Influence of substratum hydrophobicity on salivary pellicles: organization or composition?

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(Received 26 June 2014; accepted 2 October 2014)

Different physico-chemical properties (eg adsorption kinetics, thickness, viscoelasticity, and mechanical stability) of adsorbed salivary pellicles depend on different factors, including the properties (eg charge, roughness, wettability, and surface chemistry) of the substratum. Whether these differences in the physico-chemical properties are a result of differences in the composition or in the organization of the pellicles is not known. In this work, the influence of substratum wettability on the composition of the pellicle was studied. For this purpose, pellicles eluted from substrata of different but well-characterized wettabilities were examined by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The results showed that substratum hydrophobicity did not have a major impact on pellicle composition. In all substrata, the major pellicle components were found to be cystatins, amylases and large glycoproteins, presumably mucins. In turn, interpretation of previously reported data based on the present results suggests that variations in substratum wettability mostly affect the organization of the pellicle components.

Keywords: salivary pellicle; SDS-PAGE; immunoblotting; cystatin; amylase; mucin

Introduction

The term ‘salivary pellicles’ refers to the films that form immediately on any type of surface when exposed to saliva (Arnebrant 2003). The pellicle fulfils many different functions. These functions include regulation of tooth mineralization and demineralization processes, lubrication, hydration, acting as a diffusion barrier, and buffering ability (Lendenmann et al. 2000; Lussi & Schaffner 2000; van Nieuw Amerongen et al. 2004). Moreover, in an era when industry looks to nature for its ability to develop water-based lubricants, saliva stands out as an example to be mimicked due to its ability to lubricate a broad range of different surfaces (Hahn Berg et al. 2003; Bongaerts et al. 2007; Cárdenas et al. 2008). Consequently, the functions and composition of the pellicle are being studied, so that the function–composition relationship can be reproduced.

However, compositional studies in the pellicle are extremely complex. Saliva is an aqueous biological fluid containing mainly proteins/peptides but also ions, carbohydrates and lipids. The proteinaceous content of saliva is extremely heterogeneous, and more than 2,000 different proteins having been identified so far (Bandhakavi et al. 2009). The inherent surface activity of proteins is the main factor leading to pellicle formation. However, not all salivary proteins form part of the pellicle; this ability having been reported for ~160 proteins and peptides (Siqueira et al. 2012). Thus, pellicle formation is the result of a highly selective adsorption process.

Moreover, this process is influenced by many different factors including the physical and chemical properties of the substratum. It has been shown that the physico-chemical properties of the pellicle such as adsorbed amount (Vassilakos et al. 1992), formation rate (Vassilakos et al. 1992), viscoelasticity (Barrantes et al. 2014), acid-resistance (Sotres et al. 2011) and mechanical stability (Sotres, Pettersson et al. 2012) are dependent on the nature of the substratum on which it forms.

Thus, different substrata lead to different pellicles with different properties when in contact with saliva. This raises the question of whether these differences result from a difference in composition or from a difference in the organization of the constituents. Although the answer will depend on the specific system under consideration, both aspects probably have to be taken into account. It has been shown that different substrata lead to different pellicle compositions (Svendsen & Lindh 2009). But it is also known that individual salivary proteins may adsorb in different orientations on different substrata (Harvey et al. 2011).

This work aims at further exploring the relationship between pellicle composition and substratum properties. Substrata can be characterized in terms of different properties, eg wettability, roughness and charge, and all these properties will have an effect on salivary adsorption and should thus be studied separately. Specifically, this work focuses on the effect of substratum wettability. For this, pellicles which were formed in vitro on model substrata
with similar roughness and charge but with different wettabilities were collected and characterized.

Pellicle composition studies are technically difficult. First, the pellicle has to be collected. Improved harvesting techniques have recently been introduced, eg combining mechanical and chemical collecting methods (Carlén et al. 1998; Yao et al. 2001; Svendsen et al. 2008). For this, a mechanically assisted sodium dodecyl sulphate (SDS) elution procedure was employed. This method, which consists in rubbing surfaces with polyamide fibre pellets soaked in SDS solution, has been verified to be a reliable one for complete pellicle desorption (Svendsen et al. 2008). Several approaches are available for characterizing the proteinaceous composition of the pellicle, including electrophoretic, immunological, histochemical and chromatographic techniques (Hannig & Joiner 2006). Mass spectrometry techniques have facilitated more detailed characterization of the pellicle (Yao et al. 2001). In this work the harvested pellicles were characterized by means of one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Materials and methods

General

All water used was of ultra-high quality, processed in Elgastat UHQ II apparatus (Elga, High Wycombe, UK). Unless otherwise stated, all chemicals were of at least analytical grade (Amersham Biosciences, Uppsala, Sweden; Bio-Rad Laboratories, Sundbyberg, Sweden; VWR International, Stockholm, Sweden; Sigma-Aldrich, Stockholm, Sweden).

