Salivary Mucin MUC5B Could Be an Important Component of in Vitro Pellicles of Human Saliva: An in Situ Ellipsometry and Atomic Force Microscopy Study

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This paper describes a combined investigation of the salivary and MUC5B films structure and topography in conditions similar to those found in the oral cavity in terms of ionic strength, pH, and protein concentration. AFM and ellipsometry were successfully used to give a detailed picture of the film structure and topography both on hydrophilic and on hydrophobic substrata. Regardless of the substrata, the salivary film can be described as having a two sublayer structure in which an inner dense layer is decorated by large aggregates. However, the shape and height of these larger aggregates largely depend on the type of substrata used. Additionally, we show that the adsorption of MUC5B is controlled by the type of substrata and the MUC5B film topography is similar to that of the larger aggregates present in the salivary films, especially on hydrophobic substrates. Therefore, we conclude that MUC5B is a major component in the salivary film when formed on hydrophobic substrates. Furthermore, we studied how resistant the salivary and MUC5B films are against elutability by buffer rinsing and addition of SDS solution. We conclude that the adsorbed proteins contain fractions with varying binding strengths to the two types of surfaces. Specifically, we have shown that the large MUC5B biomacromolecules on the hydrophobic substrates are especially resistant to both elution with buffer solution and SDS. Therefore, these large mucins can be responsible for the increased resistance of HWS films on hydrophobic substrates and can protect the intraoral surfaces against surface-active components present in oral health care products.

1. Introduction

All types of surfaces exposed to the oral environment turn out to be coated with an organic film of adsorbed salivary biomolecules[1] denoted by the acquired pellicle.[2] The pellicle formation, which is a process of selective protein adsorption,[3] is referred to as the first event in the formation of dental plaque (biofilms). Pellicle formation has attracted substantial attention because of its wide range of barrier functions dependent on the protein composition of these films, such as lubrication,[4−6] antimicrobial functions,[7,8] protection against wear and repair of both soft and hard tissues,[9,10] and thus protecting individuals from the development of oral diseases. During the last decades, extensive research has been made to chemically characterize pellicles formed in vivo, and on various test surfaces both in vivo and in vitro (for a review, see Lendenmann et al.[11]). Members of different salivary molecular families, which participate in the formation of the in vivo enamel pellicle, have been identified as, for example, acidic proline-rich proteins, the mucin MUC5B, sIgA, statherin, histatins, and cystatin SA-1.[12−15] However, how these pellicle proteins are structurally organized on the tooth surfaces and mucosa is not yet understood.

Techniques generally utilized to collect pellicle material for analysis from dental surfaces have been elution by acids and/or scraping. Both of these techniques involve chemical and mechanical manipulations of the samples, emphasizing the need for nondestructive desorption agents and noninvasive in situ techniques for examination of the surface. Utilizing desorption techniques with no possibility to verify the degree of desorption, for example, incompletely removed surface associated material, may cause variation in results. Yao et al. showed protein composition of desorbed material largely depends on the type of compound used for desorption.[15]

During plaque control treatments, the barrier functions of the films may well be altered. Thus, it is important to obtain a better understanding of protein behavior at the interface to develop products aimed at controlling plaque growth or to develop new or improved salivary substitutes. Natural oral surfaces, as enamel, dentine, and mucosa, as well as artificial dental materials, for example, metals, porcelain, and polymers, cover a wide range of surface characteristics[16] and are all covered with a pellicle. Therefore, it is also important to acquire a better understanding of protein/biomaterial interactions. Because sodium dodecyl sulfate (SDS) is a common surfactant used in many types of oral care products, for example, toothpaste, usually in concentrations up to 2%, it was thought interesting to investigate how SDS solutions interact with adsorbed purified pellicle proteins. Previous reports show that SDS can harm mucosal surfaces[17] while mucins are known to protect intraoral surfaces.[6]

Ellipsometry is especially useful to study biological specimens as it is nondestructive, allows measuring under physiological conditions, and offers the possibility to follow and quantify adsorption/desorption processes in real time. This has been

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confirmed in several studies of various protein systems including in vitro salivary films.[18−22] So far, ellipsometry studies of adsorption from saliva and salivary fractions have reported that human whole saliva, as well as saliva gland secretions exhibit high film forming capacity[20,21,23] and a substantial variation in film forming capacity, exists among protein fractions/purified salivary proteins.[24−26] The atomic force microscope (AFM), on the other hand, is of special interest because it allows for in situ imaging of the adsorbed layers in aqueous solutions. In this way, the response of the film topography to external factors (as, for example, the effect of extensive dilution and addition of surfactants) can be followed.

Bearing in mind the fact that salivary films play a critical role with respect to the surface phenomena taking place intraorally, it was considered of importance to provide more detailed information on some key components in the formation of the pellicle. The present paper deals with adsorbed salivary films from a surface chemical point of view, where the interfacial properties of human whole saliva (HWS) and the salivary mucin MUC5B have been investigated by in situ ellipsometry and AFM imaging. In this way, structural information of the films was obtained regarding the total adsorbed amount, the mean ellipsometric layer thickness, the film refractive index, and the film topography. Furthermore, the resistance of the adsorbed layer against elution by buffer solution and the surfactant sodium dodecyl sulfate (SDS) was investigated.

