

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

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Received 27 November 2007; accepted 26 January 2008

Available online 2 February 2008

## 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.

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**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 diffu-

sion 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  $\epsilon$ -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  $1 \text{ mg ml}^{-1}$  of statherin and PRP1, respectively, in a  $10 \text{ mM Na}_2\text{HPO}_4$  buffer supplemented with  $50 \text{ mM NaCl}$  (pH 9.1) was prepared. A solution of  $100 \text{ mg FITC/ml}$  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  $2\frac{1}{2} \text{ h}$  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  $495 \text{ nm}$  with a spectrophotometer (Hitachi model 100-60, Hitachi Ltd., Tokyo, Japan). The labelling densities were then calculated by using the extinction coefficient ( $\epsilon$ ) of  $196 \text{ cm}^2 \text{ mg}^{-1}$  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  $10\text{--}20 \text{ } \Omega \text{ cm}$ ), (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  $<10^\circ$ , and the receding and advancing water contact angles for hydrophobized silica and glass were  $90$  and  $103^\circ$ , and  $78$  and  $103^\circ$ , 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  $40^\circ$  [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.

## 2.5. General

In both the TIRF and ellipsometric experiments a 10 mM phosphate buffer supplemented with 50 mM NaCl, and adjusted to pH 7.0 (denoted PBS) was used. The composition of the buffer solution was chosen to mimic the pH and ionic strength of saliva.

The anionic surfactant SDS (>99% pure, L6026, Sigma-Aldrich, Stockholm, Sweden), which is present in many oral care products at concentrations up to 70 mM [25], was used as elution agent. Critical micelle concentration (cmc) for SDS in the buffer used has been estimated to be approximately 2 mM [26]. Water treated in a Milli-Q unit (Millipore, Bedford, MA, USA including an ion exchange active carbon adsorption and reverse osmosis) was used in all experiments as well as for surface cleaning and buffer preparation. All chemicals were of at least analytical grade (Primalco, Helsinki, Finland, VWR International, Stockholm, Sweden).

## 2.6. Null ellipsometry

*In situ* null ellipsometry was used for studies of the refractive index and average film thickness of the sequential as well as the single protein adsorption experiments. The principle of this method is that the polarization of light changes upon reflection at an interface. For theoretical principles see Ref. [27]. The instrument used was an automated Rudolph Research thin-film null ellipsometer type 43603-200E (Rudolph Research, Fairfield, NJ, USA), with a xenon arc lamp as a light source, filtered to  $\lambda = 401.5$  nm [28]. Using a fixed angle of incidence ( $\sim 68^\circ$ ) and wavelength, the changes in polarization depends on the optical properties of the interface (derived from the ellipsometric angles  $\Delta$  and  $\Psi$ ).

The silica surface was mounted vertically in a quartz cuvette, thermostated to  $37^\circ\text{C}$  and containing a magnetic stirrer at constant stirring rate (325 rpm). A four-zone surface calibration, carried out in both air and buffer solution, was performed on the clean surface to determine the complex refractive index ( $N = n - ik$ ) of the substrate as well as the refractive index ( $n_0$ ) and thickness ( $d_0$ ) of the outermost oxide layer (the oxide layer was assumed to be transparent, i.e.  $k_0 = 0$ ). Statherin or PRP1 solutions were then added to the cuvette, and  $\Delta$  and  $\Psi$  were recorded *in situ*. When the optical properties of the substrate and the ambient media are known and assuming a homogeneous film, the mean thickness ( $d_f$ ) and refractive index ( $n_f$ ) of the growing protein film can be solved numerically from the changes in  $\Delta$  and  $\Psi$  [29]. The evaluation of the adsorbed protein film was performed by using a four-layer optical model, assuming isotropic media and planar interfaces. From the film thickness and the refractive index, the adsorbed amount ( $\Gamma$ ;  $\text{mg m}^{-2}$ ) can be calculated according to Cuypers model [30]. However, if the film is in-homogeneous, or if there is low level of adsorption, resulting in only minor changes in  $\Delta$  and  $\Psi$ , the refractive index and average film thickness estimations are unreliable [30]. The relative errors in  $n_f$  and  $d_f$  are quite high for low  $\Gamma$  ( $<0.5$   $\text{mg m}^{-2}$ ), however decreasing rapidly to values of 5–10% for surface coverages

