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Adsorption of lysozyme, β-casein and their layer-by-layer formation on hydrophilic surfaces: Effect of ionic strength

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ABSTRACT

The adsorbed amount and layer structure of lysozyme, β-casein and mixed layers of the two proteins were studied on hydrophilic silica and quartz surfaces using the following techniques: ellipsometry, quartz crystal microbalance with dissipation monitoring (QCM-D) and total internal reflection fluorescence (TIRF). Particular emphasis was put on the effect of solution ionic strength on the layer formation. Both lysozyme and β-casein showed a higher affinity for the silica surface when adsorbed from a solution of low ionic strength even though β-casein and silica are negatively charged at the pH used. No β-casein remained adsorbed after rinsing with a 150 mM buffer solution. The adsorbed amount of lysozyme on silica exceeded a monolayer coverage irrespective of the solution conditions and displayed a rigid structure. β-Casein forms more than a single layer on pre-adsorbed lysozyme; an inner flat layer and an outer layer with an extended structure, which largely desorbs on rinsing. The build-up through sequential adsorption of lysozyme and β-casein is favoured at intermediate and high ionic strength. The total adsorbed amount increased slightly with each deposition cycle and the mixed lysozyme/β-casein layers contain higher amounts of protein compared to those of pure lysozyme or β-casein. Sequential adsorption gives rise to a proteinaceous layer consisting of both lysozyme and β-casein. The protein layers are probably highly interpenetrated with no clear separation between them.

1. Introduction

The layer-by-layer technique was initially proposed for alternating adsorption of oppositely charged colloidal particles by Iler [1]. In the early 1990s Decher et al. [2,3] showed that this method could be used for multilayer film formation of polyelectrolytes. Over the last decades there has been growing interest in the technique and there are several reviews covering the topic [4,5]. Multilayer films are formed through the sequential adsorption of oppositely charged polyelectrolytes, through mainly electrostatic forces due to the entropic gain from the liberation of small ions but also through hydrogen bonding, hydrophobic interactions and covalent bonding. The method also permits the construction of films containing other macromolecules, such as proteins, enzymes, or nucleic acids [6]. These biopolymer films are not only of academic interest as several industrial applications have been proposed recently, mainly in the biomedical field for controlled release of drugs [7], enzyme immobilization for biosensors [8] and antibacterial coatings [9] on medical implants [10]. Further, Jessel et al. [11,12] have shown that cells can interact with proteins embedded in a polyelectrolyte multilayer film.

In the past, mixed layers of different polyelectrolytes and water soluble proteins have been successfully formed [8,13] where the secondary structure of the proteins has been found to be close to that of their native form in solution [14]. The biological activity of many proteins is therefore preserved in the multilayer film [15]. To our knowledge there are only two earlier attempts to make protein multilayers without the use of polyelectrolytes or the specific binding between an antibody and its antigen. Lvov et al. [16] carried out alternate adsorption of two globular proteins, lysozyme and glucose oxidase (GOD), of opposite net charge at the solution pH used. In their attempt GOD did not adsorb to the pre-adsorbed lysozyme layer and this behaviour was explained by the electrostatic attraction not being optimised with globular proteins. However, multilayer films were successfully formed when lysozyme was sequentially adsorbed with the anionic polyelectrolyte poly(styrene sulfonate) [16]. A more recent investigation has been made by Lindh et al. [17] of protein multilayer formation between the large glycoproteins mucin (MUC5B) and different small cationic proteins. Most successful was the build-up between MUC5B and lactoperoxidase that was explained by a good charge matching between those two. Even though the adsorbed amount

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and thickness increased with each deposition cycle the build-up resembled an adsorption–desorption behaviour upon addition of lactoperoxidase and mucin, respectively. The regular build-up versus the adsorption/redissolution process is often dependent on solution conditions when using weakly charged pH dependent polyelectrolytes as building blocks [18,19].

The aim of the present study, which is a continuation of our previous investigation [20], was to examine the film formation of two proteins having similar molecular weights, lysozyme and β-casein, β-casein is a flexible protein and can act as electrostatic glue between two lysozyme layers. A regular multilayer build-up has recently been reported for the sequential adsorption of β-casein and poly(L-lysine) on silica surfaces.[21] By controlling the pH of each protein solution such that lysozyme and β-casein remain far from their iep, the electrostatic mechanism should be optimized. At the solution pHs used, the proteins have similar magnitude of charge. For electrostatic interactions the ionic strength provides an additional control parameter and so far, no explicit study on the influence of solution ionic strength on protein multilayer formation has been performed.

As a first step it was relevant to understand the adsorption behaviour of a single protein layer of lysozyme and β-casein to hydrophilic silica at the solution conditions used here. Lysozyme is a small globular enzyme, with a positive net charge at neutral pH (iep ~ 11), that promotes hydrolysis of polysaccharides in cell walls [22]. Previous measurements have shown that adsorption of lysozyme to negatively charged surfaces is promoted in low ionic strength solutions [23], high bulk protein concentrations [23,24] and high solution pHs [25]. β-Casein is a flexible protein isolated from milk with an iep of 5.2, which has been shown to adsorb on hydrophilic surfaces at neutral solution pHs [26,27]. Apart for the latter reports, there are surprisingly few studies of β-casein adsorption to negatively charged hydrophilic substrates.

