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Detection of ochratoxin A in beer samples with a label-free monolithically integrated optoelectronic biosensor

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HIGHLIGHTS

- Optical immunosensor for label-free detection of ochatoxin A in beer.
- The sensor consists of ten integrated on silicon Mach-Zehnder interferometers.
- Ochratoxin A is determined following a competitive immunoassay format.
- The assay could be employed for ochatoxin A determination in different beer types.

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ABSTRACT

An optical biosensor for label-free detection of ochratoxin A (OTA) in beer samples is presented. The biosensor consists of an array of ten Mach-Zehnder interferometers (MZIs) monolithically integrated along with their respective broad-band silicon light sources on the same Si chip (37 mm^2). The chip was transformed to biosensor by functionalizing the MZIs sensing arms with an OTA-ovalbumin conjugate. OTA determination was performed by pumping over the chip mixtures of calibrators or samples with anti-OTA antibody following a competitive immunoassay format. An external miniaturized spectrometer was employed to continuously record the transmission spectra of each interferometer. Spectral shifts obtained due to immunoreaction were transformed to phase shifts through Discrete Fourier Transform. The assay had a detection limit of 2.0 ng/ml and a dynamic range 4.0–100 ng/ml in beer samples, recoveries ranging from 90.6 to 116%, and intra- and inter-assay coefficients of variation of 9% and 14%, respectively. The results obtained with the sensor using OTA-spiked beer samples spiked were in good agreement with those obtained by an ELISA developed using the same antibody. The good analytical performance of the biosensor and the small size of the proposed chip provide for the development of a portable instrument for point-of-need determinations.

1. Introduction

Ochratoxins are a group of mycotoxins produced as secondary metabolites by several fungi of Aspergillus and Penicillium species [1]. Amongst them, Ochratoxin A (OTA) is the most toxic member of the group [2] and can be found in a large variety of foods, such as, wheat, maize, barley, coffee, cocoa, wine, and beer [3–5]. OTA is a potent teratogen, immune suppressant, nephrotoxic and carcinogen [6–8], and was classified by the International Agency for Research on Cancer (IARC) in group 2B as a possible carcinogen to humans [9]. In order to protect consumers from risks related to mycotoxins, the European Commission has established regulatory limits for OTA levels in raw cereal grains and roasted coffee (5 μg/kg), in cereals and cereal products intended for human con-

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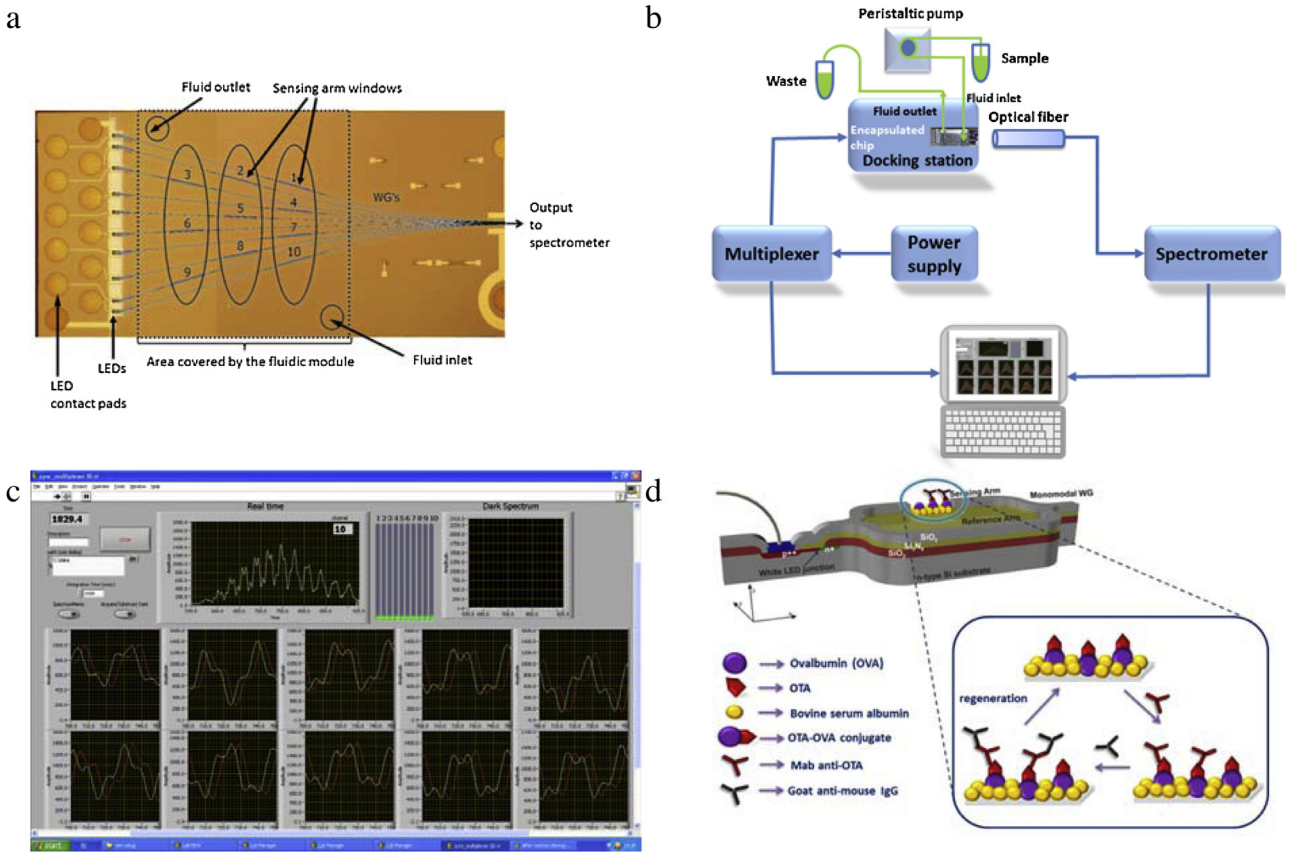

Fig. 1. (a) Microscope image of the chip overlapped by a schematic of the waveguides (not visible in the image) and an outline of the area covered from the fluidic module. (b) Schematic of the instrumentation used. (c) Screenshot showing the real-time monitoring of the output spectrum shifts of all ten BB-MZIs. A 50-nm spectral area (700–750 nm) has been isolated so as to monitor more clearly the changes in the transmission spectra. Red lines correspond to initial spectrum and white lines to spectrum after reaction. (d) Schematic of the on-chip OTA assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sumption (3.0 μg/kg), in wine and grape juice (2 μg/kg), as well as in baby food and cereal-based food intended for young children (0.5 μg/kg) [10].

