol. Beer samples that were found to be free of ochratoxin A after LC/MS analysis were spiked with the same amount of ochratoxin A as in calibrators. Beer samples were degassed in an ultrasonic bath for 20 min and then were diluted 8 times with assay buffer containing 10% methanol.

2.4. Preparation of biotinylated OTA–OVA conjugate

To a 1 mg/mL solution of OTA in 1:1 MES/methanol mixture were added 2.64 mg NHS and 4.7 mg EDC and the mixture was incubated for 1h at room temperature (RT). Then, 300 μL of this mixture were added to 500 μL of 2 mg/mL OVA solution in $NaHCO_3$ 0,1 M, pH 8,5 and incubated for 18 h at 4 ºC, followed by dialysis against $NaHCO_3$ 0,1 M, pH 8,5. Biotinylation of the OTA-OVA conjugate was performed according to a previously published method (Koukouvinos *et al.*, 2015).

2.5. ELISA for ochratoxin A determination

Microtitration wells were coated overnight with 100 μL of a 10 μg/mL anti-mouse IgG solution in coating buffer. Then the wells were washed twice with 300 μL of washing solution, and 300 μL of

blocking solution were added per well and incubated for 2 h at room temperature (RT). The wells were washed as previously and 100 µL of a 1:1 mixture of OTA calibrator or beer sample with a 20 ng/mL anti-OTA Mab solution in assay buffer were added to each well and incubated for 1h under shaking. Then, 10 µL of a 500 ng/mL biotinylated OTA–OVA conjugate solution in assay buffer in each well and they were incubated for 30 min under shaking. After that, the wells were washed four times with 300 µL of washing solution, and 100 µL of a 0.5 µg/mL streptavidin-HRP conjugate were added in each well and incubated for 30 min under shaking. The wells were washed as previously and they were incubated with 100 µL of HRP chromogenic substrate solution. The optical density of wells at 405 nm was measured after 30 min.

2.6. Ochratoxin A detection with the biosensor

Chips were cleaned/hydrophilized by oxygen plasma and then immersed in a 0.5% (v/v) aqueous APTES solution for 2 min, washed with distilled water, dried under a N_2 flow, and cured for 20 min at 120˚C. A 100 µg/mL OTA-OVA solution in coating buffer was deposited on the sensing windows of the 10 MZIs of the same chip using the microarray spotter (BioOdyssey Calligrapher MiniArrayer, Bio-Rad Laboratories Inc., California, USA). After incubation for 18 h under controlled temperature and humidity conditions, the chips were washed and immersed in blocking solution for 1 h at RT. Then the chips were washed with distilled water and dried under N_2 flow. The fluidic cover was then attached on top of each chip and the chip was placed on the measuring instrument. A peristaltic pump was used for the delivery of solutions at a constant rate of 20 µL/min. For the assay, a 1:1 mixture of OTA calibrator with the anti-OTA Mab (4 µg/mL) was run for 8 min, followed by a 4 min reaction with a 10 µg/mL of goat anti-mouse IgG antibody. Spectrum acquisition was performed in a multiplexed way in 10 x 10 s sampling cycles through the LabView Software. The recorded spectra files were off-line subjected to Discrete Fourier Transform using a specially developed MatLab program.

3. Results and discussion

In this work, three different competitive assay configurations were evaluated for the detection of ochratoxin A in beer. The first assay configuration involved immobilization of OTA-OVA conjugate onto the solid surface followed by reaction with a mixture of calibrators with the anti-OTA Mab and detection with HRP-labelled goat anti-mouse antibody. The second approach was also based on immobilization of OTA-OVA conjugate and reaction with a mixture of anti-OTA MAb with the calibrators and a biotinylated OTA-OVA conjugate. In this case, detection was performed using a mixture of enzyme-labelled streptavidin and goat anti-mouse IgG antibody. For the third approach, a goat anti-mouse IgG antibody was immobilized onto the solid support and incubated with a mixture of anti-OTA Mab with the calibrators and biotinylated OTA-OVA conjugate, whereas the detection was performed through reaction with HRP-labelled streptavidin. As is shown in Figure 1a, the most sensitive calibration curve was obtained using the third assay format, and thus this format was adopted for further experimentation. Using the selected assay format, the matrix for the preparation of calibrators and the required beer dilution in order to match the curve obtained in buffer was determined. It was found that when the calibrators were prepared in assay buffer containing 10% (v/v) methanol and the beer sample was diluted 8-times with this buffer, superimposed calibration curves were obtained, as shown in Figure 1b. The assay had a detection limit of 0.24 ng/mL in beer and a dynamic range up to 16 ng/mL.

