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Ellipsometry analysis of conformational change of immobilized protein monolayer on plasma polymer surfaces

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ABSTRACT

The conformational stability of surface immobilized protein monolayers is a key issue in applications requiring preservation of the protein bioactivity such as in biosensors and *in vivo* implants. Ellipsometry was used to detect conformational changes in a single monolayer of immobilized proteins on plasma polymer surfaces. The areal mass density of immobilized proteins was used to validate the data analysis in the protein denaturation analysis. We observed that the rate of conformation change was strongly dependent on the properties of the immobilized protein. Immobilized catalase showed a significantly slower denaturation rate than the immobilized horseradish peroxidase, indicating that the tetramer catalase is more stable than the immobilized monomer horseradish peroxidase at the surface/air interfaces. The ellipsometry results were in a good agreement with the enzyme activity analysis.

1. Introduction

The conformational stability of immobilized protein monolayers is a key issue in applications relying on the native bioactivity of proteins. Although crucial for the development of optimal platforms for the surface immobilization of bioactive protein, including enzymes and antibodies, the study of protein conformation on surfaces is impeded by the low signal to noise of most available analysis methods. Techniques, such as fluorescence spectroscopy, circular dichroism spectroscopy, and nuclear magnetic resonance spectroscopy, favoured in the study of protein denaturation in solution are not readily adapted to the studies of conformation on a wide range of surfaces [1–4]. They can be used to study proteins on surfaces if the proteins are immobilized onto nanoparticles suspended in solution and a sufficiently high concentration of the particles can be suspended to overcome signal to noise limitations. The nano-sized particles need to be transparent to the wavelengths used and must not aggregate in the solution. These requirements place substantial limits on the surfaces which can be studied.

Since single layers of proteins are often immobilized on a wide range of solid surfaces for applications such as biosensors and antibody arrays, less restrictive methods of detecting conformation changes in surface immobilized proteins are required. Of the two types of protein immobilization strategies in common use, covalent immobilization and physisorption, covalent immobilization is thought to be more promising for the retention of function. This is because physisorption relies on a large number of weak surface interactions over a large area on the protein while covalent coupling requires interaction at only one point on the protein's surface in principle. Covalently immobilized proteins on the surfaces are typically in a form of a single monolayer as subsequent layers of protein beyond the covalent immobilization interface would not be covalently attached but rather physisorbed to the first layer. Covalent immobilization is usually achieved using chemical linker groups [5–7]. In recent work, we have demonstrated a linker-free approach to achieve covalent attachment of protein monolayers onto a range of materials including metals, semiconductors and polymers [8,9]. Catalase and HRP immobilized in this way showed long lived protein activity, in the order of 3–5 weeks compared with 3–5 days for the same proteins adsorbed onto metal or polymer surfaces.

Techniques available for studying conformational changes of proteins can be classified according to whether they measure physical rearrangement of the protein molecules or rather detect changes in the function of the proteins. Techniques such as FTIR, X-ray diffraction, circular dichroism, nuclear magnetic resonance and ellipsometry are capable in principle of analysing the physical changes associated with denaturation while assays which monitor bioactivity and some types of fluorescence spectroscopy detect the associated changes in function. Results from these two types of techniques are not always correlated because not all changes in conformation result in destruction of the active site and loss of activity. Recently, an attempt was made to study protein conformation at an air/water interface using X-ray diffraction [10], which when combined with FTIR analysis suggested conformation change of proteins at the interface. It is difficult or impossible to use X-ray diffraction method to

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analyse a randomly arranged protein monolayer on solid/air interfaces.

In this work, we were particularly interested in enzyme immobilization on plasma polymers. Plasma polymers have attracted more and more attention in protein immobilization due to the simple approach and large scale capability. Changes in the conformation of immobilized proteins on solid surfaces have been studied by indirect measurements such as measurements of enzyme activity and fluorescence intensity [11–14]. Experimental studies of the kinetics of protein denaturation on a wide range of surfaces are necessary to reveal the surface properties conducive to stabilizing the conformation of the bound proteins and hence maintaining their bioactivity [15]. Correlations between the measurements which detect changes in the physical structure of immobilized proteins with those that monitor changes in bioactivity will provide important information about the degree to which the changes in conformation affect particular active sites. For this reason, ellipsometry method is advantaged as it can be sensitive only to a particular thin layer.

In the previous work we have studied protein attachment to plasma polymerized [9,16] and plasma treated surfaces [17] showing that the activity on these surfaces can be retained over a period of several weeks when the immobilized protein is kept in solution at room temperature. In this study, rather than studying denaturation in solution of the protein attached surfaces we remove the surfaces from the solution and allow it to be dried. This approximates the situation where antibodies and other proteins are spotted onto microarrays and then dried. We monitor conformational changes of the surface attached protein monolayer in real time at the solid/air interface using ellipsometry. The conformation change in a similar condition is also to be analysed using a conventional enzyme activity assay. The aim of this work is to demonstrate the usefulness and the convenience of the ellipsometry technique and the correlation with enzyme activity to observe directly the process of contraction/expansion in volume associated with changes in conformation of a surface immobilized protein monolayer and to compare the conformation change processes of two different proteins.