The occurrence of OTA in beer was first described in 1983 [11] and since then many studies have been performed [12–15] indicating the need for detecting OTA in beer. Although the higher OTA values reported for most European beers do not exceed 1.5 ng/mL, values as high as 2340 ng/mL have been determined in traditionally brewed South Africa beers [14]. OTA is transferred to beer mainly from contaminated barley and/or other adjuncts used in the brewing process since it is not destroyed during the brewing procedure [12–15]. Therefore, various analytical methods have been developed for the quantitative determination of OTA in beer, cereals and other food commodities. The most frequently used methods are chromatographic ones, such as high-performance liquid chromatography coupled with fluorescence [16,17] or mass spectrometry detection [18,19], as well as immunochemical methods including enzyme-linked immunosorbent assays (ELISA) [20–22], fluorescence-based immunoassays [23], lateral flow immunoassays [24–26], cytometric microsphere immunoassays [27–29], or flow-through immunoassays incorporated in microsystems with signal recording capabilities [30–32]. Chromatographic techniques offer sensitivity and specificity, but require time-consuming sample preparation, skilled operators and expensive equipment. Immunochemical methods usually do not require complicated sample clean-up other than filtration and dilution for application to liquid samples such as beer. Nevertheless, both analytical techniques require bench-top laboratory instrumentation and are difficult to be used at the point-of-need.

Biosensors, on the other hand, represent an upcoming trend for mycotoxins detection in a wide range of food matrices [33] due to their potential for incorporation to portable devices. With respect to OTA detection, electrochemical [34–39], piezoelectric [40], and optical biosensors [41–43] have been reported. Optical biosensors are considered more appropriate for applications in complex matrices compared to more abundant electrochemical ones since they are galvanically isolated from the sample and therefore lees prone to interferences from the sample; at the same time are capable of higher detection sensitivities than the piezoelectric ones. There are few reports for OTA determination using optical biosensors; one based on optical waveguide lightmode spectroscopy (OWLS) [41] and two based on surface plasmon resonance (SPR) [42,43]. All three approaches were label-free; the reported detection limits for calibrators prepared in buffer were: 0.5 ng/mL for the OWLS-based sensor, and 1.5 ng/mL (improved to less than 0.5 ng/mL by implementation of gold nanoparticles for signal enhancement) [42], and 0.05 ng/mL [43] for the two SPR-based sensors, respectively. Despite their excellent sensitivity, both detection principles rely on external optical components and especially laser sources for excitation that make the instruments quite bulky and expensive, appropriate for laboratory use only.

In fact, the need for external optical components is considered one of the reasons that optical sensors have not reached the point of transition from lab to the field as opposed to more flexible, in terms of instrumentation, electrochemical ones. As a solution to this bottleneck, we have developed a technology for integration onto the same substrate arrays of silicon nitride waveguides along with their respective self-aligned silicon avalanche diodes

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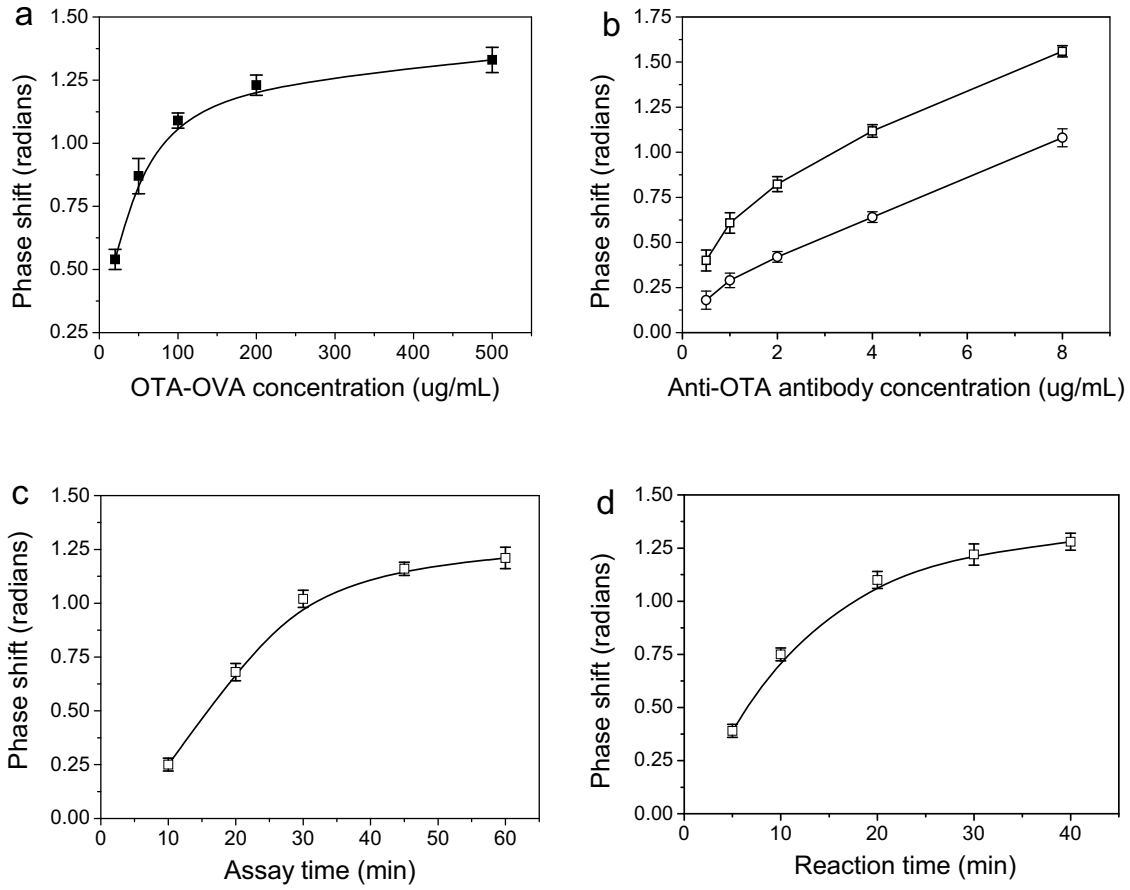

