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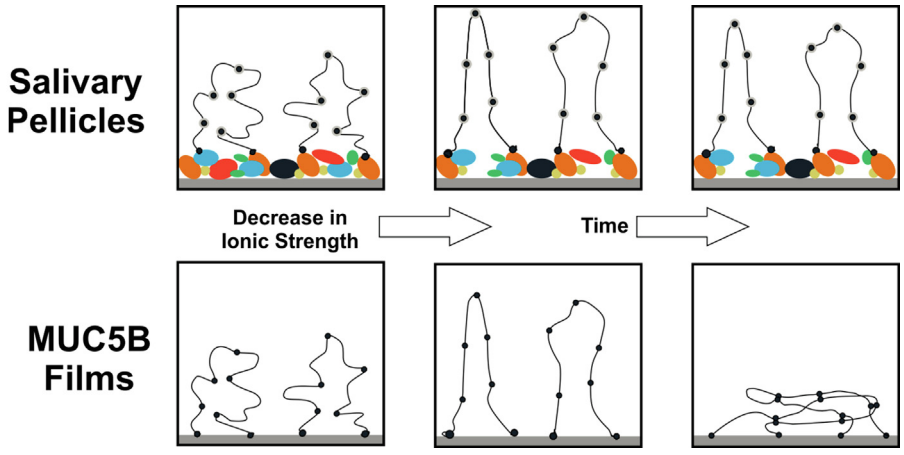
Regular Article

A comparison between the structures of reconstituted salivary pellicles and oral mucin (MUC5B) films

Hannah Boyd [a,*], Juan F. Gonzalez-Martinez [a], Rebecca J.L. Welbourn [b], Philipp Gutfreund [c], Alexey Klechikov [c,d], Carolina Robertsson [e], Claes Wickström [e], Thomas Arnebrant [a], Robert Barker [f], Javier Sotres [a,*]

[a] Department of Biomedical Science & Biofilms-Research Center for Biointerfaces, Malmö University, 20506 Malmö, Sweden
[b] ISIS Facility, STFC, Rutherford Appleton Laboratory, Chilton, Didcot, Oxon OX11 0QX, UK
[c] Institut Laue Langevin, 71 avenue des Martyrs, Grenoble 38000, France
[d] Department of Physics and Astronomy, Uppsala University, 75120 Uppsala, Sweden
[e] Department of Oral Biology and Pathology & Biofilms-Research Center for Biointerfaces, Malmö University, 20506 Malmö, Sweden
[f] School of Physical Sciences, University of Kent, Canterbury, Kent CT2 7NH, UK

GRAPHICAL ABSTRACT



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ABSTRACT

Hypothesis: Salivary pellicles i.e., thin films formed upon selective adsorption of saliva, protect oral surfaces against chemical and mechanical insults. Pellicles are also excellent aqueous lubricants. It is generally accepted that reconstituted pellicles have a two-layer structure, where the outer layer is mainly composed of MUC5B mucins. We hypothesized that by comparing the effect of ionic strength on reconstituted pellicles and MUC5B films we could gain further insight into the pellicle structure.
Experiments: Salivary pellicles and MUC5B films reconstituted on solid surfaces were investigated at different ionic strengths by Force Spectroscopy, Quartz Crystal Microbalance with Dissipation, Null Ellipsometry and Neutron Reflectometry.
Findings: Our results support the two-layer structure for reconstituted salivary pellicles. The outer layer swelled when ionic strength decreased, indicating a weak polyelectrolyte behavior. While initially the MUC5B films exhibited a similar tendency, this was followed by a drastic collapse indicating an

* Corresponding authors.
E-mail addresses: hannah.boyd@mau.se (H. Boyd), javier.sotres@mau.se (J. Sotres), javier.sotres@mau.se (J. Sotres).

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interaction between exposed hydrophobic domains. This suggests that mucins in the pellicle outer layer form complexes with other salivary components that prevent this interaction. Lowering ionic strength below physiological values also led to a partial removal of the pellicle inner layer. Overall, our results highlight the importance that the interactions of mucins with other pellicle components play on their structure.

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1. Introduction

When exposed to saliva, oral surfaces are rapidly covered by a nm-thick proteinaceous film known as the salivary pellicle that confers extraordinary protection against chemical and mechanical insults [1,2]. In this regard, the high performance of salivary pellicles as boundary lubricants has attracted significant interest. For smooth tribological contacts, they provide significantly low friction coefficients (0.01–0.25 [3–7]). Moreover, with yield strengths of ~100 MPa [8], salivary pellicles also present a high resistance to wear. What makes salivary pellicles unique among other biological lubricants is that they provide these low friction coefficients and high resistance to wear to a wide variety of tribocontacts regardless of many of their physico-chemical properties e.g., wettability. Understanding the mechanism behind this outstanding lubrication performance is of significant relevance for different applications. It would help to better understand diseases associated with loss of salivary lubrication and, therefore, for developing appropriate treatments. Moreover, it would also help in mimicking Nature's water-based lubricants like saliva, which are characterized by a performance and cleanliness that surpass that of oil-based lubricants currently used in man-made devices.