The methods used to estimate the adsorbed amount, the level of hydration and the structure of the adsorbed protein layers were ellipsometry, quartz crystal microbalance-dissipation (QCM-D) and total internal reflection fluorescence (TIRF).

2. Materials and methods

2.1. Chemicals

TRIS(hydroxymethyl)aminomethane, TRIS, 10 mM, purchased from Sigma–Aldrich (T1503), was used as a buffer for all adsorption experiments. The pH was further adjusted to pH 8.5 through addition of 0.2 M HCl. Adjustment in salt concentration was made by sodium chloride of pro analysis grade to a final ionic strength of 50 mM or 150 mM. A phosphate buffered saline (PBS) was used in the labelling of β-casein with fluorescein-5-isothiocyanate (FITC) purchased from Molecular Probes (Oregon, USA, lot 1191-4), to be used in TIRF measurements. A protein assay was used in FITC labelling and the dye reagent was purchased from Bio-Rad laboratories (catalog 500-0006).

The lysozyme (hen egg white) was purchased from Sigma–Aldrich (L6876) and used as supplied with a >90% purity where the other <10% is buffer salt such as sodium chloride. β-Casein (bovine milk) with a minimum purity of 95% was kindly provided by Hannah Research Institute, Scotland. Apart from salt (calcium and phosphate) the remaining 5% may contain trace amounts of other caseins (α, γ, κ) [28,29], which have been observed in previous studies. However, since the casein properties are quite similar the possible impurities are not expected to significantly influence the adsorption of β-casein. The final concentration of lysozyme and β-casein were in all measurements 2 mg/ml and the protein solutions were always used within 24 h of preparation. All solutions were prepared using deionised water (resistivity > 18 MΩ cm), purified with a Milli-RO 10 Plus pre-treatment unit, followed by purification with a Q-PAK unit. The outgoing water was filtered through a 0.2 μm filter.

2.2. Substrates

Ellipsometry measurements were performed on thermally oxidized silicon slides covered with a silica layer of approximately 30 nm. The slides were prepared by Stefan Klintström at Linköping University, Sweden. In TIRF and QCM-D measurements quartz glass slides and quartz crystals were used respectively. The quartz crystals were covered with approximately 50 nm of silica, and were purchased from Q-sense AB (Gothenburg, Sweden). Silica and glass wafers were cleaned in a hot mixture (80 °C) of $H_2O/HCl/H_2O_2$ (5:1:1) volume% for 5 min, followed by extensively washing with Milli-Q water. Further cleaning was carried out using a hot mixture of $H_2O/NH_3/H_2O_2$ (5:1:1) vol.% for 5 min. As a final step in the procedure the substrates were rinsed in Milli-Q water and ethanol, and stored in ethanol (99.7%) until use. QCM-D substrates were immersed in a 2% Hellmanex-11 solution (VWR) for 1 hour, and then rinsed extensively with Milli-Q water. Before measurement the substrates were placed in an air plasma cleaner (Harrick Scientific Corporation, PDC-3XG) for 5 min.

2.3. Protein labelling by FITC

For use in TIRF measurements, β-casein was fluorescent-labelled using fluorescein-5-isothiocyanate (FITC). FITC was dissolved in dimethylformamide (DMF) and mixed with the protein solution to the molar fraction 10 mol FITC/mol β-casein. The reaction was allowed to proceed with stirring for 2 h at room temperature. The FITC/β-casein solution was passed through a PD-10 column (Amersham Pharmacia Biotech), thus buffer was changed from PBS to TRIS and unbound FITC was separated from the final reaction mixture. The concentration of β-casein and the FITC labelling ratio in the sample after the column were determined from UV–vis absorbance measurements. The amount of FITC was calculated using Beer's law and a reference curve of the absorbance for β-casein/dye reagent complex at known β-casein concentrations combined with the Bradford method [30] were used to estimate the β-casein concentration. It was found that the reference curve was almost linear in the concentration range of 0–0.8 mg/ml (see supporting information). The final molar ratio was 0.3 mol FITC/mol β-casein. Prior to measurements the labelled protein solution was mixed with an equal amount of unlabelled β-casein to decrease the average number of labels per molecule in order to avoid fluorescence concentration quenching in the adsorbed protein layer. Due to the low labelling density used, we do not expect any significant change in the protein affinity for the substrate in comparison to unlabelled protein molecules in the same manner as previously shown for other dye-labelled proteins [31].

2.4. Ellipsometry

Ellipsometry is an optical technique for measurement of changes in the polarization of light caused by reflection at an interface. This technique has been extensively used for studying protein adsorption in the past and a detailed description of the technique is found elsewhere [32]. Protein adsorption and lysozyme-β-casein film build-up on silica surfaces were studied with *in situ* null ellipsometry, employing a Multiskop instrument (Optrel GdBR, Berlin, Germany), equipped with a 532 nm laser [33]. The Multiskop was used in null ellipsometry mode and the angle of incidence was 67.3°.

The data were evaluated using a four layer model (silicon, silicon oxide, adsorbed film, ambient medium) in order to determine the