Substratum preparation and wettability characterization

Silica surfaces (Semiconductor Wafer, Hsinchu, Taiwan) were used for obtaining substrata with different wettabilities. In all cases, silica surfaces were initially cleaned as described elsewhere (Wahlgren & Arnebrant 1990), rendering highly hydrophilic surfaces. More hydrophobic surfaces were obtained by liquid-phase silanization of these cleaned surfaces. This process involved immersing the surfaces in a dichlorodimethylsilane in trichloroethylene solution at room temperature (~22°C). The dichlorodimethylsilane concentration and the immersion time were varied in order to obtain different surface coverage of methyl groups (Table 1), leading to substrata with different wettabilities. Then, the surfaces were washed three times in trichloroethylene, three times in ethanol, and finally stored in ethanol. Specifically, substrata with four different wettabilities, from now on referred as S1–4, were prepared. S1 refers to the initially cleaned silica surfaces which were additionally cleaned by a 5 min plasma treatment (in a low pressure residual air glow discharge unit, PDC-32 G, Harrick Scientific Corp., Pleasantville, NY, USA). S2–4 refer to silica surfaces methylated (hydrophobized) to a gradually increasing extent. All types of substratum were characterized by means of water contact angle measurements (Table 1). For this, a DSA100 Contact Angle Measuring System (Krüss, Hamburg, Germany) was used (more specific technical details can be found in Sotres, Pettersson et al. 2012).

Saliva collection

Unstimulated human whole saliva (HWS) from four healthy adult male donors was collected by drooling into a chilled tube (Dawes 1974). Collection was performed in the morning at least 2 h after breakfast and oral hygiene procedures. The freshly collected saliva was immediately used without further treatment. The subjects gave their informed consent to participate in the study, which was approved by the committee of research ethics at Lund University (LU 2010/649).

Pellicle formation and collection

Pellicles were formed and collected following identical protocols independently on the substratum on which they were formed. Prior to the film formation, substrata were dried under nitrogen flow. Then, they were placed in shallow Petri dishes filled with freshly collected HWS. Petri dishes were placed on a shaking table and the salivary films were allowed to form for 1 h at room temperature (~22°C). Afterwards, the surfaces were rinsed three times with 1 ml of UHQ water to remove unbound proteins, and then they were blown dry to remove excess water. Pellicle collection was then carried out by rubbing the surfaces with a polyamide fibre pellet (Quick-sticks™, Dentonova AB, Huddinge, Sweden) soaked in 0.5% SDS solution in water,1 and then by two non-soaked fibre pellets (Svendsen et al. 2008). Fibre pellets from each group were pooled in a test tube with 10 µl of 0.5% SDS solution. The test tube was heated for 7 min in a water bath (temperature 85–87°C; Carlén et al. 1998; Milleding et al. 2001). The bottom of the test tube was perforated, and the solution containing the pellicle eluent was recovered in an outer test tube by centrifugation for 2 min at 1,000 rpm in a table-top centrifuge (Mini centrifuge C 7 1200, Labnet International, Wood-bridge, NJ, USA). All samples collected from the same donor on the same type of substratum were pooled and a rough estimation of their protein concentration was carried using a spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA) by measuring the absorbance at 280 nm. For this, the absorption spectrum of protein-free phosphate buffered saline (PBS) buffer was subtracted from that of the eluted pellicles (it was
also checked that SDS absorption at 280 nm was negligible. An extinction coefficient of 1 ml cm$^{-1}$ mg$^{-1}$ was employed in the calculations, a common assumption for unknown protein mixtures. Collected pellicles were designated P1–4, referring to those collected from substrata S1–4, respectively.

