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In situ ellipsometric study of surface immobilization of flagellar filaments

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ABSTRACT

Protein filaments composed of thousands of subunits are promising candidates as sensing elements in biosensors. In this work *in situ* spectroscopic ellipsometry is applied to monitor the surface immobilization of flagellar filaments. This study is the first step towards the development of layers of filamentous receptors for sensor applications.

Surface activation is performed using silanization and a subsequent glutaraldehyde crosslinking. Structure of the flagellar filament layers immobilized on activated and non-activated Si wafer substrates is determined using a two-layer effective medium model that accounted for the vertical density distribution of flagellar filaments with lengths of 300–1500 nm bound to the surface. The formation of the first interface layer can be explained by the multipoint covalent attachment of the filaments, while the second layer is mainly composed of tail pinned filaments floating upwards with the free parts. As confirmed by atomic force microscopy, covalent immobilization resulted in an increased surface density compared to absorption.

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1. Introduction

Biosensors have been under a continuous development in the past decade, while some commercial products are already on the market [1]. Biosensors for medical diagnostics or environmental monitoring are based on the specific recognition of an analyte by the bioreceptor (typically enzymes or antibodies) [2]. The recognition event is detected by means of a transducer that can be categorized into three main groups such as electrochemical, optical and mass sensing [3]. Although new and improved sensors will continue to be developed, the more crucial need in any biosensor would be the specific sensing layer. There is an essential need for biomolecular receptors (e.g. antibody, aptamer, enzyme, nucleic acid, artificially engineered receptor) for high specificity and reliable biosensing. Improvement in the affinity, specificity and mass production of the protein receptors may ultimately dictate the success of biosensor technologies in both a technical and commercial sense [4].

We have been working on the fabrication of multivalent filamentous recognition elements constructed from the protein flagellin [5,6]. In nature, polymerized flagellin forms the flagella of certain motile bacteria [7]. Each filament may comprise as many as 30 000 flagellin subunits and can grow up to about 15 μm. It has been discovered that the outermost domain of the flagellin subunits can be genetically engineered to create binding sites with specific affinity for selected analyte molecules [8], while leaving the part of the molecule involved in polymerization unchanged. Therefore, filaments containing thousands of individual binding sites can be fabricated [9,10]. However the deposition properties of the flagellar filaments are unknown, which would be essential for their immobilization on sensor surfaces.

Ellipsometry is a mature technique and can be used to study both adsorption isotherms and the adsorption kinetics on solid surfaces in aqueous solutions [12,13]. In most of these studies, oxidized silicon wafer surfaces have been used as they have good reflectivity and have a well-defined silica layer. In this work *in situ* ellipsometry was applied, which is a significant improvement to our previous ellipsometry work [5], where batch immobilization methods have been used and the samples were dried before ellipsometry measurements. In this new way the *in situ* structure of the filament layer can be studied in the native form that is in buffer solution. These two improvements (real time monitoring of the immobilization and the measurement in buffer solution) give more relevant data for further sensor applications.

For biosensor applications, different methods can be used to immobilize bioreceptors on the transducer surface, like protein

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entrapment, covalent binding, affinity binding [14,15]. Covalent immobilization is often preferred to physical adsorption to avoid leaching of the bioreceptor. Surface modification via silane chemistry is one of the most versatile means to modify surface properties of inorganic oxide surfaces [16]. The first results on our *in situ* ellipsometry presented here show that the instrumental sensitivity is sufficient to follow the immobilization of the large biomolecules such as flagellar filaments. Our previous study by optical waveguide lightmode spectroscopy (OWLS) showed that a dense near-surface layer has been formed during the immobilization, while the kinetic analysis of the process predicted self-assembly of the flagellar filaments [6]. As a continuation of our work we aimed at examining the structure of the filament layer deposited in larger extent from the surface than that was achievable by OWLS. We report here on the covalent immobilization of flagellar filaments on the surface of silicon wafers. Covalent binding of flagellar filaments on the silanized surface using glutaraldehyde (GA) crosslinking reagent was compared to physical adsorption.

2. Materials and methods

2.1. Surface preparation

Hydrophilic surfaces used for the experiments were cut from 0.3 mm thick silicon wafers as used for semiconductor manufacture. The polished surface of the wafers with an oxide layer of 20 nm was prepared by thermal oxidation. This oxide layer makes the ellipsometric measurement more sensitive as it has been shown elsewhere [17]. The surface of the substrate was cleaned using the well-known "Piranha" solution (1:3 mixture of 30% hydrogen peroxide and concentrated sulfuric acid), and it also accomplished silanol activation.

