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Protein adsorption/desorption and antibody binding stoichiometry on silicon interferometric biosensors examined with TOF-SIMS

Katarzyna Gajos^{*a}, Andrzej Budkowski^a, Panagiota Petrou^b, Varvara Pagkali^b, Kamil Awsiuk^a, Jakub Rysz^a, Andrzej Bernasik^{c,d}, Konstantinos Misiakos^e, Ioannis Raptis^{e+}, Sotirios Kakabakos^b

^aM. Smoluchowski Institute of Physics, Jagiellonian University, Łojasiewicza 11,
30-348 Kraków, Poland

^bInstitute of Nuclear & Radiological Sciences & Technology, Energy & Safety NCSR Demokritos, P. Grigoriou & Neapoleos St, Aghia Paraksevi 15310, Athens, Greece

^cFaculty of Physics and Applied Computer Science, AGH University of Science and Technology, Mickiewicza 30, 30-059 Kraków, Poland

^dAcademic Centre for Materials and Nanotechnology, AGH University of Science and Technology, Mickiewicza 30, 30-059 Kraków, Poland

^eInstitute of Nanoscience and Nanotechnology, NCSR Demokritos, P. Grigoriou & Neapoleos St, Aghia Paraskevi 15310, Athens, Greece

⁺Current address: King Abdullah University of Science and Technology (KAUST), Core Labs, Thuwal, 23955-6900, Saudi Arabia

* Corresponding author

E-mail address: katarzyna.gajos@doctoral.uj.edu.pl

Graphical abstract

Highlights

- Examining microstructured sensing areas of interferometric biosensors on silicon chip
- Step-by-step surface modifications determined with biomolecular discrimination
- Surface density of probe, blocking protein, primary and secondary antibody evaluated
- Desorption upon primary immunoreaction depends on spotting concentration of probe
- Binding stoichiometry evaluated more accurately than estimated from sensor response

Keywords

on-chip biosensor, Time-of-Flight Secondary Ion Mass Spectrometry, multi-component layers, biofunctionalization, indirect immunoassay, binding stoichiometry

Abstract

Time-of-flight secondary ion mass spectrometry has been employed to examine, with biomolecular discrimination, sensing arm areas ($20\ \mu\text{m} \times 600\ \mu\text{m}$) of integrated onto silicon chips Mach-Zehnder interferometers aiming to optimize their biofunctionalization with regard to indirect immunochemical (competitive) detection of ochratoxin A. Sensing areas are examined after: modification with (3-aminopropyl)triethoxysilane, spotting of OTA-ovalbumin conjugate (probe) from solutions with different concentration, blocking with bovine serum albumin, reaction with OTA-specific mouse monoclonal antibody followed by goat anti-mouse IgG secondary antibody. Component mass loadings of all proteins involved in immunodetection are determined from TOF-SIMS micro-analysis combined with ellipsometry of planar surfaces. These data show that partial desorption of surface-bound probe and blocking protein takes place upon primary immunoreaction to a degree that depends on probe concentration in spotting solution. Taking into account this desorption, apparent binding stoichiometry of both antibodies in immune complexes formed onto chip surface is determined more accurately than the respective evaluation based on real-time sensor response. In addition, mass loadings for probe and secondary antibody is observed to saturate for optimum probe concentrations. Also, principal component analysis of TOF-SIMS data could resolve both immunoreactions and biofunctionalization and discriminate surfaces prepared with optimum probe concentrations from those prepared using suboptimum ones.

1. Introduction

Among the large number of existing biosensing systems, optical ones have been extensively developed in the last decades due to their capability for label-free detection [1]. The lack of necessity of labelled molecules simplifies considerably the detection procedure and reduces the analysis costs. Therefore, label-free optical biosensors, including sensors based on surface

plasmon resonance [2], interferometers [3], grating couplers [4,5], micro-ring resonators [6] and photonic crystals [7], are promising candidates for development of autonomous point-of-care devices for biomedical diagnostics, environmental safety and food quality control [8–10]. Especially attractive are the interferometric biosensors which demonstrate low detection limits, are easily miniaturized and produced by mainstream silicon technology [8].

The working principle of many label-free optical biosensors [11] (*incl.* interferometers [3], surface plasmon resonance [2], and others [4,5,6]) is the detection of specific interaction between a recognition molecule immobilized on the sensing area of the biosensor with the analyte in sample, which changes the refractive index of the sensor surface region. Thus, the quality of sensor surface biofunctionalization is crucial for high sensitive and selective biosensor performance [8,11]. However, the geometry of biosensing transducers and surface microstructuring hinder the direct examination of the real transducer surface by standard surface analysis techniques [11]. The characterization of biorecognition layers formed on the sensor surface is, therefore, commonly performed on planar substrates imitating the real transducer [12–16]. Another important issue often disregarded is that the multi-step biofunctionalization and assay protocol, usually involving chemical modification of the surface, recognition molecule immobilization, blocking of free surface sites and at least one specific binding reaction, leads to a multi-component molecular coverage of the sensor surface that is the result of many interactions of solid-to-liquid phase molecules. Since many optical biosensors are sensitive to cumulative mass of all molecules on their surface the real-time signal monitoring do not provide a molecular discrimination of all these interactions. Therefore an additional verification of the formed biomolecular layers composition by an independent method should be indispensable at the stage of protocol development to ensure correct results interpretation.

Recently, Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) has been

recognized as a powerful technique to analyze biomolecular overlayers on solid supports [17], including biosensor surfaces, due to chemical specificity and surface sensitivity it exhibits. Indeed, TOF-SIMS has been successfully employed to discriminate between different molecules immobilized on the different surfaces and in particular to detect different proteins based in differences in their amino acids composition [14,18–23]. Among the reported applications of TOF-SIMS technique for biosensor surface analysis the examination of microstructured interferometric biosensors based on Mach-Zehnder interferometers monolithically integrated on silicon chips modified following a multi-step protocol has been demonstrated [19].