Understanding salivary boundary lubrication requires knowledge on both the composition and structure of salivary pellicles. However, while their composition has been extensively investigated [9,10], much is yet to be known on the structure of salivary pellicles. In the case of salivary pellicles reconstituted at solid-liquid interfaces, several works point towards a two-layer model with an inner thin dense layer, mainly formed by low molecular weight proteins, and an outer thick diffuse layer [8,11–13]. The outer layer is believed to be formed mainly by the oral mucin MUC5B. This is supported e.g., by the similarity between long-range steric normal forces exhibited under mechanical confinement by salivary pellicles and mucin films [14–17]. The presence of salivary mucins in the outer layer of reconstituted pellicles is also supported by the fact that mucin films present excellent lubrication performance similar to that of pellicles [18]. Indeed, mucin and mucin-like molecules are acknowledged as one of the key components of boundary biological lubricants. In a similar way to hydrophilic polymer brushes, it has been proposed that the strong electrostatic repulsion between anchored mucins coupled to their high hydration lowers energy dissipation when exposed to shear [19,20]. However, mucins are not the only lubricious component of salivary pellicles [21]. Moreover, mucins have a tendency to interact with other biological molecules [22,23], and it is known that the lubrication performance of mucin films can be improved, becoming closer to that of salivary pellicles, if mixed with other salivary fractions [17].

Thus, while a number of works indicate that an outer layer of anchored oral mucins mixed with other salivary components mediates the highly efficient boundary lubrication exhibited by reconstituted salivary pellicles, the nature of these additional pellicle components and the mechanisms by which they influence the structure and lubrication properties of mucins remains unknown. The goal of this work was to shed light into these aspects by comparing how ionic strength affects the structure of both salivary pellicles and oral mucin (MUC5B) films reconstituted on model solid surfaces.

2. Materials and methods

2.1. Chemicals

All water used was of ultrahigh quality (UHQ), processed in an Elgastat UHQ II apparatus (Elga Ltd., High Wycombe, Bucks, England). PBS buffer was prepared from tablets from Sigma Aldrich according to their instructions resulting in 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer solution (pH 7.4 at 25 °C). Samples were investigated either in this PBS buffer, or in what we named PBS/10 and PBS/100 where the original PBS buffer was diluted 10 and 100 times respectively in UHQ water. For neutron reflectivity experiments, deuterium oxide (D_2O) of 99.9% purity was used (ref: 151882, Sigma-Aldrich, Germany). Dichlorodimethylsilane (≥99.5%, ref: 440272), trichloroethylene (≥99.5%, ref: 251402), ammonia (≥99.95%, ref: 09682) and hydrogen peroxide solution (30 wt% in H_2O, ref: 216763) were also obtained from Sigma Aldrich. Hellmanex II was obtained from Hellma GMBH & Co (ref: 9-307-011-4-507). Unless otherwise specified, all other chemicals used were of at least analytical grade.

2.2. Cleaning and hydrophobization of silica substrates

Silica surfaces were used in all experiments. For force spectroscopy and ellipsometry, p-doped (boron) silicon wafers (Semiconductor Wafer Inc., Hsinchu, Taiwan) were used, in QCM-D experiments, gold coated quartz chips, further coated with a silica layer (Q-sense AB, Sweden) were used. Each surface was cleaned with 5 min plasma treatment, immersion into a Hellmanex II 2% v/v in water solution for 10 min, rinsed with UHQ water and a final 10 min plasma treatment. This resulted in hydrophilic surfaces with water contact angles < 5°. For Neutron Reflectivity (NR) measurements, single crystal silicon (100) blocks (polished by Sil'tronix Silicon Technologies, Archamps, France to a 5 Å RMS roughness) were used after cleaning using RCA protocol with 5:1:1 H_2O:NH_3:H_2O_2 at 80 °C for 10 min followed by additional 10 min of UV/ozone cleaning.

In MUC5B investigations, hydrophobization of the silica surfaces was achieved by means of liquid-phase silanization [24]. Specifically, clean and dried silica surfaces were immersed in a solution containing 25 μL of dichlorodimethylsilane and 50 mL of trichloroethylene for one hour. After silanization, the surfaces were washed three times in trichloroethylene and three times in ethanol. The water contact angle after hydrophobization was determined to ~90°. The surfaces were stored in ethanol until use.

2.3. Saliva collection

For saliva experiments, unstimulated human whole saliva (HWS) was collected from two healthy donors using the protocol described in [25], pooled and then used immediately. Ethical

approval was obtained from the committee of research ethics at Lund University (2018/42).

2.4. Human salivary MUC5B

MUC5B was isolated from human whole saliva as previously described [26] using a modified version of the method described in [27]. In short, whole saliva was mixed with an equal volume of 0.2 M NaCl followed by incubation overnight with stirring at 4 C. After gentle centrifugation (4400 g for 30 min at 4 uC), the supernatant was subjected to density-gradient centrifugation in CsCl/ 0.1 M NaCl (Beckman Optima LE-80 K, rotor 50.2Ti, 36 000 r.p.m., 96 h, 15 C, start density 1.45 g ml). Fractions were analyzed for density by weighing and measuring absorbance at 280 nm. MUC5B-containing fractions were identified using an antiserum, LUM5B-2, which recognizes the central domain of the MUC5B polypeptide backbone [28]. The MUC5B-containing fractions were pooled and dialysed against PBS (0.15 M NaCl, 5 mM NaHPO, pH 7) and then stored at −20 C until used. MUC5B concentration was determined by extensive dialysis against water, freeze-drying and weighing, and estimated to be 0.3 $mg{\cdot}ml^{-1}$.

2.5. Force spectroscopy

A commercial Atomic Force Microscope (AFM) setup equipped with a liquid cell (MultiMode 8 SPM with a NanoScope V control unit, Bruker AXS, Santa Barbara CA) was employed for the acquisition of force ramps. Rectangular silicon nitride levers with a nominal normal spring constant of 0.1 $N{\cdot}m^{-1}$ were employed in all the experiments (OMLC-RC800PSA, Olympus, Japan). Before every experiment, tips were rubbed against a clean freshly cleaved mica surface, a procedure that also leads to the removal of asperities and achieves a smooth tip surface [29].

