HANNAH BOYD
**ON THE STRUCTURE AND
MECHANICAL PROPERTIES OF
IN VITRO SALIVARY PELLICLES**

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OF *IN VITRO* SALIVARY PELLICLES**

Malmö University, Faculty of Health and Society Doctoral Dissertation

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HANNAH BOYD

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ABBREVIATIONS

AFM	Atomic Force Microscope
SFA	Surface Force Apparatus
QCM-D	Quartz Crystal Microbalance with Dissipation Monitoring
NR	Neutron Reflectometry
GI-SANS	Grazing Incidence Small Angle Neutron Scattering
TOF	Time of Flight
SMW	Silicon Matched Water
SLD	Scattering Length Density
CAPB	Cocamidopropyl betaine
C₁₂E₅	Pentaethylene glycol monododecyl ether
PBS	Phosphate Buffered Saline
PRP	Proline Rich Protein
IgA	Immunoglobulin A
HWS	Human Whole Saliva
vdW	van der Waals
PD	Positron Sensitive Photodetector
MAM	Membrane Associated Mucins

ABSTRACT

Salivary pellicles display exceptional hydration and lubrication performance. At present, there are still gaps in the understanding of how this is achieved. The aim of this thesis was therefore to increase our knowledge on the mechanisms underlying these properties and deepen the understanding of how they are related to the composition and structure of pellicles, with a focus on those formed under *in vitro* conditions. This has applications ranging from the development of artificial saliva and lubricating coatings for biomedical applications to methodological approaches for initial testing of oral healthcare products. For this, we also focused on developing suitable methodological approaches for these studies, centering on atomic force microscopy, quartz crystal microbalance with dissipation monitoring, ellipsometry and neutron reflectometry techniques, to investigate *in vitro* and model salivary pellicles.

First, we confirmed a two-layer structure for *in vitro* salivary pellicles and showed that the outer layer is mainly composed by the oral mucin MUC5B, but that it also contains other salivary components that enhance swelling and hydration. In the presence of bulk saliva, the outer layer also contains a reversibly and loosely bound fraction. This fraction increases the adhesiveness of the pellicle but unexpectedly has no significant effect on its lubrication performance. We also investigated the effect of mechanical confinement on model salivary pellicles by means of Neutron Reflectometry, revealing that at a pressure of 1 bar they are already completely compressed and dehydrated. Finally, with the aim to advance towards better oral healthcare products, we investigated the effect of nonionic and amphoteric surfactants on salivary pellicles, showing that they have a gentler effect on pellicle structure than the commonly employed anionic surfactants.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Saliv och speciellt den tunna salivhinnan som täcker munhålan har fenomenala egenskaper vilken utgör ett skydd för de underliggande ytorna. Denna tunna salivhinna gör att en hög hydratisering kan bibehålls i munhålan och trots att vatten är ett typiskt dåligt smörjmedel uppvisar denna hinna exceptionella smörjegenskaper. Det är idag inte känt hur denna tunna hinna kan ha dessa egenskaper men genom att öka kunskapen kring de underliggande mekanismerna i hinnan kan utvecklingen av artificiella salivsubstitut och beläggningar för biomedicinsk utrustning fortskrida. Det kan potentiellt även göra det möjligt att skapa en metod för att testa produkter för munhälsa i ett tidigt skede av utvecklingen.

Syftet med denna avhandling var därför att öka förståelsen för de strukturella och mekaniska egenskaperna hos den tunna salivhinna. Detta gjordes genom att utveckla metodiska tillvägagångssätt för att undersöka modellsystem för salivhinnan, det vill säga system som skapats av mänskligt saliv utanför munhålan eller av komponenter som separerats ut från mänskligt saliv, i ett försök att koppla ihop strukturen och sammansättning i hinnan med de egenskaper som är av intresse.

I avhandlingen så visar vi att hinnan består av två skikt, ett inre tjockare skikt och ett yttre mer hydratiserat skikt. Det yttre skiktet visade sig bestå av inte bara salivmucin (det slemämne som man tror bidrar mest till hinnans smörjande egenskaper) utan också andra ämnen som hjälper till att bibehålla hydratiseringen. Ett lager av andra ämnen fäster in till salivhinnan när den utsätts för saliv vilket tidigare studier har bortsett från. Därför fokuserar den andra studien i avhandlingen på hur detta lager påverkar hinnans egenskaper. Lagret visade sig öka vidhäftningsförmågan hos den tunna salivhinnan men hade ingen påverkan på dess smörjegenskaper. Den tredje studien fokuserade på hur mycket tryck som kunde appliceras innan salivhinnan blivit

fullständigt komprimerad så mycket att den slutat ändrat tjocklek, vilket motsvarade 1 bars tryck. Den sista studien fokuserade på hur tensider (ytaktiva ämnen, såsom tvål), en vanlig ingrediens i munhälsovårdsprodukter som till exempel tandkräm, påverkar salivhinnan. Två olika tensider testades, vilka båda anses vara snällare än den mest vanliga tensiden (sodium dodecyl sulfate, SDS), och jämfördes för att se hur de påverkade salivhinnan. Båda tensiderna uppvisade ett mildare beteende än SDS men hade olika påverkan på salivhinnan och således på salivhinnans funktioner.

Tillsammans så bidrar dessa resultat till en djupare förståelse för strukturen, sammansättningen och hydratiseringen hos salivhinnan. Detta tar oss ett steg närmare att förstå hur mekanismen bakom salivhinnans fenomenala skyddande och hydratiserande egenskaper faktiskt fungerar.

LIST OF PUBLICATIONS

Paper I

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

H. Boyd, J.F. Gonzalez-Martinez, R.J. Welbourn, P. Gutfreund, A. Klechikov, C. Robertsson, C. Wickström, T. Arnebrant, R. Barker and J. Sotres

Journal of Colloid and Interface Science, 2021, 584, 660-668

Paper II

Role of the reversibly bound fraction of *in vitro* salivary pellicles on their mechanical and lubrication properties

H. Boyd, J.F. Gonzalez-Martinez, T. Arnebrant and J. Sotres

Manuscript

Paper III

MUC5B mucin films under mechanical confinement: a combined neutron reflectometry and atomic force microscope study

J.F. Gonzalez-Martinez*, H. Boyd*, P. Gutfreund, R.J. Welbourn, C. Robertsson, C. Wickström, T. Arnebrant, R.M. Richardson, S.W. Prescott, R. Barker and J. Sotres

Submitted.

* Shared first authorship

Paper IV

Effect of nonionic and amphoteric surfactants on salivary pellicles reconstituted *in vitro*

H. Boyd*, J.F. Gonzalez-Martinez*, R.J. Welbourn, K. Ma, P. Li, P. Gutfreund, A. Klechikov, T. Arnebrant, R. Barker and J. Sotres

Scientific Reports, 2021, 11:12913

* Shared first authorship

Publications not included in this thesis:

Enabling autonomous scanning probe microscopy imaging of single molecules with deep learning

J. Sotres, H. Boyd and J.F. Gonzalez-Martinez.

Nanoscale, 2021, 13, 9193-9203

AUTHOR CONTRIBUTIONS

Paper I

HB designed and performed most of the AFM, QCM-D and Ellipsometry experimental work and data analysis, attended the NR beam times and analysed NR data. HB co-wrote the first version of the manuscript as well as the final version with contributions from co-authors.

Paper II

HB designed and performed most of the Ellipsometry and AFM experimental work and data analysis. Contact Thermal Noise AFM experiments were performed in collaboration with co-authors. HB wrote the first version of the manuscript as well as the final version after feedback from co-authors.

Paper III

HB performed AFM experiments and data analysis. HB also performed NR experiments in collaboration with co-authors. HB co-wrote the final version of the manuscript

Paper IV

HB planned and performed and analysed AFM, QCM-D and Ellipsometry experiments. HB also performed NR experiments in collaboration with co-authors. HB co-wrote the first version of the manuscript as well as the final version with contributions from co-authors.

1. THE SALIVARY PELLICLE

1.1 Saliva

Saliva, the oral fluid secreted by exocrine glands, is essential for maintaining the health and function of the oral cavity [1]. The consequences of diseases causing diminished salivary production range from difficulties in speaking and swallowing to the onset of dental caries and erosion, demonstrating the multifaceted and essential role that saliva plays [2, 3]. While saliva is highly hydrated (~99% water), it is a complex biological fluid with many components including proteins, glycoproteins, lipids and electrolytes [4, 5]. This fluid is secreted from a number of glands; the paired parotid, submandibular and sublingual glands (collectively known as the major salivary glands), as well as a number of minor glands. The secretions mixed with other fluids in the oral cavity (oral mucosa transudate and mucus from the nasal gland and pharynx) are collectively referred to as whole saliva [4].

The functions of saliva in the oral cavity are manifold. It aids efficient deglutition, mastication and speech, it is antibacterial, and it keeps oral surfaces hydrated and protected from mechanical and chemical insults [1, 6]. These functions can be categorised as either digestion or protection [3]. The composition and, therefore, function of the exocrine secretion varies depending on the gland. The gland which secretes is then dependant on stimulation or rest conditions. At rest there is a continuous flow of saliva, typically contributing to less than 20% [1] of the daily saliva production (1-1.5 L) [4]. The remaining amount is attributed to exogenous stimulation from taste, smell, or mastication. Without stimulation the majority of the salivary secretion is attributed to the submandibular glands (~60%), while only ~25% is from the parotid gland and a further ~15% from the sublingual and minor mucus glands. However, during stimulation the flow is dominated by secretion from the parotid gland (increasing to 50%) and the contribution of the submandibular glands

decreases to ~35% [1]. Multiple studies have been conducted to investigate the components of both unstimulated and stimulated saliva. These findings are summarised, e.g. by Edgar, O'Mullane and Dawes [7], where it is shown that for stimulated saliva, the total protein content drops significantly. The reduced protein content is attributed to the decrease in secretion from the mucus producing submandibular gland, resulting in a decrease in MUC5B and MUC7 glycoproteins in stimulated saliva. However, in stimulated saliva there is an increase of amylase, primarily secreted by the parotid gland. Salivary amylase contributes to the deglutination at the beginning of the digestion process by breaking down bonds between the monomeric sugar units of disaccharides, oligosaccharides, and starches [3]. So, it is expected that higher amounts of components contributing to digestion are present in stimulated saliva, while other proteins which are more likely to contribute to the protective function will decrease in amount. This is something to be considered when conducting studies on saliva and the salivary pellicle.

1.2 *In vivo* Salivary Pellicle

Within the oral cavity, the components of saliva adsorb onto oral surfaces, forming a (mostly) proteinaceous film known as the acquired salivary pellicle [8]. Within this thesis, the term *in vivo* will refer to the salivary pellicle which is formed within the oral cavity, on the teeth, mucosa or dental implants. The term *in situ* typically refers to pellicles formed on model surfaces rather than the oral surfaces, but still within the oral cavity. In this section, both *in vivo* and *in situ* pellicles will be considered. Salivary components begin to adsorb instantaneously with the initial pellicle forming within minutes. However, a maturation stage continues where further components are attached to the acquired pellicle after the initial adsorption process and thickness continues to increase for several days [9]. The pellicle composition is complex and, furthermore, depends on its location within the oral cavity. Although the salivary components tend to adsorb onto most surfaces [10], the physico-chemical properties of the underlying surfaces can have some effect on the composition of the pellicle. It is then thought that two main distinct pellicles exist depending on the location within the oral cavity; the enamel pellicle and the mucosal pellicle [8]. The enamel pellicle has been the subject of many studies resulting in it being more fully characterized than the mucosal pellicle [11]. In the oral cavity a distinct microbial film adheres to the pellicle, together these are known as dental plaque (Figure 1) [12].

Figure 1. TEM image of the in vivo salivary pellicle on enamel surfaces after 24hrs. The red line represents the initial pellicle, which typically forms within minutes. Adapted with permission from [12].

1.2.1 Function

The functions of the salivary pellicle are manifold. At the interface of the oral surfaces and bulk salivary films, the pellicle is primarily responsible for protecting and maintaining the underlying surfaces. Since the pellicle executes this role with exceptional efficiency, it has become the focus of many research studies in attempts to understand the underlying mechanisms governing this performance.

The enamel pellicle acts a semi-permeable barrier modulating ion diffusion. This facilitates mineral homeostasis by regulating the remineralisation of enamel from saliva, which is supersaturated with respect to calcium and phosphate. Without the pellicle these inorganic components would directly precipitate onto the enamel [12]. By acting as a diffusion barrier, the pellicle also provides protection against demineralisation caused by chemical assaults [13-15].

The oral cavity is heavily colonised by a vast community of microorganisms and the pellicle plays a vital role in regulating the effect of which on the underlying surfaces. Bulk saliva provides protection against the acid produced by bacteria, and the pellicle controls bacterial adherence by selective adsorption [12]. While some of the salivary proteins in the pellicle are known to have antimicrobial properties, such as cystatins, lysozyme, myeloperoxidase and histatins (making up ~8% of the enamel pellicle components [16]), some actively promote binding of bacteria [17-20]. Other proteins have been shown to regulate the metabolic activity of adsorbed bacteria. This was

true only for adsorbed proteins, suggesting their conformation change due to adsorption is important [19]. The oral commensal bacteria, which bind to the pellicle, can then prevent the colonisation by pathogenic bacteria species, maintaining oral health [21]. Some proteins within the pellicle also have functions associated with immune defence and inflammatory response, reiterating the defensive nature of the pellicle [16].

One interesting property of the pellicle is its ability to lubricate oral surfaces, greatly diminishing the need to overcome friction forces while fulfilling the processes taking place in the oral cavity as well as the wear of both hard enamel and soft oral mucosa surfaces during processes such as mastication and teeth grinding [12]. Relatively little information is available on the lubrication performance of *in vivo* pellicles due to a lack of suitable methodical approaches. However, surveys of those suffering from xerostomia and hypofunction of the salivary gland have reported difficulty with speech and swallowing [22, 23], processes that are likely to rely on lubrication for optimum performance.

1.3 *In vitro* Salivary Pellicle

The functions of the pellicle, described above, have drawn interest to the underlying mechanisms as well as pellicle structure, to gain understanding of how these functions are regulated. There are, however, limitations in experimental techniques when studying *in vivo* pellicles. It then becomes beneficial to study salivary pellicles formed *in vitro* i.e. formed outside the oral cavity on surfaces such as silica, hydroxyapatite, enamel and even cell cultures by exposing the surfaces to saliva. *In vitro* pellicles typically do not undergo the maturation period as this requires a continuous adsorption process over longer time, which then requires a constant supply of fresh saliva and this can be practically challenging. While this differs from the dynamic environment of *in vivo* conditions, resulting in some differences between *in vitro* and *in vivo* pellicles [10, 24, 25], *in vitro* salivary pellicles still preserve many of the functions of *in vivo* pellicles such as lubrication, wear and demineralisation prevention [10, 26, 27].

Aqueous lubrication is another topic that has boomed in recent years. Nature has developed water based lubricants with performances that far surpasses those developed by man, which are typically oil-based. Pellicles formed *in vitro* are one of the systems allowing lubrication studies. Of specific interest is that they are able to provide significantly low boundary friction coefficients to a wide variety of tribological contacts regardless of their physico-chemical properties [28-33].

However, the mechanisms underlying the superior performance of biogenic aqueous lubricants in general, and of *in vitro* pellicles in particular, remain largely unknown. Identifying the components and mechanisms involved in pellicle lubrication would allow a rational approach for developing aqueous lubricants for cases where those based on oil are not an option, e.g. biomedical implants or devices designed to be in contact with biological fluids or with biological barriers.

Furthermore, *in vitro* pellicles can be a means of initial evaluation for oral health products. This approach has largely been used to investigate the effect of ingredients of oral healthcare products on the structure and composition of *in vitro* pellicles, providing insights into the underlying mechanisms that govern *in vivo* performance [34, 35].

1.3.1 Formation

The formation of *in vitro* salivary pellicles is a complex process due to the high number of components involved and the variation in size and physico-chemical properties between them [10, 26]. The process is very selective [10, 26, 34, 36, 37] and governed by a combination of different interactions including electrostatic, hydrophobic and van der Waals (vdW) interactions, as well as chemical crosslinking [38]. Adsorption occurs when these interactions result in a more energetically favourable system and is therefore influenced by the properties of the substrate, such as surface charge and wettability. For example, the investigation of saliva adsorption on surfaces of different wettability has shown a higher adsorbed amount as well as a higher wear resistance on hydrophobic surfaces, indicating the importance of hydrophobic interactions [38-40]. Nevertheless, although adsorbed amounts are higher on hydrophobic surfaces, adsorption still takes place on hydrophilic surfaces. The proteins within the salivary pellicle have varying ionic character and it is therefore likely that some adsorb onto the hydrophilic surfaces under the influence of electrostatic interactions [41], and several studies on various surfaces did show that electrostatic interactions can influence adsorption of salivary proteins [42].

The initial stage of the adsorption process is thought to be diffusion controlled as the proteins must transport to the surface, meaning that more abundant and lower molecular weight proteins will arrive at the interface first. The first adsorbed proteins, known as the pellicle precursors, are those which have a high affinity for the surface and are governed by a combination of electrostatic, hydrophobic and vdW interactions [10]. After this initial adsorption, the rate will fall as availability at the interface decreases; this has been shown by a number of ellipsometry studies [26,

40]. As formation of the pellicle continues, initial adsorption is followed by protein-protein interactions and the adsorption of aggregates and larger components, e.g. bulky glycoproteins [10]. After adsorption, the proteins can undergo conformational changes either immediately or in a time-dependant manner [43].

1.3.2 Composition

The composition of *in vivo* and *in vitro* pellicles has been shown to differ slightly. However, due to methodological difficulties in collecting large enough quantities of *in vivo* pellicle to investigate the composition, many studies have focused on acquired *in vitro* pellicles instead, allowing the use of analytical techniques such as electrophoresis and mass spectrometry.

The composition of the *in vitro* pellicle is complex, dominated by a diverse range of proteins and glycoproteins but also lipids and electrolytes [12]. The predominant proteins are albumin, cystatins, histatins, statherin, secretory IgA, PRPs, lactoferrin, mucins as well as enzymes like amylase, lysozyme, peroxidase, carbonic anhydrase VI and transaminases [12, 44]. Although reports have suggested that up to 130 different proteins and peptides may occur in *in vivo* pellicles [16], the few major components mentioned above are present in pellicles formed on different substrates, as indicated by electrophoresis [45]. Furthermore, it has been reported that the pellicle composition between individuals only shows a slight variation. Therefore, *in vitro* pellicles are quite reproducible [36, 46].

Mucins

Mucins, one of the main components of both saliva and salivary pellicles, are of particular interest for the work of this thesis. Mucins are a diverse group of glycoproteins ranging in size and structure, but all contain large numbers of O-linked glycans that make up at least 50% of the molecular weight [47]. Typically, these glycosylated domains are built on a protein core that is rich in the amino acids proline, serine and/or threonine residues, i.e. the PTS domains, which anchor the O-linked glycans [47]. There are two types of mucins: membrane associated mucins (MAMs) and secreted mucins. MAMs have a transmembrane hydrophobic domain that allow them to anchor to cell membranes [48]. Secreted mucins are typically the gel forming components of mucous films, while the MAMs attach to epithelial cells and can aid in the binding of mucous films to the epithelium [49]. They also engage in signal transduction across the membrane [50]. Oral mucins include MUC1, MUC7 and MUC5B [51].

The extensive glycoprotein MUC5B has been shown to be one of the main components of the pellicle [45]. MUC5B, as with most mucins, have an elongated structure due to steric and electrostatic repulsions between the negatively charged glycans [52]. Furthermore, the high density of –OH groups allow the mucins to trap high amounts of water by means of hydrogen bonds, resulting in highly hydrated layer that is thought to be a main contributor to the lubricating properties of the pellicle and the protection against mechanical assaults [53, 54]. MUC5B has a molecular weight of about 2-4 MDa and carbohydrates account for ~80 % of its mass [55]. While the presence of MUC5B within *in vitro* salivary pellicles is certain, a full understanding of how it is incorporated into the structure of the pellicle and involved in the mechanisms governing the lubrication and protection properties have not yet been achieved.

The other oral mucins MUC1 and MUC7 are not a focus of this thesis, but are still important for the function of saliva. MUC1 is a MAM [50] and is thought to be essential to anchor the MUC5B to mucosal surfaces [49]. MUC7 is a secreted non-polymerising glycoprotein, which is also found in the mucosal pellicle [56], but its function is thought to be mainly in bulk saliva, having a protective role by interacting with pathogens [57].

1.3.3 Structure

While the composition of the *in vitro* pellicle is fairly well known, there are still gaps in the knowledge regarding the structure. This is mostly due to the methodological difficulties associated with structural analysis of thin soft films. It is generally hypothesized that the *in vitro* pellicle consists of two layers (Figure 2), with the lower molecular weight proteins forming an inner layer that is dense and the outer layer is thought to be more diffuse and highly hydrated. It is also hypothesized that the oral mucin MUC5B is a main component of the outer layer. This speculation is supported by several factors. First, it has been shown that MUC5B is a main component of salivary pellicles [45]. Moreover, similar steric repulsive forces in opposition to mechanical confinement, which are typical for surfaces with anchored polymers in good solvent conditions, have been reported by means of atomic force microscope (AFM) and surface force apparatus (SFA) studies for both *in vitro* salivary pellicles [30, 31, 58-60] and mucin layers [58, 61-64], thus indicating mucins in the outer layer [30, 31, 60, 65]. Mucin films have also shown lubrication properties similar to salivary pellicles, therefore reiterating the assumption that mucins are a main component of the outer layer [28, 66].

Furthermore, the adsorbed amount of mucins on hydrophilic surfaces (hydroxyapatite surfaces, a main component of enamel, are hydrophilic [67]) is relatively low [68], therefore the MUC5B glycoproteins may require other molecules to anchor them. This is also true for the mucosal pellicle, which requires MUC1 to anchor MUC5B [49].

Using a multitude of surface techniques, several studies have presented evidence for the two layer model. Neutron reflectometry combined with ellipsometry and AFM have shown that the *in vitro* pellicle on alumina surfaces is likely a two layer structure, where the outer layer is thought to protrude into the bulk liquid [69, 70]. However, further investigation of the structure of the pellicle is needed to confirm the two layer model, with a dense inner layer composed of low molecular weight proteins and an outer layer of mostly MUC5B.

Ellipsometry studies have shown that *in vitro* salivary pellicles carry a reversibly attached fraction, which is only observed in the presence of bulk saliva and that is removed upon rinsing with saliva free solutions [71]. However, the influence of this fraction has been overlooked during studies on the properties and functions of the pellicle.

Figure 2. Schematic of the hypothesized two layer structure of the *in vitro* salivary pellicle. Within this hypothesis, the inner layer is densely packed with low molecular weight proteins whereas the outer layer is highly hydrated and consists of mostly mucins.

1.3.4 Lubrication

The lubrication performance of the *in vivo* pellicle is exceptional, allowing it to protect the underlying oral surfaces from damage due to mechanical wear during e.g. mastication and speech. This ability has fascinated scientists as nature was able to overcome the limitations of water, i.e. low viscosity and high wettability, by the additions of a small amount of proteins, lipids, and electrolytes. A multitude of studies have been performed showing that the *in vitro* pellicle preserves the lubrication performance of *in vivo* pellicles, providing friction coefficients as low as 0.01 – 0.25 [28-30, 32, 33] as well as high resistance to wear with yield strengths of ~ 100 MPa [72] for a broad variety of surfaces with different wettability.

