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Full Length Article

Contact pin-printing of albumin-fungicide conjugate for silicon nitride-based sensors biofunctionalization: Multi-technique surface analysis for optimum immunoassay performance

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ABSTRACT

Mass fabrication of integrated biosensors on silicon chips is facilitated by contact pin-printing, applied for biofunctionalization of individual Si_3N_4-based transducers at wafer-scale. To optimize the biofunctionalization for immunochemical (competitive) detection of fungicide thiabendazole (TBZ), Si_3N_4 surfaces are modified with (3-aminopropyl)triethoxysilane and examined after: immobilization of BSA-TBZ conjugate (probe) from solutions with different concentration, blocking with bovine serum albumin (BSA), and immunoreaction with a mouse monoclonal antibody against TBZ. Nanostructure, surface density, probe composition and coverage uniformity of protein layers are evaluated with Atomic Force Microscopy, Spectroscopic Ellipsometry, Time-of-Flight Secondary Ion Mass Spectrometry and X-ray Photoelectron Spectroscopy. Contact pin-printing of overlapping probe spots is compared with hand spotted areas. Contact pin-printing resulted in two-fold increase of immobilized probe surface density as compared to hand spotting. Regarding BSA-TBZ immobilization, an incomplete monolayer develops into a bilayer as the concentration of BSA-TBZ molecules in the printing solution increases from 25 to 100 μg/mL. Upon blocking, however, a complete protein monolayer is formed for all the BSA-TBZ concentrations used. Free surface sites are filled with BSA for low surface coverage with BSA-TBZ, whereas loosely bound BSA-TBZ molecules are removed from the BSA-TBZ bilayer. As a consequence immunoreaction efficiency increases with the printing probe concentration.

1. Introduction

Surface functionalization with biomolecules is a crucial issue for the development of biosensors aiming to applications in medical diagnostics, monitoring of food safety or drug screening. The miniaturization and realization of arrays of multiple biosensors in a confined surface area often require the precise spatially resolved immobilization of probe molecules. For this purpose, besides the microfluidic-based approaches [1], surface patterning methods such as photolithography, soft lithography and printing are commonly used [2,3]. In particular, mass fabrication of integrated biosensors on silicon chips is facilitated by contact pin-printing technique, initially developed and established for the fabrication of DNA and protein microarrays [4,5]. Due to its simplicity and facile maintenance combined with high spots reproducibility contact pin-printing is nowadays the most prevalent microarrays fabrication technology [4], easily adapted for formation of arbitrary biomolecules patterns by spatially controlled solution deposition. This method has been already applied for biofunctionalization of individual transducers on silicon chips integrating arrays of interferometric biosensors [6–9].

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The strategies for probe molecule immobilization on biosensor surfaces [10], including solution deposition by contact pin-printing, involve physical adsorption, covalent binding or affinity based interactions. All these probe immobilization strategies usually require the initial modification of substrate surface. Surface modification can be realized by means of self-assembled monolayers [6–8,11] or polymer brushes [12–15]. For biosensors with silicon-based transducer, protein immobilization by physical adsorption after surface modification with organosilanes is frequently applied, since it provides a simple but efficient approach [7,8,16–19]. However, due to the fact that the probes are not covalently attached onto the surface, there is a possibility of molecule desorption during the steps that follow their immobilization, i.e., upon blocking of free surface sites with a non-functional protein [20,21] and during specific binding reactions [6,22]. Therefore, surface examination and control of protein layer composition after each successive step of the protocol is highly desirable. The amount of biomolecules on surface could be quantified applying various spectroscopic techniques [16,23–27] often supported by Atomic Force Microscopy [23–25]. However, these methods do not discriminate between different proteins in the layer covering the sensor surface, an information that can be provided by Time-of-Flight Secondary Ion Mass Spectrometry [28]. In fact, only the step-by-step surface characterization with complementary surface science techniques allows to define the structure and composition of biomolecular layer resulting from immunoassays performed on silicon surfaces [6,22].

In this work we examine the impact of *probe surface density*, obtained during the surface biofunctionalization step by *contact pin-printing*, on protein layer composition created after each assay step and how this affects the assay performance. In particular, an immunoassay for detection of the fungicide thiabendazole (TBZ) based on surface functionalization by immobilization of a TBZ-conjugate with bovine serum albumin (BSA-TBZ) and subsequent blocking with BSA, followed by immunoreaction with a monoclonal anti-TBZ antibody is studied. The contact pin-printing is compared with solution deposition by hand using a precision pipette i.e., by macro-spotting. Complementary information about nanostructure, surface density and probe composition are provided by Atomic Force Microscopy, Spectroscopic Ellipsometry (as well as X-ray Photoelectron Spectroscopy) and Time-of-Flight Secondary Ion Mass Spectrometry, respectively. As a result the optimum conditions for the immunoassay are determined and related with surface phenomena taking place upon biofunctionalization.