In order to validate the harvesting method, the pellets employed in the mechanically assisted SDS elution procedure were stored at −20°C until they were further analysed by means of one-dimensional gel electrophoresis (1-DE). Additionally, the substrata from which the pellets had already been harvested were rubbed once more, this time with polyamide fibre pellets soaked in 0.32 M HCl followed by a final rubbing with a fibre pellet soaked in water. This treatment was repeated twice. Then the solution contained in the pellets was extracted, following the same procedure as described above, for further analysis.

One-dimensional gel electrophoresis (1-DE)

Unless otherwise stated, 15% Ready Gel® Precast SDS-PAGE gels (Bio-Rad Laboratories) were used to study the protein content of the mechanically assisted SDS eluted pellicles P1–4, of the pellets used for this elution, of the HCl solution used for validation, as well as that from HWS. Gels were run at 200 V for ~35–40 min. As a molecular weight (MW) reference, an SDS-PAGE molecular weight standard (Kaleidoscope™ Prestained Standards, Bio-Rad Laboratories) was used.

Before running 1-DE gels, the protein concentrations of all eluted pellicles were spectrophotometrically estimated. Then they were diluted in PBS so that all the eluted pellicles corresponding to a given donor had the same protein concentration, which was the concentration of the pellicle sample with the lowest value collected from that donor. One additional dilution at a 1:1 ratio was performed with SDS-PAGE sample buffer (Laemmli Sample Buffer, 62.5 mM Tris-HCl, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol; Bio-Rad Laboratories) before running 1-DE. Samples were then heated at 95°C for 5 min. Finally, 15 μl of each sample were loaded in the wells of the gels. Specif-

ically, the protein concentration of the samples loaded in the gels shown in Figures 1–4 (which correspond to pellicles collected from donor #1) was ~0.43 mg ml$^{-1}$. The final concentrations of loaded samples from the additional donors are given in Supplementary Figure S1. [Supplementary information is available via a multimedia link on the online article webpage.]

SDS-PAGE was also used to detect any possible proteins that had not been eluted from the fibre pellets used for pellicle collection. For this, two pellets per sample were mixed with SDS-PAGE sample buffer (~10 μl), heated at 95°C for 5 min, and the solution containing the pellets placed in the wells of SDS-PAGE. Clean fibre pellets were also subjected to SDS-PAGE for verifying that they did not contain proteinaceous contaminants. HCl sample solutions were also subjected to SDS-PAGE following the same procedure as described for the eluted pellicles P1–4.

After SDS-PAGE, gels were washed three times with water for 10 min.

Staining procedures

Three different procedures were used for gel staining. Coomassie Brilliant Blue (CBB) was used in its two common modifications, viz. CBB R-250 and CBB G-250. The periodic acid Schiff’s (PAS) reagent was used for the specific detection of glycoproteins.

CBB G-250 staining

Gels were stained by immersing in 50 ml of CBB G-250 solution (Bio-Safe™ Coomassie G-250 stain, Bio-Rad Laboratories) for 3 h. Destaining with water was performed overnight to remove background. Further details of CBB staining can be found elsewhere (Westermeier & Marouga 2005).

CBB R-250 staining

CBB R-250 staining was carried out according to (Beeley et al. 1991). Briefly, gels were fixed and proteins in gels were then stained with 0.1% w/v CBB R-250 (BDH

Table 1. Specifications of the methylation process of the different types of substrata used in this work including (i) methyl groups available in the bulk per total area available for their adsorption and (ii) adsorption time, as well as the resulting water contact angles for each substratum.

| Substrata | Methyl groups available in the bulk per adsorption area (10$^3$ molecules nm$^{-2}$) | Incubation time (min) | Water contact angle |
|-----------|---------------------------------|---------------------|---------------------|
| S1 (clean silica) | – | – | < 5° |
| S2 | 1.7 | 30 | 38.2 ± 1.1° |
| S3 | 3.3 | 30 | 69.7 ± 0.4° |
| S4 | 14 | 90 | 98.6 ± 1.6° |
Laboratory Supplies, Poole, UK) in 40% v/v ethanol and 10% v/v acetic acid in water for 3 h. The destaining process was carried out with 10% v/v acetic acid in water.

**PAS staining**

Gels were fixed in 40% v/v ethanol and 7% v/v acetic acid in water overnight. Then gels were oxidized in 1% w/v periodic acid (Sigma) and 3% v/v acetic acid in water for 1 h. The oxidized gels were washed 10 times with water, for 10 min each wash, and were immersed in Schiff’s reagent (Schiff’s fuchsin-sulphite reagent, Sigma) in the dark for 1 h. The Schiff’s reagent was then discarded, and gels were destained with 0.58% w/v potassium metabisulphite and 3% v/v acetic acid in water until background was eliminated. Further details of PAS staining can be found elsewhere (Westermeier & Marouga 2005).