Fig. 2. (a) Effect of OTA-OVA concentration used for spotting on the zero calibrator signal. A 4 μg/mL anti-OTA antibody solution 1:1 diluted with zero calibrator was run over the chip for 30 min, followed by 20 min reaction with a 10 μg/mL secondary anti-mouse IgG solution. (b) Effect of anti-OTA antibody concentration on signals obtained for the zero calibrator (□) and a calibrator containing 20 ng/mL OTA in beer (○) from chips coated with 100 μg/mL of OTA-OVA conjugate. All other assay conditions are as described in Fig. 2a. (c) Effect of assay time on the zero calibrator signal when a mixture of zero calibrator with a 4 μg/mL anti-OTA antibody solution was run, followed by 20 min reaction with a 10 μg/mL secondary anti-mouse IgG solution. (d) Effect of reaction time with the secondary antibody solution on the zero calibrator signal. BB-MZIs were coated with 100 μg/mL of OTA-OVA conjugate and assayed using a 1:1 mixture of the zero calibrator with a 4 μg/mL anti-OTA antibody solution for 30 min. In all cases, signals are the mean value of 7 BB-MZIs ± SD.

as light sources [44]. To support label-free detection, this technology has been applied to create chips accommodating 10 integrated Mach–Zehnder interferometers (MZIs) along with their respective light emitting diodes (LEDs) on a 37-mm^2 silicon die (Fig. 1a) [45,46]. The unique characteristics of the LED with an emission spectrum that covers most of the visible and near IR region led to the demonstration of a new detection principle, that of Broad-Band Mach–Zehnder Interferometry, which allowed for sensitive detection of biomolecular interactions in label-free format [46–48]. To achieve that, the output spectra from the 10 MZIs of each chip were continuously recorded in sequence by a miniaturized spectrometer synchronized with the operation of the LEDs (Fig. 1b and c). The spectra are then analyzed by Discrete Fourier Transform and thus, the spectral shifts observed during the biomolecular reaction are converted in phase shift in both TE and TM polarization [47].

In the present study, Broad-Band Mach-Zehnder Interferometry (BB-MZI) is employed for the label-free detection of OTA in beer samples following a competitive immunoassay format. As shown schematically in Fig. 1d, the sensing arm areas of the BB-MZIs were functionalized through immobilization of an OTA-protein conjugate and the assay was realized by introducing mixtures of calibrators or samples with the antibody against OTA. An additional reaction step with a secondary antibody was employed, which upon binding to the immobilized antibody against OTA, led to the formation of one more biomolecular layer on the BB-MZI sensing arm, thus increasing the effective refractive index and enhancing the sensor response. All assay parameters have been optimized with respect to assay analytical performance (sensitivity, repeatability, accuracy). The accuracy of the developed assay was evaluated using OTA-spiked samples prepared in beers of different types (lager, pilsner, ale, dark). In addition, the potential of regeneration and re-use of biofunctionalized sensor chips was investigated, as a mean to reduce the analysis cost.

2. Materials and methods

2.1. Materials and instruments

OTA was from Aokin AG (Berlin, Germany). The mouse monoclonal anti-OTA antibody (anti-OTA mAb) was purchased from Soft Flow Hungary Ltd. (Hungary). Goat anti-mouse IgG (Fc specific)-peroxidase conjugate (anti-mouse IgG-HRP), *N*-hydroxysulfosuccinimide (sulfo-NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC), highly pure methanol (CHROMASOLV® for HPLC, ≥99.9%), ovalbumin (OVA), (3-aminopropyl) triethoxysilane (APTES), 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), goat anti-mouse IgG (affinity purified) was from Merck Millipore

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(Darmstadt, Germany), and absolute ethanol from Carlo Erba SpA (Milano, Italy). IgG Elution buffer was from Thermo Fisher Scientific Inc. (Waltham, MA). Eight-well polystyrene ELISA strips were purchased from Greiner Bio-One GmbH (Frickenhausen, Germany). The water used throughout the study was double distilled. An Elmasonic S30H sonicator from Elma Schmidbauer GmbH (Singen, Germany) was used for the degassing of beer samples. ELISA measurements were performed on a Victor3 1420 Multilabel Counter (PerkinElmer; Milano, Italy) at 405 nm wavelength. The BioOdyssey Calligrapher Mini Arrayer (Bio-Rad Laboratories Inc., Hercules, CA) was employed for spotting the chips with the OTA-OVA conjugate. A miniaturized spectrometer (QE65000; Ocean Optics Inc., Dunedin, FL 34698) was used for the collection of the integrated on chip BB-MZIs output spectrum.

2.2. Synthesis of OTA–OVA conjugate

The synthesis of OTA-OVA conjugate was performed as follows. First, 7.0 mg of sulfo-NHS and 3.6 mg of EDC were dissolved in 1 mL of 0.1 $NaHCO_3$ buffer, pH 8.5. To this solution, 20 μL of a 50 mg/mL OTA solution prepared in DMSO was added, and the mixture was incubated for 20 min at room temperature (RT). After incubation, 1 mL of 2 mg/mL OVA solution in 0.1 M $NaHCO_3$, pH 8.5, was added and incubated for 18 h at 4 °C. After that, the reaction mixture was extensively dialysed against 0.1 M $NaHCO_3$, pH 8.5, containing 0.05 mg/mL NaN_3 and stored at 4 °C. The molar ratio of OTA to OVA was calculated by measuring the absorbance of the OTA-OVA conjugate at 280 and 380 nm, which are the characteristic adsorption wavelengths of OVA and OTA, respectively. Taking into account an extinction coefficient of 30590 M^{-1} cm^{-1} for OVA at 280 nm, and 5680 M^{-1} cm^{-1} for OVA at 380 nm, a 1:1 molar ratio was determined.