However, the underlying mechanisms behind the exceptional lubrication performance of salivary pellicles are not yet fully understood. It has been hypothesized that the outer swollen layer, presumably mainly formed by the MUC5B glycoprotein, is a major contributor due to the highly hydrated –OH groups of this molecule that immobilize large amounts of water resulting in a highly solvated layer. This could facilitate lubrication by means of the mechanism known as hydration lubrication, where the hydration layers strongly held by the charged mucin glycans can support large pressures without being squeezed out, and at the same time remain highly mobile and thus have a fluid response to shear [53, 54, 73, 74]. However, studies on the friction response of individual components of the salivary pellicle have shown that MUC5B is not the only lubricious component of the pellicle [75] and in fact, the lubrication by films formed from mucin rich fractions of saliva can be improved with the addition of other salivary components [76]. This suggests that the lubrication mediated by salivary pellicles involves multiple components working together.

Lubrication is vital for movement both in nature and man-made devices, without lubrication, surfaces are subject to wear and the system will dissipate energy through friction [54], requiring more work to be done and resulting in a lower efficiency. Yet the mechanisms governing this property of salivary pellicles are still not well established, and hence further studies are essential to achieve a fundamental understanding of the phenomenon.

2. AIMS

Salivary pellicles serve multiple purposes. They hydrate and lubricate a variety of surfaces in the oral cavity with extraordinary efficiency. One of the main components, mucins, also attract significant interest as biocompatible and lubricant coating materials for implants and biomedical devices. A similar outstanding lubrication performance has been reported for salivary pellicles formed *in vitro* on a variety of surfaces. However, there is a significant knowledge gap on the relationship between the structure of these films and their properties. One of the main reasons is that structural studies of thin soft matter films present severe methodological difficulties. This thesis approaches these studies by means of dedicated methodologies with the specific aims of:

1. Understanding the relationship between structure, hydration and lubricating mechanisms of both *in vitro* and model salivary pellicles, specifically mucin films, and how this relationship is affected by oral healthcare products containing e.g. surfactants.
2. Developing methodological approaches for investigating the structure and mechanical properties of these systems, focusing on the following techniques: Neutron Reflectometry, Atomic Force Microscopy, Ellipsometry and Quartz Crystal Microbalance with Dissipation Monitoring.

Understanding the relationship between composition, structure and functionality of salivary pellicles will open new and exciting possibilities in research. The characterization of this relationship will e.g. provide knowledge on how to avoid compromising their functionality as well as enabling a rationale restoration of their functionality if this occurs. These studies will also provide information on how to develop mucin-based coatings for artificial surfaces with properties mimicking those of natural systems.

3. METHODS

3.1 Atomic Force Microscopy

3.1.1 Instrumentation

Atomic force microscopy (AFM) is a versatile tool for analysis of surfaces and thin films. Since its origin nearly four decades ago [77], many applications have been developed making it possible to measure not only topographies but also surface properties such as viscoelasticity, friction and adhesion.

AFM is an ideal technique for investigating soft or biological samples. Minimal sample preparation is required compared to other techniques such as SEM, and samples can be measured in aqueous media with minimal set-up modifications. AFM also uses very soft micro cantilevers as probes, meaning that the forces exerted onto the sample can be kept relatively low, minimising the damage of soft samples. However, AFM has a significant limitation: the investigated samples need to be anchored to solid surfaces i.e. samples in dispersion cannot be investigated by means of this technique.

The typical set up for an AFM (Figure 3a) involves using a small cantilever which is deflected by the intermolecular forces between a sharp probe attached at its free end and the sample. This deflection is monitored by a position sensitive photodetector (PD), which tracks the reflection of a laser focused on the free end of the cantilever. The PD is made of four quadrants, allowing deflections of the cantilever to be tracked in both the vertical and lateral directions. The vertical deflection can then be calculated by comparing the signals from the top and bottom halves of the detector, whereas the lateral twisting of the cantilever can be calculated by comparing the signals from the left and right halves of the detector. The relative sample-probe

position and, therefore, the probe-sample interaction and cantilever deflection, is controlled by means of highly precise positioning systems. In most setups, including the one used in this thesis (MultiMode 8 SPM with a NanoScope V control unit, Bruker AXS, Santa Barbara CA), this is achieved by a piezoelectric tube that supports the sample and can be moved in the three spatial dimensions upon application of appropriate voltages.

Figure 3. a) Schematic of a typical AFM setup. b) SEM image of a nanofabricated cantilever, showing a sharp tip typical for imaging purposes. From <https://www.nanoandmore.com/AFM-Probe-USC-F1.2-k7.3>. c) SEM image of a colloidal probe made by gluing a silica particle to the free end of the cantilever.

3.1.2 AFM Probes

AFM probes typically have a pyramidal or conical shape, with a height between 3 μm and 15 μm, and a final curvature radius of ~20 nm. They are placed at the free end of a soft cantilever that bends upon probe-sample interaction (Figure 3b). The cantilever can be considered as a spring. Thus, its deflection can be related to probe-sample forces by its normal force constant, k_N . To be able to apply low forces, cantilevers with low k_N are required. However, to avoid effects from mechanical vibrations, cantilevers should have high fundamental resonances, at least in the order of several kHz. To fulfil both requirements, AFM cantilevers require a significantly small effective mass i.e. extremely small cantilevers are required, which can be achieved by nanofabrication techniques [78]. Most AFM cantilevers used nowadays have rectangular or triangular shapes, and range from 100 – 200 μm long and 20 – 40 μm wide.

Colloidal Probes

As discussed below, a quantitative interpretation of forces measured by AFM requires knowledge on the shape and size of AFM probes. Commercial AFM cantilevers typically have sharp probes/tips. In this regard, it is possible to use values provided by the manufacturer for the size of its final apex (often considering a spherical shape). It is also possible to obtain information on these aspects of the tip final apex by imaging special grids for this purpose. However, these approaches typically result in just approximations as the actual shape is not regular and, moreover, it can change during an experiment. To overcome these problems it is a standard practice to use colloidal probes, where a colloid such as a silica micro-particle is attached to the end of the cantilever [79], resulting in a better defined geometry and contact area, useful for data analysis of probe-sample interaction forces.

To make colloidal probes in this thesis work, silica particles of $\sim 15\ \mu\text{m}$ diameter were attached to the free end of cantilevers (Figure 3c) with a two component epoxy glue. The silica particle was attached using a micromanipulator under an optical microscope before being characterised with either variable pressure Scanning Electron Microscopy (EVO LS10, Zeiss, Germany) or an optical microscope (Nikon Olympus UC90, Amsterdam, The Netherlands).

3.1.3 Normal Force Spectroscopy

AFM can be operated in the Force Spectroscopy mode, where the normal forces between the probe and sample are measured. This mode allows quantifying properties of the sample such as its elasticity and the adhesion between probe and sample. A detailed review of the method can be found in [80]. In a typical force measurement, also named force curves or force ramps, the sample is moved towards and away from the probe while registering the cantilever deflection with the PD.

A typical force curve follows the deflection vs sample vertical displacement pattern shown in Figure 4a. Far from the sample the probe-sample interaction can be neglected. Therefore, the cantilever deflection remains constant regardless of the sample vertical position ((1) in Figure 4a). As the probe approaches the surface, long range forces with the sample might develop causing the cantilever to deflect ((2) in Figure 4a). Subsequently, the probe establishes mechanical contact with the sample ((3) in Figure 4a), as observed by a drastic increase of the cantilever deflection. Upon

withdrawal, negative deflections are observed if the probe develops an adhesive interaction with the sample ((4) in Figure 4a).

Figure 4. a) Schematic of a typical cantilever deflection vs.-sample vertical position measurement. 1. Represents the cantilever far from the surface where there is no probe-sample interaction. 2. Shows the long range interactions. 3. Shows the cantilever deflection when in mechanical contact. 4. Shows the adhesive force when the cantilever is withdrawing from the sample. b) The corresponding force vs probe-sample distance curve. Adapted with permission from [80].

Fitting normal force curves to physical laws allows quantifying different properties of the sample. However, these curves need to be transformed first to a force vs. probe-sample distance representation (Figure 4b). To do this, the PD signal needs to be first converted into deflection units by scaling with the normal sensitivity, δ_N . Next the cantilever deflection, d , is scaled by the cantilever spring constant, k_N , to obtain the probed force, F .

Transforming sample vertical displacement to probe-sample distance can be a more complex process. For samples that can be mechanically deformed by the AFM probe, such as the ones investigated in this thesis, it is useful to start this process focusing on the sample deformation, δ , which can be expressed as:

$$\delta = z - z_0 - d \quad \text{Equation 1}$$

Here z is the sample displacement, z_0 is the contact point and d is the deflection of the cantilever. Finding the contact point is non-trivial, especially for samples that can also be deformed by non-contact forces [80]. In this thesis, we followed the approach from [81]. Specifically, for each force curve we selected the region that could be

unequivocally associated with mechanical contact and fitted it to the Hertz contact model for a sphere-plane geometry which relates force and deformation:

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

where E is the Young modulus of the sample, R the probe radius and ν is the Poisson's ratio of the sample. By combining Eq. 1 and Eq. 2 we obtain:

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

Fitting the deflection, d , vs. sample displacement, z , measurement to the above equation allows the contact point, z_0 , to be found. Then, using, z_0 , the sample vertical position can be converted to real probe-sample distance, d_{ts} , by adding the corresponding cantilever deflection:

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

It can also be noted that the fit to the Hertz contact model, or to other mechanical model for that matter like the Derjaguin-Muller-Topolov (DMT) or Johnson-Kendall-Roberts (JKR) models [82-85], also allows quantifying the elasticity of samples in terms of their Young modulus.

In this thesis work, force measurements were acquired by operating in the so-called Force Volume mode, where force measurement were acquired at equally spaced points within a sample area e.g. 16 x 16 measurements in a 2 x 2 μm^2 area. This allowed for statistical analysis of the results.

Analysis of steric interactions from normal force measurements

Once that force curves are in the force vs. probe sample representation, they can be fitted to physical models to quantify e.g. sample properties. In this thesis, force curves of salivary pellicles and mucin films exhibited a long-ranged force in the non-contact region that decayed exponentially with the probe-sample distance. Provided the polymeric nature of the samples investigated in this thesis (salivary pellicles and mucin films), we used the Alexander – de Gennes expression [86] for the force per unit area, f , between two surfaces with adsorbed or grafted polymers as a starting point for analysing our data:

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

where 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.

If only one of the surfaces is covered with a polymer-like film (as was the case in the works of this thesis where we used this analysis), D/L can be replaced by $D/2L$. Additionally, for $0.2 < D/L < 0.9$, the above expression is roughly exponential and can be approximated as [87, 88]:

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

The absolute force, F , can be obtained from this expression for a tip with rotational symmetry by integrating over cross-sectional areas $2\pi r dr$:

$$F \approx 2\pi \int_0^\infty f r dr = 2\pi \int_D^\infty f(z) r \frac{dr}{dz} dz \quad \text{Equation 7}$$

Assuming a parabolic shape for the tip leads to $z - D = r^2/2R$, where R is the radius of a sphere inscribed at the end of the tip. Combining Equations 6 and 7, we obtain [87]:

$$F \approx 50 k_B T R \Gamma^{3/2} e^{2\pi D/L} \quad \text{Equation 8}$$

Accordingly, we fitted the non-contact region of the acquired force curves to an exponential function¹ $F = F_0 e^{-d_{ts}/\lambda_0}$. In this scheme, the characteristic length, λ_0 , provided an estimation of the thickness of the anchored polymers in the investigated films.

3.1.4 Friction Force Spectroscopy

Friction forces were measured by raster scanning the surface of the samples while applying a constant normal force and simultaneously monitoring the photodetector lateral signal. For each scan line this signal is registered in both trace and retrace

¹ In Paper I it is stated that the Derjaguin approximation could be used to relate force per unit area to force. This is incorrect, as the Derjaguin approximation can be used to relate interaction energy to force instead. While this does not change the results of the manuscript and their interpretation, the correct modelling leading to the exponential behavior for the interacting force is the one described in this section.

directions. Then, the difference between the photodetector lateral signals, ΔV_l , for the trace and retrace is calculated for each scan line (Figure 5) and subsequently averaged for all scan lines. Finally, the average friction force, F_f , for the scan is calculated according to Equation 9, as demonstrated in [72]:

$$F_f(\Delta V_l) = \frac{k_\phi}{\delta_\phi} \frac{1}{h_{eff}} \frac{\Delta V_l}{2} \quad \text{Equation 9}$$

where k_ϕ is the torsional spring constant, δ_ϕ is the lateral sensitivity and h_{eff} is the effective height of the probe.

Figure 5. Representative example of lateral signal (volts) while scanning a line in the trace (blue) and retrace (red) directions. In this thesis work, we excluded the lateral deflection values monitored at the end of the scanned lines (dashed lines in the figure) from further $\Delta V_{Lateral}$ calculations.

In this thesis, friction force spectroscopy measurements consisted of sets of 2D-scans performed on the same area of the sample, each of them at a constant applied normal force, which was gradually increased between scans. The normal force applied by the probe during each scan, F_N , is then given by:

$$F_N = k_N \delta_N (V_{vertical} - V_{vertical0}) \quad \text{Equation 10}$$

where k_N is the normal spring constant and δ_N is the normal sensitivity. $V_{vertical0}$ is the vertical photodetector signal registered when the cantilever is far from the sample and $V_{vertical}$ is the vertical photodetector signal during the scan acquisition. $V_{vertical0}$ was measured by means of normal force curves both before and after each friction force spectroscopy experiment to account for any drift.

3.1.5 Calibration

As discussed above, many AFM applications rely on quantifying the forces that develop between probe and sample. This measurement implies converting vertical and lateral PD signals resulting from vertical and torsional deflections of the cantilevers into normal and lateral forces.

For quantifying vertical forces it is required to calculate the vertical sensitivity, δ_N , and the normal force constant of the cantilever, k_N . The vertical sensitivity, δ_N , relates the vertical PD signal to cantilever deflection in length units. The most common approach to calculate it is by displacing along the sample-probe normal direction a sample assumed to be infinitely hard in mechanical contact with the AFM probe while registering the PD vertical signal. When in contact, shifts in the PD vertical deflection will correspond to the length the sample was displaced by the piezoelectric tube, which is a known quantity given that the piezoelectric has been previously calibrated. In this thesis, clean mica and silica surfaces were used for calculating δ_N . Subsequent conversion of cantilever vertical deflections into normal forces requires determining the normal spring constant, k_N , which linearly relates deflections to forces under the assumption that the cantilever can be modelled as a spring. While different approaches can be followed, in this thesis the normal spring constant was calculated for each cantilever by means of the Sader method [89]. This involves measuring the thermal fluctuations of the cantilever from where the power spectral density is calculated. Subsequently, the first flexural mode of the cantilever is fitted to a Lorentzian function, which allows obtaining the corresponding frequency, f_N , and quality factor, Q , of the mode, which are then related to k_N by equation 11:

$$k_N = 0.1906\rho b^2 l Q (2\pi f_N)^2 \Gamma_i^N(Re_N) \quad \text{Equation 11}$$

where ρ is the density of the fluid and l and b are the length and width of the cantilever. $\Gamma_i^N(Re_N)$ is the imaginary component of the hydrodynamic load function for normal vibrations of a beam with a rectangular cross section, described in detail in [90], which only depends on the Reynolds number, Re_N ,

$$Re_N = \frac{\rho b^2 2\pi f_N}{4\eta} \quad \text{Equation 12}$$

where η is the viscosity of the surrounding fluid.

For quantifying lateral forces, it is required to calculate both the detector lateral sensitivity, δ_L , and the torsional constant of the cantilever, k_ϕ . In this thesis, k_ϕ was determined by the approach proposed by Sader and co-workers [91]:

$$k_\phi = 0.1592\rho b^4 l Q_t(2\pi f_t) 2\Gamma_i^t(Re_t) \quad \text{Equation 13}$$

$$Re_t = \frac{\rho b^2 2\pi f_t}{4\eta} \quad \text{Equation 14}$$

Again, ρ and η are the density and viscosity of the fluid and L and b are the length and width of the cantilever. $\Gamma_i^t(Re_v)$ is the imaginary component of the hydrodynamic load function for torsional vibrations of a beam with a rectangular cross section, for which an analytical expression is provided in [92], which again only depends on the Reynolds number, Re_t (Eq. 14).

The detector lateral sensitivity, i.e. the parameter that allows the conversion of the lateral detector signal to the angle of twist of the cantilever, was calculated using a combination of the Sader [91] and pivot methods [93] as proposed in [94]. The implementation of the pivot method used to calibrate the lateral sensitivity of the AFM used in this thesis involved gluing an upturned cantilever to a substrate making a pivot, then force curves were acquired with a tip-less cantilever while the moving the piezo laterally in a direction perpendicular to the long axis of the tip-less cantilever. The vertical deflection sensitivity for a load applied in the centre of the cantilever, α_0 , at a distance L away from the long axis, α_L , and the lateral or torsional sensitivity when the load is applied at a distance L from the long axis, β_L , are related to the detector lateral sensitivity, δ_L (V/rad), through the normal and torsional force constants as follows [93, 94]:

$$\frac{\alpha_L}{k_N \beta_L \alpha_0} = \frac{\delta_L}{k_\phi} L \quad \text{Equation 15}$$

Thus, if k_N and k_ϕ are known, e.g. after calibration using the Sader methods, it is possible to find δ_L .

3.1.6 Contact Thermal Noise Spectroscopy

This method, which can be used to characterize the sample viscoelasticity, is based on monitoring how the Brownian motions of cantilevers are modified due to mechanical contact with the investigated sample [95-97]. Specifically, the thermal fluctuations are first registered far from the sample and subsequently in mechanical

contact with the sample while applying a certain load (Figure 6). In both situations, the power spectral density for the registered fluctuations is calculated, and the first resonance peak fitted to a Lorentzian function to obtain both the quality factor and frequency. This methodology has been used to characterize the viscoelasticity of samples in terms of e.g. their loss tangent, upon appropriate modelling in terms of the Euler–Bernoulli model modified with a Kelvin-Voigt spring-dashpot element at the probe-sample contact [97, 98]. However, these models do not take into account possible modifications of the viscoelastic properties of samples by the applied load. In this thesis, we interpreted Contact Thermal Noise Spectroscopy data qualitatively instead. By solving the Euler–Bernoulli equation for the flexural vibrations of both non-supported and supported rectangular cantilevers on an infinitely hard surface, it can be found that for the latter the frequency of the first resonance will increase by a factor of ~ 6.5 [99, 100]. Upon a softer contact and therefore sample, mechanical contact would not be so well-defined resulting in a lower increase of the resonance frequency. The quality factor can be related instead to the dissipation of energy through the surrounding medium. When comparing the resonances while in contact with different samples, lower quality factors will indicate higher dissipation of thermal energy and, therefore, a more viscous sample character [98, 100].

Figure 6. Scheme representing the basics of Contact Thermal Noise AFM. This AFM operation mode is based on the comparison between the frequencies and quality factors of the resonances of AFM cantilevers measured (a) far from the sample and (b) in mechanical contact with the sample. Adapted with permission from [98]. Copyright 2020 American Chemical Society.

3.1.7 AFM Imaging

The pioneering method to image a sample with an AFM is by operating it in the contact mode [77]. In this mode, the sample is raster scanned with the cantilever while adjusting the sample vertical position by means of a feedback loop in order to maintain the cantilever deflection at a fixed constant value. Under the assumption that the sample is not modified by the scanning process, its topography is then provided by the inverse of the sample vertical displacement. However, this mode has some drawbacks for the visualization of soft biological samples. One being the equilibrium cantilever deflection i.e. that corresponding to zero interaction force, is subjective to drifts, especially when working in a fluid environment. If this happens, the applied force will vary during the imaging process. Additionally, while AFM cantilevers are characterized by low normal force constants, this is not the case for torsional force constants, implying that lateral forces applied while imaging in the contact mode can be highly destructive for biological materials.

The drawbacks of imaging in the contact mode can be overcome to some extent by alternative imaging modes. In the so-called dynamic modes [101], where the cantilever is oscillated at a frequency close to one of its flexural modes and the sample vertical position adjusted in order to keep one of the parameters that characterize the cantilever oscillation constant, e.g. the amplitude, can significantly reduce the invasiveness of the imaging process. A different option is to use imaging modes based on normal force measurements. These modes, while implemented in different ways under different names [102-106], have the commonality of one or more force measurements being performed at each scan point. This approach has the advantages of i) drifts in the cantilever equilibrium deflection are corrected in real time and ii) lateral forces are minimized as lateral displacements are typically performed at the point of maximum probe-sample separation. In this thesis, AFM imaging was performed by operating in the PeakForce Tapping® mode, which is actually a combination of dynamic modes and modes based on force measurements. Specifically, while imaging in this mode the sample is oscillated at a frequency in the order of kHz so that contact with the sample and, therefore, lateral forces are minimized still allowing to correct drifts in the cantilever equilibrium deflection.

3.2 Quartz crystal microbalance with dissipation monitoring

Quartz crystal microbalance with dissipation monitoring (QCM-D) is a technique that allows the adsorption process of thin homogenous films to be measured with nanogram precision as well as real time monitoring of the structural and viscoelastic properties of the films [107].

An AC voltage is applied through a gold-coated quartz crystal to stimulate the shear mode oscillation of the quartz crystal. The shifts in frequency, Δf_n , and dissipation factor, ΔD_n , of the different n sensor overtones that are induced by the adsorbed mass are monitored throughout the experiment. In the setup used in this thesis (E4 system, Q-sense AB, Sweden), the dissipation factor is calculated from the damping of the oscillations when the AC current is switched off, and is related to the ratio between dissipated and stored energy.

The frequency shift scaled by the overtone number, $\Delta f_n/n$, in the oscillations of the quartz crystal can be linearly related to the mass change on the crystal by the Sauerbrey equation [108]. However, this relationship assumes the adsorbed mass to be rigid and non-porous, which in the case of samples such as saliva and mucous films does not hold. Therefore, to take into account the viscoelastic character of such samples, the damping of the oscillations is also monitored in terms of the shifts in dissipation factor, ΔD_n . In this case, viscoelastic models, such as the Voigt model as detailed below, should be used to interpret the acquired frequency and dissipation shifts.

In this thesis, the same protocol for setup and sample adsorption, where the saliva or mucins were introduced into the cell using an Ismatec peristaltic pump IPC-N 4 at a flow rate of $0.1 \text{ mL} \cdot \text{min}^{-1}$ and allowed to adsorb for one hour, was used for all QCM-D experiments.

As mentioned, the experimental outputs of QCM-D experiments are the shifts in the frequency, Δf_n , and dissipation factor, ΔD_n , of each of the odd overtones ($n = 1, 3, 5, 7, \dots$) of the sensor. For viscoelastic films like the ones investigated in this thesis, where noticeable shifts in the dissipation factor, ΔD_n , are observed and $\Delta f_n/n$ differs for different overtones, a more reliable analysis is obtained by applying the Voigt model. The expressions obtained by applying the Voigt model for a viscoelastic film adsorbed onto a solid substrate and immersed into a Newtonian fluid are [109, 110]:

$$\Delta f_n / f_n = - \frac{d_p \rho_p}{d_Q \rho_Q} \left[1 - \eta_l \rho_l \frac{(\eta_p / \rho_p \omega_n^2)}{\mu_p^2 + \omega_n^2 \eta_p^2} \right] \quad \text{Equation 16}$$

$$\Delta D_n = \frac{1}{d_Q \rho_Q} \left[\eta_l \rho_l \frac{d_p \mu_p \omega_n}{\mu_p^2 + \omega_n^2 \eta_p^2} \right] \quad \text{Equation 17}$$

In these equations d , ρ , and ω_n stand for thickness, density, and $2\pi f_n$, the frequency of the overtone, respectively. The subscripts Q , p , and l stand for quartz crystal, protein film, and liquid medium, respectively. By a simultaneous numerical fit of the frequency and the dissipation values of at least three different overtones, not only the thickness, d , can be obtained, but also the viscoelastic properties of the film, like the shear elastic modulus, μ , and the shear viscosity η [111].