Here, such a step-by-step TOF-SIMS inspection of Mach-Zehnder interferometers (MZIs) monolithically integrated on silicon chips [19] is extended to an *indirect competitive immunoassay* for the determination of Ochratoxin A (OTA). The assay is performed on MZIs modified with protein-analyte conjugate after blocking of the surface by introducing a mixture of analyte-specific (primary) antibody with calibrators, and it is followed by reaction with a secondary antibody for signal enhancement. As in all competitive immunoassays, OTA molecules present in the calibrator or the sample compete with OTA-OVA probe molecules immobilized onto the sensor surface for binding to antibody, and hence the sensor response is inversely proportional of the OTA concentration in the calibrators or the samples, with the highest signal obtained for zero analyte concentration [24]. Several parameters determine the performance of OTA detection with the on-chip biosensors following the aforementioned competitive immunoassay format. For instance, parameters related to primary immunoreaction including assay time and anti-OTA antibody concentration, as well as the secondary immunoreaction including the duration of reaction with secondary antibody [24]. However, the main parameter, which defines both the absolute signal and the assay sensitivity is the concentration of OTA-OVA conjugate solution used for sensor biofunctionalization. It

is this parameter that is optimized in this work. A similar analysis has been previously performed on planar substrates imitating the MZI transducer [20], and the new findings are discussed in relation to those. To optimize the biofunctionalization procedure for efficient detection of analyte, the on-chip sensors where the analyte-protein conjugate (probe) has been immobilized from solutions with different concentration were examined following a strategy introduced recently [25]. Although TOF-SIMS provides a valuable information about molecular composition of multi-component layers on surfaces, it is not able to quantify directly the molecules surface density and for this reason it is complemented by ellipsometric analysis of plain surfaces prepared following the same procedure applied for the chips. As a result, component mass loadings of all proteins used, i.e. probe, blocking protein, primary and secondary antibody, are estimated based on biomolecular discrimination with TOF-SIMS. Also, protein *partial desorption* upon primary immunoreaction is revealed and effective *binding stoichiometry* of both antibodies in the immune complexes formed on the sensor surface is determined.

2. Experimental section

2.1 Fabrication of chips with integrated Mach-Zehnder interferometers

Silicon wafers were purchased from Si-Mat (Kaufering, Germany). Optoelectronic silicon chips were fabricated at the Institute of Nanoscience and Nanotechnology of NCSR “Demokritos”. A single chip consists of ten planar Si_3N_4 waveguide Mach-Zehnder interferometers monolithically integrated onto the silicon substrate along with their respective silicon avalanche diodes light sources (LEDs) (**Fig. 1a**). The rib Si_3N_4 waveguide, with a ridge of 2-nm in height, is formed on the 100-nm thick layer of silicon nitride and is therefore visible only for a short part at its beginning at the LED side where the silicon nitride layer is fully etched, as opposed to the rest of waveguide length (**Fig. 1b**). The chips fabrication

process is described in details in previous publications [26–28]. The sensing region of each Mach-Zehnder interferometer is formed by 600- μm long and 20- μm wide window etched on the 2.5 μm thick SiO_2 cladding layer (**Fig. 1a and 2a**). These windows are open over one of the arms (sensing arm) of each Mach-Zehnder interferometer, thus, exposing the particular area of the waveguides to solution supplied through a fluidic module (Jobst Technologies GmbH; Freiburg, Germany) [27,28]. A miniaturized spectrometer (QE65000; Ocean Optics Inc., Dunedin, FL 34698) was used for the collection of output spectrum from the integrated on silicon chip MZIs.

Additionally, auxiliary planar Si_3N_4 surfaces were prepared for ellipsometry measurements by low pressure chemical vapor deposition of a 1- μm thick SiO_2 layer on silicon wafer, followed by the additional deposition of a 150-nm thick Si_3N_4 layer.

2.2 Biosensor surface functionalization and assay protocol

3-Aminopropyltriethoxysilane (APTES), bovine serum albumin (BSA) and ovalbumin (OVA) were from Sigma-Aldrich (Darmstadt, Germany). The mouse monoclonal anti-OTA antibody (anti-OTA Mab) was from Soft Flow Hungary Ltd. (Pecs, Hungary) and the affinity purified polyclonal goat anti-mouse IgG antibody (anti-mIgG) was purchased from Merck Millipore (Darmstadt, Germany). The OTA-OVA conjugate was synthesized as described in the previous publication [24].

The subsequent steps of functionalization and immunoassay performed on sensing arm areas of Mach-Zehnder interferometers, are shown schematically in **Fig. 2b**. Firstly, the silicon chips surface was cleaned and hydrophilized with oxygen plasma. Then, the surfaces were silanized (1) through immersion in a 0.5% (v/v) aqueous APTES solution for 2 min, followed by washing with distilled water, drying under nitrogen stream, and backing for 20 min at 120 °C. Functionalization of the MZIs sensing arm areas with OTA-OVA conjugate (2) was performed by deposition of multiple overlapping spots of OTA-OVA solution, as it is

schematically shown in **Fig. 1a**, employing a standard microarray spotter (BioOdyssey Caligrapher Miniarrayer, Bio-Rad Inc.). OTA-OVA solutions in 50 mM carbonate buffer (pH 9.2) with three different concentrations C, namely 50, 100 and 200 $\mu\text{g/mL}$, were used for the sensing arms functionalization. After solution deposition, the silicon chips were incubated in a humidity chamber (~75% humidity) for 18 h and washed with 0.01 M Tris-HCl buffer, pH 8.25. The blocking step (**3**) was accomplished by immersing the chips in a 10 mg/mL BSA solution in 0.1 M NaHCO_3 solution, pH 8.5, for 2h, followed by washing with 0.01 M Tris-HCl buffer, pH 8.25, and distilled water, and drying in a nitrogen stream.

For the immunoassay, the functionalized chips were covered with the fluidic module and placed on the docking station of the measuring apparatus that provides for both the electrical and fluidic connections [19,24]. Firstly, the chip is equilibrated by running assay buffer (50 mM Tris-HCl buffer, pH 7.8, containing 9 g/L NaCl and 5g/L BSA). After that, a 4 $\mu\text{g/mL}$ solution of anti-OTA Mab in assay buffer was run over a chip for 30 min with a constant rate of 40 $\mu\text{L/min}$. Then, a 10 $\mu\text{g/mL}$ solution of anti-mIgG was injected and passed over a chip for 20 min with the same flow rate. This sequence provides the zero calibrator signal of the competitive immunoassay that corresponds to the highest signal obtained for the particular assay.

For TOF-SIMS analysis, silicon chips corresponding to each one of the functionalization and assay steps described above were washed with buffer and distilled water, dried under nitrogen stream and used for the measurements. In addition, a reference silicon chip with sensing arm areas covered with antibodies (**r4**) was prepared through deposition of multiple overlapping spots of a 4 $\mu\text{g/mL}$ solution of anti-OTA Mab in assay buffer.