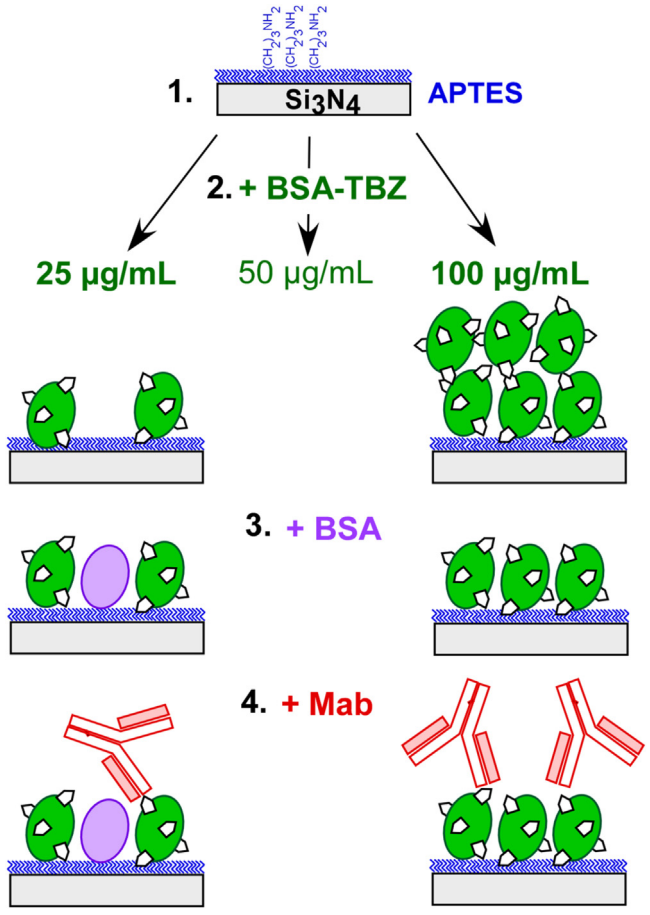

Fig. 1. Schematic of the procedure employed for biofunctionalization of Si_3N_4 surfaces and subsequent immunoassay for detection of fungicide TBZ. Si_3N_4 surfaces were modified with APTES (**1**), spotted with BSA-TBZ conjugate (probe) using solutions with different concentrations (25, 50 and 100 µg/mL) (**2**), blocked with BSA (**3**), and exposed to a solution of monoclonal antibody against TBZ (Mab) (**4**).

2. Experimental

2.1. Materials

Silicon wafers were purchased from Si-Mat (Kaufering, Germany). 3-Aminopropyltriethoxysilane (APTES) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Darmstadt, Germany). The mouse monoclonal antibody against thiabendazole (Mab clone LIB-TN3C13), and the thiabendazole conjugate with bovine serum albumin (BSA-TN3C) were purchased from the Grupo de Inmunotecnologia of Universidad Politécnica de Valencia (Valencia, Spain) [29]. The water used throughout the study was double distilled.

2.2. Surface functionalization and assay protocol

The biofunctionalization and immunoassay steps are shown in Fig. 1. As a preliminary step a 1-µm thick SiO_2 layer and an additional 150 nm thick Si_3N_4 layer were created by low pressure chemical vapour deposition on silicon substrates to mimic the composition and surface properties of the waveguides of integrated on silicon Mach-Zehnder interferometers [6,30]. Cleaned and hydrophilized by oxygen plasma Si_3N_4 surfaces **(1)** were firstly silanized through immersion in a 0.5% (v/v) aqueous APTES solution for 2 min, followed by washing with distilled water, drying under stream of nitrogen and baking for 20 min at 120 °C. Then, two different approaches of surface functionalization with BSA-TBZ **(2)** were applied involving either contact pin-printing or hand spotting of BSA-TBZ solution. Contact pin-printing involved deposition of multiple overlapping spots over an area of 7 mm × 7 mm employing a standard microarray spotter (BioOdyssey Calligrapher Miniarrayer, Bio-Rad Inc.). For hand macro-spotting a 50 µL drop of solution was deposited on the silanized surface by pipette in order to cover approximately the same area (7 × 7 mm^2). The samples resulting from both spotting approaches were then incubated at room temperature in a humidity chamber for 18 h, prior to washing with 10 mM phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl and 0.05% (v/v) Tween 20. Solutions of BSA-TBZ with concentrations of 25, 50 and 100 µg/mL in 50 mM carbonate buffer, pH 9.2, were used. After that, blocking of free surface binding sites **(3)** was performed by immersing the samples in a 2% (w/v) BSA solution in 10 mM phosphate buffer, pH 7,4, containing 0.9% NaCl (w/v), followed by washing as previously. Spotted by contact pin-printing and blocked surfaces were incubated for 1 h with a 1 µg/mL solution of mouse monoclonal antibody against TBZ **(4)** in 10 mM phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl and 0.2% BSA (w/v) and after that washed as previously. Prior to measurements with sur-