2. Materials and Methods

2.1. Chemicals. Milli-Q water (Millipore, Bedford, MA), including an ion exchange active carbon adsorption and reverse osmosis, was used in all experiments as well as for surface cleaning (see below) and buffer preparation. All ethanol used was 99.7% pure (Kemetyl AB, Haninge, Sweden). A 10 mM phosphate buffer (PBS) supplemented with 50 mM NaCl and adjusted to pH 7.0 (analytical grade Merck chemicals, Haar, Germany) was used. The surfactant sodium-dodecyl-sulfate (SDS) (>99% pure, pro, Sigma-Aldrich Chemie, Deisenhofen, Germany) was diluted in PBS to a stock solution concentration of 85 mM.

2.2. Salivary Samples. Human whole unstimulated saliva was collected from one healthy adult donor in the morning on the day of experiments as described by Dawes et al.[27] The collection was performed 2 h after breakfast. The donor was considered to be in good oral health upon clinical examination. The sampling method used has established reproducible results,[19−22] as well as the fact that no statistically significant differences were found in adsorbed amounts of salivary components from a number of individual donors.[19,20] The saliva was stored at 4 °C prior to usage for no longer than 30 min and used without further treatment. The protein content of the saliva was determined to be 1.1 mg mL^{-1} using a BioRad Micro assay (Bio-Rad laboratories AB, Sundbyberg, Sweden). The committee on research ethics at Lund University has approved this study (No: LU 518-02). MUC5B was purified from HWS following a modified version[24] of the method described in Wickström et al.[28] The concentration of MUC5B was determined by dialyzation against water, freeze-drying, weighting, and redissolving in buffer.

2.3. Surface Preparation. To cover the span of wetting properties expected for oral and bio surfaces present in the oral cavity, hydrophilic and hydrophobized silica were chosen as model surfaces for this study. Furthermore, these substrates provide smooth surfaces that are easily modified to known surface chemical characteristics.[29,30] The silicon wafers (P-type, boron doped, resistivity 10−20 Ω cm) (Okmetic OY, Espoo, Finland) used had a thermally grown oxide layer of about 30 nm thickness[31] and were cut into pieces of 10 × 30 mm. The silica surfaces were cleaned by immersion for 5 min at 80 °C in NH_4OH:H_2O_2:H_2O (1:1:5) (v/v), rinsing in water, and then further treated by immersion for 5 min at 80 °C in HCl:H_2O_2:H_2O (1:1:5) (v/v). They were then again rinsed in water and finally in ethanol. The surfaces were stored in ethanol until use. Hydrophilic and hydrophobic surfaces were then prepared as described below.

2.3.1. Hydrophilic Surfaces. The pure hydrophilic surfaces, known to possess a net negative charge,[32] were prior to use rinsed in water, ethanol, and water again, then dried in a flow of dry nitrogen, and finally plasma cleaned in low-pressure residual air using a radio frequency glow discharge unit (Harrick Plasma Cleaner PDC-3XG, Harrick Scientific, Ossining, NY).

2.3.2. Hydrophobized Surfaces. The oxidized silicon surfaces, pretreated as described above, were immersed in dichlorodimethylsilane (0.05% solution in trichloroethylene) for 1 h as described elsewere.[29] They were then rinsed and stored in ethanol. Prior to use, the surfaces were again rinsed in water, ethanol, and water, and then dried in a flow of dry nitrogen before use. These silane-treated surfaces have been previously characterized and present a remaining surface charge (ζ-potential of −45 mV) with a water contact angle ≥90°.[30]

2.4. Ellipsometry. In short, ellipsometry is an optical method for the detection of adsorption at an interface. This technique is a nondestructive and sensitive process for determination of the thickness (with angström resolution), refractive index, and mass of thin films.[33] The measurements, with a time resolution of ∼4 s, are carried out in situ without disturbing the adsorption process. The basic principle of this method is that the polarization state of an incident light beam is changed upon reflection at an interface. An automated Rudolph thin film ellipsometer (type 43603-200E, Rudolph Research Analytical, Flanders, NJ) was used. The light source was a xenon lamp, filtered to a wavelength of 4015 Å. A more detailed description of the setup of the instrument is given by Landgren and Jönsson.[34] Using a fixed angle of incidence (67.7°) and wavelength, the changes in polarization depend on the optical properties of the interface and are described by the ellipsometric angles Δ and Ψ. The silica surface was mounted vertically in a thermostated (37 °C) quartz cuvette. Prior to protein adsorption, four-zone measurements were performed in air and in buffer solution, to determine the complex refractive index ($N = n - ik$) of the substrate bulk material as well as the refractive index (n_o) and thickness (d_o) of the outermost oxide layer. HWS or MUC5B solutions were then injected into the cuvette, and the ellipsometric angles 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 film can be solved numerically from the change in the optical angles.[35] However, if the film is non-homogeneous, or there is a low level of adsorption resulting in low resolution in the ellipsometric angles, the estimations of refractive index and thickness are unreliable.[36] From the thickness and the refractive index, the adsorbed amount (mg m^{-2}) can be calculated according to Cuypers model.[36] The values of partial specific volume (ν) and molar refractivity (M/A) used in the present study are 0.75 mL g^{-1} and 4.10 g mL^{-1}, respectively, and represent average values for proteins, which have shown to be valid in previous studies of adsorption of saliva and salivary components.[19,20,26] Errors in the calculated thickness and refractive index co-vary, and, as a result, the adsorbed mass can be calculated with a much higher accuracy than the former parameters.[36] The relative errors in refractive index and layer thickness are rather high for small surface excess (<0.5 mg/m^2), decreasing rapidly to values of approximately 5−10% for surface excess >1 mg/m^2.[37]