$>1$   $\text{mg m}^{-2}$  [31]. Errors in the  $n_f$  and  $d_f$  co-vary, and as a result,  $\Gamma$  can be calculated with a much higher accuracy even at small changes in  $\Delta$  and  $\Psi$  (error of approximately 15% at  $\Gamma = 0.1$   $\text{mg m}^{-2}$  and less than 1% for  $\Gamma = 2$   $\text{mg m}^{-2}$  [31]). Cuypers model for surface excess concentration determinations assumes a known ratio of the molar weight to the molar refractivity (M/A) of the adsorbate and partial specific volume ( $v$ ). Values of M/A and  $v$  used in the present study were 4.10  $\text{g ml}^{-1}$  and 0.75  $\text{ml g}^{-1}$ , respectively. These values are average for proteins, and have shown to be valid in studies of adsorption of saliva and salivary components [14,20,26,32,33].

The adsorption of statherin or PRP1 was continuously measured for 60 min in one zone, at a final concentration of 20  $\mu\text{g ml}^{-1}$  in the cuvette. Rinsing with PBS buffer (using a continuous flow of 14  $\text{ml min}^{-1}$ ) was then performed for 5 min. After rinsing, the film stability was monitored for 30 min followed by 60 min of adsorption of 10% HWS or 50  $\mu\text{g ml}^{-1}$  MUC5B, respectively. PBS rinsing (5 min) was followed by monitoring of the film stability (30 min). Subsequently, SDS solution (17 mM in the cuvette) was added, and after 5 min, a third rinsing with PBS (5 min) was performed. The film stability was then monitored (30 min) before the measurement was stopped. As control experiments, adsorption of statherin or PRP1 (60 min) followed by buffer rinsing (5 min), film stabilization (30 min) and subsequent addition of SDS solution (5 min), a second buffer rinsing (5 min) and a final film stabilization period (30 min) were performed. Measurements were performed at least twice with a variation of  $<5\%$ .

## 2.7. Total internal reflectance fluorescence (TIRF)

This method detects fluorescent molecules adsorbed at, or near a surface. The technique is based on totally reflected light where an evanescent wave is formed at an interface, and causes a fluorescence excitation in the interfacial region. The evanescent wave decays exponentially normal to the surface, thereby restricting the fluorescence excitation to the interfacial region. The distance over which the evanescent wave amplitude decreases to  $e^{-1}$  of its initial value, is a function of the angle of incidence, and was in the present study estimated to be approximately 200 nm [21]. For further insight into theory, as well as the instrument set up and experimental procedures see Lassen and Malmsten [21]. In this study, the method was used to follow adsorption of FITC-labelled statherin or PRP1, respectively, both alone and after addition of unlabelled HWS or MUC5B, respectively. The flow cell system used enables the sample to circulate continuously. As ellipsometry, this method provides *in situ*, time-resolved information, without interfering with the measurement. However, using TIRF it is generally difficult to measure the adsorbed amounts quantitatively in a straightforward approach, as with ellipsometry, due to e.g. quenching, local pH fluctuations, uneven probe distribution across the adsorbed layer, and/or preferential adsorption of labelled/unlabelled protein fractions [22]. As a result, the TIRF evaluation in the present study is based on the relative changes in fluorescence emission that occurs during the adsorption/exchange experiments. Thus, in Section 3 the TIRF

results will be interpreted as changes in fluorescence (normalized to the signal of the fluorescein labelled statherin or PRP1, respectively, prior to addition of HWS or MUC5B, respectively). This in turn is related to the composition of the adsorbed layer at different stages in the adsorption process. Further, to minimize the effects of photobleaching, illumination during the experiments was only performed during sampling [21].

The light source of the instrument used was a 488 nm argon ion laser (model 161 B, Spectra Physics, USA), illuminating the flow cell (a dove tail prism covering the metal support and rubber seal). The surface was mounted between the metal support/rubber seal (containing the flow cell) and the prism, attached with glycerol to minimize refractive index differences. The detection part of the instrument consisted of a monochromator (Model H20 UV, Jobin Yvon, France), a photo-multiplier tube (Model R 298, Hamamatsu, Japan) and a photcounter (Model SR 400, Stanford Research System, USA).