For force spectroscopy experiments, cleaned hydrophilic (for salivary pellicles) or hydrophobized (for MUC5B films) ~1 cm^2 silica surfaces were drop-coated with 100 μL of the investigated sample and subsequently rinsed with PBS buffer after 1 h. Samples were then immediately placed on the AFM for visualization, ensuring they did not dry at any time. For subsequent AFM investigations, the solution was exchanged with diluted PBS buffers and finally with UHQ water.

Force ramps were obtained at different lateral positions by operating the AFM in the force volume (FV) mode [30] and analyzed with the FSAS software (https://github.com/JSotres/FSAS-ForceSpectroscopyAnalysisSoftware-MatLab). Specifically, FV measurements consisted of 64x64 force ramps obtained at a speed of 1 $\mu m{\cdot}s^{-1}$ over an area of 2 μm × 2 μm. Force ramps consist of successive displacements of the sample towards and away from the tip, always performed at different lateral positions when operating in the FV mode, while registering the deflection of the cantilever by monitoring a laser beam reflected at the backside of its free end with a position sensitive photodetector (PSD). The PSD signal was converted into deflection units by scaling with a factor obtained from a linear fit of the contact region of force ramps obtained on clean mica surfaces. The cantilever deflection, d, was scaled by the cantilever spring constant, k, to obtain the probed load force, F. The cantilever spring constant, k, was calculated for each cantilever by means of the Sader method [31].

Force ramps were then transformed into a force vs probe-sample distance representation by means of the procedure detailed in [32]. Specifically, the point where mechanical contact between tip and sample was established during approach ramps was found by fitting the contact region of the curve with the Hertz contact model for a sphere-plane geometry:

$$F_{Hertz}(\delta) = \frac{4ER^{1/2}}{3(1-\upsilon^2)}\delta^{3/2} \quad (1)$$

where E is the Young's modulus of the sample, R is the radius of the tip apex, ν the Poisson ratio of the sample and δ is the deformation of the sample that can be expressed by:

$$\delta = z - z_0 - d \quad (2)$$

where z is the sample displacement, z_0 is the contact point and d is the deflection of the cantilever. Thus, the sample displacement in the contact region can be rewritten as:

$$z = z_0 + d + \left[\frac{3k(1-\upsilon^2)}{4ER^{1/2}}d\right]^{2/3} \quad (3)$$

Therefore, by fitting the contact region to the above equation it is possible to determine the contact point z_0. Then, the sample vertical position was converted to real probe-sample distance, d_{ts}, by adding the corresponding cantilever deflection:

$$d_{ts} = (z - z_0) + d \quad (4)$$

As detailed below, further analysis of force ramps involved fitting the non-contact region (specifically d_{ts} > 5 nm) of the ramps in the force vs probe-sample distance representations with an exponential function.

2.6. Quartz crystal Microbalance with dissipation (QCM-D)

QCM-D measurements were performed using an E4 system (Q-sense AB, Sweden). A detailed description of the technique and its basic principles can be found elsewhere [33]. Briefly, an alternating-current voltage is applied through a gold-coated quartz chip to stimulate the shear mode oscillation of the quartz crystal. During the experiments, shifts in frequency, Δf_n, and dissipation factor, ΔD_n, of the different sensor overtones were continuously monitored. Temperature was set to 25 °C throughout all the experiments. At the beginning of the experiments, baselines for the non-coated sensors in all different solutions were registered. Then, stock (saliva or MUC5B) solutions were supplied into the QCM-D chamber using an Ismatec peristaltic pump IPC-N 4 at a flow rate of 0.1 $mL{\cdot}min^{-1}$. When the chamber was filled, the pump was stopped and the stock solutions were left to adsorb for 1 h. Then, the chambers were rinsed for 5 min with PBS buffer, followed by 55 min stabilization under non-flow conditions. This cycle was then repeated for PBS/10, PBS/100 and UHQ water. The Q-Tools software (Q-Sense AB, Sweden) was employed for fitting data to the Voigt model (details on the fit are provided in Supplementary Material Section S1.1).

2.7. Neutron Reflectometry (NR)

Neutron Reflectometry experiments were carried out on the D17 [34] and SuperAdam [35] reflectometers at the Institut Laue-Langevin, France (DOI: https://doi.org//10.5291/ILL-DATA.CRG-2539, https://doi.org//10.5291/ILL-Data.8-05-437 and https://doi.org//10.5291/ILL-DATA.9-13-797) and at the INTER [36] reflectometer at ISIS, UK (DOI: https://doi.org//10.5286/ISIS.E.RB1720420 and https://doi.org//10.5286/ISIS.E.RB1820559). Silicon blocks (100) of dimensions 76.2 × 10mm (cylindrical shape), 80 × 50 × 15mm and 50 × 50 × 10mm were used for D17, INTER and SuperAdam respectively. Measurements on INTER, a horizontal time-of flight reflectometer, used two incidence angles; 0.7 and 2.3°, where $q = \frac{4\pi}{\lambda}sin(\theta)$. D17 is also a time of flight reflectometer which scatters in a horizontal plane, here the incident angles 0.4° and 2.8° were used. SuperAdam is a monochromatic machine (λ = 5.21 Å) with a horizontal scattering plane, the reflected beam

was measured over a range of detector angles to achieve the same q-range. The measured reflected intensity, I(q), was normalised by the direct beam, I_0, to achieve the reflectivity, R(q). R(q) plots are provided as mean and standard deviation values of the reflectivities for a specific q. From the reflectivity data collected, the scattering length density, SLD(z), is calculated through data analysis in RasCAL modelling software to give the structural conformation normal to the surface. For error analysis, we used the Bayesian Markov chain Monte Carlo (MCMC) approach implemented in the refnx software [37]. Further details on the experimental method and data analysis can be found in the Supplementary Material Section S2.