**Immunoblotting**

Electroblotting of proteins onto PVDF membranes (Immun-blot PVDF Membrane, Bio-Rad Laboratories) was also performed on samples separated by SDS-PAGE. Then, the membranes were blocked in a solution of 5% bovine serum albumin (BSA) (Sigma-Aldrich) and 0.1% Tween-20 (VWR International) in Tris buffered saline (TBS) for 1 h at room temperature.

The membranes were probed with a range of antibodies, diluted in 0.2% BSA in TBS overnight. Samples were tested by using mouse monoclonal antibodies against histatin-1 (MAF487Hu21, Usen Life Science, Hubei, PRC), cystatin-S (Human Cystatin S Antibody, R&D Systems, Minneapolis, MN, USA) and amylase (Amylase (G-10), Santa Cruz Biotechnology, Dallas, TX, USA), and goat polyclonal antibodies against statherin (Statherin (N-16), Santa Cruz Biotechnology, Dallas, TX, USA), salivary agglutinin (SAG) (DMBT1 (P-20), Santa Cruz Biotechnology, Dallas, TX, USA) and proline-rich proteins (PRPs) (kindly provided by Dr A. Carlén, Göteborg University, Sweden).

The primary antibodies were diluted 1:400 (anti-histatin-1) in 0.2% BSA in TBS, 1:1,000 (anti-cystatin-S, amylase, albumin, SAG and PRPs), and 1:2,000 (anti-statherin). The membranes were then incubated in appropriate biotin-conjugated secondary antibodies (1:2,000 in 0.2% BSA in TBS) for 2 h at room temperature, and streptavidin-conjugated phosphate (1:10,000 in 0.2% BSA in TBS) for 1 h at room temperature.

Staining was performed with nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indoly-phosphate (1-Step NBT/BCIP, Thermo Scientific, Waltham, MA, USA). The membranes were washed three times with 0.1% Tween-20 in TBS in each incubation step and prior to the final staining.

**Results**

**Stained SDS-PAGE gels**

A representative SDS-PAGE gel stained with CBB G-250 of P1–4 pellicles corresponding to a single donor is shown in Figure 1 (results for pellicles from the additional three donors are shown in Figure S1). The four different pellicles share striking similarities. The more intense bands were observed at similar apparent MWs, ie ~18 kDa and ~58 kDa (the latter being indeed a double band) for all harvested pellicles independently of the hydrophobicity of the substratum. A broad band centred at MW ~190 kDa was also observed in all harvested pellicles. Interestingly, this band was not observed in HWS (Figure S2) and should therefore have originated from the degradation of high MW saliva components. Nevertheless, some differences between the eluted P1–4 pellicles could also be detected. Pellicles harvested from the more hydrophilic substrata (P1–2) exhibited several bands in the 85–115 kDa range, as well as a band at ~6 kDa, and these bands were hardly detectable in pellicles harvested from the more hydrophobic substrata (P3–4). In turn, bands in the 30–35 kDa range were slightly more intense in the pellicles harvested from the more hydrophobic substrata (P3–4). The most noticeable inter-individual difference was that the pellicles that formed from saliva from half of the donors (Figure S1)
on hydrophobic substrata exhibited a strong band at ~45 kDa. One out of four donors also showed a weaker ~58 kDa band on the pellicles collected from the more hydrophobic substrata than on those collected from the more hydrophilic substrata (Figure S1).

In order to detect the presence of carbohydrate-containing proteins, SDS-PAGE gels of the harvested P1–4 pellicles were also PAS-stained (Figure 2). PAS-stained SDS-PAGE gels showed no significant differences between the pellicles collected from the different substrata. Similar band patterns appeared in all eluted samples, presenting only two glycoprotein bands, one at MW ~180 kDa, and the other at a higher MW (> 400 kDa), which were close to the wells of gel. Interestingly, both PAS-stained bands were slightly more intense in pellicles harvested from the more hydrophilic substrata (P1–2).