Additionally, planar Si_3N_4 surfaces were prepared and functionalized as the silicon chips with the integrated MZIs to be used for ellipsometry measurements [20].

2.3 TOF-SIMS characterization

“In-situ” TOF-SIMS analysis of on-chip integrated Mach-Zehnder interferometers surface was performed using the TOF-SIMS 5 (ION-TOF GmbH) instrument. The Bi_3^+ clusters produced by a 30 keV bismuth liquid metal ion gun were used as primary ions. The ion dose density was lower than 10^{12} ion/cm² to ensure static mode conditions. A low energy electron flood gun was used for charge compensation.

Positive ion TOF-SIMS data (images and mass spectra) were acquired from several 500 μm x 500 μm areas (applied resolution was 512 x 512 points) of each silicon chip, thus covering the whole area of each sensing arm. For molecular analysis, high resolution mass spectra were extracted from an area of 15 μm x 480 μm located inside each individual sensing region. Mass calibration was performed with H^+ , H_2^+ , CH^+ , C_2H_2^+ and C_4H_5^+ peaks. A minimal mass resolution ($m/\Delta m$) > 5300 at C_4H_5^+ was obtained.

2.4 Principal Component Analysis of TOF-SIMS data

Principal Component Analysis (PCA) was performed for the TOF-SIMS positive spectra acquired from the area of individual sensing arms (15 μm x 480 μm) using PLS Toolbox (Eigenvector Research, Manson, WA) for MATLAB (MathWorks, Inc., Natick, MA). In PCA analysis only selected peaks characteristic for the proteins were included. Prior to PCA, the intensities of selected peaks from each spectrum were normalized to the sum of selected peaks and mean centered.

2.5 Ellipsometry measurements

To determine the total protein mass loading the auxiliary ellipsometry measurements were performed on planar Si_3N_4 surfaces after each step of surface biofunctionalization and assay protocol applying OTA-OVA solution with concentration 200 $\mu\text{g/mL}$. A Sentech SE800 (Sentech Instruments GmbH) Spectroscopic Ellipsometer working over a wavelength range of 320-700 nm and at a fixed angle of incidence equal to 70° was used. Results were analyzed

with the SpectraRay 3 software assuming the Cauchy dispersion formula and three-layer model consisted of silicon substrate/ Si_3N_4 /mixed SiO_2 and APTES/protein overlay as described before [20].

3. Results and Discussion

3.1 Indirect competitive immunoassay for mycotoxin detection on interferometric biosensors integrated onto silicon chip

The aim of this study was to develop an approach for “in-situ” analysis with biomolecular discrimination of the layer created onto the sensing arm area of interferometric biosensors integrated on silicon chip in the course of an indirect immunoassay. The interferometric sensors employed in the study were Mach-Zehnder interferometers (MZIs) integrated in arrays of 10 sensors in silicon chips. An image of the chip with a footprint area of $9.3 \times 4.0 \text{ mm}^2$ accommodating the 10 MZIs is presented in **Fig. 1a** [27,28]. As shown, the MZIs are non-linearly arranged onto the chip which integrates also the respective broad-band light emitting diodes (one for each MZI). The sensing region on each MZI is created by etching a $600 \times 20\text{-}\mu\text{m}^2$ window on the $2.5 \text{ }\mu\text{m}$ thick SiO_2 cladding layer covering the Si_3N_4 waveguide. A schematic of the procedure of functionalization of MZIs sensing arm areas and subsequent immunoassay for the determination of mycotoxin ochratoxin A (OTA) [24] is provided in **Fig. 2**. The procedure consists of the following steps: chemical modification with the aminosilane APTES (1), immobilization of the OTA-OVA conjugate (2), blocking of free surface sites with bovine serum albumin (BSA) (3), and two immunoreactions in sequence, the first with mouse monoclonal antibody against OTA (primary antibody) (4) and the second with a goat anti-mouse IgG antibody (secondary antibody) (5).

For the biofunctionalization of sensing arm areas with OTA-OVA solutions with different concentration C were used. On each chip, 3 out of 10 MZIs were functionalized using solution with $C = 50 \text{ }\mu\text{g/mL}$, other 3 using solution with $C = 100 \text{ }\mu\text{g/mL}$ and rest 4 using

solution with $C = 200 \mu\text{g/mL}$, as shown in **Fig. 1a**. The real-time responses of MZIs obtained by running in sequence over a chip biofunctionalized as depicted in **Fig. 1a** solutions of primary (**4**) and secondary (**5**) antibodies (after steps **1-3**) are presented in **Fig. 1c**. A baseline phase shift change rate of -0.0009 ± 0.001 radians/min is calculated when assay buffer is run over the chip. Once the solution of anti-OTA Mab is introduced, its specific binding to the immobilized OTA-OVA conjugate, resulted in a phase shift change rate equal to 0.007 ± 0.001 radians/min for the MZIs functionalized with the $50 \mu\text{g/mL}$ OVA-OTA solution and 0.011 ± 0.001 radians/min for the MZIs covered with either the 100 or the $200 \mu\text{g/mL}$ OVA-OTA solution. Then, the passing of a secondary antibody solution (anti-mIgG) resulted in a phase shift change rate equal to 0.030 ± 0.002 , 0.042 ± 0.001 and 0.048 ± 0.002 radians/min for the MZIs covered with 50, 100 or $200 \mu\text{g/mL}$ OVA-OTA solutions, respectively. These results show that the biosensor response is significantly lower when a solution with $C = 50 \mu\text{g/mL}$ of OTA-OVA was used as compared to higher concentrations, both during the primary and the secondary immunoreaction. Regarding the sensors biofunctionalized with OTA-OVA solutions of 100 and $200 \mu\text{g/mL}$, a slight difference in response is noticeable only during the secondary immunoreaction. Based on the observed phase shifts an apparent binding stoichiometry of primary and secondary antibodies in the immune complexes formed onto the chip surface can be deduced directly from the immunosensors response. The estimated values of binding molar ratio of secondary to primary antibody are about ~ 4.3 , ~ 3.8 and ~ 4.4 for MZIs functionalized with OTA-OVA solution with concentration $50 \mu\text{g/mL}$, $100 \mu\text{g/mL}$ and $200 \mu\text{g/mL}$, respectively. Output phase shifts of MZI signal reflect the changes in effective refractive index of the sensing arm, caused by cover medium and/or by the build-up biomolecular adlayer, as explained in our recent publication [29]. Assuming that the cover medium changes are negligible throughout the experiment, the experimentally determined phase shift is linearly related with the mass density of the biomolecular adlayer in accord with

de Feijter formula. For the specific geometrical-optical characteristics of the used broad-band MZIs, the minimum resolvable change of biomolecular mass has been estimated as $\sim 0.01 \text{ mg/m}^2$, calculated from the spectral response corresponding to a 5-nm thick (6.85 mg/m^2) protein adlayer that was assumed to correspond to complete coverage of MZI sensing area with protein molecules [29].