3. Results

3.1. AFM-based force spectroscopy

Results from force spectroscopy experiments on salivary pellicles and on MUC5B films are shown in Fig. 1 and Fig. 2, respectively. It can be seen that in both systems the non-contact region of the force ramps exhibited an exponential-like dependence with the probe sample distance (Fig. 1a and 2a), characteristic for steric interactions. Moreover, these steric forces extended towards longer distances when ionic strength was decreased.

Analysis of steric forces between two surfaces with adsorbed or grafted polymers usually relies on the Alexander – de Gennes expression [38]:

$$f \approx k_B T \Gamma^{3/2} \left[\left(\frac{2L}{D}\right)^{9/4} - \left(\frac{D}{2L}\right)^{3/4} \right] \quad (5)$$

where f is the force per unit area, k_B is the Boltzmann constant, T the absolute temperature, Γ the surface coverage, D the distance between the two surfaces and L the equilibrium thickness of the polymer layer. In our case, only one of the surfaces was covered with a polymer-like film i.e., a salivary pellicle. Thus, D/L could be replaced by $D/2L$. Additionally, for $0.2 < D/L < 0.9$, the above expression is roughly exponential and can be approximated as [39,40]:

$$f \approx 50 k_B T \Gamma^{3/2} e^{2\pi D/L} \quad (6)$$

Absolute forces can be obtained from this expression by means of the Derjaguin approximation for a sphere-plane geometry: $F_{sphere-plane} \approx 2\pi R_{sphere} f$. Accordingly, we fitted the non-contact region of the acquired force ramps to an exponential function$F = F_0 e^{-d_{ts}/\lambda_0}$. In this scheme, the characteristic length, λ_0, provides an estimation of the thickness of the anchored polymers in the investigated films.

Overall, we can see that for both salivary pellicles (Fig. 1a and 1c) and MUC5B films (Fig. 2a and 2c), the characteristic length of the steric repulsion, λ_0, increased when the ionic strength was lowered. This indicates that both systems swelled immediately after lowering the ionic strength. Interestingly, in the case of MUC5B films, the range of the repulsive non-contact force (and, subsequently, the fitted λ_0 values) decreased over time after solution exchange (Fig. 2b and 2c). This behavior was also observed for salivary pellicles but to a much lower extent (Fig. 1b and 1c).

We also investigated, in a final stage, the behavior of both systems when exposed to deionized water. It has previously been reported that in this environment a collapse of salivary pellicles is expected [13]. However, force spectroscopy experiments did not confirm this collapse, and a similar results to those observed in PBS/100 were obtained, which is in agreement with a different report [17].

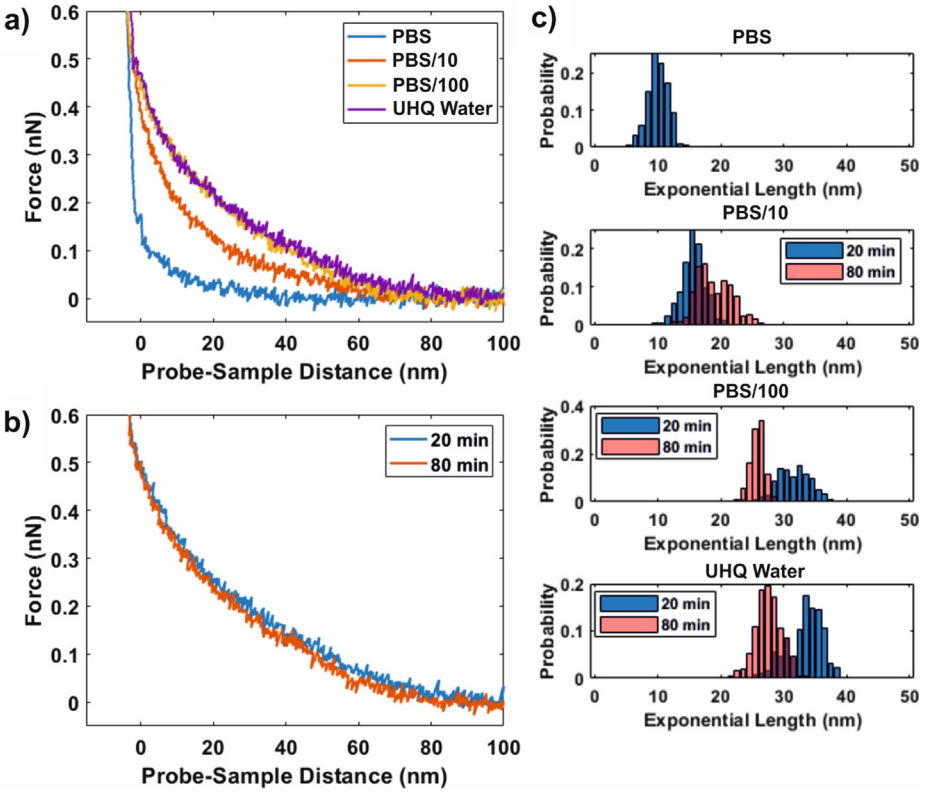

Fig. 1. a) Force vs probe sample distance ramps on salivary pellicles at different solutions (obtained after 80 min stabilization). b) Force vs probe-sample distance curves on a pellicle after 20 min and 80 min of exposure to PBS/100 buffer. c) Distributions for the characteristic length of the exponential fit of the non-contact region calculated from 4096 (64 × 64) force ramps.