The Q-Tools software (Q-Sense AB, Sweden) was employed for the fitting of the experimental data to the Voigt model. The initial input values given for fluid density and viscosity, and for the density of the adsorbed protein films are specified within the papers.

3.3 Ellipsometry

Ellipsometry is an optical technique based on changes in the polarisation of a light beam when it is reflected at an interface [112, 113]. An incident beam of polarised light will reflect of the surface, where a film can be adsorbed, the changes in the polarisation state can then be used to determine the refractive index, thickness, and hence, the adsorbed mass on an interface as it evolves over time. The polarisation of light refers to the behaviour of the electric field vector of the propagating wave. This is a result of the two components of the light wave, that oscillating parallel (p) and that oscillating perpendicular (s) to the plane of incidence. These two components have the same frequency, but can differ in both phase and amplitude resulting in a general elliptical polarisation. However, special cases where the phase difference is 0° or $\pm 90^\circ$ results in linear or circular polarisation respectively (Figure 7) [112, 113].

Figure 7. Types of polarized light: Linear is the case where both components of the light wave are in phase. Circular polarisation is the case where the components are 90° out of phase with equal amplitude. Elliptically polarised light occurs when the phase difference is a value other than 90° or if the components have a phase difference of 90° but with different amplitudes.

In this thesis, the so-called null-ellipsometry implementation was used. During a null-ellipsometry experiment, the light from the light source is first linearly polarised by the polariser, then the light passes through a quarter wave plate (the compensator in Figure 8) which results in elliptically polarised light due to the induced phase shift between the s and p wave vectors. The polarisation of the light is then further affected by reflection off the surface at, in our case, the solid liquid interface. Finally the light reaches another polariser, called analyser in this setup, before being recorded by the detector. Throughout the measurements, both the polariser and analyser are adjusted using stepper motors to minimize the signal reaching the detector (hence the name, *null* ellipsometry). At a specific elliptical state of the light it will turn linear when reflected and the linearly polarized light can be extinguished by the analyser. The positions of the polariser and analyser when minimum light intensity is obtained can be directly related to the optical properties of the sample.

When the light is reflected off the surface the state of polarisation changes and is considered in terms of the ellipsometric angles; delta, Δ , and psi, ψ , which are calculated from the polariser and analyser positions when the nulling setting is true. Δ is the phase shift, induced by reflection, of the phase difference between p-wave and s-wave ($\Delta = \delta_1 - \delta_2$), 1 and 2 represent the p and s waves respectively and ψ is the reflection induced amplitude reduction of reflected s and p waves ($\tan\psi = \frac{|R^p|}{|R^s|}$). The ellipsometric equation (equation 18) relates Δ and ψ to the *complex reflectance ratio*

where R^p and R^s are the ratios of the reflected vs. incident amplitudes. This ratio depends on the optical properties of the substrate, the film and the surrounding medium (Figure 9). ψ and Δ are measured directly.

$$\rho = \tan(\psi)e^{i\Delta} = \frac{R^p}{R^s} \quad \text{Equation 18}$$

In the work of this thesis, oxidized silicon surfaces were used for all ellipsometry experiments. Thus, a 4 layer model (accounting for a silicon/silica/sample/bulk structure) was used to find n and d for the sample. The sample adsorbed amount, Γ , was then be calculated using de Feijter's equation [114]:

$$\Gamma = \frac{(n_f - n_o)d_f}{\frac{dn}{dc}} \quad \text{Equation 19}$$

where n_f is the refractive index of the adsorbed film, d_f its thickness, n_o the refractive index of the bulk solution and dn/dc is the refractive index increment as a function of concentration of the molecules in the film. In the present work, a value of 0.18 ml/g was assumed throughout, as is typical with protein films. [114].

Figure 8. Schematic diagram of the null ellipsometry set up.

Ellipsometry investigations were performed with a Rudolph thin film ellipsometer (type 43603-200E, Rudolph Research, USA) equipped with a Xenon light source filtered to 442.9 nm. A schematic of the set-up is shown in Figure 8. During an ellipsometry experiment, the oxidized silicon surface was first characterised in both air and water ambient media, which allowed determining the complex refractive index and thickness of the silica layer on its surface. The saliva/mucins were then

adsorbed from bulk solution for 1 h, subsequently rinsed with PBS buffer for 5 min and finally allowed to stabilise for a further 55 min. In all our experiments, a flow of $15 \text{ ml} \cdot \text{min}^{-1}$ was used. The specifics of the protocol used in each study can be found in the papers included.

Figure 9. An incident light beam on a thin film anchored to a surface will reflect and refract the light beam at each interface, as can be seen here. For a silica substrate (as used in this thesis), the incident beam first hits the interface between the film and ambient media, where the light is both reflected and refracted. The refracted beam will then continue towards the silica/film interface where the same will happen and so on.

3.4 Neutron Reflectometry

Scattering techniques are methods that investigate a sample by monitoring how an incoming beam (of neutrons in this thesis work) interacts with a sample. Neutrons interact directly with the nuclei of the atoms. However, the strength of this interaction, known as the scattering length, which is proportional to the amplitude of the scattered waves, is seemingly random among different elements. There is no dependence on atomic mass and it even differs between isotopes of the same element. For instance, there is a strong scattering difference between deuterium and hydrogen, which is one of the biggest benefits of using neutron scattering, particularly for biological samples.

Specular neutron reflectivity is a surface characterisation technique to give the structure and composition, in terms of Scattering Length Density (SLD), of thin (multi-) layers along the direction normal to the interface. The reflected intensity depends on the scattering length density (i.e. the sum of coherent scattering lengths for the atoms over a volume) and thickness of the layers. A highly collimated neutron

beam is reflected at a very small angle off a highly planar surface (Figure 10a). The specular reflection results in a reflected beam with wave vector, k_r , and angle, θ_r , which is equal to the incident beam.

$$k_i = k_r = \frac{2\pi}{\lambda} \quad \text{Equation 20}$$

The momentum transfer vector, Q_z , is then defined as

$$Q_z = \|k_r - k_i\| = \frac{4\pi \sin \theta}{\lambda} \quad \text{Equation 21}$$

Reflectivity, $R(Q_z)$, is a function of the momentum transfer, where the reflected neutron beam intensity is normalised by a measurement of the direct beam intensity to give the reflectivity, $R(Q) = I/I_0$, this is the reflectivity curve. $R(Q_z)$ typically consists of a number of waves reflected from different planes within the sample. For example, in the schematic diagram of reflection shown in Figure 10a, the neutron beam is first reflected at the bottom interface of the thin layer, then a refracted beam is reflected at the top interface. The interference of these reflected waves results in oscillations of the intensity, which manifest as Kiessig fringes in the reflectivity curve. These fringes are then related to thickness by $\Delta Q = \frac{2\pi}{d}$, where ΔQ is the spacing between the fringes (Figure 10b).

During a reflectivity experiment the reflected neutrons are detected over a Q -range which is determined by i) varying the incident angle of a monochromatic beam to achieve the desired range e.g. on the SuperAdam beam line (ILL, France), or ii) by using a time of flight (TOF) beamline e.g. on the Inter and Surf beamlines (ISIS UK) and the D17 and Figaro beamlines (ILL, France). TOF beamlines use a neutron beam which contains neutrons travelling at varying speeds. The wavelength, λ , and speed, v , are related by the de Broglie relationship, $v = \frac{h}{m\lambda}$ where h is Plank's constant and m is the mass of the neutron. Therefore, by pulsing the beam with a chopper, the travel time between the fastest and slowest neutrons can be differentiated and therefore the range of wavelengths determined, resulting in a Q -range. It is then common to take measurements at two or three angles only in TOF mode.

Figure 10. a) Schematic of a neutron incident beam reflecting of a thin film on a substrate. b) An example neutron reflectivity curve where ΔQ can be seen as the fringe spacing.

3.4.1 Contrast Variation

The difference in scattering between H_2O and D_2O allows contrast variation to be used without changing the chemistry of the sample [115]. This can be done in different ways. Usually in solid liquid cells, contrast variation is achieved with the bulk liquid. The sample can be measured multiple times with H_2O , D_2O or a mix of the two as the liquid, e.g. silicon matched water (SMW) with 62% H_2O and 38% D_2O . This allows multiple data sets to be collected for the same sample, which means multiple parameters can be found from the sample model. Furthermore, manipulating the ratio between H_2O and D_2O used in the bulk can allow parts of the sample to be ‘matched out’, i.e. the SLD of the bulk is mixed to be the same as the part of the sample to be matched out, making it invisible to neutrons and other parts of the sample stand out more. This can be further exploited by deuterating parts of the sample, a common practice in samples such as surfactants or lipids.

3.4.2 Data analysis

The SLD profiles are not directly obtained from the reflectivity data, they are achieved by fitting the reflectivity curve to a model described mathematically by a combination of the Fresnel equation, Snell's law and the SLD to optical refractive index relation. The roughness, σ , is factored in by scaling the reflectivity curve with sharp interfaces, $R_0(Q)$, such as:

$$R(Q) = R_0(Q)e^{-QQ'\sigma^2} \quad \text{Equation 22}$$

where Q is the momentum transfer in the upper layer and Q' is the momentum transfer in the lower layer separated by the roughness, σ [116].

In this thesis, NR data was fitted using the RasCAL (<http://sourceforge.net/projects/rscl/>) or the refnx [117] software, which numerically fit the reflectivity to the above mentioned equations until the lowest chi squared value is found.

For fitting purposes, the sample is typically modelled by homogeneous slabs between a support (silicon) and the bulk liquid. Each slab is described by four parameters: thickness, the scattering length density (SLD), the volume fraction of the solvent (hydration of the layer) and the surface roughness. The SLD and hydration of a layer are related according to

$$SLD_{layer} = (SLD_{sample} \times (100 - Hydration)/100) + (SLD_{bulk} \times Hydration/100)$$

Equation 23

However, in some cases, e.g. in Paper III in this thesis, the sample cannot be modelled by homogeneous slabs. In the presence of in-homogeneities or different interfaces, with corresponding areas not smaller than the relevant coherent length ($\sim 10 \mu\text{m}$), mixed reflectivity models can be used. In these models, the overall reflectivity is considered to be a weighted average from different reflectivities accounting for the same number of interfaces. For instance, in the presence of two different interfaces with the corresponding reflectivities, R_A and R_B , the total reflectivity is given by:

$$R_{total} = x \cdot R_A + (1 - x) \cdot R_B \quad \text{Equation 24}$$

where $0 < x < 1$.

3.4.3 NR Sample Environment for Mechanical Confinement Studies

Within this thesis, a sample environment to mechanically confine samples during scattering experiments was used. A scheme for this sample environment, named from now on as the confinement cell, is shown in Figure 11. It consists of a stainless steel shell in which the cylindrical substrate is placed. A flexible membrane is then inflated by an external gas supply to a set input pressure, putting the sample under mechanical contact. The use of a flexible membrane is to overcome long range waviness observed in systems that press two solid surfaces together to exert confinement.

Figure 11. A Schematic diagram of the mechanical confinement cell for NR studies. A solid block e.g. silicon, covered with the sample of interest, is placed on a stainless steel support and exposed to solvent. A flexible membrane is then inflated by a known gas pressure, leading to the sample and membrane surfaces coming into close contact. A masking ring on top of the silicon block (shown in dark grey) protects the membrane from overstretching. Reprinted from Review of Scientific Instruments, 2012, 83, 113903 [118], with the permission of AIP Publishing.

3.5 *In vitro* Salivary Pellicles

3.5.1 Surfaces

While powerful in many aspects, the techniques utilised in this thesis limit the use of oral surfaces such as mucosa or enamel. AFM, QCM-D, NR and ellipsometry require highly planar surfaces. NR and ellipsometry also require them to be reflective. Additionally, QCM-D requires that the surface can be mechanically coupled to a gold-coated quartz crystal.

For accomplishing the objectives of this thesis, it was required to identify surfaces compatible with these techniques. Moreover, we also aimed at that the identified surfaces would be good models for oral surfaces, as well as for materials that could benefit from coatings mimicking *in vitro* or model salivary pellicles.

An important factor to consider for identifying good models for oral surfaces is the ionic character of the chosen surface. Oral mucosa surfaces are decorated with the MUC1 mucin [49], and thus they have an anionic character. A similar character has been reported for hydroxyapatite [67], the main component of enamel. Furthermore, *in vivo* wettability measurements have indicated the hydrophilic nature of teeth and oral mucosa surfaces [119].

Thus, we identified silica as a suitable surface for this thesis work. First, silica is a suitable surface for all techniques listed above. Moreover, clean hydrophilic silica substrates, which are characterized by an anionic character, could be considered as appropriate models for oral surfaces according to the considerations above. Additionally, silica can be modified in multiple ways to provide different physico-chemical characteristics [120], allowing its use as models for different materials that could benefit from coatings mimicking salivary pellicles.

We exploited this approach for preparing films of the oral mucin MUC5B, using hydrophobized silica surfaces as substrates following previous studies [121-123]. This enabled the investigations of MUC5B films in Papers I and III, since mucins barely adsorb on hydrophilic anionic substrates [122]. In this thesis work, silica surfaces were hydrophobized by liquid phase salinization, as described in [39].

3.5.2 Saliva Collection and *in vitro* Pellicle Formation

The saliva used in this thesis work was collected from two healthy adults a minimum of 60 minutes after cleaning their teeth and without consuming any food or drink prior to collection for a minimum 60 minutes. For unstimulated saliva, the donors drooled into a chilled falcon tube according to the method described in [124], while for stimulated saliva the donors chewed on parafilm according to the method described in [125]. The saliva from the two donors was immediately mixed, and in some instances filtered. Ethical approval for saliva collection was obtained from the committee of research ethics at Lund University (2018/42).

Depending on the technique employed, the saliva was either flowed into a cell environment or dropped onto the surface studied, where the pellicle will immediately begin to form. The pellicle was left to adsorb and stabilise for 60 minutes before rinsing with buffer solution, which was considered enough time for the initial pellicle formation as shown previously by QCM-D experiments [126].

3.6 Isolation of MUC5B mucins

In papers I and III, films formed by the glycoprotein MUC5B were studied. The isolated mucins were obtained by our collaborators Carolina Robertsson and Claes Wickström from the Department of Oral Biology and Pathology at Malmö University. The MUC5B was isolated from human whole saliva as previously described [127] using a modified version of the method described in [128]. This method prevents the denaturing of the protein, keeping its gel forming ability. To summarise, 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, the supernatant was subjected to density-gradient centrifugation in CsCl/ 0.1 M NaCl. Fractions were analysed for density by weighing and measuring the absorbance at 280 nm. MUC5B-containing fractions were identified using an antiserum, LUM5B-2, which recognizes the central domain of the MUC5B polypeptide backbone [129]. The MUC5B-containing fractions were pooled and dialysed against PBS and then stored at -20 °C until use. MUC5B concentration was determined by extensive dialysis against water, freeze-drying and weighing, and estimated to be 0.3 mg·ml⁻¹.

4. RESULTS AND DISCUSSION

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

Paper I challenged the hypothesis of the outer layer of *in vitro* salivary pellicles being mainly formed by the MUC5B mucin. For this, we investigated the response of both salivary pellicles and mucin films when changing the bulk ionic strength. Mucins, because of the high occurrence of negatively charged terminal carbohydrates, are often considered to have a polyelectrolyte-like behaviour. It would therefore be expected that the response of MUC5B films to differing ionic strength would also be similar to that of a polyelectrolyte brush, with swelling at lower ionic strengths. Following from this, if the outer layer of the salivary pellicle is in fact comprised of MUC5B, then a similar response of both the salivary pellicles and mucin films would be observed.

For this study, we combined AFM normal force measurements, QCM-D and NR to investigate salivary pellicles formed *in vitro* on silica surfaces and MUC5B films formed on hydrophobized silica surfaces. Using AFM, an immediate swelling was observed for both the pellicles and mucin films when the ionic strength was decreased. This behaviour is analogous to that of weak polyelectrolyte brushes, which can regulate their thickness in response to change in environmental ionic strength, as would be expected [130, 131]. However, when the time evolution of the response to decreasing ionic strength was explored, it became apparent that the behaviour differed between the pellicles and mucin films. There was a subsequent coiling of the mucin films, first observed with AFM force curves taken 80 minutes after exchanging to a lower ionic strength. This was not observed for salivary pellicles. Figure 12 includes histograms of the characteristic length of the steric repulsion between probe and films, an indicator of their thickness, in both PBS and PBS diluted 100 times

(PBS/100). QCM-D allows continuous monitoring of the thickness and was used in further investigations, showing that when lowering the ionic strength, the mucin swelling was an instantaneous response, which eventually reversed within the experimental time.

Furthermore, QCM-D indicated that there is an irreversible decrease in the thickness of the pellicle after the ionic strength is increased again to physiological values. NR allows structural analysis at a higher resolution and was used to further investigate the latter observation. Firstly, NR data supported the 2-layered pellicle, with a model consisting of a dense inner layer and a highly hydrated outer layer. Furthermore, using this technique it was shown that the irreversible decrease in thickness could be attributed to a partial removal of the pellicle inner layer. Considering that electrostatic interactions generally do not influence the desorption of proteins at an interface [132], the partial removal of the inner layer of the pellicle suggests that the inner layer is not only composed of proteins directly adsorbed on the substrate, but is probably a multi-component layer, despite its thickness being in the order of few nm, where electrostatic interactions between the different components are of relevance.

NR also confirmed the swelling of the pellicles with decreasing ionic strength and indicated that the thickness increase could be attributed to the outer layer.

Together, these techniques gave a fuller picture of the behaviour of the pellicle at different ionic strengths and therefore insight into its structure. Firstly, a two layer structure was confirmed, where the outer layer swells due to decreasing ionic strength. However, the clear difference between the behaviour of the mucin layer and the pellicle indicates that the outer layer is not entirely composed of mucins alone and other components are required for stability, preventing the inevitable coiling seen in the mucin films, summarised in Figure 12. It is likely that without these extra components the hydrophobic regions of the mucins are able to interact, causing the coiling.

It has long been speculated that the mucins in the salivary pellicle are responsible for the lubrication performance of the pellicle, owing to mucin behaviour being similar to that of charged polymer brushes. This study has shown that while the pellicle outer layer does behave similarly to polyelectrolytes, pellicle behaviour is not attributed to mucins alone, but a combination of mucins and other components. Furthermore, this study showed that the structure of the salivary pellicle is more complex than expected and the outer layer is not as simple as an anchored layer of mucins, but also carries other components which prevent mucin-mucin interactions. Therefore, when

considering bio-mimicking strategies for applications such as artificial saliva, anti-biofouling coatings and bio-compatible lubricants, this should be taken into account.

Figure 12. Schematic representation of the response of salivary pellicle compared to MUC5B mucin films when the ionic strength of the bulk fluid is decreased. In both cases, an initial swelling is observed, but for mucins this is followed by a subsequent coiling. This is demonstrated by the large decrease in the characteristic length of the measured long range repulsive forces, an indicator of the film thickness, after 80 minutes compared to that after 20 minutes.

4.2 Role of the reversibly bound fraction of *in vitro* salivary pellicles on their mechanical and lubrication properties

As discussed, multiple studies have been performed on the mechanical and lubrication properties of *in vitro* and model salivary pellicles. However, there is one aspect that has been overlooked in the past, i.e. how these properties are affected by the reversibly bound fraction only observed when *in vitro* pellicles are in the presence of bulk saliva. For this, we used AFM in different ways to compare the adhesion, softness, viscoelasticity and lubrication of the pellicle in both saliva and a saliva free solution (PBS buffer) to investigate if the reversibly bound fraction had any effect on the pellicle mechanical properties.

After confirming, by means of ellipsometry, the presence of the reversibly bound fraction when the *in vitro* pellicles were exposed to bulk saliva, AFM contact thermal noise spectroscopy was used to investigate the effect of this fraction on the viscoelastic character of mechanically confined pellicles, as the viscosity under mechanical confinement is one of the properties mediating the lubrication of a system. While these experiments established the viscoelastic character of *in vitro* pellicles, the difference between the pellicles in bulk saliva and saliva free solutions was negligible. This indicates that the reversibly bound fraction has no effect on the viscoelasticity of the pellicle under the applied loads (~2 nN). It was not possible to measure at lower applied loads as the background noise hid the contact resonances. Interestingly, at lower loads, force measurements showed that pellicles were softer in the presence of bulk saliva. Therefore, it cannot be excluded that the reversibly bound fraction had an effect on the viscoelasticity at lower loads than those applied in this work, something that should be investigated in the future.

Adhesion is another important factor to consider, especially when discussing lubrication. Adhesive interactions between opposing pellicles are likely due to the entanglement between the surfaces. This entanglement opposes shear forces and therefore requires energy to continuously disentangle the surfaces, and if the energy to break adhesive interactions is higher than the formation energy, dissipation of friction energy will take place. In this work, it was shown that the adhesion between opposing pellicles was typically larger in bulk saliva compared to that in saliva-free solutions. Adhesion was measured by recording AFM force curves. Representative curves are shown in Figure 13b and 13c for bulk saliva and saliva free solutions respectively. Considering this, improved lubrication would be expected after rinsing the pellicles with saliva-free solutions. However, when the AFM was used to measure the friction of the pellicle in both bulk saliva and rinsed conditions this was not observed. Whereas the measured friction coefficients were in accordance with previously reported values [28-30, 32, 33], the difference in friction coefficients between the pellicles in both investigated conditions was negligible. This is not an expected result considering the adhesion measurements. Higher adhesion should be reflected by higher friction coefficients values being observed. This may be explained by the reversibly bound fraction being removed at an early stage of the friction experiments, and due to the specifics of our experiments, where we used a colloidal probe larger than the probed area, the pellicle had no access to the saliva reservoir during the experiment, meaning the reversibly bound fraction was unable to re-adsorb. However further investigations would be required to confirm this experimentally.

Figure 13. a) Schematic drawing of lateral measurements in the presence of the reversibly bound fraction. b) Representative force curve for the pellicle in bulk saliva, where the adhesion force can be seen to be ~ 1 nN and the corresponding friction measurements. c) Representative force curve for the pellicle in saliva free solution, where the adhesion force can be seen to be ~ 0.3 nN, much less than the bulk saliva measurements. The corresponding friction measurements are similar on the other hand.

4.3 Structure of MUC5B mucin films under mechanical confinement

Mucins can form extremely efficient lubricating films. This is typically ascribed to their ability to sustain load through an increase in osmotic pressure, an aspect extensively studied e.g. by means of AFM and SFA investigations, but also to their ability to create a fluid interfacial region able to reduce friction dissipation via the hydration lubrication effect. However, the molecular details are far from being understood, especially the relationship between structure, hydration and load for the latter mechanisms, the lack of suitable experimental techniques being a key issue.

In this work, we used a novel sample environment for NR experiments to investigate mucin films under confinement, i.e. a system relevant to the study of boundary lubrication. NR reveals the structure normal to the surface for a surface anchored sample, giving information on the layer thickness, layer density and interfacial surface roughness. Hence, NR is an ideal technique to study thin films under confinement provided that a suitable environment for this condition is available. The idea of using NR to study confined samples is not new in itself. Original designs used two solid surfaces to exert pressure [133, 134]. While this was a vital first step in developing new sample cells with this function, they were not without setbacks. The main downfall being the long range waviness of the surfaces, which resulted in full mechanical confinement not being reached. The NR confinement cell used in this work involves a solid substrate, silicon in this case, onto which the sample is adsorbed, and a flexible membrane that can be inflated to pressurise the sample. Using a flexible plastic membrane will accommodate the waviness of the solid surface to reach mechanical contact [118].