3.2 Surface analysis of sensing arm areas with biomolecular discrimination

Chemical surface analysis with TOF-SIMS was performed on sensing arm areas of integrated MZIs after each functionalization and immunoassay step to study the changes in biomolecular content of the sensor adlayer. A direct inspection of sensing arm areas was possible since the MZIs sensing windows are easily resolved on the TOF-SIMS images determined with the sum of all the secondary ions (not shown). To enable biomolecular discrimination of the adlayer content, analysis of ion fragments characteristic of the substrate and each protein employed in the biofunctionalization and assay procedure (section 3.2.1), as well as a multivariate analysis of the multiple signals set (section 3.2.2) were applied.

Firstly, the uniformity of the sensing arm areas coverage with the biomolecules added in the subsequent biofunctionalization/assay steps was studied by TOF-SIMS chemical imaging. TOF-SIMS maps of ion $\text{C}_4\text{H}_8\text{N}^+$ derived from amino acids recorded for MZIs after OTA-OVA probe immobilization through spotting from solutions with different concentrations (50, 100, or 200 $\mu\text{g/mL}$) are shown in **Fig. S1** in Supplementary Material. For all the OTA-OVA solution concentrations used, the recorded maps show uniform surface coverage with the OTA-OVA molecules. This uniform surface coverage is preserved in all the subsequent steps (not shown). A comparison of intensities of $\text{C}_4\text{H}_8\text{N}^+$ ion maps reveals that the surface coverage with OTA-OVA increases with concentration of applied OTA-OVA solution.

3.2.1 Analysis based on characteristic TOF-SIMS signals

The composition of the biomolecular adlayer formed over the sensing arm areas of the integrated MZIs after each subsequent biofunctionalization/assay step for the different OTA-OVA solution concentrations applied is provided in **Fig. 3**. Reference silanized samples (**1**) and samples spotted directly with primary antibody (**r4**) are also analyzed. The intensities of secondary ions characteristic for the substrate and each of the different proteins applied in the biofunctionalization protocol are considered. Molecular fragments characteristic for OVA, BSA and antibody based on differences in their amino acid composition were selected [30,31]. Namely, the signal $C_2H_5S^+$ originating from methionine ([18], **Fig. 3e**), $C_5H_{10}N^+$ from lysine ([18], **Fig. 3f**) and $C_9H_8N^+$, $C_{11}H_8NO^+$, $C_{10}H_{11}N_2^+$ from tryptophan ([18], **Figs. 3g-i**) are assigned as characteristic to OVA, BSA and antibody, respectively, due to high percentage of these amino acids in the bulk composition of the respective proteins (**Table S1**, Supplementary Material). Additionally, the signals $C_4H_8NO_2^+$ from glutamic acid ([18], **Fig. 3b**) and $C_2H_6N^+$ from alanine ([18], **Fig. 3c**) are considered as characteristic for both albumins, OVA and BSA. However, the experimental results obtained from the reference surfaces (**1** and **r4**) show that the signal ascribed to OVA is indeed *unique*, while the TOF-SIMS peaks suggested for BSA and both albumins are characteristic but could have small contributions from antibody.

The reduction of the substrate signal ($SiOH^+$, **Fig. 3a**) upon the successive modification/assay steps reveals the building-up of the protein overlayer. On the other hand, the intensities of the ions unique for OVA ($C_2H_5S^+$ from methionine, **Fig. 3e**) as well as those of ions characteristic for both albumins ($C_4H_8NO_2^+$ from glutamic acid and $C_2H_6N^+$ from alanine, **Fig. 3b-c**) reflect the surface bound amount of OTA-OVA conjugate after the step of probe immobilization (**2**). The linear increase of intensities of these signals with the concentration C of OVA-OTA solution used for spotting reflects a progressive surface coverage with the OTA-OVA conjugate. When the free surface sites are blocked with BSA

(3), the amount of surface-bound OTA-OVA conjugate is preserved (see $C_2H_5S^+$, **Fig. 3e**). In addition, similar amounts of BSA molecules were absorbed onto the surfaces previously treated with solution of OTA-OVA of different concentration C (see signals $C_4H_8NO_2^+$, $C_2H_6N^+$, $C_4H_8N^+$, $C_5H_{10}N^+$, **Fig. 3b-d,f**).

During the primary immunoreaction (4), partial desorption of both albumins, OVA and BSA, occurs as indicated by the reduction of signals characteristic for OVA (**Fig. 3e**), BSA (**Fig. 3f**), both albumins (**Fig. 3b,c**) and proteins in general (**Fig. 3d**). This desorption process is more evident for the surfaces with the higher density of proteins adsorbed in the previous two steps. The protein binding behavior can change from irreversible to reversible as it has been shown in a previous publication [32]. Therefore, protein desorption initiated by subsequent immobilization of another macromolecule is to some extent expected. Such a behavior has been also observed on silicon surfaces modified with aminosilane at certain protein surface density values [13,25].

The efficiency of immunoreactions with the primary and the secondary antibody is indicated by the intensities of specific for antibodies tryptophan signals ($C_9H_8N^+$, $C_{11}H_8NO^+$, $C_{10}H_{11}N_2^+$, **Fig. 3g-i**). A drastic increase of all tryptophan signals is observed after the secondary immunoreaction. This result confirms directly the effective binding of secondary antibody (anti-mIgG) (5) to the immunosorbed primary antibody molecules and indirectly the presence of the primary antibody molecules (anti-OTA Mab) (4) that have been bound to the immobilized OTA-OVA probe. The intensity of tryptophan signal, that is a measure of the amount of surface-bound secondary antibody, increase as the concentration C of OTA-OVA solution used for spotting is increased from 50 $\mu\text{g/mL}$ to 100 $\mu\text{g/mL}$ and then starts to saturate as the concentration is further increased to 200 $\mu\text{g/mL}$. The dependency of the antibodies amount that binds onto the surface on the OTA-OVA solution concentration is a further evidence of reduced surface density of the OTA-OVA conjugate after the first

immunoreaction step due to partial desorption (see **Fig. 3e**), especially on the surfaces with the higher OTA-OVA probe surface densities.