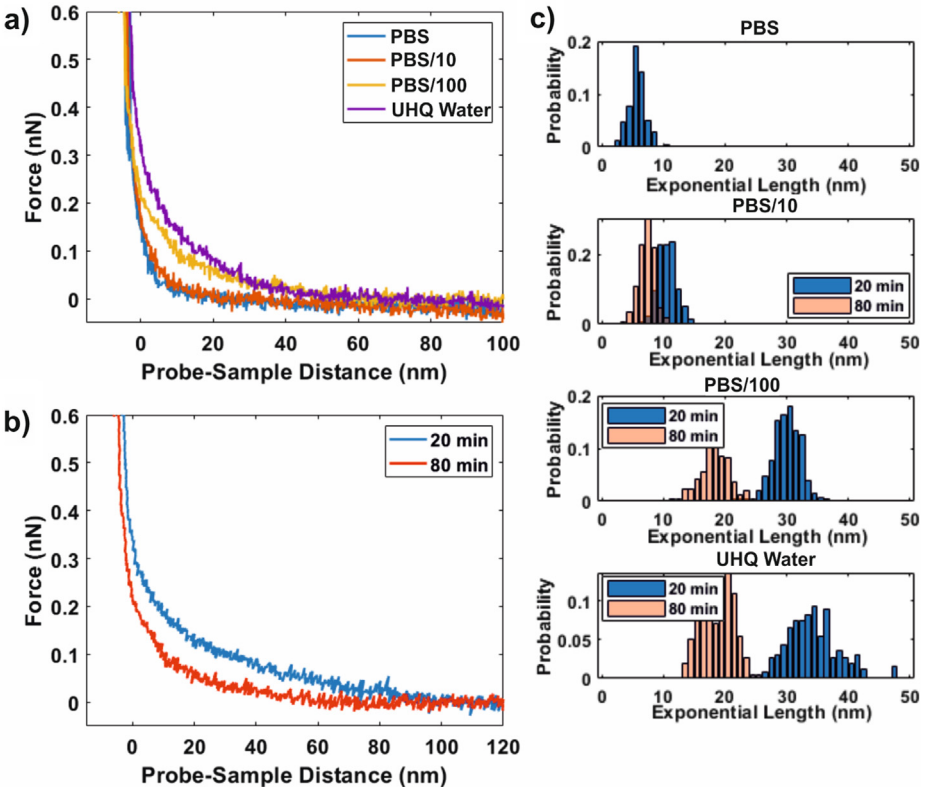

Fig. 2. a) Force vs probe sample distance ramps on MUC5B films at different solutions (obtained after 80 min stabilization). b) Force vs probe-sample distance curves on a MUC5B film after 20 min and 80 min of exposure to PBS/100 buffer. c) Distributions for the characteristic length of the exponential fit of the non-contact region calculated from 4096 (64 × 64) force ramps.

3.2. Quartz crystal Microbalance with dissipation (QCM-D)

QCM-D was also used to investigate salivary pellicles and MUC5B films under different ionic strength conditions. Representative raw QCM-D data (frequency and dissipation shifts) and thickness values derived from the Voigt model fit of the data for salivary pellicles are shown in Fig. 3a and 3b respectively. Similarly, raw QCM-D data and Voigt thickness values for MUC5B films are shown in Fig. 3c and 3d. Thickness mean and standard deviation values calculated at different steps of the experiment from two different data sets are provided in Supplementary Material Section S1.2.

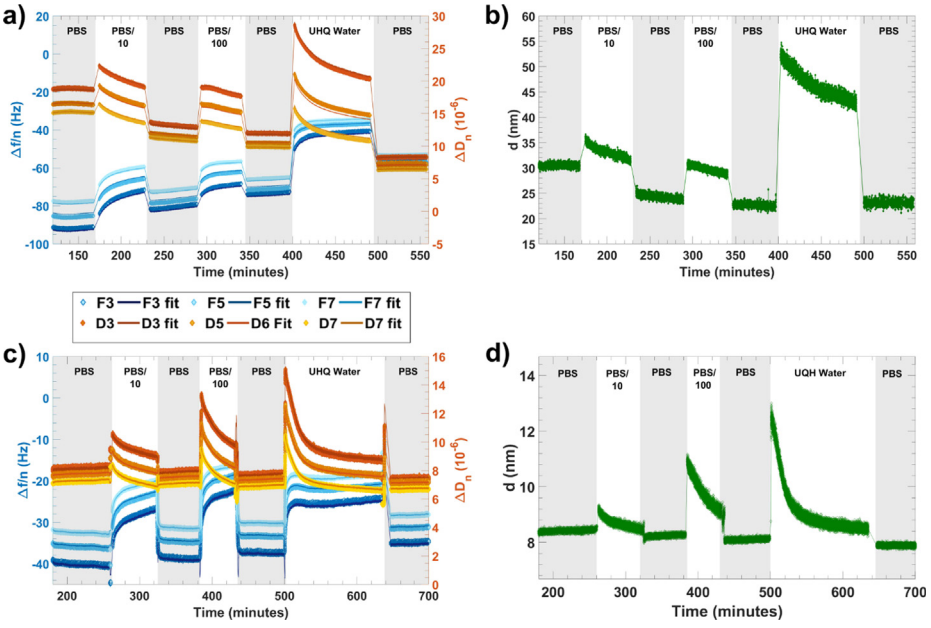

Fig. 3. a) Frequency and dissipation shifts (overtones 3, 5 and 7) obtained from QCM-D measurements and b) thickness for adsorbed salivary pellicles as obtained from fits to the Voigt model of this data. c) Frequency and dissipation data obtained from QCM-D measurements and d) the thickness for adsorbed mucin films as obtained from the Voigt model fits of these measurements.