In this work, we used this NR confinement cell to investigate MUC5B mucin films confined to pressures of both 1 and 2 bars and compared this data with that obtained for unconfined films using a standard solid-liquid cell. NR data showed a drastic decrease in thickness (by ~95% of the original thickness) when the pressure was increased to 1 bar as well as a decrease in hydration from ~92% to ~31% (Figure 14a). However, when the pressure was further increased to ~2 bar, there was little change in these parameters. The thickness values from NR while under confinement agreed with those obtained from an AFM scratch test, where a very high mechanical pressure (~10² bar) was applied in contact mode imaging. In these experiments, we obtained images that contained an area where the pellicle had been previously removed by scratching the sample with the AFM tip, allowing the thickness of the mucin film to be measured. Due to the high pressure exerted by AFM, it is likely that

the mucin film is fully compressed so it can be extrapolated that the mucin film is also fully confined at 1 bar, the minimum pressure at which the confinement cell could be operated.

AFM force measurements allowed the pressures of relevance to be deduced i.e. pressures for which minor variations would have a significant effect on the film structure. It was clear that for most measurements, a pressure of 1 bar was enough to establish mechanical contact and the region of steric repulsion between the sample and probe was below 0.5 bar, shown in Figure 14b. Therefore, while this study demonstrated the potential of neutrons to study the structure of confined mucin films, to probe this regime, the pressure must be lower than what is currently possible (minimum 1 bar) with the confinement cell.

The minimum pressure is currently determined by the flexible membrane used. There were a number of considerations and requirements that determined our choice. Most importantly, the membrane had to have a very low roughness to allow a consistent mechanical contact and for optimum reflectivity. A homogenous chemical composition (i.e. SLD for NR) was also required for simplicity of the reflectivity analysis. Furthermore, the SLD of the membrane should be higher than that of the surface used to ensure a critical angle. This promotes both alignment and reliable analysis. All these requirements were fulfilled with Melinex®, a poly(ethylene terephthalate) membrane developed by DuPont-Teijin films. However, Melinex® is quite rigid (tensile strength of ~180 MPa) and to achieve an area in smooth mechanical contact that is large enough for the neutron beam footprint at a pressure lower than 0.5 bar, a more flexible membrane that still fulfils the discussed requirements must be either identified or synthesized.

A notable challenge in this work was the analysis of NR data for the mechanically confined mucin films. Specifically, when inflating the membrane to achieve mechanical confinement, μm -sized pockets/regions where mechanical confinement was not achieved and, therefore, solvent was not completely expelled were present. This was observed in previous studies with the cell [118], characterised by an additional peak in the reflectivity profile, indicating a small contribution from another interface. Remaining water/solution pockets can occur for a number of reasons, e.g. contamination with dust particles when assembling the confinement cell. In such cases, data analysis was still possible by using a mixed reflectivity profile approach to account for additional interfaces. However, for a more simplistic data analysis, efforts to eliminate solvent pockets should be made.

Figure 14. a) Volume fraction profiles for the non-confined and confined MUC5B mucin films derived from the SLD profile. b) Force vs. probe sample distance ramp on a mucin film obtained with a colloidal probe (solid line) and fit to the Hertz contact model (dashed line). The right vertical axis indicate the pressures corresponding to the forces of the Hertz fit.

4.4 Effect of nonionic and amphoteric surfactants on *in vitro* salivary pellicles

The final paper in this thesis is relevant to oral health care. As the salivary pellicle is of such importance to maintain oral health, understanding the influence of oral health care products on salivary pellicles is vital. As previously discussed, using *in vitro* salivary pellicles is a good starting point for these studies, since a variety of experimental techniques can be used, providing meaningful understanding at the fundamental level with the potential for more effective development of formulations. Specifically, this work focused on how *in vitro* pellicles are affected by surfactants, which are found in most oral health care products [135, 136], acting as solubilising, dispersing and wetting agents. In particular, sodium dodecyl sulphate, SDS, a common anionic surfactant, is proven very successful with customers due to its high foamability, despite being related to a number of oral mucosal conditions [137]. SDS is also known to completely remove *in vitro* pellicles from surfaces [138]. Analogous behaviour *in vivo* would imply that the oral mucosa would not be protected by the pellicle, thus being more susceptible to both mechanical and chemical insults resulting in irritation. Surfactants of different ionic character such as amphoteric surfactants and also non-ionic surfactants are becoming more popular in personal care products [135, 139]. Leading from this, it is reasonable that the interactions of these surfactants with the pellicle would be less severe than that of SDS. In this work, we investigated the effect of both an amphoteric surfactant, cocomidopropyl betaine (CAPB), and a non-ionic surfactant, pentaethylene glycol monododecyl ether (C₁₂E₅), on *in vitro* salivary pellicles. To do this, AFM, QCM-D, ellipsometry and

NR were employed to investigate the pellicle before and after exposure to these surfactants.

In comparison to SDS, both surfactants demonstrated a much gentler interaction with the pellicle, with neither completely removing the pellicle. There were however some differences between the two surfactants. Both QCM-D and ellipsometry showed that some mass was removed from the adsorbed films when rinsed with both surfactants. While ellipsometry showed no significant difference between the mass removed by each surfactant, QCM-D showed a significantly larger decrease in mass due to CAPB compared to $C_{12}E_5$. This is due to the nature of the experimental techniques. Ellipsometry is sensitive only to the dry mass of the adsorbed films as the refractive index of the bulk and coupled solvent are indistinguishable. QCM-D on the other hand is highly sensitive to any solvent coupled to the system. Therefore, it was reasonable to assume that CAPB was in fact removing solvent from the pellicle. The gentler effect of $C_{12}E_5$ was reproduced by AFM force spectroscopy. The steric forces measured by AFM were not significantly changed by exposing the pellicle to $C_{12}E_5$, meaning there was little interaction between $C_{12}E_5$ and the outer layer of the pellicle. In contrast, CAPB caused a decrease in the range of the steric forces measured, indicating a strong interaction between the surfactant and the outer layer. This suggests that CAPB interacts with the extended charged glycoproteins in the outer layer. MUC5B is thought to maintain the hydration of the outer layer owing to its polymer brush like structure causing a heavily hydrated layer region. The interaction between the charged domains of the amphoteric surfactant and the charged regions of the mucins may induce coiling and a subsequent release of the trapped solvent. This was supported by NR measurements, showing a more significant decrease in thickness of the outer layer rather than inner layer after CAPB exposure. Interestingly, NR experiments also suggested that the small amount of mass removed due to $C_{12}E_5$ was mainly from the inner layer. The influence of both surfactants on salivary pellicles is summarised in Figure 15.

These results could have interesting consequences on the functionality of the pellicle since the inner layer is mainly composed of lower molecular weight proteins that typically contribute to protecting against chemical insults, while the outer layer is mostly MUC5B, known to be a vital component to maintain hydration and therefore lubrication, protecting against mechanical insults. So, while both surfactants do have some effect on the *in vitro* pellicle and, therefore, potentially its function, it is clear they are both much less aggressive than the ionic SDS. Subsequently, they may be

ideal candidates for use in oral health care products to reduce adverse effects currently caused by SDS.

Further work is required to test whether the results obtained on *in vitro* salivary pellicles can be extrapolated to *in vivo* pellicles within the oral cavity. Although it is a reasonable speculation to assume similar interactions of the non-ionic and amphoteric surfactants with the components of the pellicle, i.e. CAPB interacting with the charged mucins and $C_{12}E_5$ with the low molecular weight proteins at the pellicle-substrate interface, conducting further experiments on the mature pellicle would be beneficial and could be done using *in situ* or *in vivo* protocols for pellicle formation. This of course comes with its own drawbacks as the real time pellicle formation cannot be monitored in the mouth with the methods available today. Furthermore, testing the effectiveness of the cleaning ability of the alternative surfactants would be beneficial to determine if they comply with the functionality requirement of oral health care products. This could be done by allowing bacteria to adhere to the pellicle and then monitoring the adsorbed amount as the surfactant introduced to the cell or cuvette in QCM-D and ellipsometry. This study gives preliminary insight into surfactant pellicle interactions and therefore whether the surfactants are worth pursuing further for oral health care products.

Figure 15. Schematic of the hypothesized change in salivary pellicles under the influence of non-ionic $C_{12}E_5$ and amphoteric CAPB, along with SLD profiles calculated from NR data on the corresponding systems. $C_{12}E_5$ has mainly an effect on the inner layer. CAPB instead interacts with the mucins in the outer layer causing some coiling and water to be expelled, as shown by the SLD plot, where a decrease in thickness of the outer layer can be seen.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The purpose of this thesis was to investigate the fundamentals of the salivary pellicle, and its physico-chemical and mechanical properties. Several different methods were employed to get a fuller picture of the structure, hydration and lubrication of *in vitro* and model pellicles. This showcased the difficulties faced in studying thin, soft, highly-hydrated films, and hence, the limitations of such studies. Despite this, the combination of AFM, QCM-D, ellipsometry and NR succeeded to overcome some of these limitations and reveal new insights regarding the *in vitro* salivary pellicle.

The *in vitro* pellicle is speculated to consist of two distinct layers. The findings presented here support that hypothesis. NR experiments revealed an outer layer that is more hydrated and diffuse compared to the multi-component inner layer. Our experiments also point to MUC5B as one of the main components of the outer layer. This was determined through observing the response of salivary pellicles and mucin films to changing environments by varying the ionic strength or by rinsing with surfactants of different ionic character. However, it is clear that other components are necessary for the mucins to mimic the exact behaviour of the pellicle outer layer.

The influence of the reversibly bound fraction of the pellicle on its mechanical properties was studied. There was a clear difference in the adhesiveness of the pellicle in the presence of bulk saliva and in saliva-free solutions, with the adhesion being larger in the presence of bulk saliva, i.e. when the pellicle contained the reversibly bound fraction. Unexpectedly, no effect on the lubricative properties of the pellicle was observed when the reversibly bound fraction was present.

A novel NR sample environment was used to investigate films of MUC5B under mechanical confinement. It was shown that mucin films are almost fully compressed and dehydrated at 1 bar, but also revealed the potential of NR for mechanical

confinement studies. AFM showed that pressures of less than 0.5 bar would be of more interest and pressures lower than 1 bar should be attainable with the confinement cell after some adjustments.

Finally, alternate surfactants for oral health care products were investigated. Both the non-ionic C₁₂E₅ and amphoteric CAPB were shown to be less aggressive on the *in vitro* pellicle compared to the commonly used anionic SDS. As expected, the surfactants interacted differently with the pellicle. The non-ionic surfactant only affected the inner layer while the amphoteric surfactant affected the outer layer. So while our data suggest that either C₁₂E₅ or CAPB would be a better alternatives to SDS in oral health care products, the effect on the pellicle function for each surfactant is distinctly different.

Taken together, these findings represent several steps forward regarding our understanding of the structural features and mechanisms regulating the functions of the salivary pellicle. Drawing on the results presented, it could become possible to mimic the properties of the pellicle in man-made substances, which could be used in artificial saliva to treat those with dry mouth syndrome or as an aqueous lubricant for coatings in biomedical devices.

While some insights into the pellicle have been presented here, there are many possibilities to investigate the pellicle further and gain an even deeper understanding. As described in this thesis, the mucin MUC5B is a component that is thought to contribute to the boundary lubrication of the pellicle, likely due to it being highly hydration. Whereas it is agreed that this is a consequence of the mucin being highly glycosylated, the contribution of different glycans to these properties is not yet fully understood. By investigating the *in situ* deglycolysation of mucin films using the surface techniques described in this thesis, the role of the different glycans in the hydration of mucin films could be studied and subsequently the effect of hydration on their lubrication performance. Another study of interest would be to identify the components and interactions that prevent coiling in the pellicle outer layer, as well as those directly involved in anchoring the outer layer to the substrate. This would not only give further fundamental insights into the underlying mechanisms mediating the hydration and lubrication properties of salivary pellicles. It would also be of help in identifying and developing alternative formulations that could be used to form films with similar properties.

Another future project is to develop a sample environments that would allow the NR study of mucin films and salivary pellicles under shear. During this thesis work, the

author participated in developing a prototype where the design of the confinement cell, discussed above, has been adapted to also shear the sample by moving the sample linearly while the flexible membrane is exerting pressure. Data provided in this thesis will need to be taken into account in further developments of the shear cell. For instance, the use of more flexible membranes would allow pressures relevant to authentic pellicle conditions to be studied; in a shear cell the structure perpendicular to the surface (along the z axis) and along the x - y axis, i.e. structural changes relevant to understanding lubrication, could be observed using NR and GI-SANS respectively.

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

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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

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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 (D₂O) of 99.9% purity was used (ref: 151882, Sigma-Aldrich, Germany). Dichlorodimethylsilane ($\geq 99.5\%$, ref: 440272), trichloroethylene ($\geq 99.5\%$, ref: 251402), ammonia ($\geq 99.95\%$, ref: 09682) and hydrogen peroxide solution (30 wt% in H₂O, 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 $< 5^\circ$. 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 H₂O:NH₃:H₂O₂ 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 μ L 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 $\sim 90^\circ$. 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 °C), 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⁻¹). 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 NaHPO₄, 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·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·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 cm² 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/Sotres/FSAS-ForceSpectroscopyAnalysisSoftware-MatLab>). Specifically, FV measurements consisted of 64x64 force ramps obtained at a speed of 1 µm·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:

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

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:

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

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_{rs} , by adding the corresponding cantilever deflection:

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

As detailed below, further analysis of force ramps involved fitting the non-contact region (specifically $d_{\text{rs}} > 5$ 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·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 × 10 mm (cylindrical shape), 80 × 50 × 15 mm and 50 × 50 × 10 mm 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 ($\lambda = 5.21$ 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 rexf 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]:

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

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: $F_{\text{sphere-plane}} \approx 2\pi R_{\text{sphere}} f$. Accordingly, we fitted the non-contact region of the acquired force ramps to an exponential function $F = F_0 e^{-d_0/\lambda_0}$. 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

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

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 from the pellicle, as shown by QCM-D.

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.

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.)

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

- Supplementary Material -

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S1. Quartz Crystal Microbalance with Dissipation (QCM-D)

S1.1. Voigt modelling

The experimental outputs of QCM-D experiments are the shifts in the frequency of the resonance, f_n , and the dissipation factor, D_n , of each of the overtones of the sensor surface. If the adsorbed layer is rigid enough, the adsorbed amount and the shift in f_n are linearly related [1]. However, protein films may not be considered rigid, but viscoelastic instead. For viscoelastic films, where noticeable shifts in the dissipation factor, ΔD_n , are observed, more trustable data are obtained by applying the Voigt model. The expressions obtained by applying the Voigt model for a viscoelastic film adsorbed onto a solid substrate and immersed into a Newtonian fluid are [2]:

$$\frac{\Delta f_n}{f_n} = -\frac{d_p \rho_p}{d_Q \rho_Q} \left[1 - \eta_l \rho_l \frac{\left(\frac{\eta_p}{\rho_p} \right) \omega_n}{\mu_p^2 + \omega_n^2 \eta_p^2} \right] = -\frac{\Gamma_p}{d_Q \rho_Q} \left[1 - \eta_l \rho_l \frac{\left(\frac{\eta_p}{\rho_p} \right) \omega_n}{\mu_p^2 + \omega_n^2 \eta_p^2} \right] \quad (\text{Eq. S1.1})$$

$$\Delta D_n = \frac{1}{d_Q \rho_Q} \left[\eta_l \rho_l \frac{d_p \mu_p \omega_n^2}{\mu_p^2 + \omega_n^2 \eta_p^2} \right] \quad (\text{Eq. S1.2})$$

In these equations d , ρ , and ω_n stand for thickness, density, and $2\pi f_n$ respectively. The subscripts Q , p , and l stand for quartz crystal, protein film, and liquid medium respectively. By a numerical fit of the frequency and the dissipation values obtained at three different overtones ($n = 3, 5$, and 7) not only the adsorbed amount, Γ , can be obtained, but also the viscoelastic properties of the film, like the shear elastic modulus, μ , and the shear viscosity η [3].

We have used the Voigt model to fit our experimental data and for this purpose we employed the Q-Tools software (Q-Sense AB, Sweden). Initial input values given for fluid density and viscosity, and for the density of the adsorbed protein films are specified below:

Fixed Parameters		Parameters to fit	
Fluid density (kg/m ³)	1000	Layer η (Pa·s)	0.0001 – 0.01
Fluid η (Pa·s)	0.001	Layer μ (Pa)	$10^3 - 10^7$
Layer density (kg/m ³)	1000	Layer d (m)	$10^{-10} - 10^{-6}$

S1.2. QCM-D thickness values for salivary pellicles and mucin films on hydrophilic silica substrates

Figure S1.2. Ratios between QCM-D thickness values measured at different points of the experiment (d) and the value measured after the initial rinsing with PBS buffer (d_0) for **a)** salivary pellicles and **b)** MUC5B films. Specifically, the ticks in the x-axis correspond to the following sequential time points in the experiments: I) Initial exposure to PBS/10, II) 1h of exposure to PBS/10, III) 1h of exposure to PBS, IV) Initial exposure to PBS/100, V) 1h of exposure to PBS/100, VI) 1h of exposure to PBS, VII) Initial exposure to UHQ water, VIII) 1h of exposure to UHQ water, IX) 1h of exposure to PBS.

S1.3. QCM-D experiments for salivary pellicles on hydrophobized silica substrates

QCM-D was also used to investigate the role of ionic strength on salivary pellicles formed on silica substrates hydrophobized following the protocol detailed in section 2.2 of the main manuscript. A representative experiment is shown in Fig S1.3. It can be seen that, when adsorbed on hydrophobic surfaces, salivary pellicles exhibited both i) swelling when ionic strength was decreased, and ii) an irreversible decrease in the overall thickness after having being exposed to ionic strengths below physiological values. Thus, the wettability of the employed substrate does not have major effect in the conclusions of the manuscript regarding salivary pellicles.

Figure S1.3. a) QCM-D frequency and dissipation data (overtones 3, 5 and 7) obtained for salivary pellicles adsorbed on a hydrophobized silica substrate exposed to different ionic strengths and b) thickness values obtained from fits to Voigt model of this data.

S1.4. QCM-D experiments for MUC5B on clean hydrophilic silica substrates

QCM-D was also used to investigate the role of ionic strength on MUC5B films formed on clean hydrophilic silica substrates. A representative experiment is shown in Fig S1.4. It can be seen that, similarly to what is shown in Fig. 3c of the main manuscript for MUC5B films formed on hydrophobic surfaces, dissipation values increased immediately and drastically after lowering the ionic strength. In the same way, this initial increase was followed by a decrease. For the MUC5B data shown in Fig. S1.4, the Voigt model did not provide a good fit, which was expected because of the low dissipation values. Nevertheless, the similar behavior of the raw QCM-D data supports the fact that the conclusions of the main manuscript for the effect of ionic strength on MUC5B films were not exclusive for a given wettability of the employed substrates.

Figure S1.4. QCM-D frequency and dissipation data (overtone 5) obtained for MUC5B adsorbed on a clean hydrophilic silica substrate exposed to different ionic strengths.

S2. Neutron Reflectometry

A standard solid liquid flow cell set-up was used with hydrophilic silica substrates at room temperature. The cells were cleaned by bath sonication in ethanol, a 1:1 ethanol:UHQ mixture and finally UHQ. The silicon crystals were RCA cleaned with 5:1:1 H₂O:NH₃:H₂O₂ at 80 °C for 10 minutes.

The silica was initially characterized in buffer prepared with both H₂O ($\rho = -0.56 \times 10^{-6} \text{ \AA}^{-2}$) and D₂O ($\rho = 6.35 \times 10^{-6} \text{ \AA}^{-2}$) to achieve two contrasts before injecting the HWS into the cell and allowing it to adsorb for 60 minutes. After adsorption, the pellicle is rinsed with PBS, then characterized in the two contrasts of PBS. The bulk solvent is then exchanged for PBS diluted 100 times, before again being characterized in two contrasts. Finally, the solvent is exchanged for UHQ water and measurements are taken in the two contrasts.

For data analysis, RasCAL software (<http://sourceforge.net/projects/rscl/>) is used, which numerically fits the reflectivity to a solution consisting of Fresnel equation, Snell's law and the SLD to optical refractive index relation until the SLD profile with the lowest chi squared value is found. The roughness, σ , is factored in by scaling the reflectivity curve with sharp interfaces, $R_0(Q)$, such as:

$$R(Q) = R_0(Q)e^{-qq'\sigma^2Q^2} \quad \text{Eq. S2.1}$$

Where, q is the momentum transfer in the upper layer and q' is the momentum transfer in the lower layer separated by the roughness σ [4].

Here, the sample is considered to be composed of slabs between a support (silicon) and the bulk liquid (H₂O or D₂O). Each slab is described by four parameters; thickness, the dry scattering length density (SLD), the volume fraction of the solvent (hydration of the layer) and the surface roughness. The SLD and hydration of a layer are related according to Eq. S2.2.

$$SLD_{layer} = (SLD_{saliva} \times (100 - Hydration)/100) + (SLD_{bulk} \times Hydration/100) \quad \text{Eq. S2.2}$$

The pellicle can be considered as two slabs; the inner dense layer and an outer, highly hydrated layer. This will sit on top of a SiO₂ layer, resulting in a three layer model which is schematically represented in Fig. S2.

Figure S2. Illustration of the model used in NR fits.

The reflectivity profiles were analysed by creating this model in RasCAL and finding the optimal fitting parameters for the experimental data. Each parameter is constrained according to prior knowledge of the pellicle from previous experimental techniques and literature [5], including the SLD for saliva in each contrast liquid.

In order to improve the estimation of the errors for the fit parameters, we used the Bayesian Markov chain Monte Carlo (MCMC) approach implemented in the refnx software [6]. All parameters for each data set are shown in Table S2.

	PBS rinsed pellicle	PBS/100 rinsed pellicle	UQH rinsed pellicle
substrate roughness (Å)	5 ± 1	5 ± 1	5 ± 1
SiO ₂ thickness (Å)	11 ± 1	11 ± 1	11 ± 1
SiO ₂ hydration (%)	25 ± 1	25 ± 1	25 ± 1
SiO ₂ rough (Å)	7 ± 1	7 ± 1	7 ± 1
Pellicle inner layer thickness (Å)	32 ± 1	29 ± 1	21 ± 1
Pellicle inner layer roughness (Å)	12 ± 1	12 ± 1	19 ± 1
Pellicle inner layer hydration (%)	65 ± 1	69 ± 1	68 ± 2
Pellicle inner layer SLD (Å ⁻²) deuterated solvent	$(2.8 \pm 0.1) \text{ E-06}$	$(2.8 \pm 0.1) \text{ E-06}$	$(2.8 \pm 0.1) \text{ E-06}$
Pellicle inner layer SLD (Å ⁻²) hydrated solvent	$(1.6 \pm 0.1) \text{ E-06}$	$(1.6 \pm 0.1) \text{ E-06}$	$(1.6 \pm 0.1) \text{ E-06}$
Pellicle outer layer thickness (Å)	309 ± 1	540 ± 2	756 ± 2
Pellicle outer layer roughness (Å)	40 ± 2	30 ± 1	30 ± 1
Pellicle outer layer hydration (%)	96 ± 1	97 ± 1	98 ± 1
Pellicle outer layer SLD (Å ⁻²) deuterated solvent	$(3.1 \pm 0.1) \text{ E-06}$	$(3.1 \pm 0.1) \text{ E-06}$	$(3.1 \pm 0.1) \text{ E-06}$
Pellicle outer layer SLD (Å ⁻²) hydrated solvent	$(2.3 \pm 0.1) \text{ E-06}$	$(2.3 \pm 0.1) \text{ E-06}$	$(2.3 \pm 0.1) \text{ E-06}$

Table S2. Values obtained for NR fitting parameters.