When the ellipsometric readings for the bare surface were constant, HWS or MUC5B was added to a final concentration of 10% (v/v) HWS and 50 μg mL^{-1} MUC5B in the cuvette, respectively. After 60 min of adsorption, rinsing with buffer was performed for 5 min to remove non-adsorbed and reversibly bound salivary components. Thereafter, desorption was followed for 30 min. This was subsequently followed by addition of SDS solution to a final concentration of 17 mM (∼8.7*cmc) SDS under the experimental conditions.[19,36] After 5 min, rinsing with PBS at the same conditions as mentioned above was performed, and the measurements were then stopped after 30 min of

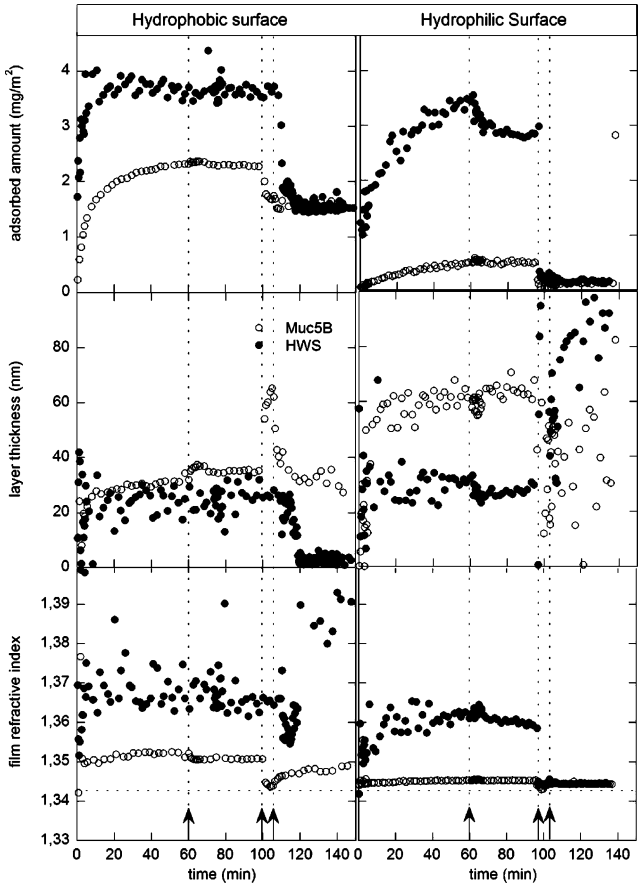

Figure 1. Time evolution of the calculated adsorbed amount, layer thickness, and film refractive index during the build-up of a MUC5B (○) and HWS (●) film on a hydrophilic as well as hydrophobized silica as measured by in situ ellipsometry. The solutions are added at time 0 min. The first arrows indicate rinsing with buffer solution, while the second and third arrows indicate addition of SDS to the cuvette and the subsequent PBS elution.

monitoring. Adsorption measurements were performed at least twice for each solution with variations <5%.

2.5. Atomic Force Microscopy, AFM. AFM imaging in a liquid cell was performed using a scanning probe microscope (Picoforce multimode SPM with a Nanoscope IV control unit, Veeco). A Picoforce z-scanner and a silicon nitride tip (Veeco, type DNP) with a cantilever constant of 0.06 N/m were used. Imaging was performed in soft-contact mode[38] by fixing the surface load to the electrostatic repulsive barrier while using the lowest possible force for imaging. In this way, the probe is not in direct contact with the surface, and imaging of soft layers is possible without alterations caused during the probing. Imaging was performed at room temperature. The height images were subjected to first-order flattening to remove offset and tilt of each line, and the mean roughness was calculated using the software provided by nanoscope. The salivary films were prepared and treated in the same way as for the ellipsometric assay. However, AFM imaging was performed after the liquid cell was subjected to extensive rinsing with buffer solution to avoid scattering problems. The experiments were repeated twice with different salivary batches, and similar results were obtained.