2.3. Calibrators preparation

An OTA stock solution of 1 mg/mL was prepared by dissolving 5 mg of OTA in 5 mL of highly pure methanol and stored in aliquots at −20 °C. Matrix matched calibrators were prepared in beer that was free of OTA (< 0.1 ng/mL), as it was determined by an ISO 17025 accredited LC–MS/MS method [28]. At first, a 20 μg/mL OTA solution was prepared in beer, from which calibrators with concentrations from 5 to 100 ng/mL were prepared by addition of 5–100 μL of this solution in 20 mL of beer. After the addition of OTA, the calibrators were degassed in an ultrasonic bath for 10 min. Calibrators were also prepared in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.9% (w/v) NaCl, 0.5% (w/v) BSA, and 5% (v/v) absolute ethanol. All calibrators were kept aliquoted at −20 °C for up to 2 months.

2.4. Chip surface activation and biofunctionalization

Chips accommodating 10 integrated BB-MZIs were cleaned and hydrophylized by a 30-s O_2 plasma treatment. Then, they were immersed for 2 min in a 0.5% (v/v) aqueous APTES solution, followed by gentle washing with water, drying under a nitrogen stream, and curing at 120 °C for 20 min. A 100 μg/ml OTA-OVA conjugate solution in 0.05 M carbonate buffer, pH 9.2 (coating buffer), was then spotted on seven out of ten MZIs of each chip, whereas the remaining 3 were spotted with a 100 μg/ml OVA solution in coating buffer to serve as a control. Coverage of the sensing arm areas was accomplished by deposition of multiple overlapping spots. During spotting the chamber humidity was set at 75% and the temperature at 15 °C to avoid drying of the deposited solution. After spotting, the chips were incubated overnight at RT under controlled humidity conditions (75%). After that, the chips were rinsed with 0.01 M Tris-HCl, pH 8.25, containing 0.9% NaCl (washing solution), blocked for 1 h with 1% (w/v) BSA solution in 0.1 M $NaHCO_3$, pH 8.5, rinsed again with washing solution and distilled water, and dried under a nitrogen stream. Dried chips were used either immediately or kept at 4 °C in a desiccator until use.

2.5. MZI immunosensor OTA assay

Prior to the assay, the fluidic module was placed on top of the chip; the encapsulated chip was placed on a handling-frame; and then inserted in the docking station of the measuring apparatus, that provided for the fluidic and electric connections (see Fig. S1, supplementary material). Aligned to the docking station is an optical fiber that collects the output transmission spectra and guides them to the spectrometer which records the spectrum of each BB-MZI with a rate of 1 spectrum/10 s (1 s integration time per BB-MZI). The spectrometer and the multiplexer, which turns on and off the LEDs, are synchronized by the control electronics. A schematic representation of the instrumentation used is provided in Fig. 1b. The spectral shifts were monitored in real-time by the dedicated software developed in LabView environment (Fig. 1c).

For the assay, the calibrators or the samples were diluted 8 times with 0.05 M Tris-HCl, pH 7.8, containing 0.9% (w/v) NaCl, 0.5% (w/v) BSA, and 10% (v/v) methanol (sample dilution buffer). Then, they were mixed at equal volumes with a 4 μg/ml anti-OTA mAb solution in 0.05 M Tris-HCl, pH 7.8, containing 0.9% (w/v) NaCl, 0.5% (w/v) BSA (assay buffer) and the mixtures were preincubated for 1 h at RT. The chip was equilibrated by running assay buffer, and upon completion of the preincubation step the mixtures of the calibrators or the samples with the anti-OTA antibody flowed over the chip for 30 min at a rate of 40 μL/min. Then, a 10 μg/ml solution of anti-mouse IgG in assay buffer was injected and allowed to flow over the chip for 20 min at the same flow rate. Finally, regeneration of the chip was performed by injecting a 0.1 M hydrochloric acid solution for 3 min followed by equilibration of the chip surface with assay buffer. A schematic of the on-chip assay is provided in Fig. 1d.

After the assay completion, the collected spectral data are subjected off-line in Discrete Fourier Transform [47]. Thus, each spectrum is transformed into two sharp peaks, one corresponding to TE and the other to TM, the phase of which constitutes the monitored signal. Despite the fact, that the phase shifts in both polarizations were calculated, the graphs showing real-time sensor responses in the present work are all referring to phase shifts in TE polarization. The analytical curve was created by plotting the signal of the different standards (S_x) expressed as percentage of the zero calibrator signal (S_0) against the standard solutions concentration in logit/log scale. The analytical curve was linearized by applying the logit transformation on the y-axis values following the formula $\text{logit}[(S_x/S_0) \times 100] = \ln[(S_x/S_0)/100 - (S_x/S_0)]$, where S_x is the signal obtained for each one of the standards and S_0 is the signal obtained for the zero standard solution. Statistical analysis was performed using the OriginPro 8 software (OriginLab Corporation, MA, USA).

2.6. OTA ELISA in microtiter plates

The microtiter plate wells were coated with 100 μL of a 4 μg/mL OTA-OVA conjugate in coating buffer overnight at RT. The coated wells were then washed twice with 300 μL of 0.01 M Tris-HCl, pH 8.25, containing 0.9% NaCl (washing buffer) and the free surface protein-binding sites were blocked by incubation with 300 μL of 1% (w/v) BSA solution in 0.1 M $NaHCO_3$, pH 8.5 (blocking solution), for 1 h at RT. OTA calibrators or beer samples were diluted 8-times with sample dilution buffer, and then mixed with an equal volume of a 200 ng/mL anti-OTA Mab solution in assay buffer. These mixtures were incubated for 60 min at RT, and then 100 μL of each mixture were added per well and incubated for 2 h under shaking. The

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wells were then washed 4 times with 300 μL of washing solution, and 100 μL of a 0.5 μg/mL anti-mouse IgG-HRP in 0.15 M Tris-HCl, pH 8.25, containing 0.5% (w/v) BSA, were added and incubated for 40 min while shaking. After that, the wells were washed 4 times with 300 μL of washing solution, followed by addition of 100 μL of the HRP substrate solution (0.03% v/v H_2O_2 and 0.0019 mM ABTS in 0.1 M citrate-phosphate buffer, pH 4.5). After 30-min incubation, the optical density of the wells was measured at 405 nm. The plot of the calibration curve was performed as described in Section 2.5 for the on-chip assay.