S3. Null-Ellipsometry

Null ellipsometry was also used to investigate the effect of ionic strength of salivary pellicles formed on clean hydrophilic substrates. Data from a representative experiment is shown in Fig. S3.

Specifically, ellipsometry experiments were performed with a Rudolph thin film ellipsometer (type 43603-200E, Rudolph Research, USA) equipped with a Xenon 442.9 nm light source, with a setup based on null ellipsometry according to the principles of Cuypers [7] and automated according to the concept of Landgren and Jönsson [8]. Theoretical principles can be found elsewhere [9]. Samples were placed in a trapezoid cuvette made of optical glass (Hellma, Germany) equipped with a magnetic stirrer and with temperature set to 25°C. The determination of the silicon complex refractive index, and of the thickness and refractive index of the silicon oxide layer was performed using air and water as ambient media [8]. Four zone measurements were conducted to minimize systematic errors. Ellipsometry experiments were performed by means of similar steps to those followed in QCM-D investigations (section 2.6 of the main manuscript). However, in this case a flow velocity of 15 ml·min⁻¹ was used. The adsorbed amount, Γ , was then calculated using de Feijter's equation assuming a value of 0.18ml/g for the dependence of refractive index on concentration [10].

As seen in Fig. S3, ellipsometry confirmed the irreversible decrease in pellicle thickness after exposure to ionic strength below physiological values observed by means of QCM-D.

Figure S3. Data from a representative null-ellipsometry experiment on the role of ionic strength on salivary pellicles formed on clean hydrophilic substrates. Specifically, the following quantities are plotted: **a)** adsorbed amount, **b)** refractive index, **c)** thickness and **d)** raw ellipsometry angles.

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III

ROLE OF THE REVERSIBLY BOUND FRACTION OF IN VITRO SALIVARY PELLICLES ON THEIR MECHANICAL AND LUBRICATION PROPERTIES

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Abstract

Salivary pellicles, the thin proteinaceous films that adsorb onto the oral surfaces, exhibit phenomenal aqueous lubrication performance. This capability has been attributed to presence of the oral mucin MUC5B on its outer surface, forming a hydrated layer that provides optimum conditions for boundary lubrication. When in bulk saliva, salivary pellicles, at least those formed *in vitro*, contain a loosely bound fraction that can be easily removed by rinsing with saliva-free solutions. This fraction quickly re-adsorbs upon subsequent exposure to saliva. The aim of this work was to investigate the effect of this reversibly bound fraction on the lubrication performance of salivary pellicles, as well as on properties expected to mediate this performance i.e., viscoelasticity and adhesiveness. For this, we used several Atomic Force Microscopy operations modes. For mechanically confined pellicles i.e., the relevant situation for lubrication studies, we did not observe that their viscoelastic character was affected by the presence of the reversibly bound fraction. However, the presence of this fraction increased the adhesiveness between opposing pellicles, indicating an entanglement between these fractions. While it would be expected that this resulted in higher friction forces between

sheared opposing pellicles, this was not the case as revealed by AFM friction measurements, which indicated a similar lubrication performance for pellicles regardless of the presence of the reversibly bound fraction.

1. Introduction

The role of saliva in the oral cavity is manifold. Hence, interest in saliva and in particular the salivary pellicle has peaked, specifically due to the extraordinary ability of the pellicle to lubricate and protect the underlying oral surfaces from mechanical wear, vital to maintaining oral health (Hahn Berg et al., 2003, Hannig, 2002, Hannig and Joiner, 2006, Lindh et al., 2014, Yakubov, 2014). Most experimental works indicate that saliva lubricates in a boundary lubrication regime (Sotres and Arnebrant, 2013, Lindh et al., 2014) i.e., salivary lubrication is mediated by the salivary pellicle, the nm-thick film that forms from the selective adsorption of salivary components onto almost any type of exposed surfaces (Lindh et al., 2014). An aspect of interest of salivary pellicles is indeed their ability to lubricate, even when formed *in vitro*, a wide variety of surfaces despite their physico-chemical properties. In this regard, friction coefficients as low as 0.01 – 0.25 (Aguirre et al., 1989a, Bongaerts et al., 2007, Hahn Berg et al., 2003, Ranc et al., 2006a, Ranc et al., 2006b) as well as high resistance to wear with yield strengths of ~100 MPa (Sotres et al., 2011) have been reported for salivary pellicles adsorbed on a variety of surfaces. Understanding the mechanisms underlying the lubrication mediated by salivary pellicles is of high interest not only for biomedical applications but also for the development of water-based lubricants. Lubrication is vital for movement both in natural systems and man-made devices. Without lubrication, surfaces are subject to wear and the system will dissipate energy through friction (Coles et al., 2010), requiring more work to be done and resulting in a lower efficiency. To date, no man made aqueous lubricants have surpassed the performance of those developed by Nature (Lee and Spencer, 2008, Spencer, 2014). Therefore understanding the underlying mechanisms governing saliva mediated aqueous lubrication will allow advances in many applications where the use of oil-based lubricants is either of environmental concern or just not a choice like in biomedical applications.

To further advance in this topic, it is instructive to consider the structure and composition of salivary pellicles. For those formed *in vitro*, previous research indicates that it consists of two layers, an inner dense layer and an outer diffuse layer (Cárdenas et al., 2007, Lindh et al., 2014). The outer layer presumably consists of large glycoproteins known as mucins, specifically the oral mucin MUC5B. This hypothesis is based on several indicators. First, the oral mucin

MUC5B has been shown as one of the main components of *in vitro* pellicles regardless of e.g., the wettability of their underlying substrate (Aroonsang et al., 2014). Mucins, because of their high density of negatively charged terminal branched carbohydrates, are often considered to have a polyelectrolyte-like behavior. As such, when anchored to a surface and in the presence of a good solvent e.g., an aqueous solution, they are expected to oppose mechanical confinement by means of steric repulsion. This has been experimentally observed by means of Atomic Force Microscopy (AFM) and Surface Force Apparatus (SFA) in multiple works (Boyd et al., 2021a, Dedinaite et al., 2005, Harvey et al., 2011, Perez and Proust, 1987, Pettersson and Dédinaite, 2008). Interestingly, *in vitro* salivary pellicles have revealed similar steric forces opposing mechanical confinement (Boyd et al., 2021a, Cárdenas et al., 2008, Hahn Berg et al., 2003, Harvey et al., 2012, Nylander et al., 1997). Additionally, oral mucin films have been shown to provide similar boundary lubrication performance as *in vitro* salivary pellicles (Aguirre et al., 1989b, Yakubov et al., 2015). Thus, it is reasonable to hypothesize that MUC5B are the main component of the diffuse outer layer of *in vitro* pellicles, even though some works point out that probably other salivary components are also present (Boyd et al., 2021a, Yakubov et al., 2015).

However, there is one aspect of *in vitro* salivary pellicles that specifically concerns its outer layer and that has been traditionally overlooked. *In vitro* salivary pellicles present a reversibly bound fraction i.e., only observed in the presence of bulk saliva but not in that of saliva-free aqueous solutions (Vassilakos et al., 1993). So far, it has barely been investigated whether this fraction plays a role in the mechanical and lubrication properties of *in vitro* salivary pellicles. For instance, little it is unknown how this fraction influences mechanical properties like adhesiveness and viscoelasticity. Regarding lubrication, macro-tribological techniques are typically suitable only for lubrication studies in the presence of the bulk lubricant solutions. Techniques like the Atomic Force Microscope (AFM) have only been used to investigate the lubrication performance of salivary pellicles rinsed with saliva-free solutions e.g. (Hahn Berg et al., 2003, Yakubov et al., 2015, Yakubov et al., 2009, Coles et al., 2010). So far, the lubrication provided by salivary pellicles in the presence of bulk saliva and saliva-free aqueous solutions has not been characterized by means of the same experimental technique and, therefore, appropriately compared. In this work, we used ellipsometry for confirming the presence of the reversibly bound fraction of *in vitro* salivary pellicles in the presence of bulk saliva and AFM for investigating the effect of this fraction of salivary pellicles on their lubrication performance. Moreover, we also

used AFM to investigate mechanical properties expected to mediate this performance, namely viscoelasticity and adhesiveness.

2. Materials and Methods

2.1. Materials

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). The samples were investigated either in bulk saliva or rinsed with PBS, referred to in this work as the bulk and rinsed pellicle's respectively.

Saliva collection

Unstimulated human whole saliva (HWS) was used for all experiments. The saliva was collected from two subjectively healthy adult donors, from whom informed consent was obtained. After collection, saliva from the two donors was pooled and filtered using a 0.45µm syringe filter (VWR) to remove any large debris such as cells from the saliva. Ethical approval was obtained from the committee of research ethics at Lund University (2018/42).

Silica surfaces

Silica was used as a substrate for salivary pellicles in all experiments. For force spectroscopy and ellipsometry experiments, p-Doped (boron) silicon wafers with a resistivity of 10–20 Ω·cm (Semiconductor Wafer Inc., Taiwan) oxidized in an oxygen atmosphere were used. These surfaces were cleaned by means of a standard RCA protocol, with 5:1:1 H₂O:NH₃:H₂O₂ at 80 °C for 10 min, followed by 10 min of plasma treatment. This cleaning procedure yielded hydrophilic surfaces with < 5° water contact angles.

2.1 Ellipsometry

Ellipsometry investigations were performed with a Rudolph thin film ellipsometer (type 43603-200E, Rudolph Research, USA) equipped with a Xenon light source filtered to 442.9 nm. The setup is based on the null ellipsometry principles of Cuypers (Cuypers, 1976) and automated according to the concept of Landgren and Jönsson (Landgren and Joensson, 1993). Theoretical principles can be found elsewhere (Lissberger, 1977). Samples were placed in a trapezoid cuvette made

of optical glass (Hellma, Germany) equipped with a magnetic stirrer and temperature set to 25 °C. First the silicon was characterized by determining the complex refractive index, thickness and refractive index of the silicon oxide layer using both air and water as ambient media (Landgren and Joensson, 1993). Four zone measurements were conducted to minimize systematic errors. Ellipsometry experiments were performed by first establishing a baseline of clean silica in PBS, then the PBS was exchanged for diluted HWS and the pellicle was allowed to adsorb and stabilize for 60 minutes. Next, the pellicle was rinsed by flowing PBS and again waiting 60 minutes for stabilization. These steps were repeated once more. A flow velocity of 15 ml·min⁻¹ was used. For ellipsometry experiments, it was necessary to dilute the filtered saliva to a ratio of 1 part HWS to 7 parts PBS. This was due to the high light scattering of the bulk saliva resulting in an optical signal too low to measure. The adsorbed amount, Γ , was then calculated using de Feijter's equation (De Feijter et al., 1978):

$$\Gamma = \frac{(n_f - n_o)d_f}{dn/dc} \quad \text{Eq. 1}$$

where n_f is the refractive index of the adsorbed film, d_f its thickness, n_o the refractive index of the bulk solution and dn/dc is the refractive index increment as a function of bulk concentration for which a value of 0.18 ml·g⁻¹ was assumed (De Feijter et al., 1978).

2.2. Atomic Force Microscopy (AFM)

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 operated, as detailed below, in different modes: contact thermal noise spectroscopy, normal force spectroscopy and friction force spectroscopy. Rectangular silicon nitride levers were used (OMLC-RC800PSA, Olympus, Japan; nominal force constant of 0.1 N·m⁻¹). Colloidal probes were prepared by attaching a silica microparticle (diameter ~15µm, PSI-15.0, G. Kisker GbR, Germany) to the free end of a rectangular silicon nitride lever with a two part epoxy adhesive (Loctite, Henkel Norden AB, Sweden). Before use, the colloidal probes were characterized an optical microscope (Nikon, Amsterdam, The Netherlands)

2.2.1. Contact Thermal Noise Spectroscopy

This AFM operation mode is based on monitoring how the Brownian motions of cantilevers are modified due to the mechanical contact with the investigated sample (Gonzalez-Martinez et al., 2019a, Tung et al., 2014). For this, the vertical deflections of the cantilevers were monitored with an external data acquisition

board (PCI-6132, National Instruments Sweden AB) at a sampling frequency of 2 MHz and a resolution of 1024 points. For each experiment, thermal fluctuations of the cantilevers were registered both far from the sample and while applying a load on the sample of ~ 2 nN. Then, the Power Spectral Density (PSD) for the registered thermal fluctuations of the AFM cantilevers was calculated. The first resonance peak from the PSD spectra was fitted to a Lorentzian function from where the frequency resonance and the quality factor were obtained. 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 (Qian et al., 2000).

2.2.2. Normal Force Spectroscopy

AFM force ramps were obtained before and after each friction experiment. This allowed characterizing the adhesiveness of opposing salivary pellicles while also checking the possibility that the probe was degraded during the friction experiment.

Force ramps were analyzed with a custom application (<https://git.io/JmEOS>). This analysis followed the same process detailed in previous works (Boyd et al., 2021a, Sotres et al., 2017). Briefly, raw force ramps were first transformed into a deflection vs sample position representation by scaling the position sensitive photodetector vertical signal by the slope of the contact region of force ramps obtained on a clean silica surface. Then, the contact point was obtained for each ramp by fitting its contact region with the Hertz contact model for a sphere-plane geometry and used as an offset for sample vertical position. Then, deflection was transformed into tip-sample interaction force by scaling the cantilever deflection by its spring constant (which was calculated for each cantilever by means of the Sader method (Sader et al., 1999)). Force ramps were then converted into a force vs tip-sample distance representation. Finally, the adhesion observed when withdrawing probe and sample was characterized in terms of the both the maximum adhesion force, F_{adh} , and the work of adhesion, W_{adh} , i.e., the area under the baseline in the withdrawal force curves.

2.2.3. Friction Force Spectroscopy

Friction measurements were carried out using the method described in (Sotres et al., 2011) and employing colloidal probes. The normal, k_N , and torsional, k_ϕ , spring constants were determined for every lever using the methods developed by Sader and co-workers (Sader et al., 1999, Pettersson et al., 2007, Green et al., 2004). The lateral sensitivity of the setup, δ_ϕ , was determined by the pivot method (Pettersson et al., 2007, Bogdanovic et al., 2000) while the normal sensitivity, δ_N ,

was calculated from the slope of the deflection of the lever while pressed against a silicon surface.

Friction measurements were performed by scanning the samples in the contact operation mode i.e., constant load force 2D scans, while continuously increasing the load force between scans. Typically, parallel lines along a square $2\ \mu\text{m} \times 2\ \mu\text{m}$ area were scanned in opposite directions at a constant tip velocity of $1\ \mu\text{m}\cdot\text{s}^{-1}$. The fast scan direction was perpendicular to the long axis of the lever. For each scan, the topography and lateral force images were simultaneously recorded in both the trace and retrace directions. Images recorded consisted of 32 points in the fast scan direction (columns) and 32 points in the slow scan direction (rows). Data analysis was performed using a custom application (<https://git.io/JEE2c>). Briefly, for each scan the mean friction force was calculated by first selecting an inner square area, with a lateral size $2/3$ of the scanned one, for successive calculations in order to avoid border effects (Figure 1). Then, the difference in the lateral signal between the trace and retrace directions, $\Delta V_{\text{Lateral}}$, was calculated for each scanning line. Subsequently, $\Delta V_{\text{Lateral}}$ was averaged over all the lines of the image. Finally, the scan total friction force, F_F , was obtained by scaling $\Delta V_{\text{Lateral}}$ through the following equation:

$$F_F(\Delta V_l) = \frac{k_\phi}{\delta_\phi} \frac{1}{h_{\text{eff}}} \frac{\Delta V_{\text{Lateral}}}{2} \quad \text{Eq. 2}$$

where h_{eff} is the effective height of the probe, which is the diameter of the silica particle plus half the thickness of the cantilever tip.

Figure 1. Representative example of lateral signal (volts), forward (blue) and backward (red), where the borders of the image (dashed line) have been excluded for calculation of $\Delta V_{\text{Lateral}}$.

For each scan, the load force applied by the lever, F_L , on the sample was determined as:

$$F_L = k_N \delta_N (V_{Vertical} - V_{Vertical0}) \quad \text{Eq. 3}$$

where $V_{Vertical}$ and $V_{Vertical0}$ are the photodiode vertical signals measured when scanning the sample and far from the sample (i.e., when probe and sample do not interact) respectively. $V_{Vertical0}$ was measured by means of force ramps at the beginning and at the end of each experiment to account for any drift.

Typically, experiments were performed as follows. First, cantilevers were calibrated in PBS and using a clean silica surface. Then, saliva was flowed into the AFM liquid cell, and let to adsorb for 60 min. Friction ramps were then obtained in at least three different areas of the sample. Subsequently, of the sample was gently rinsed with PBS buffer, and friction ramps were obtained again in at least three different areas of the sample.

AFM lift mode measurements (Walczyk et al., 2014) were performed to investigate the possibility of viscous drag forces influencing friction measurements when operating the AFM in bulk saliva. Briefly, the operation is similar to that of friction force spectroscopy. However, along each line, two pairs of scans are performed: one pair (forward and backward) in mechanical contact with the sample (contact scan) and the other pair (forward and backward) at a given distance from the sample (lift scan). During each scan i.e., contact and lift, both lateral and normal deflections were registered.

3. Results

3.1 Ellipsometry

The time evolution of the areal mass from a representative ellipsometry experiment is shown in Figure 2. The procedure in these experiments was as follows. First, a baseline of the clean silica in PBS was established. Subsequently, HWS (diluted 1 part HWS to 7 parts PBS) was introduced to the cuvette. The immediate adsorption of the salivary pellicle onto silica surfaces can be seen as the adsorbed mass quickly increases to $\sim 4 \text{ mg} \cdot \text{m}^{-2}$. A subsequent rinsing with PBS then caused a fraction of this mass to be removed, shown by a decrease of $\sim 1 \text{ mg} \cdot \text{m}^{-2}$. However, upon further exposure to bulk saliva the adsorbed mass quickly increases again to the original mass in bulk saliva. This demonstrates reversible adsorption of a salivary fraction when in bulk saliva.

Figure 2. Ellipsometry data from the adsorption of bulk filtered saliva onto a silica surface and the subsequent rinsing with PBS.

3.2. Viscoelasticity of mechanically challenged salivary pellicles

The viscoelasticity of mechanically confined pellicles was investigated by means of AFM contact thermal noise spectroscopy. Representative thermal noise PSD spectra for the free (far from the sample) cantilever both in PBS medium and in bulk saliva are shown in Figure 3a. Note that the spectra also contain a sharp mechanical peak at a frequency of ~ 8.3 kHz. The plots also show fits of the spectra to two Lorentzian functions (accounting for the cantilever resonance and for the mentioned mechanical peak). Figure 3b shows the monitored thermal noise PSD spectra when applying a load of ~ 2 nN on a clean silica surface in the presence of PBS, on the same surface when exposed to bulk saliva and after rinsing this surface with PBS. At lower applied loads, the resonances were hidden by the background noise. In the same way as for the spectra recorded far from the surface, the spectra obtained in contact with salivary pellicles showed an additional mechanical peak at a frequency of ~ 45.5 kHz. Figure 3b also shows fits of the data to Lorentzian functions (in the case of the spectra monitored in contact with salivary pellicles, the fit was to two Lorentzians accounting, in the same way as in the data from Fig. 3a, for both the cantilever resonance and the indicated mechanical peak).

Figure 3. Representative PSD plots for **a)** free (far from the sample) cantilevers in PBS buffer and whole saliva and **b)** for cantilevers in mechanical contact with a silica surface in PBS buffer, a pellicle in bulk saliva and for a pellicle rinsed with PBS buffer.

Frequencies and quality factors obtained from fits of the free and contact cantilever resonances are provided in Table 1.

Table 1. Frequencies and quality factors for the resonances depicted in Figure 3.

		f_0 (Hz)	Q
Free	PBS	13819±5	2.235±0.004
	Saliva	13056±9	1.753±0.004
Contact	Silica in PBS	89900±30	3.00±0.01
	Pellicle in saliva	45000±80	2.40±0.04
	Pellicle rinsed with PBS	48800±140	2.44±0.06

3.3. Adhesion between pellicles

The adhesiveness of opposing pellicles was investigated by means of AFM force ramps between salivary pellicles formed on both colloidal probes and silica surfaces. Specifically, in our experiments we performed force ramps first in the presence of bulk saliva and subsequently after rinsing with PBS buffer. Representative force ramps obtained in the presence of bulk saliva and PBS buffer are shown in Figures 4a and 4b respectively. It can be observed that the adhesion between opposing pellicles was higher in the presence of bulk saliva. To further quantify this observation, the mean work of adhesion i.e., the area delimited by the negative values of the force curve in the force vs probe-sample distance representation during withdrawal (W_{adh} , Fig. 4c), as well as the maximum adhesion force (F_{adh} , Fig. 4d) values were calculated for 11 different samples, each one investigated with a different colloidal probe, both in the presence of bulk saliva and PBS

buffer. It can be observed in these figures that both the maximum adhesion force and the work of adhesion were typically lower after rinsing the pellicles with PBS buffer.

Figure 4. Representative force vs probe-sample distance curves for opposing pellicles in the presence of **a)** bulk saliva and **b)** PBS buffer. **c)** Adhesion work of the rinsed pellicle vs adhesion work of the pellicle in bulk saliva (scaled by the probe radius, mean values with error bars showing the standard deviation) **d)** Maximum adhesion force of the rinsed pellicle vs maximum adhesion force of the pellicle in bulk saliva (scaled by the probe radius, mean values with error bars showing the standard deviation).

3.4. Lubrication measurements

Results from AFM friction measurements between adsorbed salivary pellicles both in the presence of bulk saliva and PBS are shown in Figure 5. Friction data on different samples exhibited a high variability. Subsequently, we represented these data as density maps of friction vs load force ramps (both quantities scaled by the specific probe radius) built from 2-3 friction measurements on 11 different samples. Specifically, Figure 5a corresponds to friction ramps obtained for in the presence of bulk saliva, and Figure 5b to friction ramps obtained in the presence of PBS buffer.

In both cases, the friction forces remain low, with a slight increase as the applied load is increased. It is clear from Figure 6 that salivary pellicles exhibit a similar

lubrication performance both in the presence of saliva and in the presence of PBS buffer. This can be quantified by fitting the data in the density maps to Amonton's law (Bhushan, 1999):

$$F_F = \mu(F_N + F_{adh}) \quad \text{Eq. 4}$$

where F_F is the friction force, μ is the friction coefficient, F_N is the normal force and F_{adh} is the adhesion force between the surfaces. It can be noted that scaling the forces by the probe ratio does not alter the linear relationship between the friction and the applied load (normal plus adhesion forces). Specifically, linear fit of the density map for the pellicle in the presence of bulk saliva results in $F_F/R=0.11F_N/R+0.05 \text{ nN}\cdot\mu\text{m}^{-1}$, and for the pellicle in the presence of PBS buffer $F_F/R=0.09F_N/R+0.05 \text{ nN}\cdot\mu\text{m}^{-1}$. Furthermore, we checked that the probe had not degraded by means of normal force measurements before and after each experiment. Force curves, specifically the adhesion, did not change confirming the probe had not degraded.

Figure 5. a) Density map of Friction vs Normal forces (scaled by the probe radius) for opposing pellicles in bulk saliva. b) Density map of Friction vs Normal forces (scaled by the probe radius) for opposing pellicles in PBS buffer.

3.5. Viscous drag in friction measurements in the presence of bulk saliva

While AFM lateral force measurements have been widely used to measure the friction force of thin films, it is important to take into account whether they can have a contribution from hydrodynamic forces arising from movement within a viscous media like saliva, especially when using probes of a relative large size ($\sim 15 \mu\text{m}$ diameter) as in our experiments. To do this, the lateral forces on the cantilever were monitored at different separations from the surfaces while moving the sample on the same way as was done during friction measurements (Figure 6). It can be seen that the friction forces measured at different separations

from the sample could be neglected. A similar result can be extrapolated for saliva-free aqueous solutions provided the higher viscosity of saliva.