3. Results

Figure 1 gives the time evolution of the three parameters measured by ellipsometry: adsorbed amount, layer thickness, and film refractive index for 10 (v/v) % HWS and 50 μg mL^{-1} MUC5B on both hydrophobic and hydrophilic silica. The reasons for diluting the saliva to 10% are dual: first, it has been shown that at 10% saliva the plateau of adsorption is approached,[20,21] and, second, it helps to avoid problems due to light scattering at higher concentrations (ellipsometry is an optical technique, and although AFM is not an optical technique it requires reflecting a laser beam on the back of the probe). The chosen concentration for MUC5B is based on the previously reported value for its content in saliva.[39,40]

Addition of HWS to both hydrophobic and hydrophilic surfaces leads to an adsorption process reaching plateau conditions already after 60 min of equilibration time at least for the hydrophobic substrate. The total adsorbed amount and film thickness seem to be independent of the type of surface used. For MUC5B, on the other hand, different adsorption patterns are found for the two different surfaces, although semi-plateau regions were found after 60 min of equilibration time in both cases. First, the adsorbed amount is much larger for the hydrophobic substrate (2.4 mg m^{-2}) than the hydrophilic one (0.5 mg m^{-2}). Larger adsorption on hydrophobic than hydrophilic substrates is indeed a common observation for proteins. The hydrophobic moieties of molecules preferably interact with hydrophobic surfaces and usually drive the adsorption process. Second, the adsorbed layer thickness is considerably larger for hydrophilic than hydrophobic surfaces. Note, however, that the ellipsometric thickness values cannot be trusted for MUC5B films on hydrophilic surfaces given that in this case the difference between the film refractive index and that of the bulk solution (indicated by a dotted line in the bottom of Figure 1) is too small. All of the studied films but HWS on the hydrophilic surface are quite resistant to dilution (performed by extensive rinsing of the ellipsometric cuvette with buffer solution). For the latter, about 20% of the film desorbs from the surface (this step is indicated in Figure 1 by the arrow occurring at $t \approx 60$ min). Addition of SDS solution for 5 min and subsequent rinsing with buffer solution (indicated by the second and third arrows in Figure 1, respectively) leads to the removal of a major fraction of the films as observed by the large decrease in adsorbed amount. However, the adsorbed film on hydrophobic surfaces seems to be more resistant to SDS treatment than those on hydrophilic ones. Previous ellipsometric studies on the effect of SDS on salivary films onto hydroxyapatatite indicate that further increasing the concentration of SDS above its cmc does not have an increased cleaning effect.[19] This indicates that the remaining adsorbed fraction has a high binding affinity to the surface and cannot be solubilized by action of this surfactant. The ellipsometric thickness for hydrophilic surfaces after SDS treatment, on the other hand, cannot be trusted because the difference between the film refractive index and that of the bulk is not significantly large (see Figure 1).

Soft-contact mode AFM imaging is a valuable tool to study the topography of HWS and MUC5B on both hydrophilic and hydrophobic silica surfaces. In this mode, the probing force is fixed to the electrostatic repulsive one, minimizing the effect of the probe on the film structure.[38] For both hydrophilic and hydrophobic surfaces, a film covers the entire surface as observed by AFM imaging. A typical representation of the HWS films on the hydrophobic surface is given in Figure 2. The film contains a few larger aggregates that protrude toward the solution. Interestingly, the surface density, size, and shape of these larger aggregates are substrate dependent. Line cuts from the height AFM images are given in Figure 2c for both HWS on hydrophilic and hydrophobic substrates. Note that size measurements in the lateral AFM image can have systematic errors, which can differ from tip to tip.[41] AFM height measurements, on the other hand, do not suffer from this problem and