Before each experiment, the system was rinsed with PBS buffer for 10–15 min until a steady baseline was observed. FITC-labelled statherin or PRP1 was then added to a final concentration of  $20 \mu\text{g ml}^{-1}$  in PBS in the cell and allowed to adsorb. The outline of the experiments was the same as described in the ellipsometry section. Protein solutions were kept at constant flow rate of  $1 \text{ ml min}^{-1}$  through the flow cell. The TIRF experiments were performed at  $27^\circ\text{C}$ .

### 3. Results and discussion

Studying sequential adsorption of salivary proteins and secretions at the solid/liquid interface by both ellipsometry and TIRF gives information on total adsorbed amounts, average film thickness as well as the compositional changes of the adsorbed films, thereby gaining insight about possible exchange processes that take place at the surface. The results of this study may be divided into three main sections, i.e. (i) effects on the adsorption behaviour of HWS or MUC5B due to pre-adsorbed statherin or PRP1, (ii) exchange of statherin or PRP1 by HWS or MUC5B, and (iii) SDS elution of mixed films in comparison with single protein films.

#### 3.1. Effects on the adsorption behaviour of HWS/MUC5B due to pre-adsorbed statherin/PRP1

The ellipsometric experiments of the adsorbed amount of statherin and PRP1 on both types of substrates are shown in Table 1, and are in agreement with previous studies [15].

For statherin and HWS in sequence on hydrophilic substrates, the increased adsorbed amount of HWS and the somewhat smaller total mean film thickness as compared to HWS adsorption alone ( $3.3 \text{ mg m}^{-2}$  and  $230 \text{ \AA}$  as compared to  $2.8 \text{ mg m}^{-2}$  and  $250 \text{ \AA}$ , see Tables 1 and 2 and Fig. 1), indicates an increased favourable interaction between proteins in HWS and adsorbed statherin compared to HWS and the bare hydrophilic silica. As both the hydrophilic silica surface [34] and statherin are negatively charged at pH 7, statherin most likely adsorbs with positively charged amino acids (amino acids

Table 1

Adsorbed amounts ( $\Gamma$ ,  $\text{mg m}^{-2}$ ) and average film thickness ( $d$ ,  $\text{\AA}$ ) of statherin, PRP1, HWS and MUC5B on hydrophilic and hydrophobized surfaces, measured by *in situ* ellipsometry. Values are taken 30 min after end of adsorption and buffer rinse

	Hydrophilic silica		Hydrophobized silica	
	$\Gamma$ ( $\text{mg m}^{-2}$ )	$d$ ( $\text{\AA}$ )	$\Gamma$ ( $\text{mg m}^{-2}$ )	$d$ ( $\text{\AA}$ )
Statherin adsorption	0.2	– <sup>a</sup>	0.8	– <sup>a</sup>
After SDS elution	0	– <sup>a</sup>	0	– <sup>a</sup>
PRP1 adsorption	0.6	– <sup>a</sup>	1.3	30
After SDS elution	0.1	– <sup>a</sup>	1.0	15
HWS adsorption	2.8 <sup>b</sup>	250 <sup>b</sup>	3.7 <sup>b</sup>	250 <sup>b</sup>
After SDS elution	0.1 <sup>b</sup>	– <sup>b</sup>	1.5 <sup>b</sup>	50 <sup>b</sup>
MUC5B adsorption	0.5 <sup>b</sup>	– <sup>b</sup>	2.3 <sup>b</sup>	350 <sup>b</sup>
After SDS elution	0.1 <sup>b</sup>	– <sup>b</sup>	1.5 <sup>b</sup>	300 <sup>b</sup>

<sup>a</sup> – indicates too low adsorption for any firm interpretation of data.

<sup>b</sup> Cárdenas et al. [14].

Table 2

Adsorption data (total adsorbed amounts ( $\Gamma_{\text{total}}$ ,  $\text{mg m}^{-2}$ ), total film thickness ( $d_{\text{total}}$ ,  $\text{\AA}$ ), as well as net increase in adsorbed amount ( $\Delta\Gamma$ ,  $\text{mg m}^{-2}$ ) and film thickness ( $\Delta d$ ,  $\text{\AA}$ ) of HWS or MUC5B, respectively) for the sequential experiments. Values are taken 30 min after the end of adsorption and buffer rinse