3. Results and discussion

3.1. Optimization of the MZI immunosensor assay

The main parameters optimized regarding the development of OTA immunoassay on the BB-MZIs were the OTA-OVA conjugate concentration, the anti-OTA antibody concentration, the assay time, as well as the duration of the reaction with the secondary goat anti-mouse IgG antibody. The selection was based on the zero calibrator signal (maximum assay signal) as well as on the assay sensitivity as determined by the percent inhibition values corresponding to specific calibrators.

For the selection of the optimum OTA-OVA concentration used for coating of the integrated BB-MZIs, different sensors on the same chip were spotted with OTA-OVA solutions with concentrations ranging from 20 to 500 μg/mL. As shown in Fig. 2a, maximum zero calibrator signal values were obtained for OTA-OVA concentrations equal to or higher than 100 μg/mL. At the same time, the intra-assay signal variation was greatly improved when the OTA-OVA concentration in the spotting solution was increased to 100 μg/mL. Thus, this concentration was selected for further experimentation. Another important parameter was the concentration of the anti-OTA antibody. In Fig. 2b, the zero calibrator signals as well as the signals corresponding to a calibrator containing 20 ng/mL of OTA in beer are provided. As shown, both signals increased continuously as the anti-OTA antibody concentration increased from 0.5 to 8 μg/mL; however, the ratio of the signal obtained for the 20 ng/mL OTA calibrator solution to the signal of the zero calibrator was also increased as the anti-OTA antibody concentration increased, thus reducing assay sensitivity. Therefore, as a trade-off between zero calibrator signal and assay sensitivity, an antibody concentration of 4 μg/mL was selected which provided adequate zero calibrator signal (approximately 1 rad) and a percent inhibition of 57% for the calibrator containing 20 ng/mL OTA in beer. Lower antibody concentrations provided slightly higher sensitivity with an inhibition of approximately 45–50% for the 20 ng/mL calibrator. However, the zero calibrator signals ranged from 0.4 to 0.8 with signal coefficient variations higher than 10%, thus affecting negatively the assay sensitivity and dynamic range. Using chips coated with 100 μg/mL and the selected anti-OTA antibody concentration, the assay time was optimized. The reaction times of OTA Mab with the immobilized OTA as well as of secondary antibody with bound OTA Mab were optimized. As it can be concluded from Fig. 3c, the zero calibrator signal increased as the assay time increased and maximum plateau values were obtained for assay duration equal to or higher than 60 min. However, more than 80% of the maximum plateau signal value was obtained for assay time equal to 30 min. Thus, in order to keep the assay time as short as possible, a 30-min assay time was selected. Similarly, for the reaction with the secondary anti-mouse IgG antibody, a 20-min reaction step was selected since only a moderate increase of approximately 15% was obtained for the zero calibrator signals when the reaction time was increased up to 40 min (see Fig. 2d).

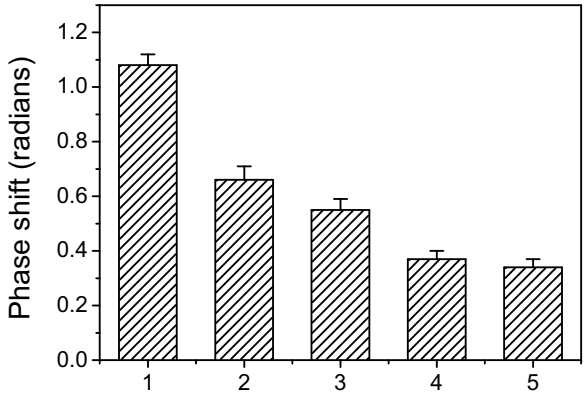

Fig. 3. Net mean signal values obtained from chips spotted with OTA-OVA for zero calibrator without pre-incubation with the antibody solution (column 1) and a calibrator containing 40 ng/mL OTA in beer without pre-incubation (column 2), or with pre-incubation for 30 (column 3), 60 (column 4) or 120 min (column 5) with the anti-OTA Mab. Signals are the mean values of 7 BB-MZIs ± SD.

Another parameter investigated was the effect of pre-incubation of the calibrators with the anti-OTA Mab on the assay sensitivity. For this purpose, a calibrator containing 40 ng/mL of OTA in beer was diluted 8-times with assay buffer and then mixed at 1:1 volume ratio with the anti-OTA Mab solution and incubated for 30, 60 and 120 min prior to the introduction on the chip. The responses obtained were compared with that obtained for the same calibrator without pre-incubation, i.e., by mixing with the Mab solution just prior to the introduction of the mixture on the chip. The responses obtained with the specific calibrator were also compared with those obtained with the zero calibrator. It should be noted that the pre-incubation did not affected the zero calibrator signal. Therefore in Fig. 3, the mean signal values corresponding to zero calibrator without pre-incubation along with those corresponding to a calibrator containing 40 ng/mL in beer both without pre-incubation and with pre-incubation for 30, 60 and 120 min, are provided. As is obvious from the responses corresponding to beer calibrator containing 40 ng/mL of OTA, the pre-incubation step improved considerably the assay sensitivity. In fact, the % inhibition obtained by pre-incubating the calibrator with the Mab solution for 30, 60 and 120 min was 50.9, 34.3, and 31.5, respectively, as compared to 61.1% inhibition received without pre-incubation. In other words, a significant improvement in the assay sensitivity was achieved when a 60-min pre-incubation of the calibrators with the anti-OTA Mab was employed; whereas pre-incubation for 120 min, resulted in a moderate improvement. Thus, in order to keep the assay time as short as possible a 60-min pre-incubation was adopted in the final protocol.