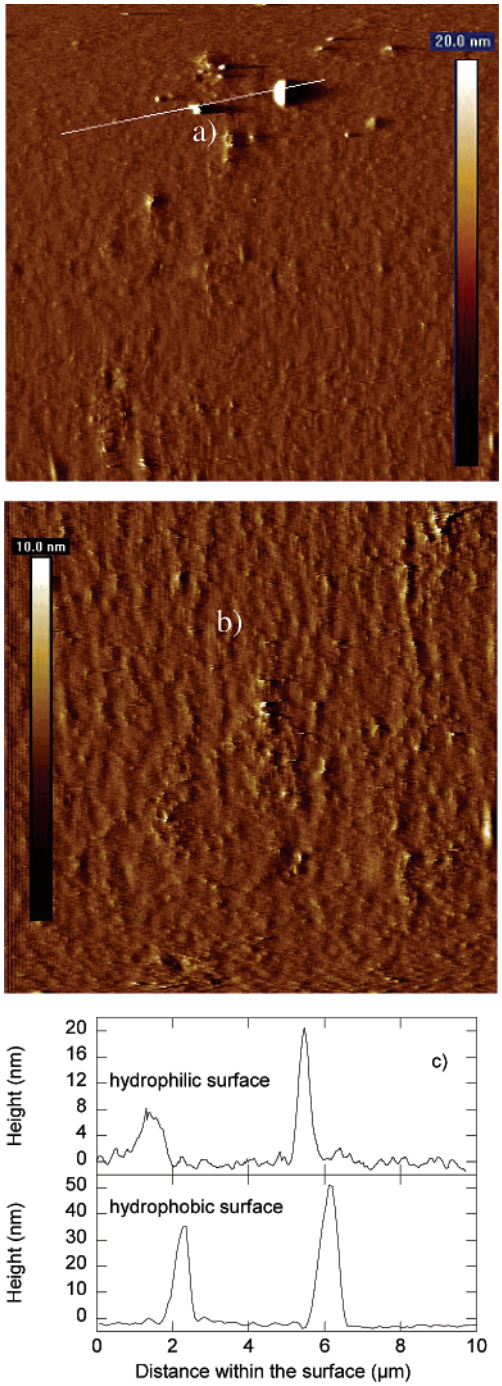

Figure 2. Soft-contact AFM deflection images of a HWS film on the hydrophobic substrate. The image represents an area of 20*20 μm^2 (a). A detail of the surface morphology on a hydrophobic surface (10*10 μm^2) is given in (b). Note the difference in the scale bars. Representative line profiles (from the corresponding height image) for HWS on hydrophilic and hydrophobic surfaces are given in (c) that include the large aggregates present in the film (as an example, see the cut indicated by the white line in (a)).

hence are more reproducible irrespective of the tip geometry.[41] Therefore, only the height measurements are used to systematically compare the difference in the size of the large aggregates present in various films studied. Large elongated aggregates are present on hydrophobic surfaces, while smaller globular aggregates are present on hydrophilic silica. Additionally, the root square roughness of the areas between the large aggregates is at least twice the one for clean surfaces prior addition of HWS, regardless of the type of surface used (see Table 1). Such

Table 1. Root Square Roughness of the Height AFM Images Excluding the Regions Where Large Aggregates Are Present

	root square roughness (nm)[a]	
	hydrophobic substrate[b]	hydrophilic substrate[b]
clean surface prior to adsorption	0.35 ± 0.13^3	0.25 ± 0.09^3
HWS	0.65 ± 0.12^1	0.85 ± 0.31
after SDS treatment	$0.41 \pm 0.11^{1,3}$	0.31 ± 0.10^3
MUC5B	1.17 ± 0.21^2	0.72 ± 0.20
after SDS treatment	0.83 ± 0.25^2	0.46 ± 0.12

[a] The root square roughness is the average between the height deviations and the mean line/surface, taken over a determined length/area. [b] Values are averages and standard deviations of at least seven different height images. Values that are statistically equal to each other are marked with equal symbols:[1,2,3].

increased roughness corroborates the formation of an inner denser film on the surface. A closer look of the HWS film on the hydrophobic surface (10*10 μm^2 image is given in Figure 2b) reveals the rich topography of the inner film. Similar topographical features for the inner film were found on the hydrophilic surface.

Figure 3 gives deflection soft-contact AFM images of MUC5B films on (a) hydrophilic (10*10 μm^2) and (b) hydrophobic substrates (40*40 μm^2). The topography of the MUC5B films is dramatically different depending of the substrate used. Only a few globular aggregates are present on the hydrophilic surface. Note that these aggregates do not form a uniform film but instead adsorb as individual "droplets" on the surface. On the hydrophobic surface, however, a uniform film is found that is decorated by large, elongated aggregates similar to those in the HWS film formed on the same type of surface. Image shadows of the large aggregates are consistently present regardless of the scan direction and angle (see Figure 3b). Velegol et al.[42] found the same type of shadows when imaging bacteria. The authors demonstrated that these shadows are artifacts that arise from the interaction of the sides of pyramidal tip with substrates that have large height relative to the size of the tip. Representative line profiles from the height images are given in Figure 3c. It is evident the remarkable difference in topography between the films on the two different types of surface. The maximum height in the adsorbed layer on hydrophobic substrates reached 120 nm. On the hydrophilic surface, however, the droplets do not exceed 10 nm in height. Moreover, the root square roughness for HWS on the hydrophilic surface is larger than that of the clean surface (see Table 1). The same effect is observed for the HWS film on hydrophobic surfaces (calculated, in this case, by excluding the large aggregates present in the film).