SDS-PAGE gels were also stained with CBB R-250 (Figure S3), as this methodology has proven helpful in detecting PRPs (which are stained as pink-violet bands; Beeley et al. 1991). However, whereas such bands were detected in HWS at MW ~28 and 30 kDa, they were not observed in the eluted samples.

In order to verify that the harvesting technique did effectively remove the entire sample from the substrata, different controls were carried out (Figures S4.1 and S4.2). First, SDS-PAGE gels were run with the polyamide fibre pellets used in pellicle collection (Svendsen et al. 2008). Additionally, after harvesting the substrata were rubbed with pellets soaked in diluted HCl (Bennick et al. 1983), and then the solution contained in these pellets was extracted as already described and used to run SDS-PAGE gels also. These gels were then CBB G-250 and PAS stained. The results showed no detectable protein bands in any of the samples, validating the harvesting methodology.

**Immunoblotting**

SDS-PAGE gels were immunoblotted for a variety of salivary proteins. Immunoreactive bands against amylases and cystatins were present in all the harvested pellicles at MW ~56–59 and 18 kDa, respectively (Figure 3a, b), which is consistent with their expected MW and electrophoretic mobility. Immunoblotting for statherin (Figure 3c) resulted in bands (~ 6 kDa) which were more strongly stained in the pellicles harvested from the more hydrophobic substrata (P3–4) than in the pellicles harvested from the more hydrophilic substrata (P1–2).

Blotting with anti-PRP antibodies gave rise to several bands at MW ~28, 30, 40 and 70 kDa (Figure 4). Previous investigations using the same antibody showed a similar band pattern (Carlén et al. 1998). In that study, the band at ~30 kDa was found to be acidic PRPs whereas the bands at 40 and 70 kDa were associated with glycosylated PRPs. Interestingly, all PRP bands were present in the pellicles harvested from all the substrata except for the 40 kDa glycosylated PRPs which were present only in the pellicles collected from the more hydrophilic substrata (P1–2).

In immunoblots of P1–4 pellicles probed with the SAG antibody, only weakly stained band patterns could be observed, whereas a strongly stained band (~250–300 kDa) was present in HWS (Figure S5). Immunoblots for histatin-1 antibody showed that this protein was not detectable in any of the eluted pellicle samples. An
immunoreactive band of histatin-1 was, however, present in HWS at MW ~6 kDa (Figure S5).

**Discussion**

Studying the effect of the substratum wettability on salivary pellicle composition involves three methodological steps: (1) working with substrata with a well-defined wettability and that only differ in this property; (2) formation and collection of pellicles on these substrata; and (3) compositional analysis. Silica surfaces differing in their methylation degree were used as substrata; in this way a broad wettability range can be achieved without significantly modifying either the surface potential (Malmsten et al. 1998) or the roughness (Sotres, Pettersson et al. 2012) of the surfaces. Furthermore, this strategy allows the surface wettability span of most materials exposed in the oral cavity to be achieved (Glantz et al. 1991). Regarding the sample, freshly collected saliva was used to avoid alteration and degradation of the salivary components due to freezing and thawing (Francis et al. 2000). Saliva from different donors was studied separately, instead of pooling saliva, in order to resolve inter-individual differences. Studying pellicles formed in situ might have given additional information as they differ from those formed in vitro (Carlén et al. 1998, 2003). Thus, whereas this work is a reasonable starting point for studying the effect of substratum wettability on pellicle composition, studies on pellicles formed in situ should follow. Nevertheless, in vitro studies are highly interesting per se. For instance, a large amount of research is devoted nowadays to developing lubricants which mimic the lubricity of in vitro formed pellicles. Pellicles were rinsed before collection. This was done to remove unbound, ie bulk, saliva components; however, rinsing also removes the more loosely bound fraction of the pellicle (Vassilakos et al. 1993). In order to minimize this effect, rinsing was done with UHQ water which, due to its extremely low ionic strength, induces a collapse of the pellicle (Macakova et al. 2010) which reduces the effect of rinsing on pellicle composition. The mechanically assisted SDS elution procedure was chosen for pellicle collection as this has been reported to be an efficient method for the complete removal of pellicles formed on enamel both in situ and in vitro (Svendsen et al. 2008). In this study the same technique has been validated for hydrophilic as well as hydrophobic silica surfaces (Figures S4.1 and S4.2).