Sequence	Hydrophilic surfaces				Hydrophobized surfaces			
	$\Gamma_{\text{total}}$	$\Delta\Gamma$	$d_{\text{total}}$	$\Delta d$	$\Gamma_{\text{total}}$	$\Delta\Gamma$	$d_{\text{total}}$	$\Delta d$
Statherin + HWS	3.5	3.3	230	– <sup>a</sup>	3.9	3.1	195	– <sup>a</sup>
Statherin + MUC5B	0.6	0.4	– <sup>a</sup>	– <sup>a</sup>	2.2	1.4	340	– <sup>a</sup>
PRP1 + HWS	3.3	2.7	270	– <sup>a</sup>	3.5	2.2	275	245
PRP1 + MUC5B	1.2	0.6	1200	– <sup>a</sup>	2.4	1.1	370	340

<sup>a</sup> – indicates too low adsorption for firm interpretation of data (see Table 1).

6–13 [35]) in contact with the surface, and both the small negatively charged N-terminal (amino acids 1–5) and the uncharged C-terminal (amino acids 14–43) protrudes away from the surface. Such a conformation has indeed been proposed for adsorbed statherin by computer simulations [35]. Onto this initial statherin layer (although the adsorbed amounts are low;  $0.2 \text{ mg m}^{-2}$ , see Table 1), proteins in HWS may adsorb with hydrophobic interactions, and/or positively charged proteins in HWS may adsorb. For statherin and MUC5B on hydrophilic surfaces the adsorbed amounts of statherin did however not notably affect the adsorption of MUC5B ( $0.4 \text{ mg m}^{-2}$  as compared to  $0.5 \text{ mg m}^{-2}$ ; Tables 1 and 2). The adsorbed amount of only MUC5B on hydrophilic surfaces is quite low ( $0.5 \text{ mg m}^{-2}$ ; Table 1) which indicates that there is only a weak attraction between the hydrophilic surface and MUC5B that probably is not much affected by the low adsorbed amounts of statherin. Previous studies [14] indicated that electrostatic repulsion between MUC5B and hydrophilic silica determined the adsorption process, which supports the present findings.

Pre-adsorbed statherin on hydrophobized surfaces decreased the adsorbed amounts of both HWS (from  $3.7$  to  $3.1 \text{ mg m}^{-2}$ ) and MUC5B (from  $2.3$  to  $1.4 \text{ mg m}^{-2}$ ) (Tables 1 and 2 and Fig. 1). The adsorbed amounts of statherin are much higher on hydrophobized surfaces compared to hydrophilic ones ( $0.8 \text{ mg m}^{-2}$  as compared to  $0.2 \text{ mg m}^{-2}$ ; Table 1), which indicates a more densely packed layer and therefore less avail-

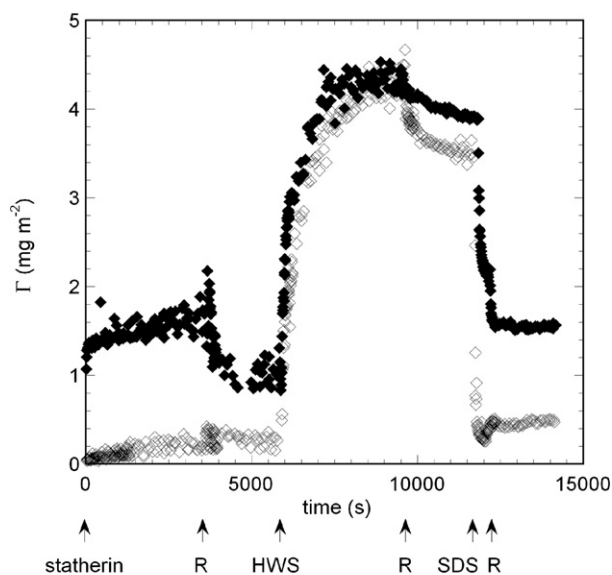


Fig. 1. Kinetics (total adsorbed amount ( $\Gamma$ ;  $\text{mg m}^{-2}$ ) versus time (s)) of the sequential adsorption of statherin and HWS on hydrophilic silica ( $\diamond$ ) and hydrophobized silica ( $\blacklozenge$ ). Addition of statherin and HWS are indicated by arrows. R = PBS rinsing. SDS = addition of SDS solution.