3.2. Sample preparation and effect of sample matrix

Beer is a complicated sample with respect to immunochemical analysis since, besides to other ingredients, it contains a considerable amount of carbon dioxide and has a pH of about 4.5, which might affect negatively the assay performance. Usually the effect of beer matrix is alleviated by removal of carbon dioxide, in other words by degassing, and dilution with the selected assay buffer that adjusts also the pH of the sample. For the degassing of the beer samples, we found that sonication for 10 min in an ultrasonic bath prior to the analysis enabled removal of diluted carbon dioxide as witnessed by the fact that no further gas bubbles were produced by vigorous shaking of the beer sample. It should be noted that if degassing was omitted, the creation of bubbles within the fluidic cell during pumping of the sample, affected negatively the repeatability of measurements, resulting to intra-assay coefficients

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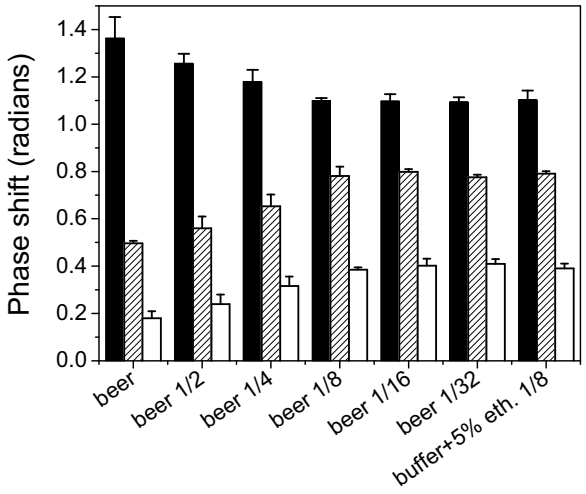

Fig. 4. Effect of beer dilution on the signal obtained for the zero calibrator (black columns), as well as for calibrators containing 10 (hatched striped columns) or 40 ng/mL of OTA (white columns) prepared in undiluted beer, or in beer diluted 2, 4, 8, 16 and 32-times with assay buffer containing 10% (v/v) methanol. To obtain the same final OTA concentration in the differently diluted samples, the OTA-free beer used was spiked with different OTA amounts. The respective signals obtained using calibrators prepared in buffer containing 5% (v/v) ethanol diluted 8 times with assay buffer containing 10% (v/v) methanol are also provided. Signals are the mean value of 7 BB-MZIs ± SD.

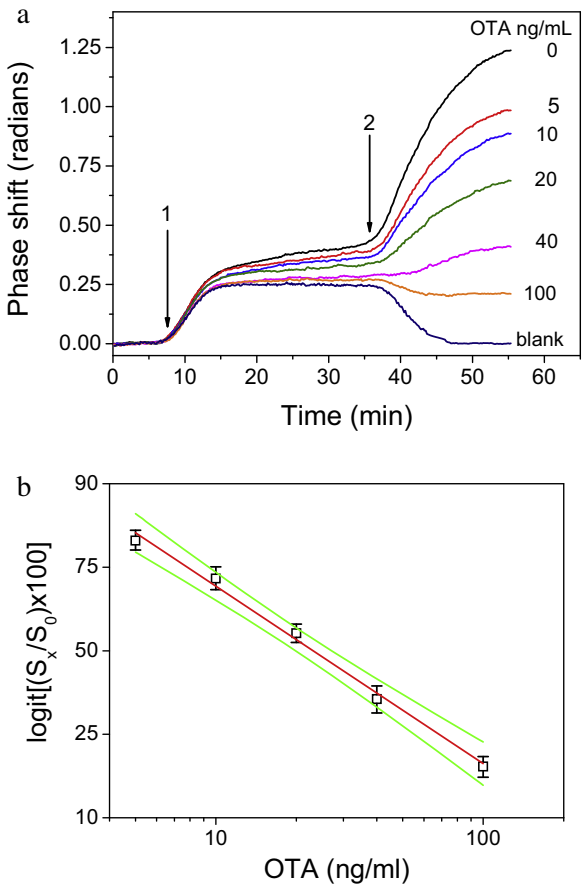

Fig. 5. (a) Characteristic responses from a chip where 7 out of 10 BB-MZIs have been coated with OTA-OVA conjugate and the rest 3 with OVA (blank) when passing over the chip: from start to arrow 1: assay buffer; from arrow 1–2: mixtures of calibrators prepared in beer and diluted 8-times with assay buffer with the anti-OTA antibody (4 μg/mL in assay buffer); from arrow 2 to end: goat anti-mouse IgG antibody solution (10 μg/mL in assay buffer). Each line corresponds to mean response of 7 BB-MZIs for the specific reaction and of 3 BB-MZIs for the control. (b) Typical OTA calibration curve. Each point is the mean value of 3 chips (21 BB-MZIs) ± SD. Red line corresponds to linear regression; Green lines represent the 95% confidence limits of the curve. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of variation values for replicate determinations higher than 20%. Therefore, a 10-min degassing step was included in the sample preparation procedure. In addition to degassing, dilution of beer with assay buffer was also tested as a mean to minimize matrix effect on the assay performance. As shown in Fig. 4, the beer should be diluted at least 8 times with the assay buffer that contains 10% (v/v) methanol in order to minimize the beer matrix effect on the zero calibrator signal and the assay sensitivity. Although the less diluted or even undiluted beer provided higher absolute zero calibrator signals and more sensitive inhibition curves it was found that the absolute values could vary significantly depending on the beer type (lager, ale, dark, etc.) and thus, it would not be possible to use one kind of beer or an appropriate buffer for the preparation of calibrators. On the other hand, further dilution, up to 32 times, did not affected neither the calibrators absolute signal nor the% inhibition values; nevertheless, extensive dilution of the sample is not desirable since this way the lower detectable OTA concentration in beer samples will increase and beers with low OTA content will be considered as OTA-free. In addition, it was found that when the calibrators were prepared in buffer containing 5% (v/v) ethanol (to resemble the beer alcoholic content), the least dilution that provided absolute calibrator values and% inhibition values similar to those obtained with the 8-time diluted beer were obtained when these calibrators were diluted also 8-times with assay buffer containing 10% (v/v) methanol. Thus, by adopting this 8-time beer dilution of the samples, calibrators prepared either in OTA-free beer or buffer containing 5% (v/v) ethanol can be used.

