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Comparison of the adsorption kinetics and surface arrangement of "as received" and purified bovine submaxillary gland mucin (BSM) on hydrophilic surfaces

Maria Lundin [a,*], Tomas Sandberg [b], Karin D. Caldwell [b], Eva Blomberg [a,c]

[a] Surface and Corrosion Science, Department of Chemistry, Royal Institute of Technology, Drottning Kristinas väg 51, SE-10044 Stockholm, Sweden
[b] Department of Physical and Analytical Chemistry, Division of Surface Biotechnology, Box 577, BMC, Uppsala University, SE-75123 Uppsala, Sweden
[c] Institute for Surface Chemistry, P.O. Box 5607, SE-114 86 Stockholm, Sweden

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ABSTRACT

The effect of bovine serum albumin (BSA) as impurity in a commercial bovine submaxillary gland mucin preparation (BSM; Sigma M3895) on the adsorption of BSM to hydrophilic surfaces (mica and silica) has been studied in terms of adsorption kinetics, amount and structure of the formed adlayer. The Surface Force Apparatus (SFA) was used to gain information about the extended and compressed structure of adsorbed "as received" BSM, purified BSM, BSA extracted from the "as received" BSM and mixtures of the latter purified proteins. The adsorbed amount was estimated using a combination of X-ray Photoelectron Spectroscopy (XPS), Enzyme-Linked Immuno Sorbent Assay (ELISA), Enzyme-Linked Lectin Assay (ELLA), Dual Polarization Interferometry (DPI) and Quartz Crystal Microbalance (QCM-D) measurements. Under the used conditions, purified BSM showed very low affinity for silica and only small amounts were found to adsorb on mica. Initially, the BSM molecules adopted an extended conformation on the mica surface with tails extending into the bulk phase. These tails were irreversibly compressed into a very thin (10 Å) layer upon applying a high load. "As received" BSM formed considerably thicker compressed layers (35 Å); however, the extended layer structure was qualitatively the same. When mixtures of purified BSM and BSA were coadsorbed on mica, a 9 wt-% albumin content gave a comparable layer thickness as the "as received" BSM and from XPS data we draw the conclusion that the albumin content in the layer adsorbed from "as received" BSM was approximately 5 wt-%. Adsorption from an equal amount of BSM and BSA revealed that even though the amount of BSM is scarce in the mixed layer, the few BSM molecules have a drastic effect on the adsorbed thickness and structure. Clearly, this study shows the importance of characterizing the mucin used since differences in purity give rise to different adsorption behaviours in terms of both adsorbed amount and layer structure.

1. Introduction

Mucins are large glycoproteins (>10^6 Da) with important functions in the mucus gel, which covers the epithelial cell surfaces. One of the key functions of the mucins is to act as a steric barrier and prevent non-specific interaction of proteins and cells with the underlying cell membrane [1]. Detailed studies of mucin adsorption are therefore of great relevance to the area of biomaterial coatings [2–4]. Further, the mucus layer is interesting from the point of view of being the primary target for oral drug delivery [5,6]. A wide range of mucins isolated from different regions and species [7] has been studied in terms of adsorption to solid surfaces. Thus, it has been shown that the adsorbed mucin layer structure depends on the mucin source [8], solution conditions [9,10] and type of substrate [11,9,12] (non polar, polar and polar-charged). Besides mucin there are a large number of other smaller proteins present in mucous with important functions. For example, Raynal et al. [13] have shown that although purified human salivary mucin (MUC5B) self associate at high bulk concentration to form gels, such gels do not replicate the native mucus. Other proteins such as eg. albumin, lysozyme, lactoferrin and IgG associate with mucin naturally.

The mucin used in this work was bovine submaxillary gland mucin (BSM). This protein has shown adsorption properties that are similar to MUC5B when adsorbed on hydrophilic surfaces from low protein concentrations (<100 μg mL^{-1}) [11]. Previous measurements on the adsorption of BSM have shown that the adsorbed BSM molecules forms extended and highly hydrated layers [14] with a low friction coefficient [15] on negatively charged hydrophilic surfaces. Further the pre-adsorbed BSM forms complexes with polysaccharides [14] and salivary proteins [16,17] at the solid–liquid interface and also multilayers can be built with mucin–chitosan [14,12] and mucin–lactoperoxidase [16] using the layer-by-layer deposition method.

* Corresponding author. Fax: +46 8 20 8998.
E-mail address: maria.lundin@surfchem.kth.se (M. Lundin).

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Specifically, commercial grade BSM (Sigma M3895) has displayed substantial batch-to batch variations, which has necessitated a significant quality control prior to analytical work with this material [8]. Albumin was identified as the predominant contaminant in the mentioned BSM starting material. However, this impurity could be highly eliminated upon applying additional purification. From an adsorption point of view the presence of mucin aggregates [18] and non-mucin components eg. globular proteins in "as received" Sigma BSM are likely to affect the adsorption event.