When the three assay configurations were tested on the chip (Figure 2a), it was found that the first one provided the most sensitive assay. A typical calibration curve obtained with chips is provided in Figure 2b. The detection limit achieved in this case was 5 ng/mL in beer and the dynamic range extended up to 50 ng/mL. The assay was precise with intra- and inter-assay CVs lower than 10%. The accuracy of the method was assessed by recovery experiments using spiked beer samples. Recovery values ranged from 86 to 118%, confirming the accuracy of the method developed. In addition, the assay with the sensor was completed in less than 20 min, including washing prior to and after the assay, whereas the respective ELISA assay required almost 2 hours.

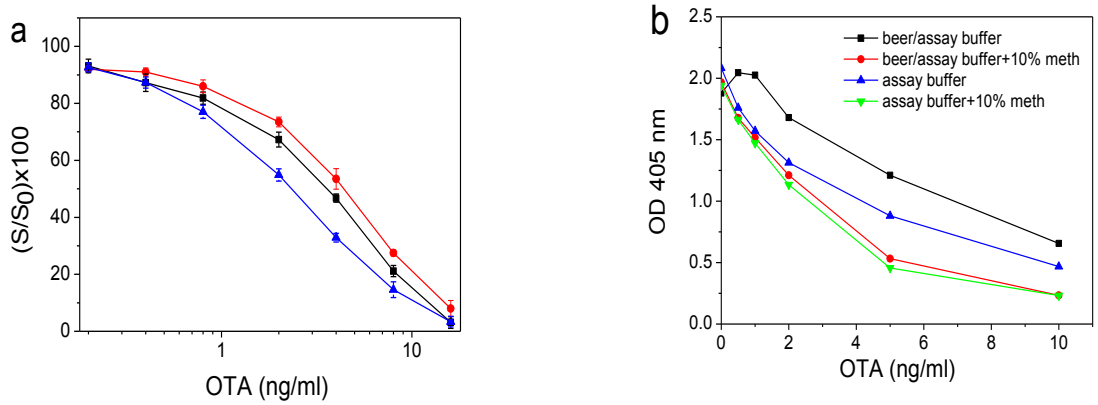

Figure 1: (a) Calibration curves obtained following the first (black line), the second (red line) and the third (blue line) ELISA configuration for the detection of ochratoxin A in beer. (b) Calibration curves obtained with calibrators prepared either in beer and diluted 8-times with assay buffer (black line) or with assay buffer containing 10% (v/v) MeOH, or in assay buffer and further diluted 8-times either with the same buffer or assay buffer containing 10% (v/v) MeOH.

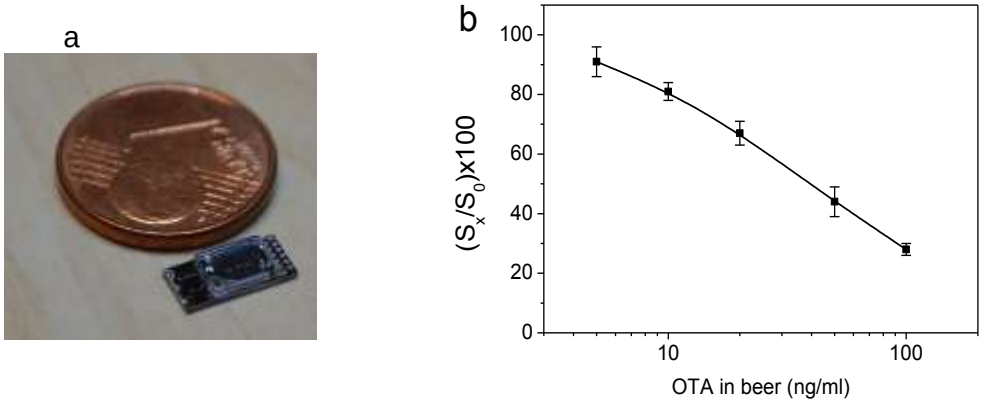

Figure 2: (a) Image of the chip with the fluidic on top. (b) Typical calibration ochratoxin A curve obtained with the integrated on chip MZI sensors. Each point is the mean value of three chips (7 MZIs per chip). Error bars correspond to ± SD.

4. Conclusions

A label-free optical immunosensor based on integrated on chips Mach-Zehnder interferometers for the determination of ochratoxin A in beer samples was developed. The assay was sensitive, repeatable and accurate. Given the analytical performance of the sensor and its small size (4.2x8 mm^2), that could facilitate the development of portable devices, the sensor could be employed for the detection of ochratoxin A in beer samples as well as in other foodstuffs at the point-of-need.

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