Optical sensing methods such as Mach-Zenhder interferometry that rely on changes in the effective refractive index on the transducer surface for the monitoring of biomolecular reactions could be affected by the sample matrix when the sample has a very different refractive index from the buffers running prior to or after the assay. This was the case with the beer samples. In Fig. 5a, real-time responses obtained for a series of calibrators prepared in 8-times diluted beer along with the response of a blank BB-MZI (functionalized with OVA) are provided. As shown, in all cases the introduction of the calibrator/antibody mixture over the sensors (arrow 1) that have been equilibrated with assay buffer, leads to a sharp response increase that is mainly attributed to increase of the medium refractive index, as indicated also by the control BB-MZI response. After this, a slower signal increase was observed from the BB-MZIs functionalized with OTA-OVA that is mainly due to the specific antibody/immobilized analyte reaction (arrow 1 to arrow 2), whereas stable signal values were obtained from the control BB-MZIs functionalized with OVA. Then, when the secondary antibody solution in assay buffer is run over the chip (arrow 2 to end), removing the beer calibrator, the signal of the control BB-MZI returned to values close to zero. On the other hand, the responses obtained from the OTA-OVA modified BB-MZIs are the result of a combination of decrease in medium refractive index with an increase of the refractive index on the sensing surface due to reaction of secondary antibodies with the immobilized anti-OTA Mab molecules. This is more obvious in the case of high concentration calibrator (100 ng/mL OTA) and less pronounced in the lower concentration ones. Nevertheless, after 10-min reaction with the secondary antibody, the response observed is solely due to the reaction since the medium refractive index effect has been fully removed as evidenced by the response of the control BB-MZI.

Based on the values received for the different calibrators after 30-min assay and 20-min reaction with the secondary antibody (Fig. 5a), a typical linearized calibration curve obtained with cali-

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Table 1
Recovery values.

Beer sample	Amount added (ng/mL)	Amount determined (ng/mL)		% Recovery	
		BB-MZI	ELISA	BB-MZI	ELISA
Amstel lager	15.0	15.7	15.5	105	103
	30.0	29.6	30.5	98.7	102
	45.0	42.0	43.5	93.3	96.7
ZEOS pilsner	15.0	13.6	14.1	90.7	94.0
	30.0	33.2	32.9	111	110
	45.0	49.7	48.6	110	109
Fix dark	15.0	17.4	16.8	116	112
	30.0	27.2	27.6	90.6	92.0
	45.0	42.2	41.9	93.8	93.1
Vergina pale ale	15.0	16.3	15.9	109	106
	30.0	27.6	28.4	92.0	94.7
	45.0	48.1	47.8	107	106
Brink's dark	15.0	16.3	17.0	109	113
	30.0	31.2	33.2	104	111
	45.0	43.6	46.2	96.9	103
KEO lager	15.0	13.6	14.2	90.7	94.7
	30.0	27.4	28.9	91.3	96.3
	45.0	41.6	43.1	92.4	95.8

brators prepared in beer, as well as, its 95% confidence limits, are shown in Fig. 5b. The linear regression equation is:

$$\text{logit}[(S_x/S_0) \times 100] = -1.01(\pm 0.06)\log C + 1.39(\pm 0.08),$$

$R^2 = 0.992$, $p < 0.0001$,

where C is the OTA concentration in ng/mL, S_0 the phase shift corresponding to zero calibrator, and S_x the phase shift corresponding to calibrators containing OTA at concentrations ranging from 5 to 100 ng/mL.

The assay detection limit was determined as the concentration corresponding to mean value of zero calibrator −3 standard deviations (SD; n = 21), and was 2.0 ng/mL in beer (0.25 ng/mL in the diluted beer). The quantification limit was determined as the concentration corresponding to mean value of zero calibrator − 6SD (n = 21), and was 4.0 ng/mL in beer (0.50 ng/mL in the diluted sample).

To determine the assay precision, three control samples with low, medium and high OTA concentration to cover the range of the calibrators were prepared in an OTA-free beer different from that used for the preparation of 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 9% and 14%, respectively.

In order to evaluate the possible effect that different beer matrices, i.e., lager, pilsner, ale, dark, etc., could have on the sensor performance, different types of beers were tested. All the beers used were free of OTA as was determined by an ISO 17085 LC–MS/MS analysis [28] of these samples and confirmed by the sensor developed. The samples were fortified with 3 different concentrations of OTA (15, 30 and 75 ng/ml). The samples were analyzed in triplicate both prior to and after the addition of OTA by applying the degassing and dilution procedure followed for the calibrators. The recoveries were calculated by the following equation: % recovery = [concentration determined/concentration expected] × 100, and are provided in Table 1 along with the values determined by an ELISA developed using the same immunoreagents (see Supplementary material Fig. S2 for the calibration curve of the ELISA). Due to the fact that the ELISA calibration curve had a dynamic range that extended from 1 to 40 ng/mL, a 16-times beer dilution was adopted for the analysis of spiked beer samples. As shown, the results obtained by the two methods are in good agreement. Moreover, the recovery values determined for the sensor ranged between 90.6 and 116%, indicating the good accuracy of the immunosensor.