Figure 6. Friction measurements, obtained by operating the AFM in the lift mode, in the presence of bulk saliva at different separations between a colloidal probe and a pellicle formed *in vitro* on a silica surface.

Discussion

The aim of this work was to investigate whether the reversibly bound fraction of *in vitro* salivary pellicles had an effect on their mechanical properties, with a specific interest in their lubrication performance. For this purpose, we first confirmed the presence of this reversibly bound fraction by means of ellipsometry. Subsequently, we focused on two of the quantities expected to mediate the boundary lubrication performance of salivary pellicles: their viscoelasticity and adhesiveness. In this regard, the viscoelasticity of mechanically confined pellicles was investigated by means of AFM contact thermal noise spectroscopy. AFM was also used to investigate the adhesiveness of the pellicles by means of normal force measurements as well as their lubrication by means of lateral force measurements.

These surface techniques require planar, and for ellipsometry, reflective surfaces, meaning the use of oral surfaces such as oral mucosa or enamel is prevented. To select an alternative, the key surface properties affecting the pellicle formation must be addressed e.g., the ionic character and wettability (Sotres et al., 2011, Lindh et al., 2014). In this regard, hydroxyapatite, the main component of enamel, exhibits a hydrophilic anionic character (Kawasaki et al., 2003). The MUC1 decorated oral mucosa (Ukkonen et al., 2017) has also proved a hydrophilic character when investigated *in vivo* (Glantz et al., 1991). Subsequently, we used silica,

characterized as well by a hydrophilic and anionic character, as a substrate for forming salivary pellicles.

The first step of this work consisted in confirming the presence of a reversibly bound fraction on the *in vitro* salivary pellicles. Following previous works (Vassilakos et al., 1993), ellipsometry was used to monitor the adsorbed mass of the salivary pellicle formed on silica substrates upon successive steps of exposure to (diluted) saliva and subsequent rinsing with PBS buffer. The rapid formation of the pellicle was indicated by the quick increase in adsorbed mass to $\sim 4 \text{ mg} \cdot \text{m}^{-2}$ upon exposure of the silica surface to the diluted saliva. Despite using diluted saliva, the obtained adsorbed amounts are similar to those reported for salivary pellicles on similar surface formed from non-diluted saliva (Boyd et al., 2021b). After 1 h of pellicle formation, PBS was flowed through the cuvette to rinse the pellicle resulting in a decrease of $\sim 1 \text{ mg} \cdot \text{m}^{-2}$. However, when this process of exposing the pellicle to bulk saliva and then rinsing with PBS was repeated, this mass was quickly readsorbed and desorbed respectively. This is in agreement with previous reports (Vassilakos et al., 1993), which showed that there is a fraction of the salivary pellicle which is less tightly bound, and therefore is easily removed during rinsing, on top of the tightly bound rinsed pellicle.

AFM Contact Thermal Noise spectroscopy was used to investigate the viscoelastic character of mechanically confined pellicles, which is the system of relevance for e.g., lubrication studies i.e., the higher the viscous character of a surface the higher the expected dissipation of friction energy. In these experiments, we used standard AFM cantilevers with pyramidal tips, as with colloidal probes we could not discern the resonances from the background noise. Thermal spectra recorded for the cantilevers far from the sample indicated that their first eigenmode was characterized by a slightly lower frequency and a significant lower quality factor in the presence of bulk saliva than in the presence of PBS (Figure 3a, Table 1), indicating the higher density and viscosity of bulk saliva as expected. The first eigenmode recorded for the cantilevers in mechanical contact with a clean silica surface in PBS was characterized by a significant higher frequency and quality factor than the modes recorded in contact with salivary pellicles. According to the Euler–Bernoulli model for the flexural vibrations of a cantilever, the frequency of the first eigenmode will increase by a factor ~ 6.25 (Gonzalez-Martinez et al., 2019a, Landau and Lifshitz, 1986) when in contact with an infinite hard surface with respect to that for a free cantilever (actually this factor holds for a rectangular cantilever but can be used as an approximation for the triangular cantilevers used in this work). It follows from Table 1, that the frequency when in contact with the silica surface increased by a factor ~ 6.5 , which is in reasonable

agreement with what was expected. Interestingly, when in contact with the salivary pellicles, both in the presence of bulk saliva and PBS, the frequency of the first eigenmode only increased by a factor of ~ 3.5 . This indicates a softer contact and, therefore, a softer interface (Gonzalez-Martinez et al., 2019a, Gonzalez-Martinez et al., 2019b). It is also pointed out by our data that the quality factor of the resonances increased when in contact with the surfaces, indicating a lower dissipation of thermal energy. It can also be observed from our results that the quality factor when in contact with the hard silica surface was higher than when in contact with the salivary pellicles, indicating a higher dissipation of thermal energy through the softer contact with the pellicles. This is, our results indicate a higher viscous character for the pellicles than for silica surfaces. However, no significant differences neither in the frequency nor in the quality factor of the contact resonance were observed for the salivary pellicles in the presence of bulk saliva and in the presence of PBS. This implies that, whereas our experiments proved the presence of a soft viscous film on the surface i.e., the salivary pellicles, no contribution from the reversible attached fraction to the viscoelasticity when mechanically probed was observed. This suggests that in our experiments this fraction was completely compressed by the AFM probe and that just its mass was not enough to provide a measurable difference in viscoelasticity. This correlates with the fact that, at the range of forces applied during AFM contact thermal noise measurements (~ 2 nN, we were not able to measure resonances at lower loads, as in this case they were hidden by the background noise), the force vs. distance behavior measured in bulk saliva and PBS buffer overlapped (Supplementary Information SI.1). However, at lower applied forces i.e. below 2 nN, force measurements differed, indicating a softer and more swollen interface in the presence of bulk saliva (Supplementary Information SI.1). Whereas these force measurements did not show a significant hysteresis between forward and backward ramps i.e., an indication of viscous character, it cannot be excluded that the presence of the reversibly bound fraction had an effect on the viscoelasticity of the pellicles at applied loads lower than those investigated in this work.

We also investigated how the presence of the reversibly bound fraction affects the adhesion between pellicles. Adhesion is of relevance for lubrication as it can lead to friction between two sheared surfaces. When two surfaces come into close contact, adhesion forces might eventually develop. If these surfaces are then sheared, adhesive interactions will be formed and broken constantly. If the energy required to break adhesive interactions is higher than that gained when forming them, dissipation of friction energy will take place (Bowden and Tabor, 2001,

Blancher, 2012). Specifically, we investigated adhesion by means of AFM normal force measurements. Our experiments (Figure 4) indicated that the adhesion between pellicles was significantly higher in the presence of bulk saliva than in the presence of PBS buffer. This indicates that the reversibly bound fractions of opposing pellicles entangle upon mechanical contact.

Finally, we also investigated whether the reversibly bound fraction of salivary pellicles had an effect on their lubrication performance. For this, friction measurements over a ramp of increasing normal forces were made using contact mode AFM (Figure 5). Salivary pellicles proved to be highly lubricious both in the presence of bulk saliva and PBS buffer, with a friction coefficient of ~ 0.1 in good agreement with previously reported values (Aguirre et al., 1989a, Bongaerts et al., 2007, Hahn Berg et al., 2003, Ranc et al., 2006a, Ranc et al., 2006b). Moreover, we also showed that these measurements were not influenced by a possible viscous drag in the presence of bulk saliva (Figure 6). However, no significant differences were observed between the lubrication performance investigated in bulk saliva and in PBS buffer i.e., the reversibly bound fraction of salivary pellicles had no effect on lubrication, at least within the resolution and procedures used in our experimental approach.

A similar lubrication performance of salivary pellicles in the presence of bulk saliva and PBS buffer is not what one would expect when considering our additional experiments on the adhesiveness and viscoelasticity of pellicles. Specifically, the presence of the reversibly bound fraction resulted in overall higher adhesiveness between interacting pellicles. While for some probes the adhesion force (scaled by the probe radius) was ~ 0.05 nN/ μm (Fig. 4d) in good agreement with the value obtained from linear fits of friction vs. normal force data (Fig. 5), Fig. 4d also shows that for a significant number of experiments the pellicle adhesiveness in bulk saliva was even twice as high than in PBS buffer, something that did not have an impact on the friction experiments. As discussed above, higher friction forces and, therefore, lower lubrication performance would be expected in this case. Regarding viscoelasticity, more viscous interfaces typically result in higher dissipation of friction energy when sheared as the energy used to deform the interfaces is not compensated by an instantaneous full recovery in the trailing region (Blanchet, 2012). However, we did not observe that the presence of the reversibly bound fraction resulted in a different viscoelastic character of pellicles when mechanically confined (Figure 3, Table 1). Nevertheless, it should be noted that the pressure applied in these experiments was probably higher than in friction measurements, as they were performed with standard AFM probes (radius ~ 40 - 200 nm after the smoothing pre-treatment as we previously characterized

(Gonzalez-Martinez et al., 2018)) applying a load of 2 nN. Thus, it cannot be discarded that the presence of the reversibly bound fraction had an effect on the viscoelasticity of the sheared pellicles in our friction experiments. However, if this was the case, it would be expected that the pellicle viscoelasticity would be higher when exposed to bulk saliva than to PBS, provided that this quantity follows the same trend as the pellicle softness (Supplementary Information SI.1). If this was the case, viscoelasticity would still point in the same direction as the adhesiveness results i.e., higher friction would be expected in the presence of the reversibly bound fraction.

Different hypothesis could be formulated for explaining the lack of impact of the reversibly bound fraction on the lubrication properties of *in vitro* salivary pellicles. For instance, this could be the result of the interacting surfaces being in constant mechanical contact during the lubrication measurements, as in our experiments we investigated pellicles formed on planar surfaces and colloidal probes with a diameter of $\sim 15\ \mu\text{m}$ sheared over areas of $2\ \mu\text{m} \times 2\ \mu\text{m}$. In this situation, if the reversibly bound fraction is removed in the early stages of the experiment it would not be able to rebind again as the interacting surfaces were not exposed to bulk saliva i.e., the reservoir of this fraction, until the end of the experiment. Further work to test this hypothesis is planned. As an example, by lifting the AFM cantilever between each of the scans of a friction measurement for enough time for the reversibly bound fraction to re-attach. Nevertheless, further work would be required to provide a solid explanation for the reported lack of influence of the reversibly bound fraction in the lubrication performance of salivary pellicles.

Conclusions

In this work, we investigated whether the reversibly bonded fraction of *in vitro* salivary pellicles had an effect on their mechanical properties, specifically on their viscoelastic character, adhesiveness and lubrication performance. For this, we used different AFM operations modes as well as complimentary ellipsometry measurements to investigate salivary pellicles in the presence of bulk saliva i.e., a situation where the pellicles contain the reversibly bound fraction, and in the presence of PBS buffer i.e., a situation where this fraction is not present.

Ellipsometry confirmed the presence of the reversibly bound fraction in the presence of bulk saliva. Upon mechanical confinement, the presence of this fraction did not affect, within our experimental resolution and using our experimental pro-

tocol, the viscoelastic character of the pellicles. This suggests that in our experiments the reversibly bound fraction was fully compressed. The presence of this fraction had an effect instead on the adhesiveness of opposing pellicles, indicating entanglement between the opposing reversibly bound fractions. Finally, we also showed that the presence of this fraction did not significantly affect the lubrication performance of the pellicles. This was an unexpected observation, as higher friction forces would have been expected from higher adhesiveness. This could indicate different situations due to the experimental details. It might be that the reversibly bound fraction detaches at an early stage when exposed to a friction challenge and that it cannot re-adsorb until the sheared region has access to the reservoir of this fraction i.e., bulk saliva. However, further work would be required to test this hypothesis.

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Role of the reversibly bound fraction of in vitro salivary pellicles on their mechanical and lubrication properties

-Supplementary Information-

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SI.1 Normal force measurements on salivary pellicles

The figure below shows representative normal force measurements obtained while approaching salivary pellicles (both in the presence of bulk saliva and after rinsing with PBS buffer) with the same cantilevers used for AFM contact thermal noise spectroscopy experiments.

III

MUC5B MUCIN FILMS UNDER MECHANICAL CONFINEMENT: A COMBINED NEUTRON REFLECTOMETRY AND ATOMIC FORCE MICROSCOPY STUDY

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Abbreviations

NR: Neutron Reflectometry; AFM: Atomic Force Microscopy; PBS Phosphate Buffered Saline; SLD: Scattering Length Density; PS: Polystyrene; PEO poly(ethylene oxide).

Abstract

Hypothesis: Among other functions, mucins hydrate and protect biological interfaces from mechanical challenges. Mucins also attract interest as biocompatible coatings with excellent lubrication performance. Therefore, it is of high interest to understand the structural response of mucin films to mechanical challenges. We hypothesized that this could be done with Neutron Reflectometry using a novel sample environment where mechanical confinement is achieved by inflating a membrane against the films.

Experiments: Oral MUC5B mucin films were investigated by Force Microscopy/Spectroscopy and Neutron Reflectometry both at solid-liquid interfaces and under mechanical confinement.

Findings: NR indicated that MUC5B films were almost completely compressed and dehydrated when confined at 1 bar. This was supported by Force Microscopy/Spectroscopy investigations. Force Spectroscopy also indicated that MUC5B films could withstand mechanical confinement by means of steric interactions for pressures lower than ~ 0.5 bar i.e., mucins could protect interfaces from mechanical challenges of this magnitude while keeping them hydrated. To investigate mucin films under these pressures by means of the employed sample environment for NR, further technological developments are needed. The most critical would be identifying or developing more flexible membranes that would still meet certain requirements like chemical homogeneity and very low roughness.

Keywords

Neutron Reflectometry; Atomic Force Microscopy; Force Spectroscopy; Mechanical Confinement; Mucins.

1. Introduction

Mucins, long glycoproteins with a central region that contains a high density of oligosaccharide side-chains (accounting for ca. 80% of their mass) [1], attract an enormous amount of research interest both because of their manifold role in biological systems and because of their multiple possibilities as biocompatible coatings. In living systems, mucins are the main component of mucus blankets [2] i.e., the viscous and hydrated gel that forms the outermost part of mucosal barriers. Mucus serves multiple purposes. It is a lubricious barrier that keeps mucosal surfaces hydrated and protects them from harsh chemical conditions, mechanical insults and pathogenic organisms [2, 3]. Because of the difficulties associated with performing mucus studies, this component of mucosal barriers has been less investigated than the underlying mucosal layers. In this regard, *in vitro* physico-chemical studies of mucin and mucin-based films have significantly contributed to our understanding of these systems [4]. However, we still do not fully understand the mechanisms by which mucus fulfills many of its functions. For instance, little is known on the structure and hydration of mechanically challenged mucus.

Expecting a similar performance as that achieved in biological systems, mucins have also been investigated as coatings in biomedical applications. The performance of many medical implants and devices e.g., contact lenses, catheters, ventilation tubes, or stents is still challenged by non-satisfactory anti-biofouling and tribological properties. The use of mucins as coatings of the typically hydrophobic synthetic materials of which medical devices are made from has provided promising results. Mucin coatings not only efficiently suppress biofouling events, partially by conferring them with a hydrophilic character, but also can significantly reduce friction and wear [5-9]. However, in many cases passively adsorbed mucin coatings do not satisfactorily resist the mechanical stresses to which biomedical devices can be exposed. Subsequently, achieving stable resistant mucin-based coatings is attracting significant interest [10, 11].

From the above discussion, it follows that understanding how the structure and hydration of mucin films changes when mechanically confined would be of high interest not only for understanding how mucous films protect biological barriers, but also for the rational design of mucin-based coatings. The study of soft matter films under mechanical confinement was originally made possible by the emergence of the Surface Force Apparatus and the Atomic Force Microscope [12]. These techniques provide accurate interaction forces at different separations. Subsequently, they have been extensively used to study how mucus, mucins and

mucin-based films respond to mechanical challenges [13-23]. However, they do not provide structural information. Indeed, going beyond measurements of forces and additionally measuring the near-surface structures has been a challenge for a number of decades. The main experimental difficulty has been to attain reproducible and accurate confinement within the restrictions of the analytical technique to be employed e.g., geometry, interference with the sensing probe, etc. The development by Cosgrove and collaborators of a confinement apparatus to be used in neutron reflectometry (NR) was a milestone in this direction [24-26]. Neutron reflectometry (NR) is indeed an ideal tool for extracting structural properties perpendicular to the surface of films under confinement, providing high resolution information on layer thickness, interface roughness, and layer density. In the mentioned setup, mechanical confinement was achieved by means of two quartz plates between which the sample was placed, and the pressure was controlled by a hydraulic ram. Since then, different variations of this mechanical confinement cell for NR studies have been proposed [27, 28]. However, these setups suffered from some limitations such as the tendency to misalignment of the plates, the long range waviness of the plates and the presence of entrained dust limiting the achievable confining separations. More recently, a setup with the potential of overcoming these limitations was developed by de Vos *et al.* [29]. In this setup (Fig. 1), from now on referred as confinement cell, one of the rigid surfaces was replaced by a flexible membrane that can conform to long range waviness or bend around any entrained dust. This setup has been successfully used in a number of investigations on the structure of soft matter systems under confinement e.g., for poly-electrolyte multilayers [30, 31], polymer brushes [32-34] and lipid bilayers [35].

Figure 1. Schematic diagram of the flexible-film confinement cell for NR studies [29]. A silicon block is placed on a stainless steel support, and exposed to solvent. A flexible membrane is then inflated by a known gas pressure, leading to the silicon and membrane surfaces coming into close contact. A masking ring on top of the silicon block (shown in dark grey) protects the membrane from overstretching. Reprinted from Review of Scientific Instruments, 2012, 83, 113903, with the permission of AIP Publishing.

The aim of this work was to test the potential of the presented mechanical confinement cell for NR studies to investigate the structure of mechanically confined mucin films. Specifically, films of the oral mucin MUC5B, one of the key components of salivary pellicles i.e., thin films formed upon selective adsorption of saliva that protect oral surfaces against chemical and mechanical insults [36].

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). For neutron reflectivity experiments deuterium oxide (D₂O) of 99.9% atom% d was used (ref: 151882, Sigma-Aldrich, Germany). Dichlorodimethylsilane ($\geq 99.5\%$, ref: 440272), trichloroethylene ($\geq 99.5\%$, ref: 251402), ammonia ($\geq 99.95\%$, ref: 09682) and hydrogen peroxide solution (30 wt. % in H₂O, ref: 216763) were also obtained from Sigma Aldrich. Polystyrene (MW: 62300 Da ref: ps22031) was purchased from PSS (Germany). Unless otherwise specified, all other chemicals used were of at least analytical grade.

2.2. Human salivary MUC5B

MUC5B was isolated from human whole saliva as previously described [37] using a modified version of the method described in [38]. 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 °C), the supernatant was subjected to density-gradient centrifugation in CsCl/ 0.1 M NaCl (Beckman Optima LE-80K, 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 [39]. 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·mL⁻¹. Ethical approval was obtained from the committee of research ethics at Lund University (2018/42).

2.3. Cleaning and Hydrophobization of Silica Substrates

Silica surfaces were used in all experiments. For force microscopy/spectroscopy, p-doped (boron) silicon wafers (Semiconductor Wafer Inc., Hsinchu, Taiwan) were used. For Neutron Reflectivity (NR) we used single crystal silicon (100) blocks (polished by Sil'tronix Silicon Technologies, Archamps, France to a 5 Å RMS roughness). Surfaces were cleaned using a RCA protocol with 5:1:1 H₂O:NH₃:H₂O₂ at 80 °C for 10 minutes followed by additional 10 minutes of plasma cleaning in the case of AFM surfaces and UV/ozone cleaning in the case of NR surfaces. Regardless of the cleaning procedure, in all cases we obtained highly hydrophilic surfaces with water contact angles <5°.

Subsequent hydrophobization of the silica surfaces was achieved by means of liquid-phase silanization [40]. 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 ~90°. The surfaces were stored in ethanol until use.

2.4. Neutron Reflectometry (NR)

Neutron Reflectometry (NR) experiments were carried out at the D17 [41] reflectometer at the Institut Laue-Langevin, France (doi:10.5291/ILL-DATA.8-05-437; doi:10.5291/ILL-DATA.9-13-604; doi:10.5291/ILL-DATA.9-13-797) and at the INTER [42] reflectometer at ISIS, UK (doi:10.5286/ISIS.E.RB1810616; doi:10.5286/ISIS.E.RB1720422; doi:10.5286/ISIS.E.RB1610131), both time of flight reflectometers.

Initially, the silicon blocks (hydrophobized as detailed above) used as substrates were clamped against a home made PEEK trough, specifically designed for round disks, and characterized in PBS buffer prepared with both H₂O (hPBS, SLD = $-0.56 \times 10^{-6} \text{ Å}^{-2}$), and D₂O (dPBS, SLD = $6.36 \times 10^{-6} \text{ Å}^{-2}$). Then, a 0.3 mg·ml⁻¹ MUC5B in hPBS solution was flowed through the PEEK trough and left to adsorb for 1 h. Then the blocks were extensively rinsed with dPBS and subsequently characterized once more in hPBS and dPBS.

Afterwards, the mucin-coated blocks were mounted on the NR confinement cell (Fig. 1), not allowing the coated side to dry at any moment. Full details for the experimental setup are provided in [29]. Briefly, the coated silicon blocks were mounted on a stainless steel support. The blocks were held in place by a stainless steel masking plate with an aperture in the center, exposing the mucin-coated

silicon side. Attached to this mount is a pressure chamber with a flexible poly(ethylene terephthalate) (PET/Melinex[®], DuPont-Teijin films) membrane on one of its faces. A masking plate provides support for the membrane when inflated and prevents it from overstretching and entering the beam path. The membrane is inflated against the silicon block by raising the internal pressure using a digital pressure regulator and a dry N₂ gas source. The masking ring contains two small holes (not shown in Figure 1) that can be used for solvent to leave the cell. Under pressure, solvent can also easily leave the cell where the masking ring and the silicon block are in contact. At a pressure of ~1 bar, the membrane and substrate were in good contact in a circular area of 35-45 mm in diameter. After a confining pressure was applied/incremented, the sample was always given at least 60 min to equilibrate. By doing short reflection experiments of only 10 min we could follow this equilibration process and only performed a full characterization if equilibrium was reached. After the experiments, both samples and membranes were checked for damage. Overall, the samples did not show visual evidences of mechanical damage after the experiments. However, membranes did not recover their original shape. For this reason, samples were only investigated using increasing pressure ramps.

In our experiments, the Melinex was coated with a PS layer. This was achieved by spin coating 5 mL of a 10 g/L PS-toluene solution to the planar side of the Melinex while rotating it at 2000 rpm. The PS-coated Melinex was also characterized by means of AFM and NR.

NR Data Analysis: The measured reflected intensity, $I(q)$, was normalized by the direct beam, I_0 , to achieve the reflectivity, $R(Q)$, where $Q = \frac{4\pi}{\lambda} \sin(\theta)$, Q being the scattering vector, λ the neutron wavelength and θ the incident angle. Fitting of the $R(Q)$ data was achieved using an optical matrix method [43] using the refnx software [44]. Specifically, the interface between the silicon support and the bulk solvent was considered as a stratified medium composed by different slabs.

For fitting NR data from mechanically confined surfaces we used a mixed reflectivity model accounting for the fact that the overall interface comprised areas with different media in contact. In this approach, provided that the areas are not smaller than the relevant coherence length (~10 μm), the overall reflectivity was considered a weighted average of the reflectivity from a type A contact (silicon/Melinex) and a type B contact accounting for the formation of pockets mainly filled with solvent when inflating the membrane (silicon/pockets) so that $R_{Total} = x \cdot R_A + (1 - x) \cdot R_B$ where $0 \leq x \leq 1$.