3.2.2 Compositional multivariate TOF-SIMS analysis

To examine simultaneously multiple TOF-SIMS signals and to enhance detection of subtle changes in the composition of the biomolecular adlayer formed on the sensing arm areas of the MZIs, Principal Component Analysis was applied. The analysis was performed on a set of 42 positive ion fragments (listed in **Fig. S2**, Supplementary Material) originating from both proteins and APTES molecules. Thirty-six spectra recorded from sensing arm areas of MZIs functionalized with OTA-OVA conjugate (**2**) using solutions with concentration 50, 100 or 200 $\mu\text{g/mL}$, as well as from thus functionalized MZIs after the subsequent steps of blocking (**3**) and primary and secondary immunoreaction (**4** and **5**) have been included in PCA. Multivariate PCA provides the directions of uncorrelated major variations (so-called Principal Component) within the analyzed data set thus enables us to reduce dimensionality while maintaining all the essential information.

The biomolecular composition of functionalized MZIs is expressed in **Fig. 4** by the first PC, capturing the majority of the variance (61.96%), and the third PC (7.97% of variance). As revealed from the loadings plot (**Fig. S2**), where Principal Components are correlated to particular TOF-SIMS signals, PC1 distinguishes between aminosilane and amino acids characteristic for albumins, which yield negative loadings, and those characteristic for antibodies, which yield positive loadings (see **Table S1**, Supporting Information). The data points in PC1 scores plot, corresponding to TOF-SIMS spectra recorded for different samples, are clearly separated for positive and negative PC1 values. Points corresponding to the steps of OTA-OVA immobilization (**2**) and blocking (**3**) are characterized by negative PC1 values, while those corresponding to both immunoreaction steps (**4** and **5**) have positive PC1 values. This result provides a direct confirmation of the anti-OTA Mab binding to the surface

immobilized OTA-OVA during the primary immunoreaction. The subsequent immunoreaction with the secondary antibody results in even higher PC1 values reflecting the effective binding of anti-mIgG onto the anti-OTA Mab molecules that have been immunosorbed onto the surface. In addition, PC3 differentiates sensing arm areas functionalized with a 50 $\mu\text{g/mL}$ OTA-OVA solution from those functionalized with solutions with higher concentration. Although the interpretation of the PC3 in terms of biomolecular composition is not unequivocal, the results are indicative of the difference in surface composition obtained when applying a 50 $\mu\text{g/mL}$ OTA-OVA solution concentration instead of a higher probe concentration. This difference is also visible for single characteristic TOF-SIMS signals (**Fig. 3**) and is reflected in the real-time response of the MZIs (**Fig. 1c**). Therefore, the higher concentrations of OTA-OVA solution, i.e., 100 or 200 $\mu\text{g/mL}$, are considered as optimal for biofunctionalization of MZIs sensing arms with the OTA-OVA probe.

3.3 Determination of the component mass loadings of different molecules

To quantify the biomolecular composition of sensing arm areas, component mass loadings of different molecules are evaluated (**Fig. 5**). This analysis is based on both TOF-SIMS results from the chip sensing arm areas (**Fig. 3**) and auxiliary ellipsometric data from their planar counterparts (**Table S2**, Supplementary Material) in order to provide the total protein surface density. The considered intensities of TOF-SIMS signals, $I'(\mathbf{n}) = I(\mathbf{n}) - I(\mathbf{1})$, are determined always with reference to bare silanized surface (**1**). Since TOF-SIMS spectra show linear correlation with organic surface composition [33,34], the assumption that each component contribution to any TOF-SIMS signal $I^{\text{component}}(\mathbf{C}, \mathbf{n})$ is proportional to component mass loading for the samples (**n**) prepared using the same probe concentration $\mathbf{C}$ is made. The ratio of these two quantities, $I^{\text{component}}(\mathbf{C}, \mathbf{n}) / \Gamma^{\text{component}}(\mathbf{C}, \mathbf{n})$, is determined for the surfaces

prepared using an OTA-OVA solution of $C = 200 \mu\text{g/mL}$ (for which the total mass loadings have been determined) with pure components (with total mass loadings equivalent to component mass loadings): OTA-OVA (**2**) for the (unique) signal $\text{C}_2\text{H}_5\text{S}^+$, albumins (**3**) and primary antibody (**r4**) for the (non-unique) signals $\text{C}_4\text{H}_8\text{NO}_2^+$, $\text{C}_2\text{H}_6\text{N}^+$, $\text{C}_5\text{H}_{10}\text{N}^+$ or $\text{C}_4\text{H}_8\text{N}^+$. Also, we take advantage of the discussion of TOF-SIMS data presented in **Fig. 4**, from which we assume that the addition of new component upon blocking (**3**) and secondary immunoreaction (**5**) is not accompanied by some distinct change of existing multi-component coverage. Therefore for these two surface modification steps, an increase in both total protein mass loading and in TOF-SIMS signal is attributed to the newly added component, and the ratio of both increments is determined from the data obtained from the analysis of surfaces spotted with OTA-OVA solution of $C = 200 \mu\text{g/mL}$. With the above assumptions made, the component mass loadings of all molecules involved at the different biofunctionalization/assay steps are calculated for the samples prepared using different OTA-OVA concentration C (**Fig. 5**), except for the chip sensing areas after the primary immunoreaction (**4**). For these samples, similar calculations can be made only for the chips prepared using the OTA-OVA solution with concentration $C = 200 \mu\text{g/mL}$ case, since for these samples the total mass loadings enable evaluation of multi-component contributions to non-unique TOF-SIMS signals.

3.3.1 Albumin desorption upon primary immunoreaction

As revealed from the intensity of the characteristic TOF-SIMS signals (see **Section 3.2.1**) the primary immunoreaction step is accompanied by partial desorption of both albumins, OTA-OVA and BSA. Based on the estimated component mass loadings (**Fig. 5**), the amount of desorbed protein can be evaluated for each albumin and for surfaces prepared using different OTA-OVA conjugate concentrations in the spotting applied in the probe immobilization step (**2**). The amount of OTA-OVA conjugate desorbed during the primary immunoreaction strongly depends on the OTA-OVA surface density (**2**) achieved by spotting the solutions of

different concentration. For C equal to 50 or 100 $\mu\text{g/mL}$, the desorbed OTA-OVA amount is calculated to about 0.05 mg/m^2 , a value that is in the margin of error. In turn, for $C = 200 \text{ }\mu\text{g/mL}$, a considerably higher degree of OTA-OVA desorption of about 0.3 mg/m^2 is observed. However, the desorption of BSA molecules observed during this step is significantly more extensive than that of the OTA-OVA molecules. In particular, the BSA mass loading after the surface blocking step is comparable for all surfaces treated with the OTA-OVA solution of different concentration and is about 1.25 mg/m^2 . The amount of BSA desorbed during immunoreaction increases as the OTA-OVA concentration increases (see **Fig. 3f**) reaching a value of about 1.1 mg/m^2 for $C = 200 \text{ }\mu\text{g/mL}$. As discussed in **Section 3.2.1** a similar phenomenon was observed for BSA adsorbed on APTES modified substrates [25].

