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A comprehensive study of lysozyme adsorption using dual polarization interferometry and quartz crystal microbalance with dissipation

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ABSTRACT

Protein adsorption plays a crucial role in biomaterial surface science as it is directly linked to the biocompatibility of artificial biomaterial devices. Here, elucidation of protein adsorption mechanism is effected using dual polarization interferometry and a quartz crystal microbalance to characterize lysozyme layer properties on a silica surface at different coverage values. Lysozyme is observed to adsorb from sparse monolayer to multilayer coverage. At low coverage an irreversibly adsorbed layer is formed with slight deformation consistent with side-on orientation. At higher coverage values dynamic reorientation effects are observed which lead to monolayer surface coverages of 2–3 ng/mm^2 corresponding to edge-on or/and end-on orientations. These monolayer thickness values ranged between 3 and 4.5 nm with a protein density value of 0.60 g/mL and with 50 wt% solvent mass. Further increase of coverage results formation of a multilayer structure. Using the hydration content and other physical layer properties a tentative model lysozyme adsorption is proposed.

1. Introduction

Protein adsorption at the liquid/solid interface has been the focus of research interest in the field of biomaterials surface science for decades [1,2]. One of the reasons is that it is considered as the first step in acute biological response to artificial materials and closely linked to biocompatibility [3]. Previous work has investigated how protein is adsorbed onto various surfaces under different conditions [4–7], the underlying mechanism [8–10] and possible conformational changes of proteins during adsorption [11–13]. Despite years of effort in this field, there are still unresolved questions in protein adsorption, such as the understanding of quantitative structure–property relationships between protein and substrate surface chemistry. Only few studies examined the role of coupled solvent within the adsorbed layer despite the fact that hydration is closely linked to the response of protein at surfaces [14]. Moreover, there have been only a few models systematically and quantitatively describing protein adsorption [3]. All of those issues need more attention as they connect directly to the biocompatibility of artificial biomaterial application design, which can only be resolved through more experimental and theoretical work.

A number of techniques have been employed to study protein adsorption at surfaces, examples including quartz crystal microbalance with dissipation (QCM-D) [15,16], atomic force microscopy [17,18], ellipsometry [19,20], grazing angle infrared spectroscopy [21,22], and dual polarization interferometry (DPI) [23,24]. QCM-D is a well-established bio-sensing technique for studying protein adsorption measuring the adsorbed protein mass as well as the coupled solvent within the layer [25]. QCM-D is in effect a high-resolution weighing device that senses mass deposition of less than 1 ng/cm^2 [12]. The induced change of frequency due to mass adsorption on the quartz crystal is measured together with the dissipation energy in real time, which are then used to convert to other parameter output such as the hydrated adsorbed layer mass, thickness, density and viscoelastic properties of the layer. The hydrated mass is probed because the adsorbed protein layer also couples water trapped in and around it to the oscillating sensor surface. DPI, on the other hand, is a more recent optical technique utilizing a waveguide interferometer. The technique measures the changes in effective refractive index of a sensing waveguide, in two different polarizations of light, caused by the adsorption of molecules to the waveguide surface [26]. The detected changes of signals can be expressed as the average thickness and refractive index (RI) of an equivalent uniform adsorbed layer, from which the layer density and adsorbed mass can be resolved. One of the advantages of DPI over other optical 'dry mass' sensitive techniques such as ellipsometry is that it gives two independent measurements, i.e. the RI and thickness, which enables a more detailed characterization of the adsorbed layer [27].

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The work described here is aimed at providing a better understanding of protein adsorption using a well-known protein, human milk lysozyme. The study is carried out using both DPI and QCM-D techniques in parallel to investigate protein adsorption of different solution concentrations on a silica surface. As mentioned above, each of the two techniques have been utilized to study protein adsorption in the past, nevertheless, limited work has focused on combining the data from the two techniques [28]. The study presented here focuses on the information obtained from each instrument and important knowledge such as the percentage layer solvation and information on mass distribution within the layer has been extracted by combining DPI with QCM-D data. A detailed structure–property relationship between surface coverage and lysozyme adsorption has been constructed. In addition, a systematic model has been proposed for lysozyme adsorption to the hydrophilic silica surface.