The proteinaceous content of the pellicles was analysed by SDS-PAGE. This strategy was chosen because the present work aimed at shedding light on the mechanisms underlying the dependence of several physicochemical pellicle properties (eg adsorption kinetics, thickness, viscoelasticity, and mechanical stability) on the wettability of the substratum. It is reasonable to hypothesize that these properties, because they are macroscopic observations, are mediated by those components accounting for most of the pellicle content. While SDS-PAGE cannot be used for a precise quantitative determination of the proteinaceous content of a sample, the intensity of a protein band would still be dependent on the quantitative binding of the dye and, therefore, on the amount of protein.

A first look at the stained SDS-PAGE gels of P1–4 pellicles reveals that they share a similar pattern. In CBB stained gels, the more intense bands, ie those corresponding to MW ~190 kDa, 56–59 kDa and 18 kDa, were found for all the investigated pellicles. Blotting experiments suggested that the double band observed at 56–59 kDa could be attributed to amylases, specifically to the non-glycosylated and glycosylated isoforms, which is consistent with previous reports which employed immunological identification and mass spectrometry methods (Beeley et al. 1991; Carlén et al. 1998; Yao et al. 2001). Blotting experiments also attributed the band observed at ~18 kDa to cystatins (a value fairly close to its MW, and to the SDS-PAGE MW reported in the literature; Henskens et al. 1994). However, even though a monoclonal antibody against cystatin-S was used, ~15% cross-reactivity with cystatin-SA and cystatin-SN is advised by the antibody provider.

The most noticeable difference in the CBB stained gels is the presence of a ~6 kDa protein in the pellicles harvested from the more hydrophilic substrata (P1–2). This MW is similar to that of statherin, a known pellicle component. However, in agreement with Gibbins et al. (2014), blotting experiments showed that statherin adsorbed preferentially on more hydrophobic substrata. The more substantial statherin adsorption on hydrophobicized silica surfaces is consistent with previous reports (Santos et al. 2008; Vitorino et al. 2008). This is explained by the prevalence of the hydrophobic interaction developed between the hydrophobic substrata and the C-terminal part of the protein which also has a hydrophobic character. Moreover, both the silica substrata (Malmsten et al. 1998) and the statherin N-terminal region (Raj et al. 1992) are negatively charged. Thus, as proposed by Lindh et al. (2002), the resulting electrostatic repulsion could be responsible for the weaker presence of statherin in pellicles formed on more hydrophilic substrata. Blotting experiments also showed that the 6 kDa band could not be attributed to histatin-1. Thus, further investigations are needed to identify the pellicle component responsible for this band. Nevertheless, since adsorption on the negatively charged hydrophilic silica substrata would be mainly governed by electrostatic interactions, it is reasonable to expect the 6 kDa band would correspond to a cationic molecule.

The bands stained at MWs in the 30–33 kDa range represent another difference between the harvested
pellicles, as they were more intensively stained in the pellicles harvested from the more hydrophobic substrata. Considering the MW, these bands could be in principle attributed to PRPs. Indeed, blotting confirmed the presence of PRPs in this MW range for all harvested pellicles. However, these proteins should be heavily stained as well with CBB R-250 but they were not in the present experiments. This suggests that, on the pellicles formed on the more hydrophobic substrata, other proteins of similar MW as PRPs should be present as well.

PAS stained SDS-PAGE gels of all the eluted samples demonstrated positive glycoprotein bands at high MW (>400 kDa, close to the wells of gels) and at MW ~180 kDa. It is reasonable to attribute the high MW band to MUC5B and the 180 kDa band to MUC7 (in agreement with previous reports; Soares et al. 2012). Interestingly, whereas the presence of glycosylated PRPs was proved by immunoblotting, their presence was not observed in the PAS-stained gels of any of the pellicles, suggesting that they were only present in a very small amount.

As previously mentioned, in SDS-PAGE gels the more intense bands correspond to the more abundant components. Although some compositional differences between pellicles collected from different substrata and donors were observed, the results here indicate that amylases, cystatins and mucins account for most of the content of the pellicles formed on the substrata employed in this work, independently of their hydrophobicity.

There are some differences in these results compared to those of a recent study (Gibbins et al. 2014), where very small amounts of amylases and MUC5B were found on pellicles formed on similar substrata. The time allowed for pellicle formation is the most noticeable difference between the cited study (20 min) and the present study (1 h). Gibbins et al. (2014) studied pellicles formed on (curved) particles, whereas the present work focused on pellicles formed on planar substrata. It is not known in detail whether differences in surface composition exist between the substrata used in Gibbins et al. (2014) and those used in the current study. These differences suggest that maturation processes and substrata roughness/surface properties might play an important role in pellicle composition.

