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Studies on the exchange of early pellicle proteins by mucin and whole saliva

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Abstract

Adsorption of small pellicle proteins statherin or proline-rich protein 1 (PRP1), respectively, and subsequent adsorption of human whole saliva (HWS) or salivary mucin MUC5B, respectively, was studied using ellipsometry and total internal reflectance fluorescence. Differences in elution (using sodium dodecyl sulphate (SDS) solutions) between mixed and single protein films were also investigated. On both hydrophilic and hydrophobized surfaces HWS and MUC5B were found to adsorb to pre-adsorbed layers of statherin and PRP1, respectively. Statherin adsorption on both substrate types showed no or minor exchange by HWS or MUC5B and no change in SDS elution between mixed and single protein films. Small amounts of PRP1 were exchanged by HWS on both surface types and the SDS elutable fractions were similar or larger for mixed films compared to single protein films. PRP1 and MUC5B in sequence showed minor exchange of PRP1 on hydrophilic surfaces, while no exchange could be established on hydrophobized substrates. SDS elutable fractions decreased for PRP1 and MUC5B mixed films compared to single protein films. In conclusion, minor amounts of statherin and PRP1 are exchanged during the time course of the experiments, which indicates that these proteins may to a large extent remain incorporated in the pellicle.

Keywords: Total internal reflectance fluorescence; Ellipsometry; Hydrophilic surfaces; Hydrophobic surfaces; FITC; SDS; MUC5B; Proline-rich protein; Statherin

1. Introduction

The acquired pellicle is the thin salivary film that covers all oral surfaces, formed by selective adsorption of primarily salivary proteins. The pellicle is known to have many important functions, such as protection against hazardous microbes, lubrication to facilitate e.g. speech and mastication, and preservation of the integrity of the teeth by mineral homeostasis (for reviews see [1,2]). As saliva contains many different types of proteins, with varying molecular weights and at different concentrations, the pellicle build-up is believed to occur by exchange processes, where high molecular weight proteins, e.g. mucins (high adsorption affinities but low diffusion rates) may partly replace smaller components, such as statherin and acidic proline-rich proteins, present at higher concentrations (and have high diffusion rates) with time. This process is analogous to "the Vroman effect" [3] observed on surfaces in contact with blood.

The flexible, low molecular weight proteins statherin (4.5 kDa (43 amino acids), pI 4.2) and PRP1 (16 kDa (150 amino acids), pI 4.7) have been identified in the initial pellicle formed *in vivo* (for a review see [1]), where they are believed to be involved in the calcium homeostasis activity [4], lubrication [5,6] and mediation of microbial adhesion [7,8].

The charge distributions in statherin and PRP1 are very asymmetric, consisting roughly of a non-polar (statherin) or slightly positively charged (PRP1) C-terminal and a negatively charged N-terminal domain [4]. It is the negatively charged domain with phosphorylated serines that has been shown to govern these proteins high adsorption affinities to hydroxyapatite (HA, the main constituent of enamel) ([1,9] and references therein).

The mucin MUC5B is a large glycoprotein (>11 MDa) known for e.g. tissue coating and antimicrobial abilities [10]. It has shown to appear after approximately 2 h in the pellicle [11], which is probably due to the large size of MUC5B, resulting in

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a slow adsorption process. MUC5B has shown to adsorb to HA *in vitro* [12]. Indeed, the affinity constants of MUC5B binding to HA is higher than those of e.g. statherin and PRP1 ([9] and references therein), which indicates that MUC5B have the potential to replace these initially adsorbed low molecular weight pellicle proteins.

Many studies have been performed on the composition and ultrastructure of salivary pellicles (see e.g. [1] and references therein). However, studies aimed at elucidating exchange processes of proteins in the pellicle, particularly with a more physicochemical approach, are few. In the present study sequential adsorption and possible exchange processes were investigated, over time, when adsorbed statherin or PRP1 interacted with human whole saliva (HWS) or MUC5B, respectively. This was performed on two types of well-defined model surfaces, one hydrophilic and one methylated (hydrophobized), both with a net negative charge, to elucidate the effect of surface wettability on the sequential adsorption processes. Further, elution by sodium dodecyl sulphate (SDS) solution was performed to investigate the stability of the mixed films on the two types of surfaces. Null ellipsometry, an optical method previously used in studies of adsorption concerning salivary secretions and purified salivary proteins (for a review see [13]) was used to study the total adsorbed amount and mean layer thickness in these experiments. The technique of total internal reflectance fluorescence (TIRF) was utilised to study the sequential adsorption process of fluorescein-labelled statherin or PRP1, with unlabelled HWS or MUC5B. By combining these two techniques, it is possible to obtain information about the composition of the adsorbed layer. The individual adsorption/elution behaviour of statherin and PRP1 on hydrophilic and hydrophobized surfaces using ellipsometry was also investigated in the present study. Data on HWS and MUC5B single protein systems (obtained from [14]) are also presented to elucidate possible differences in adsorption and elution of the single proteins as compared to the sequential protein systems.