2. Materials and methods

2.1. Materials

Human milk lysozyme (14.4 kDa), HPLC grade water (resistivity > 18 MΩ cm), monobasic and dibasic phosphate, and sodium deodecyl sulphate (SDS) were purchased from Sigma (Sigma & Aldrich, UK) and used as received. Millex syringe filter (pore size = 0.22 μm) was obtained from Fisher (Fisher Scientific, UK). Hellmanex® III (Hellma Analytics, Germany) is an alkaline liquid concentrate and the diluted solution (2%, in deionized water) was used to clean the DPI injection loops.

Buffer used was 10 mM phosphate and was prepared using monobasic and dibasic phosphate and HPLC grade water to the desired concentration. The pH was adjusted to 7.4. Lysozyme solution of 5 mg/mL stock solution was prepared in the same buffer solution and then filtered to remove any un-dissolved particles. The filtered stock solution was then diluted to the subsequent concentrations as needed. The concentrations of diluted protein solutions were checked by a UV–Vis spectrometer (Varian Cary® 300 UV–Vis Spectrometer, Agilent Technologies, UK) at 280 nm. Silicon oxynitride (Farfield-Biolin Scientific AB, Sweden) and silicon dioxide (Q-Sense-Biolin Scientific AB, Sweden) sensor chips were used for the DPI and QCM-D experiments respectively. The cleaning procedure was performed in the following way: 2% SDS (in deionized water) immersion for 30 min, followed by excessive rinsing with deionized water, ethanol, deionized water, and HPLC grade water. The chips were then blown dry with nitrogen gas and UV/ozone cleaned for 15 min to remove any impurities. Contact angle measurements were performed to check the polarity of the chips. The values were determined with a goniometer (CAM200, KSV NIMA-Biolin Scientific, Finland). The sessile drop mode was adopted: drops of 5 μL of water were applied on the DPI and QCM-D chips after cleaning, and the angle between the air/liquid and liquid/solid interfaces was measured as the contact angle. The measurements were repeated three times on each type of chip, and three batches of chips of each type were used. A contact angle of 8.77° ± 0.82 was obtained for the DPI chips and a value of 8.55° ± 0.75 was obtained for the QCM-D chips, showing a good similarity of polarity between the two surfaces. The surfaces are considered as 'super-hydrophilic' since the contact angle is less than 10° [29] and such values are in agreement with previous studies investigating the effect of different cleaning treatments on the polarity of the silica surface [30].

2.2. DPI

An *Ana*light® dual polarization interferometer (*Ana*light® 4D, Farfield-Biolin Scientific AB, Sweden) was used to optically characterize lysozyme adsorption on a sensor chip with a silicon oxynitride surface (FB80, Farfield Scientific, UK). The sensor chip comprises dual slab waveguides, one above the other, with the upper being exposed to the analyte solution. Polarized light travels down the two monomode waveguides and combines to give interference fringes at the output. The evanescent field at the interface of the exposed upper waveguide responds to changes in the refractive index above it and causes a change in the propagating phase of the light which is observed as a change in the output interference fringes and recorded as a real-time phase change signal. The instrument illuminates the chip with two orthogonal polarizations of light that excite transverse electric (TE) and transverse magnetic (TM) waveguide modes, which generates two different phase change signals. These are analysed to provide two independent parameters which are the RI and thickness of the adsorbed layer.

Each chip has two fluidic channels, channel 1 and channel 3 where the sensing waveguide is allowed to contact with liquid samples. In addition, there is a glass clad channel 2 that acts as an instrument reference channel. The exposed waveguide surface area within each channel was 15 mm^2 (1 × 15 mm). The fluidic channel volume was defined by a kalrez gasket which was 0.5 mm thick and gives a total channel volume of approx. 10 μL. The thicker than standard gasket was used to more closely match the fluidic volume with those of the QCM-D.