Finally, 40*40 μm^2 deflection soft-contact AFM images were acquired after the various salivary films were treated with SDS and subjected to extensive rinsing with buffer solution. The topography of the SDS-treated HWS (Figure 4a) and MUC5B (not shown) films on hydrophobic surfaces dramatically differs from that prior to SDS treatment. For HWS, an apparent increase in the number of large aggregates occurs, although they are smaller in height after SDS treatment. Only relatively few large aggregates are left on the surface for MUC5B. These aggregates are much smaller in length, although they have the same height as before the SDS treatment (compare the line profiles given in Figure 4b to Figure 2c/Figure 3c). Moreover, the root square roughness for HWS and MUC5B films on hydrophobic surfaces is not affected by SDS treatment, being larger than that for the bare clean surfaces at least for MUC5B (Table 1). This indicates that the inner layer is not fully removed. For a hydrophilic

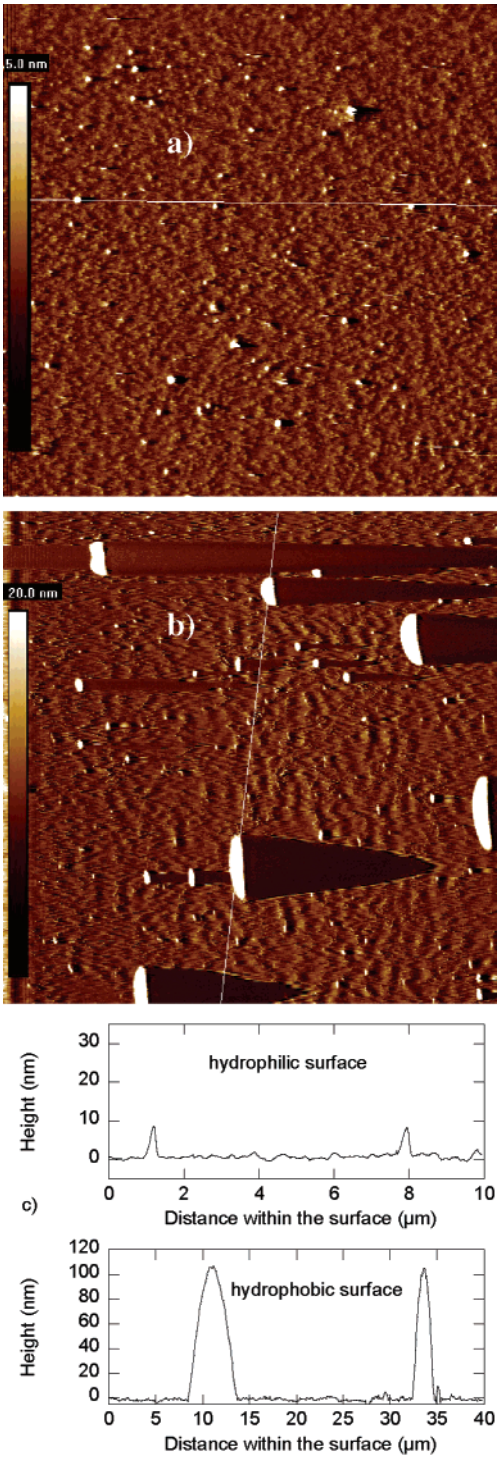

Figure 3. Soft-contact AFM deflection images of a MUC5B film on (a) hydrophilic (10*10 μm^2) and (b) hydrophobic surfaces (40*40 μ^2). Note the difference in the scale bars. Line profiles (from the corresponding height image) of the cuts indicated by a white line are given in (c).

surface, on the other hand, SDS treatment and the subsequent rinsing with buffer induce complete desorption of the films, yielding the same square mean roughness as for clean bare surfaces (Table 1).

4. Discussion

4.1. MUC5B Films. The structure of the MUC5B films largely depends on the type of substrata used (Figures 1 and 3). The measured adsorbed amounts for MUC5B films both on hydrophilic and on hydrophobic substrata are in agreement with previous studies.[24] The higher affinity of MUC5B to hydrophobic substrates (larger adsorbed amount for the same bulk concentration) is typical of proteins due to favorable hydrophobic interactions between protein segments and the surface.[43] AFM images show large elongated aggregates only on hydrophobic surfaces. The length of these aggregates can reach up to 8 μm. Such tremendous dimension of the aggregates is not surprising given the structure of MUC5B. MUC5B are giant biomacromolecules (>11 MDa) having an oligomeric structure composed by different regions of peptide chains with various degrees of glycosylation (carbohydrates comprise ~80% of MUC5B).[28,44] The radius of gyration for MUC5B isolated from HWS was reported to be 230 nm,[45] the molecule having properties similar to a rigid coil as measured by light scattering[46] with length that can reach up to 10 μm. These values are well in accordance with the dimensions of the observed larger aggregates on the hydrophobic substrate (Figure 3b), although smaller globular and elongated aggregates are also present. Adsorption to the hydrophobic substrate can induce large conformation changes in the MUC5B macromolecules. Moreover, the observed adsorbed structures could be smaller fractions of MUC5B that break off during the purification procedures. The MUC5B biomacromolecule can be disassembled into its constituent units (2−3 MDa) and then into high molecular mass glycopeptides (0.3−0.5 MDa) commonly known as T-domains. The length of the subunits is reported to be 200−600 nm.[46]