able area for adsorption of HWS and MUC5B. A conformation where the negatively charged N-terminal of statherin is extended into solution could also explain a decrease in adsorption of HWS and MUC5B due to increased repulsion between segments of adsorbed molecules and components in solution. Computer simulations of statherin adsorbed on a negatively charged hydrophobic surface indicated such a conformation on the surface [35]. Furthermore, the structure of the HWS and MUC5B films may be altered due to the amount of pre-adsorbed statherin. Previous reports on MUC5B using atomic force microscopy illustrated large elongated aggregates on hydrophobized surfaces, whereas on hydrophilic substrates smaller globular aggregates were dominating [14]. As a consequence of the pre-adsorbed statherin, there may be a shift in the MUC5B film structure towards the smaller globular aggregates due to an increased hydrophilic character of the pre-adsorbed statherin covered hydrophobized surface.

Although adsorbed amounts of PRP1 on hydrophilic surfaces were larger than those of statherin ( $0.6 \text{ mg m}^{-2}$  as compared to  $0.2 \text{ mg m}^{-2}$ ; Table 1), pre-adsorbed PRP1 did not notably affect the adsorbed amounts of HWS or MUC5B ( $2.7 \text{ mg m}^{-2}$  as compared to  $2.8 \text{ mg m}^{-2}$  for HWS and  $0.6 \text{ mg m}^{-2}$  as compared to  $0.5 \text{ mg m}^{-2}$  for MUC5B; Tables 1 and 2 and Fig. 2). As described above, the larger adsorbed amounts may lead to less area accessible for HWS or MUC5B adsorption. In addition, the structure of PRP1 may be of importance. As PRP1 has a larger negatively charged N-terminal domain (amino acids 1–30) compared to statherin, it is possible that the PRP1 film would be more negatively charged, and thus explain why we cannot see any increased interaction between PRP1 and HWS as observed for statherin and HWS.

On hydrophobized substrates, pre-adsorbed PRP1 decreased the adsorbed amounts of HWS and MUC5B while the mean

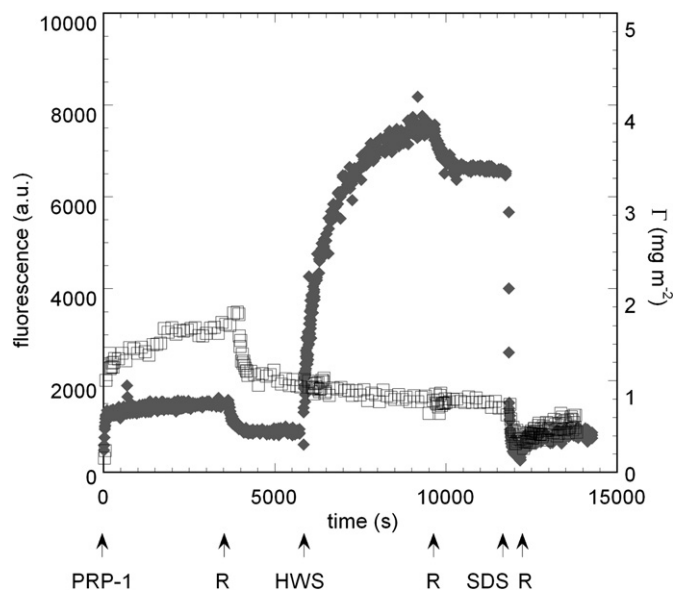


Fig. 2. Kinetics (fluorescence (a.u.) ( $\square$ ) and total adsorbed amount ( $\Gamma$ ;  $\text{mg m}^{-2}$ ) ( $\blacklozenge$ ) versus time (s)) of the sequential adsorption of PRP1 and HWS on a hydrophilic surface. Addition of PRP1 and HWS are indicated by arrows. R = PBS rinsing. SDS = addition of SDS solution.

film thicknesses remained the same (from  $3.7 \text{ mg m}^{-2}$  and  $250 \text{ \AA}$  to  $2.2 \text{ mg m}^{-2}$  and  $245 \text{ \AA}$  for HWS, and from  $2.3 \text{ mg m}^{-2}$  and  $350 \text{ \AA}$  to  $1.1 \text{ mg m}^{-2}$  and  $340 \text{ \AA}$  for MUC5B; Tables 1 and 2). These results also point towards an effect of decreased area for adsorption and/or an increased repulsion between molecules in HWS or MUC5B, and the pre-adsorbed PRP1 film as described above for statherin.