2.2. Protein solution

Flagellin (molecular weight 51.5 kDa) was from *Salmonella typhimurium* wild type strain SJW1103 and purified as described in [18]. The monomers were repolymerized to form flagellin filaments by adding ammonium sulfate to a 3 mg/mL monomer solution to give a final concentration of 0.8 M. The filaments were washed twice by centrifugation and suspending them in 10 mM sodium phosphate buffered saline, pH 7.45. After the last centrifugation the pellet was kept at 4 °C and used as the stock of material. Flagellar filament solutions of 0.3 mg/m1 were prepared in sodium phosphate buffered saline (PBS).

For a preliminary biosensing experiment, we used a polyclonal IgG solution obtained from rabbit serum raised against Salmonella flagellin. Antibodies were purified by affinity chromatography using a Sepharose-ProteinA column (Amersham). Small aliquots of the IgG solution were stored at −20 °C (c = 2.2 mg/mL) until use. Before the ellipsometric experiment the IgG sample was taken out of the deep-freezer, and diluted to the working concentration (c = 25 μg/mL) with PBS.

2.3. Immobilization

The sample and reagent solutions were made up using Milli-Q (MQ) water with 18.2 MΩ cm resistivity. Reagents were obtained from Sigma–Aldrich and used as received. Sodium phosphate buffered saline solutions were prepared from tablet, dissolving it in the appropriate volume of MQ water (PBS, containing 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The protein was covalently crosslinked to the Si wafer surface using glutaraldehyde (GA), following the procedures described by Cass and Ligler [15]: surfaces were aminated by immersing them for 1 h in a 2.5% solution of 3-aminopropyltriethoxysilane (APTES) in MQ water, followed by curing at 120 °C for 2 h. The silanized surfaces were then placed in a 2% solution of glutaraldehyde (GA) in PBS and allowed to stand at 20 °C for 2 h. The glutaraldehyde-activated surfaces were mostly used immediately, but if needed could be kept in PBS for up to two days before use. The multilayer structure of an activated sample surface is shown in Fig. 1.

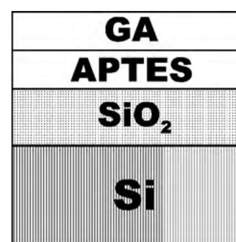

Fig. 1. Schematic representation of the multilayer structure of the activated Si wafer used for the immobilization of flagellar filaments. The immobilization procedure composed of the primer silane layer (APTES stands for 3-aminopropyltriethoxysilane) and the subsequent crosslinker layer (GA stands for glutaraldehyde).

2.4. Ellipsometry and atomic force microscopy (AFM)

Ellipsometry was used in this work to follow the adsorption and immobilization process of flagellar filaments on the surface of Si wafers. The measurements were performed using a SOPRA ES4G rotating polarizer spectroscopic ellipsometer. The immobilization experiments were carried out and monitored in a flow cell (purchased from Woollam Co. Inc.) at 25 °C during the experiments. The volume of the flow cell was approximately 5 mL, the incoming light beam entered and left the cell through quartz windows. The cell was fixed to the substrate surface using an O-ring seal. Nominal incidence angle of 75° was set on the instrument (SOPRA ES4G) arms so as the probing beam hits the windows perpendicular. Peristaltic pump (Ismatec, Type ISM834C) was used to send the solutions through the flow cell with a flow rate of 2.5 mL/min. At the outset of *in situ* experiments, we compared the best fits obtained with and without the flow cell in order to calibrate the window effect. The phase shift caused by birefringence of the window results in refractive index errors of about 0.0001 that is negligible.

Both cleaned hydrophilic Si wafer surface and glutaraldehyde (GA) activated surface were used: in this way the physical adsorption and covalent immobilization can be compared. Prior to the kinetic measurements, ellipsometric spectra were collected in air as well as in the PBS solution for the determination of the complex refractive index of the substrate and for the careful alignment. After collecting the spectra, a single wavelength (at 600 nm) measurement was carried out with the PBS–Protein–PBS sequence of solutions, while recording the values of Ψ and Δ. The time resolution in the sensogram (the plot of Δ versus time) is 16 s which was sufficient to follow the adsorption/immobilization process. Finally, the ellipsometric spectrum was measured again on the deposited protein layer at the end of the experiment (in PBS solution).

The mass of flagellar filaments deposited during the experiment was calculated using the de Feijter equation [19]. For this calculation the thickness (d) and the filament content were determined first by finding the best fit of an optical model (Wvase32 software). Our model involves different layers corresponding to the subsequent steps in sample preparation: a Si wafer is normally considered as crystalline Si and a native oxide (SiO_2) on its surface. The thickness of this surface oxide was increased by thermal oxidation in this work, and found to be 20 ± 0.5 nm by ellipsometry. The subsequent APTES and GA layers gave very similar thickness to be ∼1 nm on the top. The layer of deposited filaments was represented

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