Several LC/MS–MS methods have been developed for the determination of OTA in beer samples with detection limits ranging from 0.005–0.1 ng/mL [16–19]. Almost all of them employ complex sample preparation procedures (degassing, extraction with organic solvents, centrifugation, concentration by solvent evaporation, and/or dilution) and expensive instrumentation for use in well-controlled laboratory environment. Immunochemical techniques based on different detection principles, ranging from microtitration plate ELISA [20] to colloidal gold flow-through immunoassay strips [20] and gel-based immunoassay columns [24], are less sensitive compared to LC/MS–MS based ones; nevertheless the sample preparation is reduced to degassing and dilution with appropriate buffer. In particular, Wang et al., reported the development of an ELISA and a colloidal gold flow-through immunoassay with detection limits for beer of 1.75 and 25 ng/mL, respectively [20]. On the other hand, a qualitative OTA enzyme immunoassay based on immunoaffinity columns (detection performed by naked eye), that permitted analyte pre-concentration from 10 mL of sample, could discriminate OTA-contaminated from non-contaminated beers with a cut-off value of 0.2 ng/mL. In addition, an electrochemical sensor based on screen-printed electrodes offered a detection limit of 5 ng/mL for OTA in beer, achieved after 50-times dilution with buffer. Compared to these methods, the proposed sensor provides comparable or higher detection sensitivity (0.25 ng/mL in assay buffer, 2.0 ng/mL in beer), especially when compared to the label-free electrochemical sensor.

3.3. Regeneration and re-use of the biofunctionilized chips

The potential to regenerate and re-use the biofunctionalized chips was also evaluated. This aspect is significant for the application of the proposed biosensor for real samples analysis since it could considerably suppress the analysis cost. Therefore, at first several solutions were tested to find the most appropriate regeneration medium. The solutions tested were: a commercially available IgG Elution buffer for immunoaffinity chromatography; 0.1 M glycine/HCl buffer, pH 2.5; 0.05 M NaOH; 0.05 M HCl; and, 0.1 M HCl. To evaluate the different regeneration solutions, the chips were assayed with a mixture of zero calibrator with the anti-OTA Mab, followed by reaction with the secondary antibody (Fig. 6a, black columns). After that the different regeneration solutions were run over the chip for 1 (white columns), 2 (hatched striped columns), or 3 min (horizontally striped columns) followed by reaction with the secondary anti-mouse IgG antibody to determine the residual anti-OTA Mab remaining on the surface after regeneration. As shown, the efficient regeneration solution with respect to removal of the bound onto the surface antibody molecules was 0.1 M HCl solution. In particular, the signals obtained after passing the 0.1 M HCl solution for 3 min led were almost negligible. Using all the other regeneration solutions, the signals obtained after reaction of the remaining anti-OTA antibody with the secondary antibody ranged between 6 and 12% of the signal provided by the zero calibrator. Thus, 3-min regeneration with 0.1 M HCl was adequate to remove completely the bound antibody molecules from the chip surface.

In a next step, the stability of the immobilized onto the sensor surface OTA-OVA conjugate with respect to repetitive regenerations using 0.1 M HCl solution was evaluated. In Fig. 6b, the mean zero calibrator values obtained from 7 BB-MZIs of a single chip after 20 repetitive assay/regeneration cycles performed in three consecutive days are provided. As shown, all values fall within the mean value ± 2SD range, showing the excellent stability of the immobilized OTA-OVA conjugate versus regeneration.

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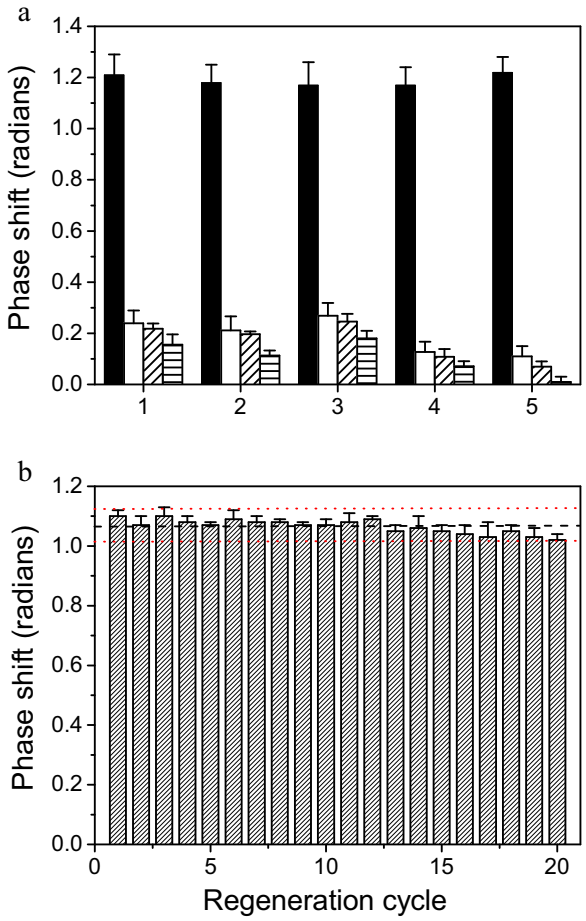

Fig. 6. (a) Mean signal values obtained from OTA-OVA modified chips for the zero calibrator (black columns) and signals obtained after regeneration for 1 (white columns), 2 (hatched stripped columns) or 3 min (horizontally striped columns) by running the anti-mouse IgG antibody. The solutions tested were: IgG Elution buffer (column set 1), 0.1 M glycine/HCl buffer, pH 2.5 (column set 2), 0.05 M NaOH (column set 3), 0.05 M HCl (column set 4), and 0.1 M HCl (column set 5). (b) Mean zero calibrator signal values (± SD) from a chip regenerated 20 times in 3 consecutive days. The black horizontal line corresponds to mean value of the 20 measurements and the dashed red lines to mean value ± 2SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

A miniaturized biosensor based on monolithically integrated arrays of Broad-Band Mach-Zenhder interferometers was developed for the label-free determination of OTA in beer samples. The assay was completed in less than 1 h (including regeneration), and had a detection limit of 2.0 ng/mL in beer, which corresponded to 0.25 ng/mL in the 8-times diluted sample. The proposed biosensor was compared in terms of analytical accuracy with a competitive ELISA developed in microtitration plates using the same monoclonal antibody by analyzing different beers spiked with known amounts of OTA. The values determined with the two methods were in good agreement supporting the accuracy of the measurements performed by the developed immunosensor. In addition, the proposed immunosensor could be regenerated and re-used multiple times without any loss of immobilized immunoreagents activity. The good analytical performance of the sensor developed in combination with its small size and the ability for multiple determinations makes the proposed sensor suitable for incorporation into a portable instrument for point-of-need applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2016.03.019.

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