Previous work has concerned the adsorption and layer structure of either purified BSM or "as received" BSM. Most often these experiments have been carried out using different solution conditions and types of substrates. The aim of the present work was therefore to compare the adsorption behaviour of "as received" BSM from Sigma (M3895), with that of our purified BSM under identical experimental conditions onto (negatively charged) hydrophilic and hydrophobic model surfaces. Further, we elucidate the effect that the presence and absence of bovine serum albumin (BSA) has on the adsorption of BSM onto negatively charged hydrophilic surfaces. Thus, adsorption of "as received" BSM is compared to that of pre-equilibrated mixtures of our purified BSM and BSA. A combination of six different techniques was employed in order to obtain a many-sided view of the adsorbed layer structure and on the kinetics of adsorption. On mica, a highly negatively charged surface, the adsorption of proteins was evaluated in terms of layer thickness, structure and adsorbed amount using the Surface Force Apparatus (SFA), X-ray Photoelectron Spectroscopy (XPS), Enzyme-Linked Immuno Sorbent Assay (ELISA) and Enzyme-Linked Lectin Assay (ELLA). On silica, the in situ adsorption of proteins was followed with Dual Polarization Interferomety (DPI) and Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). From the combination of these techniques it was possible to elucidate the structure of the adsorbed layers at different length scales. For instance, the SFA and DPI both provide information about the layer thickness. However, DPI is an optical technique and determines the thickness on the basis of the assumption that the layer is homogenous and has constant optical properties throughout its thickness. In contrast, the film thickness derived from the interferometric SFA accounts for the presence of even a small fraction of loops and tails extending out from the surface. Further, the XPS technique provides detailed information of the chemical composition of the adsorbed layer and allows quantification of the adsorbed amount.

2. Materials and methods

2.1. Solutions

Generally, all measurements were performed with solutions of 20 mM phosphate-buffered saline of pH 7.4 supplemented with 98 mM $NaNO_3$ (PBS20) to a total solution ionic strength of 150 mM. The $NaNO_3$ salt was used instead of NaCl since Cl^- interacts with the silver coating, which is used in force measurements (SFA). However for the XPS measurements, NaCl was used to avoid interference of nitrogen atoms from a $NaNO_3$ electrolyte solution. Solutions were filtered twice through filters of pore size 0.2 μm. Sodium nitrate ($NaNO_3$) and sodium chloride (NaCl) of Suprapur grade (>99.99% purity) were obtained from Merck and used "as received". Milli-Q water was prepared from deionised water (resistivity > 18 MΩ cm), using a Milli-RO 10 Plus pre-treatment unit (Milli-Q) in combination with a Q-PAK unit (Milli-Q).

2.2. Proteins

Pre-fractionated bovine submaxillary gland mucin (BSM) with an average molecular weight of about 7 MDa, [19] was purchased from Sigma (M3895). This commercial mucin, was used as received or purified according to the "mild" purification protocol recently described by Sandberg et al. [20]. To exclude nonmucin components this protocol makes use of anion exchange chromatography and size exclusion chromatography. In the present work, the high molar mass, essentially contaminant-free, BSM fraction (BSM-I) was used. This mucin product (in the following referred to as"purified BSM") contains approximately 5 wt-% of aggregated material, has an average molar mass of 2.9 MDa and exists mainly as random coils in solution, as determined by static light scattering measurements.

The bovine serum albumin (BSA) used in the present study was extracted in the anion exchange chromatography step during the purification process, transferred to PBS20, and positively identified using combined polyacrylamide gel electrophoresis (PAGE)–Western blot analysis. Compositional PAGE analysis showed this BSA preparation to be highly pure, with essentially no protein contamination. Stock solutions of BSA at 0.24 mg/mL were prepared and kept frozen until use. The BSA stock solutions were thawed, diluted and equilibrated with measurement buffer for approximately 3 h prior to further use. Freeze–dried BSM was solubilised in measurement buffer at 0.1 mg mL^{-1}. The solution was equilibrated for at least 5 h, and passed through a PD-10 (Amersham Pharmacia Biothech, Sweden) desalting column, followed by dilution to its final concentration. Mixtures of BSM and BSA were equilibrated for at least 5 h.

2.3. Substrates

Muscovite Mica (Axim Enterprises Inc., USA) was used as a substrate for SFA, XPS and ELLA/ELISA measurements. QCM-D measurements were performed using AT-cut 5 MHz quartz crystals covered with either silica or polystyrene (Q-Sense AB, Sweden). The waveguide chips for the DPI measurements were composed of silica doped with nitrogen (Farfield Sensors Ltd., UK).

2.4. Surface Force Apparatus (SFA)

Interactions between BSM and BSA coated surfaces were investigated using the MARK-IV Surface Force Apparatus. With this interferometric surface force technique the total force acting between two macroscopic molecularly smooth surfaces in a crossed cylinder configuration is measured as a function of surface separation [21,22]. The resolution in distance determination is about 2 Å while the detection limit of the force is about 10^{-7} N, corresponding to a normalized force of about 10 μN/m. The interaction force, F, between crossed cylinders normalized by the undeformed geometric mean radius of the surfaces, R, as a function of the surface separation, D, is related to the free energy of interaction per unit area, G_f, between two flat surfaces via the Derjaguin approximation [23]:

$$\frac{F(D)}{R} = 2\pi G_f(D) \tag{1}$$

This equation is valid provided that $R \gg D$, which is the case in these experiments. The mica sheets were silvered on one side, and thereafter glued on to silica discs with the silvered side down. Prior to protein deposition the mica–mica contact position was established in air, a procedure which also assures the surface cleanness. All layers were prepared ex-situ by adsorption of protein from PBS20 buffer solutions containing 98 mM $NaNO_3$ for 30 min in order to precisely control the deposition time and to avoid having proteins present in the bulk solution during force measurements. The ex-situ adsorption procedure has been suggested previously to eliminate adsorption of BSM aggregates by Dedinaite et al. [14]. The used protein concentrations were 50 μg mL^{-1} for mea-