At this point, it would be enlightening to discuss some of the recent major physico-chemical findings on salivary pellicles taking into consideration the results of this work. For a wide variety of substrata of different hydrophobicities, there is growing evidence (Cárdenas, Arnebrant et al. 2007; Macakova et al. 2010; Sotres et al. 2011; Harvey et al. 2012) that, at least in vitro, pellicles consist of an inner thin dense layer (formed mainly of proteins of relatively low molecular weight) and an outer thick diffuse layer (mainly composed of mucins). The presence of mucins in the outer layer is further supported by studies showing that pellicles and mucin films exhibit similar long-range steric normal forces (Nylander et al. 1997; Dedinaite et al. 2005; Pettersson & Dedinaite 2008) and friction coefficients (Hahn Berg et al. 2003; Pettersson & Dedinaite 2008). Additionally, by means of ellipsometry it has been shown that pellicles formed on hydrophilic and hydrophobized silica exhibit no substantial differences in thicknesses and refractive indices (Cárdenas, Elofsson et al. 2007). Adsorbed amounts depend on substratum wettability. However, they vary by a factor of at most two (Vassilakos et al. 1992) and are therefore in line with the above-mentioned scheme. QCM-D did not reveal any major differences between the viscoelastic properties of pellicles formed on these substrata (Barrantes et al. 2014). Thus, pellicles formed on substrata of different hydrophobicities not only exhibit a similar composition, as shown in the present work, but also a similar organization, as inferred from the literature.

The results of the present work can be used to speculate on the composition of the widely accepted two-layer structure. This work has shown that cystatins, amylases and mucins account for most of the content of pellicles formed from saliva from all donors and on substrata of different hydrophobicities. When protein adsorption occurs from a mixture of proteins, such as saliva, the molecules with highest diffusion coefficients (low MW molecules) and with the highest concentration in the mixture arrive at the surface first (Vroman & Adams 1986). Cystatins, amylases and mucins are highly abundant in HWS (Figure S2). Cystatins and amylases, as they have a lower MW, will diffuse to the substratum faster than mucins. Thus, it is reasonable to speculate that the inner thin dense layer of the pellicles would be composed mainly by cystatins and amylases, whereas the present finding of mucins in all types of pellicles would support them as the major component of the outer thick diffuse layer.

It has also been reported that pellicles formed on substrata of different hydrophobicities exhibit marked differences. Analysis of these differences, taking into account the present results, allows further speculation on the structure of the pellicle inner layer. First, it is known that pellicles form faster on hydrophobized than on hydrophilic silica (Vassilakos et al. 1992; Cárdenas, Elofsson et al. 2007). Cystatins, as they have a lower MW, will diffuse to the substratum faster than amylases. Cystatins are globular proteins which contain plenty of acid and basic residues on their surface (Ramasubbu et al. 1996; Bell et al. 1997). They also contain a wedge-shaped group of mostly hydrophobic residues in their outer surface that constitutes the binding region of cysteine proteases (Grizonka et al. 2001). It is thus expected that cystatins would adsorb on almost any type of surface, becoming the major component of the
fraction of the inner dense layer interacting directly with the substratum. In this scheme, cystatins would interact with the hydrophilic silica surfaces mainly by means of electrostatic forces, whereas the interaction with the hydrophobized surfaces would be primarily driven by hydrophobic forces. This scheme is in agreement with the previously reported kinetic data. The initial rate of adsorption would be governed by that of cystatins, which would be mainly driven by hydrophobic forces, which are of longer range and stronger magnitude than the electrostatic forces that would govern adsorption on hydrophilic surfaces.

It is also relevant that pellicles formed on hydrophilic substrata have a higher mechanical stability than those formed on hydrophobized substrata (Sotres et al. 2011; Sotres, Pettersson et al. 2012). Interestingly, a similar mechanical stability dependence on substratum wettability has been reported for monolayers of proteins with an amphiphilic character (Sotres, Lindh et al. 2012). In the previously described scheme, most of the pellicle proteins interacting directly with the substratum would be cystatins, which also have an amphiphilic character. This similarity further supports cystatins as being the major component of the inner layer of pellicles formed on both hydrophilic and hydrophobized silica substrata.