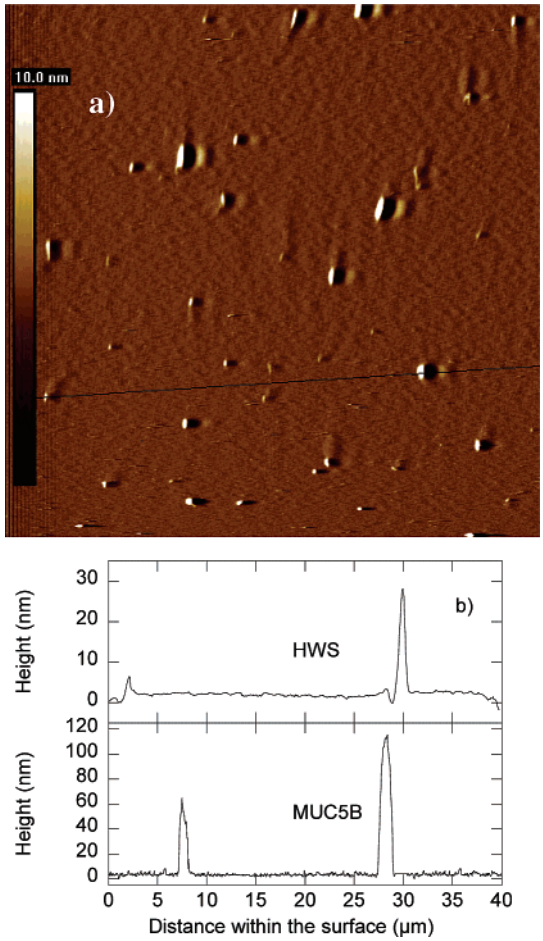

Figure 4. Soft-contact AFM height image for the remaining HWS after treatment with SDS on a hydrophobic surface (a). The image represents an area of 40*40 μm^2. Representative line profiles for HWS and MUC5B films after SDS treatment are given in (b).

The fact that only small globular aggregates are present on the hydrophilic surface indicates that the electrostatic repulsion between the negatively charged MUC5B biomacromolecules and the similarly charged surface determines the adsorption process in this case. Thus, only smaller fractions of the less glycosylated domains should be able to adsorb on the hydrophilic surface. Interestingly, earlier static light scattering studies show that rigid coils are expected for whole MUC5B and its subunits while random coils typically describe the structure of T-domains in bulk solution.[47] Such rigid coils for the T-domains are indeed in agreement with the smaller elongated adsorbed structures observed. However, we cannot disregard changes in the MUC5B biomacromolecule conformation upon binding to the surface that could partially compact the molecule in a similar way to the adsorption of DNA on hydrophobic surfaces.[48]

4.2. Salivary Films. HWS addition to both hydrophilic and hydrophobic surfaces leads to the formation of a film as indicated both by ellipsometry (Figure 1) and by AFM imaging (Figure 2). Such films present quite a complex topography. The root square roughnesses calculated from the AFM images (excluding the areas where aggregates with >20 nm in height are present) indicate that an inner dense film covers the entire surface for both types of substrata (Table 1). This inner film is decorated by large elongated aggregates that protrude toward the bulk. The length and height of these aggregates depend on the type of substrata used. They are larger both in length and in height on hydrophobic substrata (they can reach up to 120 nm). The ellipsometric thickness, however, is ~30 nm regardless of the substrata used. Note that the model used to interpret the ellipsometric data assumes an even layer of constant refractive index. Such assumption is incorrect as demonstrated by AFM. Therefore, the ellipsometric thickness represents an "average" thickness over the whole adsorbed layer, and thus it is not surprising that similar values are found for both types of substrate: smaller (typically 10 nm in height) but considerably denser aggregates are present on the hydrophilic surfaces as compared to the hydrophobic one. The inner layer is more likely to be a monolayer of various globular proteins present in saliva, whereas mucins are most likely involved in the outer layer. AFM imaging cannot provide the real thickness of this inner layer as it only probes the outer lateral morphology of the film. In this regard, efforts to open a scratch in the film by applying large force loads (within the compliance region) were unsuccessful, corroborating the large resistance that these films have against mechanical stress.[4] Recent neutron reflectivity measurements of salivary films on sapphire show that the film is also composed of two sublayers, the inner being denser and with a thickness of 4 nm, while the outer is diffuse and thicker (~30 nm).[49] These data suggest that the inner layers should be a monolayer of small globular proteins. Adsorption of HWS has previously been studied by ellipsometry on various model surfaces, that is, enamel, HA, silica, and hydrophobically modified silica.[18−22,26] These studies, however, only report the total adsorbed amount to the various surfaces. It must be pointed out that our results are well in accordance with these earlier reports. In a separate study, Nylander et al.[50] measured the thickness of adsorbed films from HWS on mica surfaces using the surface force apparatus. Their force−distance curves show the onset of a nonelectrostatic repulsive force at 80 nm likely to be due to steric hindrance between the large aggregates present within the film, well in accordance with our results. Interestingly, earlier transmission electron microscopy and AFM studies on the in vivo formed pellicles on enamel surfaces that were dried prior to imaging also indicate that the salivary pellicle on enamel is composed of two types of layers: a basal layer on top of which larger proteins were adsorbed, the latter having a height larger than 30 nm.[51,52] Rykke et al.[53−55] showed that large aggregates are present in parotid saliva both in bulk saliva and in the acquired pellicle on enamel surfaces. These aggregates were negatively charged and presented a mean hydrodynamic radius of 100−150 nm but increased in size with time when resting after sampling. The authors claimed that such aggregates are micelle-like structures built up by amphiphilic salivary proteins. We believe, however, that the giant negatively charged mucins should be largely responsible for this type of structure, although cationic proteins such as lactoferrin and lysozyme have been shown earlier to be present in the mucus gels of saliva.[21] The presence of such cationic proteins can contribute to cross-linking of different mucin molecules and thus inducing particle aggregation and eventually precipitation.