The instrument was set to operate at 20.0 °C ± 0.1. A fluidic system comprising an external syringe pump was used to provide a continuous controlled flow at a rate of 25 μL/min unless otherwise stated. The buffer was degassed prior to the experiment and sample injections (apart from the protein solution) were also manually degassed to avoid air bubbles. An initial waveguide calibration was carried out by injecting 80 wt% ethanol in water mixture whilst the instrument was running under degassed HPLC water. This was then followed by switching the buffer to 10 mM phosphate (degassed) with a flow rate of 25 μL/min for the lysozyme adsorption study. A 2% SDS injection was done prior to each experiment. The volume of each lysozyme solution injection was also fixed at 125 μL. During each injection the flow was interrupted at almost the end of the injection to prolong the stabilization during lysozyme injection. This lasted for 5 min before the flow was started again. The buffer rinsing was also maintained for 15 min after each injection and the injection loops were cleaned by rinsing through 3–4 volumes of 2% Hellmanex, followed by 3–4 volumes of water. The adsorbed lysozyme layer was removed by injection of 2% SDS and the baseline was observed to come back to the original start. Each step was repeated for the next concentration of lysozyme solution. Data was analysed using *Ana*light explorer (Farfield-Biolin Scientific AB, Sweden) to calculate layer RI, thickness, mass and density. The bulk solution exchange rate was examined by injecting NaCl solution whilst running HPLC grade water as bulk solution. The time between the end of the injection and both TM and TE signals back to the baseline was taken as the bulk exchange time (approximately 1 min). During lysozyme adsorption, correction of the bulk RI is needed in order to obtain accurate values of layer density and mass. This is because the free lysozyme molecules in the bulk solution need to be taken into consideration as it would influence the raw signal data output. The new RI is the sum of the bulk solution RI and the protein solution RI which can be obtained by multiplying the concentration of the lysozyme solution used by the RI increment of protein (dn/dc). The standard value of 0.182 cm^3/g is used as the RI increment of protein in all calculations.

2.3. QCM-D

In parallel to DPI, QCM-D was also performed to record real-time frequency and dissipation value of lysozyme adsorption (QCM-D E4, Q-Sense-Biolin Scientific AB, Sweden). The technique utilized AT-cut quartz crystals coated with silicon dioxide that were stabilized in HPLC grade water after the cleaning procedure (fundamental frequency = 4.95 MHz, overtone n = 3, 5, 7, 9, 11 and 13). The temperature was controlled at 20.0 °C ± 0.1 and the buffer was 10 mM phosphate (degassed). Liquid injections were transported into each of the cells through the coupled peristaltic pumping system at a fixed flow rate of 100 μL/min. For lysozyme solution injection, the volume was 500 μL per injection and the flow was stopped close to the end of the 5 min-injection to ensure a stable recording during each of the injection. The flow was continued and buffer solution was replaced at the end of the injection followed by 15 min rinsing. The flow rate and the injection volumes were 4 times higher than the DPI setup because the QCM-D chamber volume is 40 μL as compared to the DPI sample volume of 10 μL. Each sensor chip was re-used for the next higher concentration and the adsorbed lysozyme layer was removed by running a 5 min 2% SDS injection, followed by 10 min buffer rinsing. The return of both frequency and dissipation shift was used as a standard for complete removal of adsorbed proteins. The bulk solution exchange rate was examined in the same way as described for DPI, and a similar exchange time (approximately 1 min) was found.

Both the Sauerbrey equation and Voigt model were valid approaches in the present study to calculate the lysozyme adsorption at the studied solution concentration range [31]. Details are discussed in Section 4.1. The Sauerbrey equation relates frequency shift (Δf) to the sensed mass per unit area (Eq. (1)).

$$\Delta M = -C\Delta f/n \tag{1}$$

where ΔM, C, and n represent the adsorbed mass per unit area, mass sensitivity constant (17.7 $ng/cm^2\ Hz^{-1}$), and the overtone number, respectively. The fifth overtone is used for data analysis.

The Voigt model was used to obtain the viscoelastic properties of lysozyme adsorbed layer. The fixed parameters were bulk solution density and bulk solution viscosity, which were assumed as 1000 kg/m^3 and 0.001 kg/ms, respectively. The parameters needed to fit were the layer viscosity, layer shear modulus and layer thickness, which were set in the range of 0.0001–0.1 kg/ms, 1 E^4 and 1 E^8 Pa, and 1 E^{-10} and 1 E^{-6} m, respectively. Overtone n = 3, 5, 7, 9, 11, and 13 are employed for the modelling. Finally, the mass obtained from the Voigt model was compared with the Sauerbrey equation calculated mass (Fig. S1, Supporting information). It is found that for most of the data, the difference between the two mass values is less than 15% with dissipation values less than 1×10^{-6}, i.e. no significant over-estimation or under-estimation of the adsorbed mass which is often problematic if the model used is not suitable.

For the percentage layer solvation calculation, the mass obtained from the DPI (ΔM_{ads}) was subtracted from the Sauerbrey equation calculated mass (ΔM_s), then divided by ΔM_s (Eq. (2)).

$$\text{wt\% solvation} = (\Delta M_s - \Delta M_{ads})/\Delta M_s \tag{2}$$