In this scheme, amylases, because of their higher MW, will probably diffuse towards the substratum at a later stage than cystatins. The interaction between amylases and the already adsorbed cystatins could be due to the interplay of electrostatic and hydrophobic forces, this being highly dependent on the specific orientation of the cystatins in the inner layer. Moreover, amylases are known to interact with mucins also (Iontcheva et al. 1997; Bruno et al. 2005). Thus, in this scheme, they would provide anchoring points for the glycoproteins detected at MWs of 180 and >400 kDa, and associated in this work with MUC7 and MUC5B respectively, which would be the main constituents of the outermost diffuse layer.

The present work investigated how hydrophobizing a negatively charged substratum affects the composition of the adsorbed pellicle. It could be speculated that the observed results could be extrapolated to the case of a positively charged substratum. In this case, the abundant and heterogeneously charged cystatins could still interact with the substratum by means of electrostatic forces, and it would therefore be plausible that they would be the main constituents of the pellicle fraction directly adsorbed on the substratum. However, the situation might be completely different if, for instance, the substratum possessed a specific surface chemistry, as in the case of hydroxyapatite and enamel surfaces. Although these are highly hydrophilic surfaces, they lead to pellicles with a significant amount of phosphorylated proteins such as histatins, PRPs and statherin (Arnebrant 2003), whereas in the present study their presence was found to be minimal if not non-existent. This is because the phosphorylated residues of these proteins develop a strong attractive interaction with the exposed calcium groups on the hydroxyapatite surface (Sotres, Barrantes et al. 2014). Thus, the specific surface chemistry of hydroxyapatite/enamel, not its wettability, would be the main factor contributing to the presence of phosphorylated proteins in the salivary films formed on these surfaces.

Finally, the results presented are relevant for the development of water-based lubricants, especially those mimicking saliva. It is widely accepted that mucins contained in the pellicle play an important role in its low friction coefficients (Sotres & Arnebrant 2013). However, a good lubricant not only reduces friction but also prevents wear, and mucins by themselves do not form strongly attached layers on any type of surface (Sotres, Madsen et al. 2014). Thus, any lubricant containing mucin-like proteins for reducing friction should also contain other components to anchor these proteins to the interacting surfaces (ideally to as many different types of surface as possible); a strategy for this can be suggested from the results presented. Specifically, a lubricant containing a large amount of small heterogeneously charged amphiphilic molecules (such as cystatins) would rapidly lead, upon contact with almost any type of surface, to a layer formed by such molecules. This inner layer could then function, either by itself or by an additional layer such as that constituted by amylases, as the anchoring layer for the lubricious outer layer made-up of mucin-like molecules.

Conclusions

In this work the proteinaceous content of salivary pellicles formed on silica surfaces with step-wise increasing hydrophobicity was characterized by means of SDS-PAGE, the resulting gels being analysed by diverse staining (CBB G-250, CBB R-250 and PAS) as well as by immunoblotting procedures. Whereas some compositional differences were observed between the pellicles harvested from the different substrata, the major components of all substrata, independently of their hydrophobicity, were found to be cystatins, amylases and two large glycoproteins, presumably mucins.

In addition, the well-accepted two-layer model of the pellicle in terms of the results is discussed. A scheme is proposed where the inner dense layer of the pellicle is composed mainly of cystatins and amylases, cystatins accounting for most of the molecules directly adsorbed on the substratum. Mucins are instead the main component of the outer diffuse layer. In this scheme, the hydrophobicity of the substratum does not significantly affect
the composition of the pellicle, but the nature and magnitude of the forces driving the adsorption of the components of the inner layer and, subsequently, their orientation. Despite its simplicity, how this scheme would account for most of the reported physicochemical properties of adsorbed salivary pellicles is discussed.

Acknowledgements

The Anandamahidol Foundation in collaboration with the Swedish Defence Materiel Administration (FMV), Malmö University, the Gustaf Th. Ohlsson Foundation and the Swedish Defence Materiel Administration (FMV), Malmö University, the Gustaf Th. Ohlsson Foundation and the Swedish Defence Materiel Administration (FMV), Malmö

Note

1. On silica surfaces, complete SDS-induced desorption takes place already at a concentration close to the critical micelle concentration (in water 0.25% w/v) (Hahn Berg et al. 2001).

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