2. Materials and methods

2.1. Protein purification

Statherin and PRP1 were purified according to Lindh et al. [15] and MUC5B was purified according to Wickström et al. [16] and further treated as described in Lindh et al. [17]. The protein concentrations were chosen based on the ones present in saliva [15,18].

2.2. Saliva collection

Unstimulated HWS was collected according to Dawes [14,19] from one healthy donor considered to be in good oral health. The collection was performed at least two hours after last food intake. This study has been approved by the committee of research ethics at Lund University (No. LU 518-02). The saliva was diluted to 10% in the experiments. This was done to facilitate *in situ* time resolved experiments by avoiding light scattering at higher concentrations. It has previously been shown that 10% of saliva gives similar total adsorbed amounts as 100% of saliva [20].

2.3. Fluorescein labelling of statherin and PRP1

For the TIRF experiments, statherin and PRP1 were labelled with fluorescein-5-isothiocyanate (FITC, Isomer 1, Molecular Probes, Leiden, the Netherlands). FITC labels the ϵ-amino group of the amino acid Lysine (Lys) and also the amino terminus in proteins. Due to the low amounts of Lys in statherin (1 Lys) and in PRP1 (2 Lys), the labelling procedure was modified for these proteins. A stock solution of 1 mg ml^{-1} of statherin and PRP1, respectively, in a 10 mM Na_2HPO_4 buffer supplemented with 50 mM NaCl (pH 9.1) was prepared. A solution of 100 mg FITC/ml dimethylformamide was added to the protein stock solutions, yielding a FITC protein ratio of 1–4 mg FITC/1 mg protein. The solutions were incubated at room temperature on a shaking table for 2½ h in the dark. Unbound FITC was then separated from the labelled proteins by means of a sephadex PD-10 column (Amersham Bioscience, Uppsala, Sweden). The UV absorbencies of the labelled protein solutions were measured at 495 nm with a spectrophotometer (Hitachi model 100-60, Hitachi Ltd., Tokyo, Japan). The labelling densities were then calculated by using the extinction coefficient (ϵ) of 196 cm^2 mg^{-1} for FITC [21]. The average molar ratio of FITC to protein was 0.012 and 0.148 mol FITC/mol statherin and PRP1, respectively. Due to the low labelling densities, no large effects of quenching or self-quenching would be expected [22], nor any change in the adsorption behaviour [21]. The protein concentrations were determined by the BioRad protein microassay (BioRad Laboratories, Sundbyberg, Sweden).

2.4. The solid surfaces

The silicon wafers (P-type, boron doped, resistivity 10–20 Ω cm), (Okmetic OY, Espoo, Finland), used for the ellipsometric experiments were prepared as described in Cárdenas et al. [14] to obtain hydrophilic and hydrophobic characteristics, respectively. For the TIRF measurements, glass slides (Gold Seal Rite-on, Microslides, Clay Adams, NY, USA) were cleaned and modified in the same way as the silicon slides to obtain hydrophilic and hydrophobic character, respectively. The water contact angles at hydrophilic silica and glass have been reported to be $<10°$, and the receding and advancing water contact angles for hydrophobized silica and glass were 90 and 103°, and 78 and 103°, respectively [22]. TIRF requires transparent surfaces, while ellipsometry gives higher sensitivity on silicon, which is the reason for using different substrates. However, surface examination have shown no significant differences in contact angles and composition as obtained by ESCA between the two substrate types [22]. Studies have shown a water contact angle of tooth enamel of 40° [23] and that the enamel surface has a slight negative charge [24], which means that its wettability will be between that of the two model surfaces used in this study, but with a lower charge. Furthermore, the wettability range spanned by the model substrates used cover most dental materials, such as porcelain, metals and polymers.