As a result of the dependence of desorption phenomena on the concentration of OTA-OVA solution used for spotting the MZIs sensing arm areas, protein overlayers with different composition regarding OTA-OVA and BSA existed on the MZIs sensing arm areas during the primary immunoreaction step. After this step, however, the OTA-OVA mass loading is similar for the surfaces spotted with OVA-OTA solution with concentration C equal to 100 or 200 $\mu\text{g/mL}$, whereas is significantly lower for $C = 50 \text{ }\mu\text{g/mL}$ (**Fig. 5**). This dependence on OTA-OVA solution concentration C is reflected as well in the surface mass loadings of secondary antibody achieved after the second immunoreaction. Since the amount of bound secondary antibody saturates for OTA-OVA solution concentrations equal to or higher than 100 $\mu\text{g/mL}$, this concentration range can be proposed as optimal for the MZIs biofunctionalization.

3.3.2 Antibodies binding stoichiometry

The estimated component mass loadings can be expressed as component molar mass

loadings when divided by the molar mass of each particular protein (**Fig. 5**). Based on the molar mass loadings of OTA-OVA probe, anti-OTA Mab primary antibody and anti-mIgG secondary antibody determined after each biofunctionalization/assay step, the apparent binding stoichiometry of immune complexes formed onto the biosensor surface were evaluated (**Fig. 6**). When a OTA-OVA solution with concentration $C = 200 \mu\text{g/ml}$ was applied for probe immobilization, a molar binding ratio of primary antibody to probe of about ~ 1.3 was determined based on the anti-OTA Mab (averaged for different TOF-SIMS signals) and OTA-OVA mass loadings after the primary immunoreaction. This binding ratio could be the result of multivalence of OTA-OVA conjugate (more than one OTA molecules per OVA) that could lead to binding of some of the antibodies molecules with both their binding sites to the same or neighbor surface-bound OTA-OVA molecules. This very strong binding is indicated by the relative harsh regeneration conditions required (running a 0.1 M HCl solution for 3 min) in order to remove the antibodies bound onto the immobilized OTA-OVA probe [24]. The molar binding ratio of secondary antibody to the primary one determined also for chips functionalized with an OTA-OVA solution with concentration $C = 200 \mu\text{g/ml}$ is about ~ 2.5 . In turn, the respective binding ratio estimated directly from the sensors response is about ~ 4.4 . Nevertheless, the MZI biosensor response is proportional to the total mass density of molecules bound onto the sensing arms region without discrimination. Thus, the signal observed in real-time during the anti-OTA Mab solution flow is the sum of two procedures that affect differently the total protein surface density; the partial desorption of OTA-OVA and BSA molecules decreases the total protein surface density whereas the binding of anti-OTA Mab to surface-bound OTA-OVA probe increases the total protein surface density values. As a result the value of binding ratio between primary and secondary antibodies that is calculated based onto the real-time MZIs responses is overstated.

The effective molar binding ratio of anti-mIgG secondary antibody and OTA-OVA

probe was calculated for all the concentrations of OTA-OVA solution applied to MZIs functionalization. Consistent values of about ~2.9, ~2.9 and ~3.2 were obtained (see **Fig. 6**) for chips functionalized by spotting OTA-OVA solutions with concentration C equal to 50, 100 and 200 $\mu\text{g/mL}$. This result is very important because it means that the signal enhancement achieved by the reaction with the secondary antibody is independent of the concentration of the OTA molecules available for reaction with the primary antibody onto the surface. Thus, in the course of the indirect assay where the signal is reversibly proportional to the analyte concentration in the sample, the reaction with the secondary antibody would not affect the signal due to primary immunoreaction and hence the assay sensitivity.

3.4. The examination of microstructured biosensors extended from the analysis of planar surfaces

This study focuses on protein desorption and antibody binding stoichiometry aiming to optimization of the sensor biofunctionalization procedure. To this end, the probe is immobilized (from solutions with different concentration) to the microstructured MZI transducers, which are examined with TOF-SIMS. This is an extension of our recent analysis [20] of molecular arrangement, composition and orientation, examined (for one probe concentration) with AFM and other surface techniques for the planar surfaces, mimicking real transducers, in the course of the same (competitive) indirect immunoassay for the determination OTA. The main findings of the previous [20] and the current work are related. First, the molecular arrangement of multicomponent monolayer covered with secondary antibodies [20] enables the evaluation of component mass loading (**Section 3.3** and **Fig. 5**) with TOF-SIMS, as the TOF-SIMS signals decay exponentially with layer thickness [20] and would, therefore, fail to sample thicker overlayers. Second, the determination of the molecular composition, demonstrated in [20] for probe and blocking protein, is extended here (with 8

instead of 2 characteristic TOF-SIMS signals) for all components (incl. antibodies), and different probe concentrations (**Figs. 3 and 5**). Also, partial protein desorption during primary immunoreaction, concluded in [20], is shown here to depend on the probe spotting concentration, confirming the generality of this recently reported phenomenon [25]. Third, the molecular orientation of immunoabsorbed primary (side-on) and secondary antibody (head-on) [20] corresponds well to their binding stoichiometry in the immune complexes formed onto the MZI transducer (with the ratio of ~ 1.3 for primary antibody to probe, and ~ 2.5 for secondary to primary antibody), evaluated here with TOF-SIMS more accurately than estimated from sensor response.

4. Conclusions

TOF-SIMS technique was employed for “in-situ” examination of sensing arm areas of integrated on silicon chips MZIs after each subsequent step of biofunctionalization and immunoassay protocol applied for the determination of ochratoxin A. The application of TOF-SIMS technique allows the discrimination of the different proteins involved in the sensor biofunctionalization/assay protocol by analysis of ion fragments characteristic for these protein as well as by multivariate PCA analysis. In addition, the mass density of different proteins, i.e. OTA-OVA, BSA, primary and secondary antibody, in the multi-component molecular overlayers formed is estimated based on TOF-SIMS data supplemented by ellipsometry measurements.