Overall, QCM-D data indicated that both surface adsorption of saliva and MUC5B lead to thin (nm-thick) but highly viscous layers in agreement with previous reports [13,41]. Moreover, QCM-D data supported that obtained by means of force spectroscopy investigations. Decreasing ionic strength led to an immediate swelling of both salivary pellicles and MUC5B films. After this initial swelling, an eventual coiling was observed for both systems. However, in agreement with force spectroscopy data, the eventual coiling of MUC5B films was significantly higher than for salivary pellicles, up to an extent that they eventually coiled down to a thickness only slightly larger than that observed for physiological ionic strength. In fact, this eventual coiling of MUC5B films was significantly more pronounced than that observed in force spectroscopy experiments.

QCM-D revealed, because of its sensitivity to the whole films and not only to their outer layer, a structural aspect of salivary pellicles exposed to low ionic strength that could not be observed in force spectroscopy investigations. In the case of salivary pellicles, lowering the ionic strength led to an irreversible decrease of ~25% of the overall thickness. This, which was also observed when investigating salivary pellicles by means on null-ellipsometry (Supplementary Material Section S3), was not observed for MUC5B films.

3.3. Neutron reflectometry (NR)

Salivary pellicles were investigated by means of Neutron Reflectometry (NR) in order to gain insight into the irreversible thickness change originated by exposure to low ionic strength revealed by QCM-D data. Reflectometry profiles and corresponding fits for salivary pellicles in deuterated solvents are shown in Fig. 4a. Corresponding scattering length density (SLD) profiles from the fits are shown in Fig. 4b. SLD is a parameter that determines how neutrons are scattered and it provides information on the composition of the sample. The results from fitting NR data are often presented as an SLD profile i.e., a representation of how the SLD of the sample varies along the direction perpendicular to the sample surface. As detailed in Supplementary Material Section S2, fitting NR data on salivary pellicles required a two-layer model, in agreement with [11]. NR confirmed the swelling of salivary pellicles when decreasing the ionic strength. Moreover, NR indicated that this swelling is due to only the outer layer. The SLD profiles show that the outer layer swelled along the substrate normal direction, while also increasing the SLD towards that of the deuterated solvents as the ionic strength was lowered. As inferred from the parameters given in Table 1, this could be quantified as an increase in thickness and hydration of the outer layer. Furthermore, NR fits also indicated that the inner layer decreased in thickness after lowering the ionic strength. This suggests that lowering the ionic strength resulted in a gradual partial removal of the inner layer of salivary pellicles, which would explain the irreversible change in thickness observed by means of QCM-D. The optimal fit for the inner layer also suggests a large increase in the roughness to a value of similar magnitude to the layer thickness, although this may be in accordance with the removal of material with the layer and indicate the formation of holes where the proteins have been entirely removed, it is not possible to confirm this with NR alone. However, the general conclusion from the NR data agrees with the removal of material form the pellicle, as shown by QCM-D.

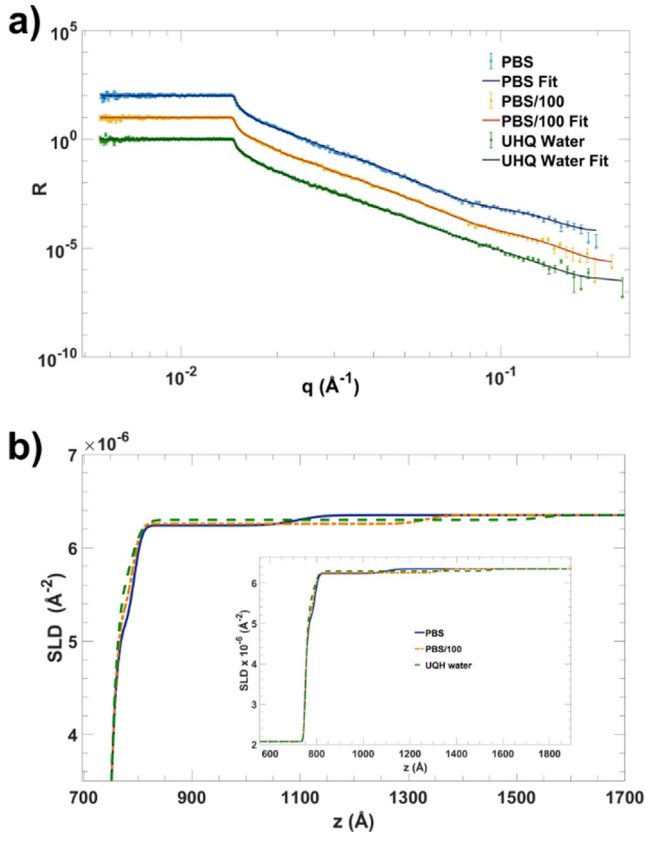

Fig. 4. a) NR curves (markers) and fits (lines) for the data collected at D17, ILL. Curves for the salivary pellicle in PBS (blue), the pellicle in PBS/100 (yellow), both using D_2O as solvent, and the pellicle in deionized D_2O (green) are shown. b) The region of interest of the corresponding SLD plots for the NR fits, with the full profile (inset). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Parameters for the pellicle inner and outer layer obtained from the NR fits shown in Fig. 4.