### 3.2. Exchange of statherin/PRP1 by HWS/MUC5B

As the adsorbed amounts of statherin were very low on hydrophilic substrates (Table 1), the fluorescence emissions in the TIRF experiments were too low for any reliable evaluation (data not shown), and no conclusions on exchange phenomenon could be drawn.

On hydrophobized substrates, the TIRF data indicated minor exchange of pre-adsorbed statherin by HWS and MUC5B, respectively (Fig. 3a), which may be explained by the strong binding strengths of proteins that are usually observed on hydrophobic surfaces [36]. The low labelling density of statherin may also result in limited sensitivity and hence possibilities to detect minor exchange.

For PRP1 in sequence with HWS, a partial exchange took place on both types of substrates, where a larger fraction was displaced from the hydrophilic surface (Fig. 3b). This is in line with general observations where pre-adsorbed proteins have shown to be more readily displaced from hydrophilic surfaces [37].

A minor fraction of PRP1 was displaced by MUC5B on hydrophilic surfaces (Fig. 3c). The TIRF measurement of MUC5B added to pre-adsorbed PRP1 on hydrophobized surfaces revealed an increase in fluorescence emission upon adsorption of MUC5B (Fig. 3c). The origin of this increase is at the present unknown. However, it is generally known that the fluorescence

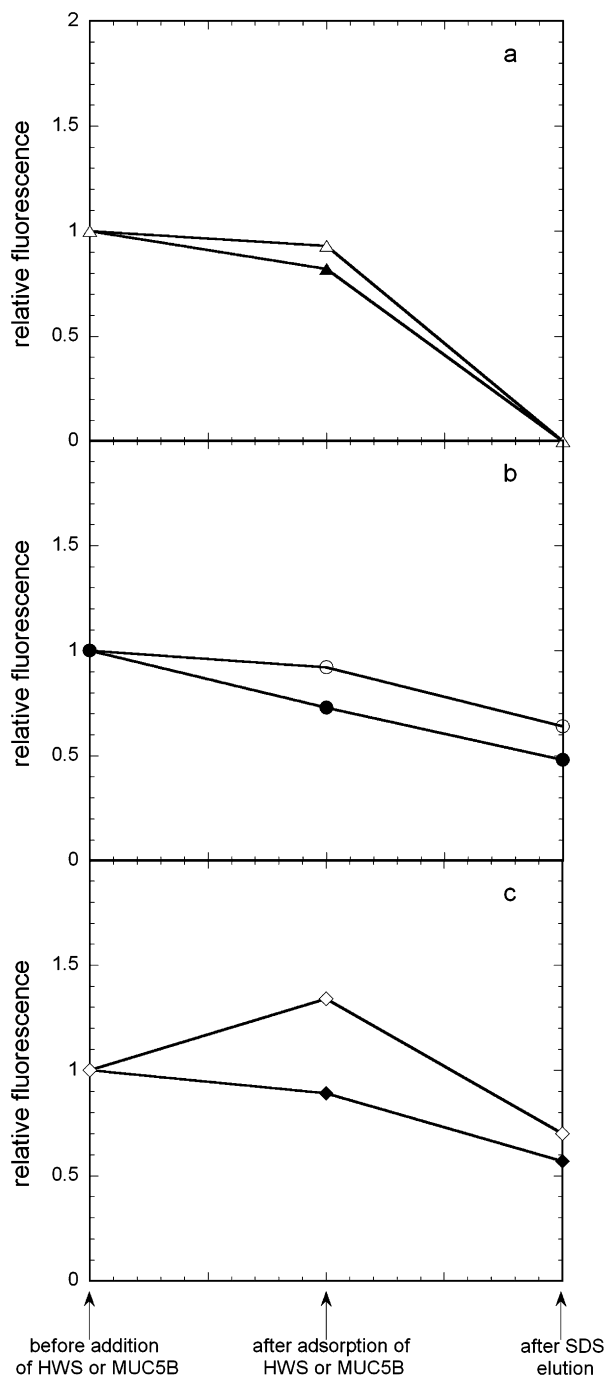


Fig. 3. Relative fluorescence after buffer rinsing of FITC labelled PRP1 from the sequential adsorption of (a) statherin and HWS (▲) and statherin and MUC5B (Δ) both on hydrophobized substrates, (b) PRP1 and HWS on hydrophilic surfaces (●), and on hydrophobized surfaces (○), (c) PRP1 and MUC5B on hydrophilic surfaces (◆), and on hydrophobized surfaces (◇). The arrows indicate changes in the conditions during the experiments. Values are taken 30 min after adsorption and end of rinse. The signal is normalized to the fluorescence emission of adsorbed statherin or PRP1 (recorded after buffer rinsing), respectively, before addition of HWS or MUC5B, respectively.