The effectiveness of all steps following biofunctionalization, including both immunoreactions, was confirmed by the changes in the biomolecular composition of adlayer formed on the MZIs sensing arm areas that were revealed by the TOF-SIMS analysis. Moreover, partial desorption of proteins adsorbed in preceding steps was observed during the primary immunoreaction (4). The investigation of this phenomenon revealed that the

desorption degree increases with the concentration of the OTA-OVA solution applied to probe immobilization (2), which in turn corresponds to higher protein surface density prior to immunoreaction. The amount of desorbed OTA-OVA conjugate and BSA molecules, estimated based on component mass loadings, indicates that desorption affects much more blocking protein than the probe. The dependence of the formed adlayers composition on the OTA-OVA concentration in the spotting solution is reflected in the amount of antibodies bonded onto the surface upon the two immunoreactions. Since the final mass loadings of both probe and secondary antibody saturates for concentration equal to or higher than $C = 100 \mu\text{g/mL}$, this concentration range of OTA-OVA solution is proposed as optimal for probe immobilization.

In addition, the molar binding ratios of primary antibody to OTA-OVA conjugate (~ 1.3) and of secondary antibody to the primary one ($\langle a\text{-mIgG} \rangle / \langle a\text{-OTA Mab} \rangle \sim 2.5$) were evaluated from the component mass loadings corresponding to optimal OTA-OVA concentration for chip functionalization, i.e., $C = 200 \mu\text{g/mL}$. It was shown that the determination of antibodies binding stoichiometry in the immune complexes formed onto the chip surface following the proposed approach is more accurate than estimation based on sensor response ($\langle a\text{-mIgG} \rangle / \langle a\text{-OTA Mab} \rangle \sim 4.4$), since the last does not take into account the desorption phenomenon of molecules already immobilized onto the surfaces that accompanies the primary immunoreaction. This finding indicates the great importance of the step-by-step analysis with biomolecular discrimination of the adlayers formed onto biosensors during both functionalization and assay procedure to improve their performance.

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Appendix A. Supplementary Material

Additional data on: i) TOF-SIMS chemical imaging of OTA-OVA immobilization on sensing arms of Mach-Zehnder interferometers integrated on silicon chips, ii) amino acid composition of OVA, BSA and antibody proteins, iii) PCA analysis of TOF-SIMS spectra, iv) total protein surface density on planar samples.

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Fig. 1. (a) Image of the chip showing the arrangement of the sensing arm windows of the 10 MZIs and the areas spotted with OTA-OVA solutions of different concentration. The waveguides are indicated by the dashed yellow lines that could be considered as a guide to the eye. (b) A magnifying image of the chip showing the transition from fully etched to rib waveguide (outlined by the dashed yellow line) and the respective sensing arm window etched at the SiO₂ cladding layer. (c) Output phase shifts obtained from a chip where 3 out of ten MZIs (No 3, 6 and 9) were functionalized with OTA-OVA using a C=50 µg/mL solution (red curves), other 3 (No 2, 5 and 8) using the C=100 µg/mL solution (blue curves), and the rest 4 (No 1, 4, 7 and 10) using a C=200 µg/mL solution (black curves). The sequence of solutions run over the chip are: start to arrow 1: assay buffer; arrow 1 to 2: anti-OTA Mab

solution; and arrow 2 to end: anti-mIgG. The molar ratio of secondary to primary antibody, determined based on averaged responses, $\langle a\text{-mIgG} \rangle / \langle a\text{-OTA Mab} \rangle$ equal to ~ 4.3 , ~ 3.8 and ~ 4.4 for $C = 50$, 100 and $200 \mu\text{g/mL}$ solutions, respectively.

Fig. 2. (a) Schematic of on-chip integrated MZI sensor showing the window over the Si₃N₄ waveguide defining the sensing arm area that is examined after each step of the procedure for biofunctionalization and indirect (competitive) immunoassay to detect the mycotoxin OTA. (b) Sensing Si₃N₄ areas were modified with APTES (1), spotted with OTA-OVA conjugate (2), blocked with BSA (3), and exposed to anti-OTA Mab (zero calibrator) (4), and then to goat antibody against mouse IgG (anti-mIgG) (5). Reference samples were spotted with anti-OTA Mab (r4). OTA-OVA solutions with different concentration $C = 50$, 100 and $200 \mu\text{g/mL}$ were applied during the step (2) to prepare chips with sensing areas covered with different amounts of OTA-OVA.

Fig. 3. Analysis of multi-component molecular coverage of the Si_3N_4 MZIs sensing areas after each successive surface modification/assay step (2-5) based on characteristic TOF-SIMS signals. Intensities of TOF-SIMS signals, determined at identical conditions, are plotted for 3 series of surfaces spotted with OTA-OVA solutions of different concentration. Results from reference surfaces including amino-silanized areas (1) spotted directly with anti-OTA Mab (r4) are also included. The TOF-SIMS signals correspond to: (a) substrate (SiOH^+), (b, c) the two albumins, OVA and BSA ($\text{C}_4\text{H}_8\text{NO}_2^+$ from glutamic acid and $\text{C}_2\text{H}_6\text{N}^+$ from alanine), (d) proteins ($\text{C}_4\text{H}_8\text{N}^+$ ions from arginine, proline and valine), (e) OVA (unique signal $\text{C}_2\text{H}_5\text{S}^+$ from methionine), (f) BSA ($\text{C}_5\text{H}_{10}\text{N}^+$ ions from lysine), and (g, h, i) antibody ($\text{C}_9\text{H}_8\text{N}^+$, $\text{C}_{11}\text{H}_8\text{NO}^+$ and $\text{C}_{10}\text{H}_{11}\text{N}_2^+$ ions from tryptophan).

Fig. 4. PCA analysis of TOF-SIMS spectra recorded for the sensing arm window areas of integrated BB-MZI after each successive surface modification step (2-5) for three series of samples spotted with OTA-OVA solution of concentration C equal to 50, 100 and 200 $\mu\text{g/mL}$. Scores on PC1 (a) and PC3 (b), for corresponding loadings see Fig. S2 in Supporting Information. Please note, that the primary (4) and secondary (5) immunoreactions are resolved by PC1. In addition, the PC3 differentiates the surfaces spotted with a 50 $\mu\text{g/mL}$ OTA-OVA solution from those spotted with OTA-OVA solutions with concentrations 100 and 200 $\mu\text{g/mL}$.