	PBS		PBS/100		UHQ Water	
	Inner	Outer	Inner	Outer	Inner	Outer
Thickness (Å)	32 ± 1	309 ± 1	29 ± 1	540 ± 2	21 ± 1	756 ± 2
Roughness (Å)	12 ± 1	40 ± 2	12 ± 1	30 ± 1	19 ± 1	30 ± 1
Hydration (%)	65 ± 1	96 ± 1	69 ± 1	97 ± 1	68 ± 2	98 ± 1

4. Discussion

In this work, we have used different advanced surface techniques to gain insight into the structure of salivary pellicles and MUC5B films. Some of these techniques e.g., QCM-D, NR and ellipsometry, require highly planar, macroscopic and, for the latter, reflective solid surfaces. This requirement prevented the use of substrates more representative of the oral cavity e.g., oral mucosa. An important factor to consider for choosing an alternative surface to use instead was the ionic character, as this is known to play an important role on the formation of salivary pellicles [2,8]. Oral mucosa surfaces are decorated with the MUC1 mucin [42]. Thus, they have an anionic character. A similar character has been reported for hydroxyapatite [43], the main component of enamel. Therefore, we employed silica surfaces in our experiments, which also have an anionic character that is not altered by the hydrophobization protocol used in this work [44]. While there is certain controversy regarding wettability of oral surfaces, *in vivo* wettability measurements indicated a hydrophilic nature for both teeth and oral mucosa [45] surfaces. Thus, for the study of salivary pellicles we used clean hydrophilic silica substrates. However, isolated mucin fractions barely adsorb on hydrophilic substrates [41]. This agrees with the fact that in the two-layer pellicle model, the outer mucin layer needs an inner layer that anchors it to the substrate.

Thus, we followed instead the common approach of employing hydrophobic substrates (silica methylated with dichlorodimethylsilane, an approach that preserves the anionic character of silica [44]) to investigate oral mucin films [41,46,47]. Nevertheless, as shown in Supplementary Material Sections S1.3 and S1.4, the wettability of the employed substrates did not have a drastic influence on the reported observations.

AFM-based force spectroscopy is an excellent tool to probe steric forces on polymer-coated surfaces [48] i.e., those originating from the change in entropy of the chain molecules induced by mechanical confinement. While no comprehensive theory is available to describe steric forces, most current approaches are based on the increase of osmotic pressure in the confined space when surfaces approach each other. These approaches relate steric forces to quantities like polymer layer thickness, density, temperature, etc. Thus, the measurement of steric forces can provide valuable structural information on polymer-like coatings. In this work, we followed this approach and investigated steric forces on both reconstituted salivary pellicles and MUC5B films at different ionic strengths to gain insight into their structure.

Specifically, we used force spectroscopy to characterize the range of steric forces, which was quantified in terms of their characteristic/exponential decay length. These results indicated swelling of both salivary pellicles and MUC5B films when decreasing the ionic strength for all investigated values, i.e., a transition between osmotic and salted regimes was not observed. In agreement with previous reports [49], this suggests that both systems behave like weak polyelectrolytes [50] i.e., they were able to regulate their thickness according to changes in the environmental ionic strength for all investigated conditions. The time evolution of the characteristic length was also investigated. Because of the time required to setup a force spectroscopy experiment, it was not possible to perform experiments immediately after solution exchange. Moreover, because the relatively high probability of events that could lead to probe contamination, it was not possible to continuously monitor the characteristic/exponential decay length over long periods of time. Therefore, we characterized this quantity at two time points, 20 min and 80 min after solution exchange. These experiments revealed that, after the initial swelling when ionic strength was decreased, bare MUC5B films exhibited an eventual collapse. This was barely observed for salivary pellicles and, indeed, is not expected for bare polyelectrolytes. Collapse when decreasing ionic strength has instead been reported e.g., for polyzwitterionic polymers, an effect known as anti-polyelectrolyte effect [51–53]. This effect can be explained by the presence, at high ionic strength, of hydration shells of counter ions that prevent electrostatic inter/intra-chain interactions. At low ionic strength this effect is weakened resulting in the collapse of the polymers. Like most mucins, MUC5B have a bottlebrush structure consisting of a long polypeptide chain heavily decorated by, mostly, negatively charged terminal carbohydrates [54]. Thus, they have a highly anionic rather than a polyzwitterionic character. However, they also have hydrophobic (unglycosylated) domains. It is reasonable that the more extended conformation of MUC5B molecules at low ionic strengths facilitates interactions among hydrophobic mucin domains that, gradually, lead to the collapse observed after the initial swelling. The assumption that MUC5B mucins are present in the outer layer of salivary pellicles is extensively supported by the literature as well as by the similar steric repulsion that we observed for both systems. Our results then suggest that in salivary pellicles MUC5B is complexed with other salivary components that prevent the interaction between mucin hydrophobic domains. Indeed, the ability of MUC5B to form complexes with other salivary component has been reported [55,56]. These other components might be lost during the MUC5B isolation process, resulting in significant differences between the outer layer of in-vitro salivary pellicles and bare MUC5B films that should be considered when using the latter as models for pellicle and for that matter, bio-lubrication studies. Additionally, these two fields would benefit from further investigations towards identification of these additional components.

