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Indirect immunoassay on functionalized silicon surface: Molecular arrangement, composition and orientation examined step-by-step with multi-technique and multivariate analysis

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ABSTRACT

The arrangement, composition and orientation of immunoreagents employed in an indirect immunoassay for determination of mycotoxin OchraToxin A (OTA) are specified for Si_3N_4 substrate, aiming to imitate biosensor transducers made of the same material. Si_3N_4 surfaces are examined after modification with (3-aminopropyl)triethoxysilane, spotting with OTA-ovalbumin conjugate (probe), blocking with bovine serum albumin, reaction with a mouse monoclonal antibody against OTA and, finally, reaction with a goat anti-mouse secondary antibody. Atomic force micrographs, their autocorrelation and height histogram parameters, show the stepwise development of a multi-component monolayer covered by groups of secondary antibody molecules. Time-Of-Flight Secondary Ion Mass Spectrometry reveals the composition of probe and blocking protein, as well as their partial desorption during the primary immunoreaction. Ellipsometry provides surface amount of all proteins, increasing step-by-step from 0.7 to 6.9 mg/m^2. In addition, ellipsometry combined with TOF-SIMS reveals the mass loadings of different molecules in the intermediate and the final overlayer. Based on this, some orientations of the immobilized molecules are proposed and a molar ratio of ~2.5 for secondary to primary antibody is calculated. The orientations of the primary and secondary antibody are further clarified by Principal Component Analysis of TOF-SIMS data, through which a side-on and a head-on orientation is deduced for the primary and the secondary antibody, respectively. These findings demonstrate how the combination of multiple surface analysis techniques can provide insight on the arrangement, composition and orientation of biomolecules in the course of multi-step procedures employed in biosensors.

1. Introduction

Since their discovery over 50 year ago, immunochemical methods have become one of the most powerful analytical tools [1], further exploited by the massive development of immunosensors which have found application in areas ranging from biomedical diagnostics to food safety and drug screening [2].

For silicon-based immunosensors, the surface should be appropriately functionalized prior to the immobilization of the specific immunoreagents, whereas a blocking step should be performed prior to immunoreaction to eliminate non-specific binding of reagents during the immunoreaction. The immunosensor analytical performance depends on the arrangement/orientation of immunoreagents on its surface as well as the amount of immobilized molecules. Commonly, *molecular arrangement* is concluded from spectroscopic [3–6], quartz crystal microbalance [7] or Atomic Force Microscopy data [4,6,8,9]. Nevertheless, all these approaches seem not sufficient to provide insight of what is happening onto the surface, when different molecules are involved which are

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not only introduced but also partly removed from the surface during the successive modification/immunoreaction steps [10]. Therefore, surface examination with a method that could provide *discrimination between immobilized onto surface molecules*, such as Time-of-Flight Secondary Ion Mass Spectrometry, is expected to provide valuable information and help to improve immunosensors performance. In addition, such a surface examination after each functionalization and assay steps is rarely provided by the usually applied surface characterization methods [11].

Here, such a *step-by-step surface characterization* is applied in order to define *molecular arrangement* and *composition* during the course of an *indirect immunoassay* for the detection of mycotoxin, OchraToxin A (OTA). The assay is accomplished by immobilization of an OTA conjugate with ovalbumin on the surface, followed by immunoreaction with a monoclonal anti-OTA antibody (primary antibody) and a secondary antibody as a mean to increase the specific signal. It should be noted that although several investigations have been performed to define the orientation of immobilized antibodies (e.g. [5–7,9,12–14]), this is the first time the research is extended to cover *molecular orientation of* both *the primary and secondary antibody*, observed directly on functionalized surface in the course of an indirect immunoassay, and compared with the situation on reference surfaces spotted with these antibodies. Thus, in the present study, direct determination of antibody orientation is provided by Principal Component Analysis of TOF-SIMS data [4,12], performed for multi-component protein overlayers.

2. Experimental section

2.1. Materials

Silicon wafers were purchased from Si-Mat (Kaufering, Germany). 3-aminopropyltriethoxysilane (APTES), bovine serum albumin (BSA), and ovalbumin (OVA) were purchased form Sigma-Aldrich (Darmstadt, Germany). The synthesis of OTA-OVA conjugate was performed as described in a previous publication [15]. The mouse monoclonal anti-OTA antibody (anti-OTA Mab; isotype IgG1, κ) was purchased from Soft Flow Hungary Ltd. (Hungary). Affinity purified polyclonal goat anti-mouse IgG antibody (secondary antibody) was from Merck Millipore (Darmstadt, Germany). The water used throughout the study was double distilled.