may change upon the unfolding of proteins, or upon changes in the local surroundings, e.g. pH and hydrophobicity. One possible explanation could therefore be that the adsorption of MUC5B to pre-adsorbed PRP1 results in changes in the conformation of PRP1, or in the local environment surrounding the

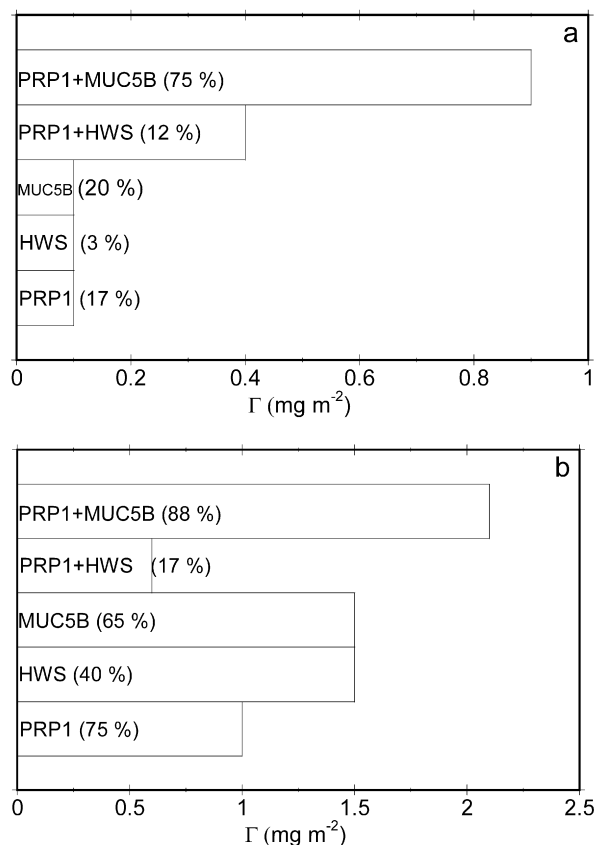


Fig. 4. Amounts adsorbed ( $\Gamma$ ;  $\text{mg m}^{-2}$ ) after SDS elution followed by buffer rinsing of PRP1, HWS, MUC5B, PRP1 + HWS and PRP1 + MUC5B on hydrophilic surfaces (a) and on hydrophobic surfaces (b). Values are taken 30 min after end of rinse. The remaining fraction (in %) as compared to amount adsorbed prior to addition of SDS are also given.

fluorescent molecules, leading to a higher fluorescence signal on this particular surface type.

The pellicle build-up is a selective and dynamic adsorption process and is initially dominated by adsorption of small salivary proteins and peptides, which with time are replaced at the surface by other larger salivary components. However, the findings from the model systems used in the present study indicate that this exchange may be quite limited during the first hour of pellicle formation (i.e. the time course of the experiments). This further indicates that an exchange similar to the one observed in blood, i.e. the Vroman effect [3] does not apply to the same extent in saliva, at least at these experimental conditions. As seen in Fig. 3, the largest exchanges observed were with HWS, which indicate that salivary components other than MUC5B (at least in the purified form used) may be more prone to exchange statherin or PRP1. Possibly, MUC5B is more prone to complex bind with the pre-adsorbed proteins (as described in the next paragraph), than to exchange them at the surface.

### 3.3. SDS elution of mixed films in comparison with single protein films

Generally, adsorbed amounts remaining after SDS elution were larger on hydrophobized substrates as compared to hydrophilic ones (Fig. 4). Mechanisms for SDS elution on sur-

faces with different wettabilities are described in detail in Ref. [38].

On hydrophilic surfaces, SDS elution followed by buffer rinsing of statherin and HWS mixed films resulted in an “irreversibly” adsorbed fraction that was larger than for HWS alone ( $0.25 \text{ mg m}^{-2}$  as compared to  $0.1 \text{ mg m}^{-2}$ ; Table 1 and Fig. 1). This could be due to the higher adsorbed amount of HWS to pre-adsorbed statherin compared to the clean hydrophilic surface, as described above. For statherin adsorption followed by adsorption of MUC5B on the same type of surface, there was no visible difference in elution with SDS between the sequential adsorption and the adsorption of only MUC5B (data not shown).