2. Experimental details

Horseradish peroxidase (HRP) and bovine liver catalase were purchased from Sigma. The proteins were immobilized onto pulsed plasma deposited polymeric surfaces. The plasma polymer surfaces were deposited using a system described in detail in previous reports [9,16]. In summary, acetylene (10 sccm), argon (4 sccm) and nitrogen (4 sccm) were injected into the plasma chamber and the pressure was maintained at 20 Pa. The depositing plasma was generated by capacitively coupling 13.56 MHz RF pulsed power to the gas mix. Substrates used were double-side polished p-type silicon wafers (100) with roughness less than 1 nm. The substrates were held on a pulse biased electrode, which was immersed in the RF plasma with pulse bias of 500 V to yield surfaces more resistant to aging than those deposited with lower pulse bias [18]. The thickness of the deposited plasma polymers was approximately 40 nm with refractive index close to 1.7. The physical properties and the biocompatibility of the plasma polymers were reported in Refs. [9,16,18].

Proteins (either HRP or catalase) were diluted to 50 ug/ml in 10 mM sodium phosphate buffer pH 7 and the plasma polymer surfaces were incubated in this solution for about 12 h with rocking in sterile Petri dishes. After incubation, samples were transferred to a new Petri dish and incubated with Milli-Q water for 20 min then rinsed 3 times in Milli-Q water. The samples were dried by placing the surfaces onto a dry Kimwipe tissue and lightly contacting the dry tissue 3 times on the surface for 5 s each time. The covalent attachment of close to a monolayer on these surfaces was previously reported using a number of analysis methods including quartz crystal microbalance technique, ellipsometry combined with AFM, and ATR-FTIR method as reported previously [8,9,16].

In HRP activity analysis, after protein incubation, samples were transferred to a new Petri dish and incubated with fresh phosphate buffer for 20 min. The washing step in the Petri dishes was repeated four times in fresh buffer, until the wash solution had no activity detected in the final wash solution. The sample was then applied to a Kimwipe to dry for 30 s, and then placed protein side up for various amounts of time. HRP activity of the samples was measured by the method described in Ref. [8]. In catalase activity analysis, hydrogen peroxide (75 µl, 6 mM) in phosphate buffer instead of TMB was added to the stainless steel surface and incubated for 6 min. During this time the plates were added to the surface on a tissue culture plate that was clamped to an ELISA plate shaker and was shaken. After 6 min, 3 µl was removed and the remaining peroxide was assayed. The hydrogen peroxide was added to 0.25 ml of solution consisting of a mixture of 0.6 N H2SO4 and 10 mM FeSO4 and 20 µl of 2.5 M KSCN was added to develop colour. Absorbance was measured at 475 nm. Another surface drying method was to use mild nitrogen flow for comparison in the activity analysis.

Spectroscopic ellipsometry (J.A. Woollam M-2000) was used to study the plasma deposited layer and the surface immobilized protein layer. Ellipsometric data was collected after the plasma polymer was deposited onto the silicon substrate and then again after protein immobilization and drying. The first measurement was made immediately after drying and subsequent measurements were taken at regular time intervals without moving the sample position until all data were collected. The ellipsometry analysis was done at 70° incident angle. The simulation was carried out using a so called uniqueness range method recommended by Hilfiker et al. [19] in the wavelength range between 540 and 600 nm with the mean squared error (MSE) less than 0.1. The step of the wavelength was 1.5 nm.

The fitting of the ellipsometric data with a two-layer system model was used to determine the time dependent effective thickness and refractive index of the immobilized protein layer. The optical constants of the plasma polymer were determined prior to the protein immobilization.

3. Results

Fig. 1 shows the refractive index and thickness of the immobilized monolayer of HRP as a function of time after drying. The refractive index was lower compared with some reported results. This is not

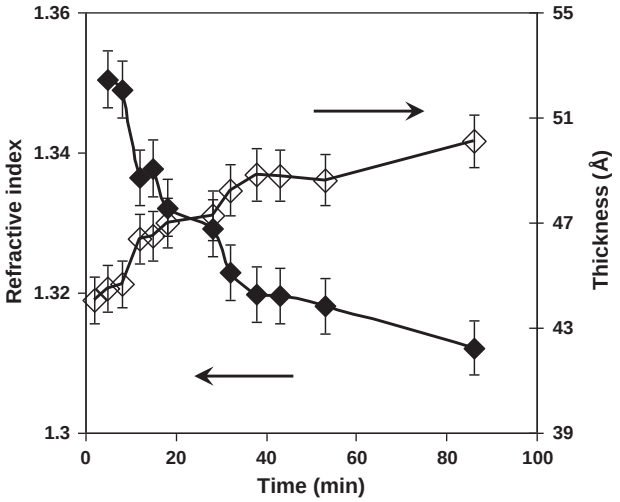

Fig. 1. The time dependences of the fitted refractive index and thickness of a surface immobilized HRP protein layer on a plasma polymer surface obtained from ellipsometry.