Parameters obtained by fitting NR data along with the SLD profiles allowed approximating the Volume Fraction Profiles (VFP) of the different components of the interfaces using a modified version of the routine included in the *refnx* software for calculating SLD profiles as indicated in [45].

2.5. Atomic Force Microscopy and 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 used. For imaging experiments, we used triangular silicon nitride cantilevers with a nominal normal spring constant of $0.7 \text{ N}\cdot\text{m}^{-1}$ (ScanAsyst-Fluid, Bruker AFM Probes, Camarillo CA). For force spectroscopy experiments, we used rectangular silicon nitride cantilevers with a nominal normal spring constant of $0.76 \text{ N}\cdot\text{m}^{-1}$ (OMCL-RC800PSA, Olympus, Japan) were employed in all experiments. AFM images were represented and analyzed with the WSxM software [46].

Scratching experiments were performed by scanning in contact mode an area of mucin films while applying a high load ($\sim 100 \text{ nN}$). Then, a wider area was visualized by operating in the Peak Force Tapping Mode while applying a load of $\sim 1 \text{ nN}$.

Force ramps were acquired by using colloidal probes instead. For this, spherical silica particles with a nominal diameter of $15 \text{ }\mu\text{m}$ (PSI-15.0, G. Kisker GbR, Germany) were attached to the free end of the cantilevers by using a two part epoxy adhesive (Loctite, Henkel Norden AB, Sweden). A micromanipulator (TransferMan NK 2, Eppendorf, Germany) was utilized to place glue, and subsequently the silica particle, on the free end of the cantilever. This was done with the help of an optical microscope (Nikon, Amsterdam, The Netherlands). Colloidal probes were visualized by variable pressure SEM (EVO LS10, Zeiss, Germany) for characterizing their shape and size. Force ramps (speed of $1 \text{ }\mu\text{m}\cdot\text{s}^{-1}$) were obtained at different lateral positions by operating the AFM in the force volume (FV) mode [47] and analyzed with the FSAS software (<https://git.io/JmEOS>). Analysis of force ramps was done following a process similar to that detailed in previous works [14, 15, 23]. Briefly, raw force ramps were first transformed into a deflection vs sample position representation by scaling a position sensitive photodetector signal by the slope of the contact region of force ramps obtained on a clean mica surface. Then, deflection was transformed into probe-sample interaction force by scaling the cantilever deflection by its spring constant, which was calculated for each cantilever by means of thermal noise method [48]. Then, the region

of the force ramp associated with mechanical contact was fitted with the Hertz contact model for a sphere-plane geometry. Extrapolating the Hertz fit to zero deflection provides the contact point i.e., the offset for the sample displacement values. The Hertz fit also allowed estimating the contact area for each cantilever deflection and, therefore, the pressure applied.

3. Results

3.1. Polystyrene-coated Melinex

The membrane to be used in the confinement cell, or at least the side in contact with the sample, needs to be characterized by a very low roughness (ideally below 1 nm) in order to achieve a smooth mechanical contact over large areas and to have a reasonable reflectivity, as this quantity decreases exponentially with increasing roughness. It is also beneficial if the membranes are characterized by a Scattering Length Density (SLD) higher than that of the sample support, typically silicon ($\text{SLD} = 2.07 \times 10^{-6} \text{ \AA}^{-2}$). This will result in the presence of a critical angle, which will confirm whether confinement is achieved when applying a pressure. Following previous works [29-33], we used a 50 μm thick poly(ethylene terephthalate) (PET) film, traded under the name Melinex®, which meets these characteristics. Melinex can be acquired from DuPont Teijin films Ltd. without going through the roughing process that most films for commercial use are subjected to. This results in a film with one of its sides with a roughness in the $\sim\text{nm}$ order. However, AFM imaging revealed that that this planar side was also easily contaminated and could not be satisfactorily cleaned with standard protocols e.g., water/ethanol/water washing steps followed by N_2 drying (features with sizes in the 10-200 nm range could still be observed after cleaning as shown in Supplementary Material Section S1). In order to make sure that the membrane was completely cleaned (critical for NR experiments, as it was not possible to characterize the AFM experiments just before them), plasma or ozone/UV cleaning was required. However, this step increased Melinex roughness, ending up with a RMS roughness for its planar side of $\sim 3 \text{ nm}$ (Supplementary Material Section S1).

In order to achieve a lower roughness while keeping the flexible membrane clean from contaminants features, just after ozone/UV cleaning we spin-coated the Melinex with polystyrene (PS). As shown by AFM imaging (Fig. 2a), this resulted in a RMS roughness for its topography of $\sim 3 \text{ \AA}$. In order to characterize the thickness of the PS-coating as well as to experimentally determine the SLD

of the Melinex (required for modelling NR data from mechanical contact experiments), NR was used to characterize the PS-coated Melinex in air. NR raw and fitted data is provided in Fig. 2b. The corresponding SLD profile along with the values for all fitting parameters are provided in Supplementary Material Section S2. Overall, NR indicated a thickness for the PS coating of 33 ± 2 Å and a SLD for the Melinex of $(2.53 \pm 0.01) \times 10^{-6}$ Å⁻².

Figure 2. a) AFM image of a PS-coated Melinex surface. The inset shows the height profile along the highlighted line. b) Fitted NR data for the PS-coated Melinex in air.

3.2. NR of non-confined mucin films

MUC5B mucins rapidly and irreversibly adsorb to hydrophobized silica surfaces [14]. Nevertheless, before mechanical confinement experiments we always characterized by means of NR the mucin-coated hydrophobized silicon blocks in a solid-liquid setup. Representative experiments, fitted to a Si/SiO₂/Silanes/Mucins/Solvent model and corresponding SLD profiles in both hydrogenated and deuterated PBS buffer are shown in Fig.3.

Relevant fit parameters are provided in Table 1 (values for all fitting parameters are provided in Supplementary Material Section S3). It can be observed that mucins films extended up to ~ 440 Å from the surface, being highly hydrated ($\sim 92\%$, in good agreement with previous reports [4]) and exhibited a high tendency for deuteration in good agreement with previous reports [49].

Figure 3. a) Fitted NR data and **b)** corresponding SLD profiles for a MUC5B mucin coated silicon block in both dPBS and hPBS solvents.

3.3. NR of mechanically confined mucin films

NR data obtained while mechanically confining the MUC5B mucin films at both 1 and 2 bar is presented in Fig. 4. A clear critical angle was observed at $Q \sim 0.0049 \text{ \AA}^{-1}$ for both investigated pressures. The location of this critical angle is what is expected for a silicon/Melinex interface (provided a SLD of $2.53 \cdot 10^{-6} \text{ \AA}^{-2}$ found from the fit in Fig. 2). Hence, this clearly shows that most dPBS was expelled and that the silicon and the Melinex could be brought into close proximity. The NR curve obtained at 1 bar shows also another clear feature at $Q \sim 0.012 \text{ \AA}^{-1}$. This was unlikely due to a layer of dPBS trapped between the silicon and Melinex as secondary fringes were not observed. Instead, it was more likely due to a small contribution of reflection from a different interface. This additional feature/peak was also observed in previous experiments with the employed confinement cell e.g., [29], and was associated with the presence of pockets where complete mechanical confinement was not achieved containing, therefore, trapped (deuterated in this particular case) solvent. This hypothesis was supported by the fact that the mentioned feature was less pronounced after incrementing the pressure from 1 bar to 2 bar so that the solvent trapped in these pockets was eventually squeezed away. Therefore, for fitting NR data obtained under mechanical confinement we used a mixed reflectivity model, as indicated in the Materials and Methods section, accounting for the reflection from:

- *Reflectivity A*: Silicon/SiO₂/Silanes/Mucins/PS/Melinex interface.
- *Reflectivity B*: Si/SiO₂/Silanes/Mucins/Pockets interface (accounting for the areas where the Melinex was kept away from the silicon).

As shown in Fig. 4a, this model provided a good fit for the experimental data, supporting our hypothesis. Specifically, the contribution from the pockets decrease from 1.3% at 1 bar to 0.4% at 2 bar, supporting that the solvent trapped in the pockets was squeezed away when incrementing the pressure. SLD profiles for *Reflectivity A* i.e., for the areas where the mucins were mechanically confined, at both 1 and 2 bar are shown in Fig. 4b. For comparison, Figure 4c shows the Volume Fraction Profiles obtained for the mucin layer from the SLD profile of *Reflectivity A* i.e., for the mechanically confined mucins, along with the Volume Fraction Profile obtained from the respective SLD profiles for the non-confined mucins (Fig. 3b). Thickness and hydration values used for fitting the non-confined mucins, and for the mucin layers confined at 1 and 2 bar are shown in Table 1 (values for all fitting parameters are provided in the Supplementary Material Section S4).

Figure 4. a) NR data obtained while mechanically confining the MUC5B mucin films in dPBS at both 1 and 2 bar. Critical edges for the Si/Mellinex and the Si/"pockets" interfaces are highlighted. NR data for the non-confined mucin films in dPBS is included for comparison. b) Corresponding SLD profile for the films confined at 1 and 2 bar. c) Volume fraction profiles for the non-confined and confined mucin films derived from the SLD profile.

Table 1. Thickness and hydration values obtained by fitting NR data for the non-confined mucin films as well as for the mucin films mechanically confined (*Reflectivity A*) at 1 and 2 bar.

	Thickness (Å)	Hydration (%)
MUC5B 0 bar (non-confined)	440.0 ± 70.0	92 ± 1
MUC5B 1 bar	23.9 ± 1.6	31 ± 6
MUC5B 2 bar	22.1±1.2	25 ± 4

Overall, the NR data for the mucin films confined at 1 bar could be fitted with a thickness and hydration for the films of ~2 nm and ~30% respectively, in contrast with the ~44 nm thickness and the ~90% hydration for the non-confined films. Increasing the confining pressure to 2 bar had barely an effect on the thickness and hydration that resulted from fitting the NR data to a similar model.

3.4. AFM scratching of MUC5B mucin films

In order to validate NR data, the thickness of mechanically confined MUC5B films was also measured by scratching an area of the films with the AFM tip by operating the AFM in the contact mode at high loads (~100 nN), and then by visualizing the scratched area at lower loads (~1 nN) while operating the AFM in the Peak Force Tapping mode minimizing, therefore, the destructive lateral forces. Results from a representative scratch are shown in Fig. 5. It can be seen that the thickness obtained in these experiments, 2-3 nm, is in good agreement with the thickness provided by NR for mechanically confined MUC5B films. Incrementing the load did not lead to lower thickness values, but to dragging of mucins along the scratched area instead. As discussed below, the exerted pressure during AFM imaging was higher than in NR experiments provided the small contact area. While the mechanical pressure applied on the films during AFM imaging cannot be accurately estimated because of the uncertainty in the contact area, it is possible though to make a rough estimation. For an applied load of ~1 nN and a contact area of ~100 nm², the applied pressure would be ~10² bar, a higher value than that applied with the confinement cell in our NR experiments.

Figure 5. a) AFM topography image of a scratched mucin film and b) height profile along the highlighted line in the topography image.

3.5. Force spectroscopy on MUC5B mucin films

To further understand our NR data on confined MUC5B mucin films, we investigated this system by means of AFM-based force spectroscopy. AFM force scans provide accurate measurements acting on the AFM probe at different relative positions from a sample. When in mechanical contact, several models are available for describing the interaction between probe and sample. Among those, we have used the Hertz model. The choice was based on the fact that adhesions were almost non-existent in our experiments. We used this approach as mechanical contact models do not only describe the force vs sample indentation behavior, but also provide an estimation of the contact area at different forces. Thus, fitting force ramps will also allow the estimation of pressure values that can be compared

to those applied in NR experiments. However, this approach requires a probe with a well-defined geometry. Subsequently, we used colloidal probes with an average diameter of 15 μm (according to the manufacturer). Nevertheless, we always characterized the probes by means of SEM imaging before use in order to have an accurate estimation of the radius of each probe (Fig. 6a). Figure 5b shows a force vs. probe sample scan (solid line) representative from these experiments. Two regimes are clearly differentiated at short separations. Initially, the force exhibits an exponential dependence with the separation, previously reported for force scans on mucin films [14] and representative of a steric repulsion. At shorter separations, forces exhibit a dependence with separation that is well fitted (dashed line in Fig. 6b) by the Hertz contact model:

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

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 [50]. Deviations from the Hertz model occurred though at high forces, which is not unexpected as the applicability of the Hertz model is based on the assumption that the thickness of the film is lower than the radius of the probe. Nevertheless, the use of the Hertz model still allowed estimating the pressures exerted by the probe when in mechanical contact. Specifically, in the Hertz model, the contact radius, r_c , can be estimated by $r_c = \sqrt{\delta R}$ where δ is the indentation/deformation. Thus, the pressure, P , can be estimated as $P = F / \pi r_c^2$. In the right vertical axis of Fig.

6b the pressures corresponding to the Hertz fit are shown. In this example, it can be seen that the pressure for which the force entered the regime described by the Hertz model is well-below 1 bar. Figure 6c shows a histogram, calculated from 256 force ramps, of the pressures for which the force ramp entered the Hertz mechanical contact regime. Again, this shows that for the vast majority of measurements this threshold value is well below 1 bar i.e., the minimum pressure for which we were able to perform NR confinement experiments. We can also see from Fig. 6b that the indentation of the sample after the curves entered the Hertz regime barely increased with force/pressure, even less than that predicted by the Hertz model, which is expected providing that the thickness of the film was significantly smaller, according to our NR data, than the probe size.

Figure 6. **a)** Colloidal probe used in our experiments. **b)** Force vs. probe sample distance ramp on a mucin film obtained with a colloidal probe (solid line) and fit to the Hertz contact model (dashed line). The right vertical axis indicate the pressures corresponding to the forces of the Hertz fit. **c)** Histogram of the pressures, according to the Hertz model, for which the force scans entered the region defined by the Hertz model.

4. Discussion

NR experiments performed with the confinement cell showed that, when confined at 1 bar, the MUC5B mucin films were almost completely compressed. Specifically, the film thickness and hydration parameters in the models used to fit the NR data decreased from ~ 440 Å to ~ 24 Å and from $\sim 92\%$ to $\sim 31\%$ respectively. The values of these parameters barely changed when fitting the NR data obtained for the films confined at 2 bar. Interestingly, the value of the thickness parameter obtained after fitting the NR data of the mechanically confined films were in good agreement with the ~ 2 -3 nm thickness provided by AFM imaging of scratched areas of the films. As discussed above, the mechanical pressure applied on the films during AFM imaging was in the order of $\sim 10^2$ bar. This is a higher value than that applied with the confinement cell in our NR experiments so that the thickness provided by AFM also corresponds to that of fully compressed mucin films.

In order to estimate the pressures that would originate relevant changes in e.g., the thickness and hydration of the mucin films, colloidal probe spectroscopy experiments were performed. In good agreement with previous results, force ramps performed on these systems exhibited two differentiated regimes when approaching probe and sample: an initial repulsive regime with an exponential-like dependence with separation, characteristic of steric repulsions, followed by a regime where the force exhibited a steeper dependence with the separation that could be well fitted by the Hertz contact model. Fitting to the contact model combined with the knowledge of the size and geometry of the probe allowed a reasonable estimation on the relationship between applied pressure and deformation to be made. Specifically, we found out that for the vast majority of performed force ramps, for a pressure of 1 bar i.e., the minimum value at which we were

able to operate the confinement cell, mechanical contact was already established, and above this point, deformation barely increased with applied pressure. From this data, it could also be inferred that the pressures for which the probe and sample interact by means of steric repulsion typically lay below 0.5 bar. This would be the pressures for which mucin films could protect interfaces from mechanical challenges while keeping them highly hydrated. It would be of high interest to investigate the films at these pressures by means of the NR confinement cell, not only because then significant structural changes with pressure would be observed but also because of its relevance for *in vivo* situations. For example, pressures applied during tongue and labial movements have been reported to be in the order of ~ 0.1 bar [51, 52]. Nevertheless, the pressures that can be achieved with the current setup can be of relevance for some other situations. For example, teeth, which are covered by a thin layer of salivary components including MUC5B, can be exposed to biting pressures in the order of ~ 10 bar [53, 54].

We only studied MUC5B mucin films formed by adsorption from $0.3 \text{ mg}\cdot\text{mL}^{-1}$ bulk solutions. It is reasonable to expect that structural changes induced by confinement will depend on the surface concentration of mucins. However, $0.3 \text{ mg}\cdot\text{mL}^{-1}$ was the highest concentration obtained during purification, and increasing the concentration would have required procedures that could irreversibly damage the mucins. Nevertheless, previous studies showed that the surface amount of MUC5B mucins had already a weak dependence with bulk concentration at these values [55]. Thus, it is unlikely that different results than those reported would have been obtained by investigating films formed from bulk solutions of higher mucin concentrations.

As mentioned, the minimum pressure that we could apply during a NR experiment with the confinement cell was ~ 1 bar. This is originated by the requirement that the area confined by the membrane needs to be bigger than the area illuminated by the neutron beam ($\sim 1\text{-}10 \text{ cm}^2$). Thus, the minimum pressure that can be applied during an experiment is that for which the membrane is deformed enough to achieve a mechanical contact area with the sample of $\sim 1\text{-}10 \text{ cm}^2$. This is determined by the flexibility of the membrane. However, flexibility is not the only characteristic that the membrane needs to fulfill. The membrane, or at least the side in contact with the sample, needs to be characterized by a very low roughness (ideally below 1 nm) in order to achieve a smooth mechanical contact over large areas and to have a reasonable reflectivity, as this quantity decreases exponentially with increasing roughness. The membrane needs to be characterized as well by a homogeneous chemical composition. If μm -sized domains of different

chemical composition are present, the resulting data will be a combination of different reflectivities. This would enormously increase the complexity of the analysis of the data, to an extent where obtaining reliable information would be almost impossible. Moreover, in these experiments, where studies at different contrasts are impractical because of the impossibility to change the solvent once mechanical contact is established, a reliable analysis of the data is highly dependent on the presence of a critical angle. For this to happen, the membrane needs to be characterized by a SLD higher than that of the sample support, typically silicon ($\text{SLD} = 2.07 \times 10^{-6} \text{ \AA}^{-2}$). These characteristics are fulfilled by Melinex, which has been so far the membrane used in all reported experiments performed with the confinement cell [29-34]. However, for the minimum pressure we were able to operate using Melinex as a membrane i.e., 1 bar, the MUC5B mucin films were fully compressed. This was also observed e.g., for uncharged polymer brushes when investigated with the confinement cell [32]. This suggests that for studying these type of soft matter systems by means of the confinement cell for NR studies, one would need to identify and use different membranes, more flexible than Melinex so that a wide area in mechanical contact could be establish for pressures well below 0.5 bar (as indicated by our force spectroscopy experiments), but that would still fulfill all the characteristics indicated above.

5. Conclusions

The goal of this work was to investigate the potential of a recently developed confinement cell for investigating by means of NR the structure of MUC5B mucin films under mechanical confinement. In this setup, by inflating a Melinex membrane against mucin films we were able to establish mechanical confinement at a pressure of 1 bar. Overall, NR indicated that already at this pressure the mechanically confined mucin films were almost completely compressed and dehydrated. Increasing the pressure to 2 bar significantly decreased the contribution from the solvent containing pockets. However, this increase in pressure had almost no effect on the mechanically confined mucins. A similar observation i.e., full compression at 1 bar, was also reported for PEO polymer brushes when investigated with the confinement cell [32]. AFM investigations provided a thickness for the fully compressed mucin films i.e., 2-3 nm, that was in good agreement with the film thickness parameter used to fit NR data. AFM also confirmed that for a pressure of 1 bar the interaction between AFM probe and mucin films could be described by a mechanical contact model, where the deformation of the films barely changed with applied pressure.

As indicated by AFM force spectroscopy, for investigating the mucin films in a regime where steric repulsion could withstand the mechanical pressure exerted by the membrane in NR experiments, it would be needed to achieve a good mechanical contact at pressures of 0.5 bar and below. This would be the pressure range where the mucin films would be able to provide mechanical protection to interfaces while keeping them hydrated. As we discussed, for this it will be needed in the future to identify or develop membranes more flexible than Melinex but that still would meet certain requirements like very low roughness and chemical homogeneity.

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MUC5B MUCIN FILMS UNDER MECHANICAL CONFINEMENT: A COMBINED NEUTRON REFLECTOMETRY AND ATOMIC FORCE MICROSCOPY STUDY

- Supplementary Information -

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S1. Cleaning of Melinex membranes

We initially explore the possibility of a gentle cleaning procedure for the Melinex membrane consisting of successive water, ethanol and water rinsing steps followed by N₂ drying. However, we were not able to completely clean the membrane following this procedure, and often regions contained big (height values in the 10-200 nm range) particles as shown by AFM imaging (Fig. S1a). Additional cleaning of these surfaces by means of ozone effectively removed the contamination observed in (a) leading, however, to an average RMS roughness value of ~ 3.1 nm (Fig. S1b). In order to further lower the roughness, we spin-coated this sample with polystyrene as detailed in the main manuscript.

Figure S1. AFM images of **(a)** Melinex cleaned with successive water, ethanol and water rinsing steps followed by N₂ drying. **(b)** Melinex cleaned as in (a) followed by 10 min ozone cleaning. The inset shows the height profiles along the highlighted lines. Color height scales: (a) 160 nm, (b) 16.9 nm.

S2. NR for PS-coated Melinex

Figure S2. SLD profile for the PS-coated Melinex in air

Table S2. Fit parameters for the NR data obtained for the PS-coated Melinex in air (fig. 2 in the main manuscript).

	SLD ($10^{-6} \text{ \AA}^{-2}$)	Thickness ($\text{\AA}$)	Roughness ($\text{\AA}$)
Air	0	-	-
PS	1.4	33.0 ± 1.7	6.4 ± 1.3
Melinex	2.53 ± 0.01	-	23 ± 2

S3. NR of non-confined mucin films

Table S3. Fit parameters for the NR data obtained for the MUC5B mucin films at solid liquid interfaces (Fig. 3 in the main manuscript).

	SLD ($10^{-6} \text{ \AA}^{-2}$)		Thickness ($\text{\AA}$)	Roughness ($\text{\AA}$)	Hydration (%)
Si	2.07		-	-	-
SiO ₂	3.47		13.9 ± 1.2	2.2 ± 1.4	1.6 ± 0.4
Silanes	0.7		23.5 ± 0.6	10.8 ± 1.8	18.9 ± 1.7
MUC5B	dPBS	5.56 ± 0.04	440 ± 70	6.2 ± 0.2	92.0 ± 0.7
	hPBS	1.90 ± 0.10			
Solvent	dPBS	6.36	-	190 ± 40	-
	hPBS	-0.56			

S4. NR of mechanically confined mucin films

Table S4a. Fit parameters for the NR data obtained for the MUC5B mucin films mechanically confined at 1 bar.

1 bar	Scale	Slab	SLD ($10^{-6} \text{ \AA}^{-2}$)	Thickness ($\text{\AA}$)	Roughness ($\text{\AA}$)	Hydration (% dPBS)
Structure 1	0.987	Si	2.07			
		SiO ₂	3.47	13.9	2.2	1.6
		Silanes	0.7	23.5	10.8	18.9
		MUC5B (Compressed)	5.60±0.30	23.9±1.6	11.7±0.5	31±6
		PS	1.4	33.0	22±3	1.3
		Backing (Melinex)	2.61±0.01	-	12.4±1.6	-
Structure 2	0.013	Si	2.07	-	-	-
		SiO ₂	3.47	13.9	2.2	1.6
		Silanes	0.7	23.5	10.8	18.9
		MUC5B (Pockets)	5.60±0.30	93.0±25.0	11.7±0.5	33±11
		Backing (Pockets)	4.94±0.02	-	23.0±4.0	-

Table S4b. Fit parameters for the NR data obtained for the MUC5B mucin films mechanically confined at 2 bar.