Fig. 5. Evaluated component mass loadings and molar mass loadings of different molecules: OTA-OVA probe (a), BSA blocking protein (b), primary anti-OTA Mab (c), secondary anti-mIgG antibody (c), plotted for the 3 sample series spotted with OTA-OVA probe solutions of different concentration C. Dashed lines are a guide to eye. Please note that the primary immunoreaction (step 4) is accompanied by drastic desorption of the blocking protein (b). A

similar but smaller in magnitude phenomenon that depends on probe solution concentration C is observed for the OTA-OVA probe (a). As a result component mass loading of the probe saturates for concentration equal to or higher than $C = 100 \mu\text{g/mL}$. This leads to similar dependence on probe concentration, C , of the secondary antibody mass loadings (c). Cf. PCA differentiation of the 50 $\mu\text{g/mL}$ series from both the 100 and 200 $\mu\text{g/mL}$ samples series (Fig. 3).

Fig. 6. Molar binding ratio of primary antibody to probe, $\langle \text{a-OTA Mab} \rangle / \langle \text{OTA-OVA} \rangle$, based on the respective averaged values of molar coverage of anti-OTA Mab and OTA-OVA (up-pointing triangle), secondary to primary antibody $\langle \text{a-mIgG} \rangle / \langle \text{a-OTA Mab} \rangle$ (down-pointing triangle), and effective molar ratio of secondary antibody to probe $\langle \text{a-mIgG} \rangle / \langle \text{OTA-OVA} \rangle$ (diamonds), evaluated from Fig. 5 and plotted versus the probe concentration C used for spotting the samples. Cf. The molar ratio of secondary to primary antibody has been determined directly from the sensors response, $\langle \text{a-mIgG} \rangle / \langle \text{a-OTA Mab} \rangle$ (Fig. 1).

Supplementary Material

Protein adsorption/desorption and antibody binding stoichiometry on silicon interferometric biosensors examined with TOF-SIMS

Katarzyna Gajos^a, Andrzej Budkowski^a, Panagiota Petrou^b, Varvara Pagkali^b, Kamil Awsiuk^a,
 Jakub Rysz^a, Andrzej Bernasik^{c,d}, Konstantinos Misiakos^e, Ioannis Raptis^{e+}, Sotirios
 Kakabakos^b

^aM. Smoluchowski Institute of Physics, Jagiellonian University, Łojasiewicza 11,
 30-348 Kraków, Poland

^bInstitute of Nuclear & Radiological Sciences & Technology, Energy & Safety NCSR
 Demokritos, P. Grigoriou & Neapoleos St, Aghia Paraksevi 15310, Athens, Greece

^cFaculty of Physics and Applied Computer Science, AGH University of Science and
 Technology, Mickiewicza 30, 30-059 Kraków, Poland

^dAcademic Centre for Materials and Nanotechnology, AGH University of Science and
 Technology, Mickiewicza 30, 30-059 Kraków, Poland

^eInstitute of Nanoscience and Nanotechnology, NCSR Demokritos, P. Grigoriou & Neapoleos
 St, Aghia Paraskevi 15310, Athens, Greece

⁺Current address: King Abdullah University of Science and Technology (KAUST), Core
 Labs, Thuwal, 23955-6900, Saudi Arabia

* Corresponding author
E-mail address: katarzyna.gajos@doctoral.uj.edu.pl

Fig. S1. TOF-SIMS chemical images (using $C_4H_8N^+$ ions) of chip areas including part of the integrated MZI sensing arm windows after immobilization of OTA-OVA conjugate by spotting solutions with concentration C of (a) 50, (b) 100, or (c) 200 $\mu\text{g/mL}$. The sensing arm window area subjected to micro-analysis by TOF-SIMS (see **Figs. 3, 4**) is marked by dashed lines.

Table S1. Comparison of per cent amino acid composition (%) of OVA [S1], BSA [S1] and model antibody [S2].

amino acids	OVA (%)	BSA (%)	antibody (%)
alanine / ala	9.1	8.0	5.1
arginine / arg	3.9	4.0	2.8
asparagine / asn	3.9	2.4	4.6
aspartic acid / asp	4.4	6.9	4.8
cysteine / cys	0.3	6.0	2.0
glutamine / gln	3.9	3.4	4.3
glutamic acid / glu	8.8	10.1	5.1
glycine / gly	4.9	2.7	5.4
histidine / his	1.8	2.9	1.9
isoleucine / ile	6.5	2.4	3.4
leucine / leu	8.3	10.5	5.9
lysine / lys	5.2	10.1	6.2
methionine / met	4.4	0.7	2.0
phenylalanine / phe	5.2	4.6	3.9
proline / pro	3.6	4.8	6.3
serine / ser	9.8	4.8	12.5
threonine / thr	3.9	5.7	8.8
tryptophan / trp	0.8	0.3	2.0
tyrosine / tyr	2.6	3.4	4.6
valine / val	8.0	6.2	8.3

Fig. S2. PCA analysis of TOF-SIMS spectra recorded for the sensing arm window areas of integrated BB-MZI after each successive surface modification step (2-5) for three series of samples spotted with OTA-OVA solution of concentration C equal to 50, 100 and 200 $\mu\text{g/mL}$. Loadings on PC1 (a) and PC3 (b).

Table S2. Auxilairy data on total protein mass loading Γ obtained through ellipsometry measurements on planar Si_3N_4 surfaces biofunctionalized with an OTA-OVA solution of $C = 200 \mu\text{g/mL}$ and analyzed after each biofunctionalization/ assay step.

Functionalization step	Γ [mg/m^2]
2) OTA-OVA	0.67 ± 0.05
3) +blocking	1.97 ± 0.05
4) +anti-OTA Mab	2.35 ± 0.06
5) +anti-mIgG	6.9 ± 0.1
r4) anti-OTA Mab	1.36 ± 0.05

References

- [S1] Universal Protein Resource, (n.d.). <http://www.uniprot.org> (accessed April 22, 2015).
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Highlights

- Examining microstructured sensing areas of interferometric biosensors on silicon chip
- Step-by-step surface modifications determined with biomolecular discrimination
- Surface density of probe, blocking protein, primary and secondary antibody evaluated
- Desorption upon primary immunoreaction depends on spotting concentration of probe
- Binding stoichiometry evaluated more accurately than estimated from sensor response