The TIRF data of the mixed films of statherin and HWS or MUC5B on hydrophobized substrates indicated that SDS elution resulted in complete removal of adsorbed statherin (Fig. 3a). After SDS elution and the subsequent rinse the adsorbed fraction remaining on the surface therefore seemed to consist exclusively of HWS or MUC5B components, respectively. This suggests that the elution of statherin by SDS was not affected by co-adsorption of either HWS or MUC5B.

On hydrophilic surfaces, elution with SDS followed by buffer rinse of the mixed films of PRP1 and HWS resulted in a fraction of “irreversibly” adsorbed proteins that was similar compared to fractions remaining adsorbed after SDS elution of either PRP1 or HWS alone (Fig. 4a). For the sequence of PRP1 and MUC5B the remaining adsorbed fraction after SDS elution was found to be higher than for either MUC5B or PRP1 adsorption alone (Fig. 4a), indicating that the mixed films were less readily desorbed. PRPs/MG1 complexes in solutions have been reported [39], and the present results further suggests that these complexes might form on surfaces as well and are profoundly resistant to SDS elution. One may speculate that this could be due to the fact that these complexes are of higher mass and have more anchoring sites to the surface and furthermore that the complexation might lead to “crosslinking” in the layer. The fluorescence data obtained by TIRF showed that about half or more of the pre-adsorbed PRP1 was still adsorbed after SDS elution for both sequences (Figs. 2 and 3b–3c). This supports the above hypothesis that the mixed films (at least for PRP1 and MUC5B) are less susceptible to SDS elution, and further that both HWS and MUC5B protects pre-adsorbed PRP1 from elution with SDS.

For the mixed films composed of PRP1 and HWS on hydrophobized substrates, the fluorescence signal showed that after SDS elution and rinsing a smaller “irreversibly” adsorbed fraction of PRP1 was left compared to SDS elution of PRP1 alone (65% as compared to 75%; derived from Table 1 and Fig. 3b). Following the ellipsometric data on the total adsorbed amount (which was lower after SDS elution than for either PRP1 or HWS alone (Fig. 4b)), corroborates that adsorbed HWS promotes desorption of pre-adsorbed PRP1 and perhaps also vice versa. As described earlier, pre-adsorbed PRP1 decreased the adsorbed amount of HWS while the average film thicknesses were the same as compared to HWS adsorption alone. These results could indicate the formation of a dif-

fuse/loosely associated film that may be easily penetrated by SDS.

As pointed out above, the fluorescence signal for PRP1 and MUC5B on hydrophobized surfaces was difficult to elucidate, and no conclusions could be drawn on the fraction of PRP1 remaining in the film after SDS elution. From the ellipsometric data (Fig. 4b) it can be established that a similar fraction remained adsorbed after SDS elution of the mixed film as compared to the films of the single components.

#### 4. Summary

The adsorption experiments showed that components from HWS or MUC5B adsorbed to pre-adsorbed layers of statherin or PRP1, respectively. The pre-adsorbed proteins affected the subsequent adsorption of HWS or MUC5B and the effect depended on the underlying surface characteristics as well as the characteristics of the interacting proteins. The adsorbed amounts of statherin and PRP1 on the two types of surfaces, as well as the conformation of these proteins are factors included in this context. Statherin and PRP1 were exchanged to a small extent by HWS and MUC5B, respectively. Further, both HWS and MUC5B appeared to protect PRP1 to some degree from elution with SDS on hydrophilic substrates. These results could have great clinical importance, as these proteins may be protected against oral care products containing SDS.

#### Acknowledgments

We are grateful to Professor Niklas Strömberg at Umeå University for the gift of PRP1 and statherin, and Dr Claes Wickström at Malmö University for purification of MUC5B. This study was supported by research grants from Malmö University, the Institute for Research and Competence Holding AB (IRECO), the Knowledge foundation (KK stiftelsen, Biofilms—research centre for biointerfaces), The Swedish Dental Society, and The Swedish Patent Revenue Fund for Research in Preventive Dentistry.

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