2.2. Surface functionalization and assay protocol

The biofunctionalization and immunoassay steps are shown schematically in Fig. 1. Firstly, Si_3N_4 surfaces **(1)**, cleaned and hydrophilized with oxygen plasma, were immersed in a 0.5% (v/v) aqueous APTES solution for 2 min, followed by gentle washing with distilled water, drying under N_2 stream and curing for 20 min at 120 °C. Then, a 200 μg/mL OTA-OVA solution in 50 mM carbonate buffer, pH 9.2 was spotted **(2)** (over 7 mm × 7 mm areas) on the surfaces using the BioOdyssey Calligrapher Mini Arrayer (Bio-Rad Laboratories, Inc.) [10]. The spotted samples were incubated in a humidity chamber for 18 h and washed with a 10 mM Tris-HCl buffer, pH 8.25, and distilled water. Blocking **(3)** was performed by immersing the samples in a 10 mg/mL BSA solution in 0.1 M $NaHCO_3$, pH 8.5, for 2 h followed by washing and drying as previously. Reaction with the primary antibody **(4)** was performed through incubation of the samples for 1 h with a 4 μg/mL solution of anti-OTA Mab in 50 nM Tris-HCl buffer, pH 7.8, containing 9 g/L NaCl and 5 g/L BSA (assay buffer). After that, the samples were washed and dried as previously, and then incubated with a 10 μg/mL secondary antibody solution in assay buffer **(5)** for 1 h. Additionally, reference samples were prepared by spotting directly solutions of 4 μg/mL of anti-OTA Mab **(r4)** or 10 μg/mL of secondary antibody in 50 mM carbonate buffer, pH 9.2 **(r5)**. The reference samples were washed with buffer and distilled water and dried under N_2 stream.

2.3. AFM surface examinations

Agilent 5500 microscope working in a non-contact mode (resonant frequency ~70 kHz, tip radius ~7 nm, spring constant ~2 N/m) under ambient conditions was used to acquire AFM images from different areas of each sample. The WSxM software was used (downloadable at http://www.nanotec.es) for processing of the images.

2.4. TOF-SIMS surface characterization

A TOF.SIMS 5 (ION-TOF GmbH) instrument equipped with Bi_3^+ ion clusters (30 keV liquid metal ion gun) was used. Ion dose density was lower than 10^{12} ion/cm^2 (static mode). A low energy electron flood gun was used for charge compensation. High mass resolution positive ion static TOF-SIMS spectra were acquired from six non-overlapping 100 μm × 100 μm areas of each sample. 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. To investigate differences in molecular coverage rather than changes in relative composition, TOF-SIMS data that have been obtained with dose density and primary beam current identical for all measurements are used without normalization. Surface sensitivity of TOF-SIMS experiments using Bi_3^+ primary ions was described [16] by exponential decay of signal intensity as a function of organic overlayer thickness, and characterized by escape depth of secondary ions of ~4 nm [16]. This value corresponds to uniform protein layer [11] with surface density of ~5.5 mg/m^2.

2.5. Multivariate TOF-SIMS analysis with PCA

Principal Component Analysis was performed for the positive TOF-SIMS spectra using the PLS Toolbox (Eigenvector Research, Manson, WA) for MATLAB (MathWorks, Inc., Natick, MA). The intensities of selected peaks from each spectrum were normalized to the sum of selected peaks and mean-centred prior to running PCA.

2.6. Ellipsometry measurements

The Sentech SE800 (Sentech Instruments GmBH) Spectroscopic Ellipsometer (wavelength range of 320–700 nm, fixed angle of incidence ~70°) along with the SpectraRay 3 software was used. Average thickness was estimated by fitting model predictions to Ψ and Δ relations, assuming the Cauchy dispersion formula. The three-layer model consisted of silicon substrate/Si_3N_4/mixed SiO_2 and APTES layer/protein overlayer. Fixed refractive index values n = 3.87 for Si, 2.01 for Si_3N_4 [17], 1.46 for both SiO_2 and APTES [18], and 1.53 for proteins [19] were used. The thickness of Si_3N_4 layer (122.5 nm), as well that of mixed SiO_2 and APTES layer (6.3 nm), both obtained from measurements performed on a bare silanized surface, were taken into account to fit the thickness of protein layer.

3. Results and discussion

3.1. Multi-step procedure for silicon surface modification leading to indirect assay

The reagents and assay procedure employed in the present study have been previously applied for the determination of OTA in beer samples employing an array of integrated on silicon Mach-Zehnder Interferometric sensors based on Si_3N_4 waveguides [10,15]. Thus,

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