In order to gain further insight into the differences exhibited by salivary pellicles and MUC5B films, we investigated both systems by means of QCM-D. In contrast to AFM, this technique allowed continuous monitoring of the thickness of these systems. These experiments confirmed the tendency for swelling upon decrease of ionic strength for salivary pellicles and MUC5B films. QCM-D data also confirmed the tendency of MUC5B to collapse after the initial swelling. However, this technique also indicated an irreversible change of salivary pellicles after being exposed to low ionic strengths i.e., the overall thickness measured at physiological values was significantly lower after this exposure, an observation that was also confirmed by null-ellipsometry investigations (Supplementary Material Section S3). To further understand this result, we investigated the role of ionic strength on salivary pellicles by means of NR, an ideal tool for extracting structural properties perpendicular to the surface of adsorbed films. Because of the relatively long acquisition times required for NR experiments compared to the timescale of change observed in the AFM and QCM-D experiments, it was not possible to monitor the time evolution of the structure of salivary pellicles upon changes of the ionic strength. Therefore, NR characterization was initiated in each case 1 h after external solution exchange and, thus, represents the asymptotic structure. In agreement with previous investigations [11], NR confirmed a two-layer structure for salivary pellicles. NR data also confirmed that lowering the ionic strength led to the swelling of the outer layer. Interestingly, NR also indicated that lowering the ionic strength leads to a significant decrease of the pellicle inner layer thickness along with an increase in roughness. This explains the irreversible change of salivary pellicles observed in QCM-D and null-ellipsometry investigations in terms of a partial removal/desorption of the inner layer. Overall, this implies that electrostatic interactions play a role in the stability of the pellicle inner layer. Considering that it is generally accepted that electrostatic interaction do not play a significant role in desorption of proteins adsorbed on solid-liquid interfaces [57], this observation suggests that the pellicle inner layer is not only composed of proteins directly adsorbed on the substrate, but is probably a nm-thin multi-component layer where electrostatic interactions between the different components are of relevance.

We have presented results for salivary pellicles and MUC5B films reconstituted on hydrophilic and hydrophobized silica substrates respectively. These solid substrates can be considered good models for e.g., dental implants. Moreover, they also mimic up to a reasonable extent the electrostatic character of oral surfaces. Nevertheless, further studies are required in order to extrapolate our findings to *in vivo* salivary pellicles. For instance, it has been shown that the presence of the transmembrane mucin MUC1 expressed by oral epithelium cells enhances the binding of salivary components, specifically MUC5B mucins [58], by means of a combination of electrostatic and hydrophobic interactions [59]. Further work on e.g., MUC1 coated substrates would provide further insights into this aspect.

5. Conclusions

In this work, we investigated the response to changes in the environmental ionic strength of salivary pellicles and MUC5B films reconstituted at solid-liquid interfaces. Specifically, both systems were studied by means of AFM-based force spectroscopy and QCM-D. NR was also employed in the characterization of reconsti-

tuted salivary pellicles. Previous studies point towards a two-layered structure for reconstituted salivary pellicles with an inner thin dense layer and an outer thick diffuse layer [2]. In this regard, there are also evidences that the outer layer is mainly formed by MUC5B mucins [11]. However, some studies also indicate that mucins in the outer pellicle layer might be complexed with other salivary components [17]. The hypothesis that initiated this work was that further insight into these differences could be obtained by comparing how ionic strength affects the structure of both systems.

Overall, our results supported the two-layer model for reconstituted salivary pellicles and that the outer layer is mainly formed by MUC5B mucins. However, significant differences between MUC5B films and the outer layer of reconstituted salivary pellicles were also found. Reconstituted salivary pellicles exhibited significant swelling when ionic strength was decreased, resembling the behavior of weak polyelectrolyte brushes. Whereas MUC5B films also showed swelling immediately after decreasing ionic strength, this was followed by an eventual coiling. This suggests that interactions between their hydrophobic domains were facilitated upon the more extended conformation achieved when decreasing ionic strength, resulting in the eventual coiling. The absence of this behavior in the case of reconstituted salivary pellicles indicated that MUC5B molecules in their outer layer are complexed with other salivary components that prevent interactions between mucin hydrophobic domains.

We also present evidences for a partial removal of the pellicle inner layer when the ionic strength is decreased below physiological ionic strength values. As electrostatic interactions do not typically play a relevant role in desorption of proteins adsorbed at solid liquid interfaces, our results suggest that the pellicle inner layer is not only formed by components directly adsorbed on the substrate.

Further work towards identification of these additional components, both those that anchor mucins to solid interfaces and those that prevent interactions between mucin hydrophobic domains, would be a milestone in our understanding not only of the structural aspects of reconstituted salivary pellicles but also, from a more general perspective, of biological aqueous lubricants. Additionally, in order to extrapolate our findings to *in vivo* salivary pellicles, it would be needed to investigate more relevant but also complex substrates e.g., hydroxyapatite and mucins/MUC1 decorated surfaces.

CRediT authorship contribution statement

Hannah Boyd: Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Juan F. Gonzalez-Martinez:** Investigation, Writing - review & editing. **Rebecca J.L. Welbourn:** Investigation, Writing - review & editing. **Philipp Gutfreund:** Investigation, Writing - review & editing. **Alexey Klechikov:** Investigation, Writing - review & editing. **Carolina Robertsson:** Investigation, Writing - review & editing. **Claes Wickström:** Investigation, Writing - review & editing. **Thomas Arnebrant:** Conceptualization, Writing - review & editing, Funding acquisition. **Rob Barker:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition. **Javier Sotres:** Conceptualization, Methodology, Software, Writing - original draft, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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