At this point, we would like to stress the striking similarity between the larger aggregates present for HWS and MUC5B films on hydrophobic substrata. They are not only similar in shape but in height (note the different scan sizes in Figures 2a and 3b: 20 and 40 μm, respectively). This might indicate that MUC5B is present within the salivary film and thus can be responsible for many of the film protective functions such as anti-microbial protection and/or lubrication given that it imparts a high negative charge to the surface as well as it protects the inner, denser salivary layer by, for instance, steric effects. The length of the large aggregates is smaller for HWS than MUC5B films. This can be explained by the proteolytic degradation of MUC5B that readily occurs in fresh saliva.

4.3. Elutability by Buffer and Surfactant. Eluting the salivary and MUC5B films with buffer solution and the anionic surfactant SDS provides information on the binding strength of the adsorbed proteinaceous films at the solid−liquid interface, which in turn is of fundamental interest in studies of adhesion phenomena related to oral biofilm formation. Moreover, it is known that SDS removes proteinaceous films in general[31] and is commonly used in dental hygiene products. The mechanism behind this was suggested to be repulsion between highly soluble protein−surfactant complexes and the negatively charged silica surface.[31] In the present study, rinsing the proteinaceous films with buffer solution had a minor effect on the adsorbed amount and layer thickness in general. A small fraction of the HWS film desorbs from hydrophilic surfaces (Figure 1), in line with earlier reports.[20,21] Such desorption is accompanied by a decrease in the film thickness. The presence of a loosely bound fraction that desorbs upon rinsing can explain such desorption and has been described earlier in the literature.[56] On the other hand, addition of SDS led to dramatic changes in the film structure. Both ellipsometry and AFM show that SDS efficiently removes the adsorbed layers or both HWS and MUC5B on hydrophilic surfaces. These results agree with previous studies of SDS elution on salivary films on silica surfaces.[19] On hydrophobic substrata, however, SDS causes partial desorption of the adsorbed layer for both HWS and MUC5B. AFM shows that, indeed, SDS only induces partial desorption of the film under these experimental conditions where the larger aggregates seem to resist SDS addition although their shape changes: they are not only smaller but also less elongated. Interestingly, the surface density of these large aggregates seems to increase upon SDS addition to the HWS film on the hydrophobic surface (compare Figures 2a and 4a). Such apparent increase in surface density of the larger aggregates could be due to the solubilization by complexation with SDS of some proteins that act as compacting (or cross-linking) agents. Such agents can change the structure

of mucin layer in a manner similar to their bulk behavior.[57−59] In this regard, recent studies performed in our group[60] on the interactions between MUC5B and various small cationic salivary proteins at surfaces show that specifically lactoperoxidase induces large structural changes in the adsorbed layer of MUC5B. Indeed, lactoperoxidase and MUC5B can be used to build multilayers at hydrophilic and hydrophobic surfaces.[60] We speculate that removal of such small cationic proteins by SDS from the salivary film can therefore release constrains within the large mucin biomacromolecule as observed in the AFM images. Interestingly, the same adsorbed amount after SDS treatment of HWS and MUC5B films on hydrophobic surface (Figure 1) is found, which, in combination with the AFM data, indicates that indeed the same type of aggregates remains adsorbed in both cases, some of the large MUC5B biomacromolecules. In conclusion, the large mucin MUC5B may be a very important component of the salivary pellicle because it protects the various oral surfaces against SDS, a typical surfactant present in oral health products.

5. Conclusions

The structures of the salivary and MUC5B films are presented in this study in terms of total adsorbed amount, layer thickness, and layer morphology as studied by AFM and ellipsometry. The salivary film can be described as having a two sublayer structure in which an inner dense layer is decorated by large aggregates. The shape and size of these aggregates largely depend on the type of substrata used. Moreover, our results indicate that MUC5B might be the main component of the salivary film on hydrophobic substrates. Furthermore, the different elutability upon buffer rinsing and addition of SDS solution indicates that the adsorbed proteins contain fractions with varying binding strengths to the two types of surfaces. Specifically, we have shown that the large MUC5B biomacromolecules are especially resistant to both elution with buffer solution and SDS. Therefore, these large mucins can be responsible for the increased resistance of HWS films on hydrophobic substrates and might protect the intraoral surfaces against surface-active components present in oral health care products.

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