2 bar	Scale	Slab	SLD ($10^{-6} \text{ \AA}^{-2}$)	Thickness ($\text{\AA}$)	Roughness ($\text{\AA}$)	Hydration (% dPBS)
Structure 1	0.996	Si	2.07			
		SiO ₂	3.47	13.9	2.2	1.6
		Silanes	0.7	23.5	10.8	18.9
		MUC5B (Compressed)	5.60±0.30	22.1±1.2	12.2±0.2	25.0±4.0
		PS	1.4	33.0	21.0±0.3	1.3
		Backing (Melinex)	2.61±0.01	-	14.0±4.0	-
Structure 2	0.004	Si	2.07	-	-	-
		SiO ₂	3.47	13.9	2.2	1.6
		Silanes	0.7	23.5	10.8	18.9
		MUC5B (Pockets)	5.60±0.30	49±30	12.18±0.14	15±11
		Backing (Pockets)	5.55±0.06	-	15±10	-

IV

OPEN

Effect of nonionic and amphoteric surfactants on salivary pellicles reconstituted in vitro

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Surfactants are important components of oral care products. Sodium dodecyl sulfate (SDS) is the most common because of its foaming properties, taste and low cost. However, the use of ionic surfactants, especially SDS, is related to several oral mucosa conditions. Thus, there is a high interest in using non-ionic and amphoteric surfactants as they are less irritant. To better understand the performance of these surfactants in oral care products, we investigated their interaction with salivary pellicles i.e., the proteinaceous films that cover surfaces exposed to saliva. Specifically, we focused on pentaethylene glycol monododecyl ether (C₁₂E₅) and cocamidopropyl betaine (CAPB) as model nonionic and amphoteric surfactants respectively, and investigated their interaction with reconstituted salivary pellicles with various surface techniques: Quartz Crystal Microbalance with Dissipation, Ellipsometry, Force Spectroscopy and Neutron Reflectometry. Both C₁₂E₅ and CAPB were gentler on pellicles than SDS, removing a lower amount. However, their interaction with pellicles differed. Our work indicates that CAPB would mainly interact with the mucin components of pellicles, leading to collapse and dehydration. In contrast, exposure to C₁₂E₅ had a minimal effect on the pellicles, mainly resulting in the replacement/solubilisation of some of the components anchoring pellicles to their substrate.

Surfactants are a common ingredient of oral hygiene products like toothpastes and mouthwashes^{1,2}. They act as solubilizing, dispersing and wetting agents. Moreover, they promote foaming, which is preferred by consumers since it provides a perception of cleanliness³. Sodium dodecyl sulfate (SDS) is the most common surfactant present in oral products since the development of synthetic surfactants³, nowadays being one of the most commonly used surfactants in oral health products at concentration of up to 2%⁴. However, the use of ionic surfactants like SDS has been related to exacerbation of periodontal disease, initiation and progression of gingival recession and recurrent oral ulceration⁵. In this regard, non-ionic and amphoteric surfactants attract significant interest as they have been shown to be less irritant⁶. While it is difficult to compete against the use of SDS because of its foaming properties, taste and low cost, amphoteric surfactants, especially cocamidopropyl betaine (CAPB) are becoming more popular in personal care products, including toothpastes⁷. Nonionic surfactants are instead nowadays preferred in mouthwashes¹. The goal of this work was to increase the scientific basis of the mechanisms of action of the less-irritant non-ionic and amphoteric surfactants so that the formulation of oral care product could be improved. Specifically, we have focused on the effect that these two types of surfactants have on reconstituted salivary pellicles.

Salivary pellicles are thin (thickness of few nanometers) organic (mostly proteinaceous) films that form rapidly on oral surfaces upon exposure to saliva⁸. Salivary pellicles fulfil many functions. These include e.g., regulation of tooth mineralization and demineralization processes, lubrication, hydration, acting as a diffusion barrier and buffering ability^{8,9}. Thus, it is reasonable that the use of oral products with minimal effect on salivary pellicles would also be gentler towards oral mucosa surfaces. Indeed, ionic surfactants, especially SDS, are known to highly damage salivary pellicles^{10,11}. This agrees with the fact that electrostatic interactions have been shown to

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Figure 1. (a) QCM-D data (markers) and corresponding fits to the Voigt model using overtones 3rd, 5th, and 7th (solid lines) for a representative experiment where human whole saliva (HWS) was adsorbed on silica surfaces, rinsed with PBS buffer, exposed to C_{12}E_5 in PBS solution and finally rinsed with PBS buffer again. (b) Adsorbed mass calculated from fitting the data in (a) to the Voigt model. (c) QCM-D data (markers) and corresponding fits to the Voigt model using overtones 3rd, 5th, and 7th (solid lines) for a representative experiment where human whole saliva (HWS) was adsorbed on silica surfaces, rinsed with PBS buffer, exposed to a CAPB in PBS solution and finally rinsed with PBS buffer again. (d) Adsorbed mass calculated from fitting the data in (c) to the Voigt model.

play an important role in pellicle structure^{12,13}. In this work we explored whether the less-irritant nonionic and amphoteric surfactants exhibit different interactions with salivary pellicles than ionic surfactants.

For this purpose, we investigated salivary pellicles reconstituted on model solid surfaces before and after exposure to a nonionic surfactant (pentaethylene glycol monododecyl ether, C_{12}E_5) and an amphoteric surfactant (cocamidopropyl betaine, CAPB) by means of different surface techniques i.e., quartz crystal microbalance with dissipation (QCM-D), null-ellipsometry, force spectroscopy and neutron reflectometry. All of these techniques have already proven to be highly useful for pellicle structure investigations^{4,13–15}. QCM-D, NR and ellipsometry, require highly planar, macroscopic and, for the latter, reflective solid surfaces. Because of these requirements, it was not possible to use more relevant oral cavity substrates e.g., oral mucosa and enamel, to reconstitute salivary pellicles. An important factor to consider for choosing the right model surface is its charge, as this is known to play an important role on the formation of salivary pellicles^{16,17}. Oral mucosa surfaces are decorated with the MUC1 mucin^{18,19}. Thus, they have an anionic character. A similar character has been reported for hydroxyapatite²⁰, the main component of enamel. Therefore, we employed in our experiments silica surfaces, which also have an anionic character²¹, as substrate for salivary pellicles.

Overall, we show that both C_{12}E_5 and CAPB are gentler towards salivary pellicles reconstituted on silica surfaces than SDS, which removes them completely. However, CAPB and C_{12}E_5 affected the reconstituted pellicles to different extents and by means of different mechanisms.

Results

Quartz crystal microbalance with dissipation (QCM-D) characterization. Raw QCM-D data, corresponding fits to the Voigt viscoelastic model and areal masses obtained from these fits, from representative experiments showing the formation of salivary pellicles on hydrophilic silica surfaces, and their subsequent exposure to C_{12}E_5 and CAPB in PBS solutions are shown in Fig. 1.

QCM-D data confirmed the formation of viscoelastic (as inferred from the high dissipation shift values) salivary pellicles upon the exposure of the silica substrates to saliva in a time frame of minutes in agreement with previous reports^{13,15}. Subsequent exposure to both C_{12}E_5 and CAPB led to a decrease in both frequency and dissipation shifts. However, these quantities did not return to zero values indicating that the investigated surfactants did not entirely remove the pellicles. This is in contrast with the effect of SDS, which completely removes pellicles formed on hydrophilic surfaces^(11, Supplementary Information Section S4).

The specific effect on QCM-D signals was different for the two investigated surfactants. Data registered at the end of the PBS buffer rinsing steps performed before and after exposure to surfactant solutions indicated a

Figure 2. Areal mass calculated from representative ellipsometry experiments where salivary pellicles were formed, rinsed with PBS and subsequently exposed to (a) C₁₂E₅ and (b) CAPB solutions followed by a final PBS rinsing step.

Figure 3. Comparison between the adsorbed mass as measured by QCM-D and ellipsometry. The mean ratio of the mass of the pellicle before to the mass after treatment by the surfactants over three data sets are shown. Error bars correspond to standard deviation values.

decrease in frequency shift of ~ 7 Hz in the case of C₁₂E₅ (Fig. 1a) and of ~ 18 Hz in the case of CAPB (Fig. 1c), with changes in dissipation shifts following the same trend. Areal masses calculated from Voigt model fits revealed the same tendency i.e., exposure to C₁₂E₅ resulted in a decrease of ~ 3 mg·m⁻² (Fig. 1b) while exposure to CAPB resulted in a decrease of ~ 7 mg·m⁻² (Fig. 1d). The ratio, averaged over 3 different experiments, between QCM-D mass measured after and before exposure to surfactants evidenced the same behaviour (Fig. 3).

Ellipsometry characterization and comparison with QCM-D results. Areal masses from representative ellipsometry experiments where salivary pellicles were exposed to C₁₂E₅ and CAPB solutions are shown in Fig. 2. Corresponding refractive index and thickness values for these experiments are shown in Supplementary Information S5. Up to some extent, CAPB caused a larger decrease in both the areal mass and thickness of sali-

Figure 4. (a) Force vs probe-sample distance ramps on salivary pellicles (reconstituted on silica substrates) before (blue) and after (green) exposure to $C_{12}E_5$ for 1 h and subsequently rinsed with PBS buffer. (b) Probability distributions for the characteristic length obtained from exponential fits of the non-contact regions of force ramps on pellicles before and after exposure to $C_{12}E_5$. (c) Force vs probe sample distance ramps on salivary pellicles (reconstituted on silica substrates) before (blue) and after (red) exposure to CAPB for 1 h and subsequently rinsed with PBS buffer. (d) Probability distributions for the characteristic length obtained from exponential fits of the non-contact regions of force ramps on pellicles before and after exposure to CAPB.

vary pellicles compared to $C_{12}E_5$. However, as shown in Fig. 3, when averaged over data from different experiments the difference in amount desorbed by each surfactant was not as significant as that shown by QCM-D.

Figure 3 shows the ratio of both ellipsometry and QCM-D areal masses of salivary pellicles measured in PBS after and before surfactant exposure averaged over three different data sets. According to QCM-D, less than ~60% of the pellicle mass is remaining after treatment with CAPB compared to ~87% after $C_{12}E_5$ exposure. However, according to ellipsometry the difference is much lower with only ~5% more mass being removed by CAPB compared to $C_{12}E_5$. Although differences between experimental techniques are to be expected due to different sensitivities and environments, for the specific comparison of these two techniques differences are expected (as discussed later on) as ellipsometry is basically sensitive to the solid content of adsorbed layers (often called “dry mass”) whereas QCM-D is also sensitive to the mass of solvent coupled to the layers (thus, the mass monitored by QCM-D is often referred as “wet mass”).

Force spectroscopy characterization. Salivary pellicles before and after exposure to $C_{12}E_5$ and CAPB solutions were investigated by means of AFM-based force spectroscopy. Data from a representative experiment are shown in Fig. 4. Force ramps (representative examples shown in Fig. 4a,c) from all experiments (pellicles before and after exposure to both surfactants) exhibited long-range exponential-like repulsive forces, characteristic for steric interactions, when mechanically confining the samples with the AFM probe. When confined enough, the force-distance behaviour drastically changed to one that could be modelled by the Hertz mechanical contact model instead (specifics on the fitting procedure are detailed in Supplementary Information Section S3). Interestingly, exposure to $C_{12}E_5$ did not have a significant effect on the repulsive long-range steric forces (Fig. 4a). This was clearly seen in the characteristic length, λ_{exp} (Eq. 2), found when fitting to exponential functions the region of the ramps where these forces were observed (Fig. 4b).

For CAPB-treated pellicles, imaging of the samples by means of AFM revealed (Supplementary Information Section S6) that a fraction of ~5% of the overall surface area became covered with a μm -domains (absent before exposure to this surfactant) of a material that we hypothesized to be aggregated CAPB. For force spectroscopy

experiments on these samples, we avoided areas covered by these aggregates. Force ramps performed on the remaining homogeneous CAPB-treated pellicle (accounting for ~95% of the surface area) revealed that exposure to this amphoteric surfactant drastically lowered, by a factor ~3, the distances to which the repulsive steric forces opposing mechanical confinement extended (Fig. 4c,d).

Neutron reflectivity characterization. To further investigate the mechanisms by which $C_{12}E_5$ and CAPB interact with reconstituted salivary pellicles, neutron reflectivity (NR) was used. The non-destructive and highly penetrating nature of neutron beams allows extracting structural properties perpendicular to the surface of films, providing high resolution information on layer thickness, interface roughness, and layer density (in terms of hydration and scattering length density, a quantity equivalent to a refractive index that is determined by the composition of specific regions of the sample).

Each of the silicon blocks used as pellicle substrates were first characterized in three different contrasts: PBS buffer prepared with H_2O (hPBS, $SLD = -0.56 \times 10^{-6} \text{ \AA}^{-2}$), with silicon matched water ($0.38:0.62 D_2O:H_2O$, smwPBS, $SLD = 2.07 \times 10^{-6} \text{ \AA}^{-2}$) and with D_2O (dPBS, $SLD = 6.36 \times 10^{-6} \text{ \AA}^{-2}$). Similar characterizations were performed after formation of salivary pellicles on each block and after treatment of the pellicles with the surfactants (except for CAPB-treated surfactants, for which only dPBS and hPBS characterizations were performed). Specifically, we report here experiments on salivary pellicles treated with chain deuterated $C_{12}E_5$ ($dC_{12}E_5$), with fully hydrogenated $C_{12}E_5$ ($hC_{12}E_5$) and with hydrogenated CAPB.

For NR data analysis, the *refnx* software was used²². In all experiments, the interface between the silicon support and the bulk solvent was considered as a stratified medium composed by different slabs. The reflectivity originating from such an interface can be described according to the optical matrix method²³, where each of the slabs included in the model is characterized by four output parameters: the layer thickness, the scattering length density (SLD), the volume fraction of solvent (hydration) and the surface roughness. In all cases, fits of NR for each of the investigated systems were co-refined using data from all contrasts available. However, while similar, different salivary pellicles exhibited slight differences that did not allow to co-refine fits from different pellicles i.e., it was not possible to simultaneously fit reflectivities from different pellicles with identical fitting parameters (even though similar results as those reported in¹² were obtained in all cases), and, therefore, from pellicles treated with $dC_{12}E_5$ and $hC_{12}E_5$.

For fitting NR data on the clean silicon blocks, a simple Si/SiO₂/Solvent model was used. For fitting NR data obtained for salivary pellicles, we used a two-layered structure for the pellicles (Fig. 5a) in agreement both with previous NR studies^{12,16} and with a model reported in multiple other studies where pellicles consist of an inner thin dense layer and an outer thick diffuse layer^{13,16,24,25}. For fitting NR data from the pellicles after being exposed to the surfactants, we used the same model allowing for changes in the inner and outer pellicle layers. Moreover, we allowed the pellicle layers to include a percentage of surfactant (Supplementary Information Section S7).

Raw and fitted NR data for a silicon block and a salivary pellicle subsequently formed on that block before and after treatment with $dC_{12}E_5$ are shown in Fig. 5b. Corresponding SLD profiles are shown in Fig. 5c. Similarly, raw and fitted NR data for a different silicon block and a pellicle subsequently formed on that block before and after treatment with CAPB are shown in Fig. 5d, with corresponding SLD profiles in Fig. 5e. Relevant parameters obtained from the fits are shown in Tables 1 and 2 for the effect on the pellicles of $dC_{12}E_5$ and CAPB respectively (the complete set of fit parameters is available in Supplementary Information Section S7).

In agreement with observations from other techniques, $dC_{12}E_5$ had a minor effect on the salivary pellicle. Mainly, the pellicle outer layer exhibited slight coiling and the hydration of the inner layer increased by ~10%. Fitting NR data from the pellicle treated with $hC_{12}E_5$ (Supplementary Information Section S7) revealed a similar tendency, even though in this case the hydration of the inner layer only increased by ~5%. The obtained SLD values might support that the pellicles retained some $C_{12}E_5$ both in the pellicle inner and outer layers. However, when considering the errors this data was not conclusive.

In contrast, exposing the pellicles to CAPB induced a significant coiling of the pellicle outer layer (relative decrease in thickness by a factor ~3, in good agreement with Force Spectroscopy data). The pellicle inner layer was hardly influenced by CAPB though. The SLD profile also suggested that, after rinsing, a higher amount of surfactant was retained, mostly by the pellicle outer layer.

Discussion

In this work, we investigated the effect of a model nonionic surfactant, $C_{12}E_5$, and a model amphoteric surfactant, CAPB, with salivary pellicles reconstituted at solid-liquid interfaces. Both types of surfactants are considered to be less aggressive towards oral surfaces than the most commonly used ionic surfactants, especially SDS, in oral care products. Salivary pellicles i.e., the mostly proteinaceous films that adsorb on almost any surface upon exposure to saliva, constitute a protective barrier for oral surfaces. Thus, our aim was to investigate whether the gentler effect of nonionic and amphoteric surfactants correlated with a different effect on salivary pellicles from that of ionic surfactants. For this, we employed different surface techniques: force spectroscopy, QCM-D, null-ellipsometry and neutron reflectometry. The latter require the use of highly planar reflective surfaces, preventing the use of oral surfaces like mucosa or enamel to reconstitute salivary pellicles. For identifying a substrate that we could use instead, we considered two properties known to influence the properties of salivary pellicles: the substrate wettability²⁶ and ionic character^{8,25}. In vivo wettability measurements indicated a hydrophilic nature for both teeth and oral mucosa²⁷ surfaces. Hydroxyapatite, the main component of enamel/teeth surface has an anionic character³⁰. Because of being decorated with the MUC1 mucin¹⁹, the same applies to oral mucosa surfaces. Therefore, we used silica surfaces as substrates for salivary pellicles because of their hydrophilic anionic character and their suitability for the employed experimental techniques.

Figure 5. (a) Illustration of the two-layered pellicle model used to fit NR data (illustration created with CorelDRAW, Corel Corporation, Canada). (b) NR curves, in dPBS contrast, and corresponding fits (solid lines) for a clean silicon block and a pellicle adsorbed on that block before and after treatment with dC₁₂E₅. (c) Corresponding SLD profile for both dPBS and hPBS contrasts. On the right hand side, zooms for the regions corresponding to the pellicle outer layer are shown. (d) NR curves, in dPBS contrast, and corresponding fits (solid lines) for a clean silicon block and a pellicle adsorbed on that block before and after treatment with CAPB. (e) Corresponding SLD profiles for both dPBS and hPBS contrasts. On the right hand side zooms for the regions corresponding to the pellicle outer layer are shown.

In agreement with previous reports¹¹, we confirmed (Supplementary Information Section S4) that exposure of salivary pellicles reconstituted on silica surfaces to SDS concentrations above the CMC value led to a complete removal of salivary pellicles reconstituted on these surfaces. In contrast, the investigated nonionic and amphoteric surfactants had a gentler effect on the pellicles. However, these surfactants had different effects on the pellicles.

The areal mass of salivary pellicles before and after being exposed to both types of surfactants was investigated by ellipsometry and QCM-D. While ellipsometry is sensitive mostly to the dry content of the adsorbed material, QCM-D also is highly sensitive to the mass of the solvent coupled to the adsorbed films^{28,29}. In our experiments, the ellipsometry mass of salivary pellicles before and after being exposed to C₁₂E₅ and CAPB solutions did not show significant differences (Fig. 3). However, QCM-D (Fig. 3) revealed that CAPB removed a significantly higher fraction than C₁₂E₅. This indicated that CAPB had a stronger interaction, with a highly hydrated fraction of the salivary pellicle at least. We can interpret this observation in terms of the two-layer model proposed for reconstituted salivary pellicles^{12,13,16,24,25}. In this model, reconstituted pellicles consist of an inner thin dense layer (formed mainly of low molecular weight proteins) and an outer thick diffuse highly hydrated layer (mainly composed by the highly charged oral mucin MUC5B). In this scheme, it is reasonable to hypothesize that the amphoteric surfactant CAPB developed a strong interaction with the highly hydrated

		Salivary pellicle before treatment with $dC_{12}E_5$	Salivary pellicle after treatment with $dC_{12}E_5$
Pellicle inner layer	Thickness (Å)	46 ± 1	45 ± 1
	Hydration (%)	62.0 ± 0.8	70.1 ± 0.5
	Salivary content (%)	100	92 ± 2
	Surfactant content (%)	–	8 ± 2
Pellicle outer layer	Thickness (Å)	286 ± 12	277 ± 16
	Hydration (%)	97.1 ± 0.2	98.0 ± 0.1
	Salivary content (%)	100	96 ± 5
	Surfactant content (%)	–	4 ± 5

Table 1. Parameters obtained from fits of NR data of a salivary pellicle adsorbed on a silicon block before and after treatment with $dC_{12}E_5$. The parameters were obtained from co-refined fits of 3 measurements of the Si block (in dPBS, smwPBS and hPBS), 3 measurements of the pellicle before exposure to $C_{12}E_5$ (in dPBS, smwPBS and hPBS) and 3 measurements after exposure to $C_{12}E_5$ (in dPBS, smwPBS and hPBS). Errors correspond to those calculated by means of the Bayesian Markov chain Monte Carlo (MCMC) approach implemented in the refnx software²².

		Salivary pellicle before treatment with CAPB	Salivary pellicle after treatment with CAPB
Pellicle inner layer	Thickness (Å)	46 ± 1	35 ± 1
	Hydration (%)	55.7 ± 0.3	54.4 ± 0.9
	Salivary content (%)	100	99.4 ± 0.7
	Surfactant content (%)	–	0.6 ± 0.7
Pellicle outer layer	Thickness (Å)	325 ± 11	117 ± 8
	Hydration (%)	97.5 ± 0.2	95.5 ± 0.5
	Salivary content (%)	100	92.0 ± 2.0
	Surfactant content (%)	–	8.0 ± 2.0

Table 2. Parameters obtained from fits of NR data of a salivary pellicle adsorbed on a silicon block before and after treatment with CAPB. The parameters were obtained from co-refined fits of 3 measurements of the Si block (in dPBS, smwPBS and hPBS), 3 measurements of the pellicle before exposure to $C_{12}E_5$ (in dPBS, smwPBS and hPBS) and 2 measurements after exposure to $C_{12}E_5$ (in dPBS, and hPBS). Errors correspond to those calculated by means of the Bayesian Markov chain Monte Carlo (MCMC) approach implemented in the refnx software²².

and charged outer pellicle layer, resulting in its coiling and the subsequent release of trapped solvent. This was supported by force spectroscopy and NR experiments. Force spectroscopy probes the pellicle outer surface. The long-range repulsive forces observed with this technique, when the salivary pellicles are mechanically confined, agree with the presence of steric forces that would counteract the increase of osmotic pressure resulting from the confinement of a diffuse polymer-like layer^{30,31}. After exposure to CAPB solutions, the range (quantified in terms of the characteristic length obtained from the exponential fit of the non-contact region) to which steric forces extended decreased (Fig. 4c,d), by a factor ~ 3, suggesting a partial coiling/collapse of the pellicle. NR fits (Fig. 5d,e, Table 2) supported this observation by indicating that the pellicle outer layer collapsed by a similar factor. Moreover, because of the high hydration of the pellicle outer layer, its coiling also supported the significant decrease of the pellicle “wet” mass indicated by QCM-D experiments. The main component of the pellicle outer layer, the MUC5B mucin, comprises of a heavily O-glycosylated polypeptide chain resulting in a net negative charge at physiological pH as most of these glycans contain terminal sialic acids or sulfated sugar residues³². Thus, it is expected that electrostatic forces would play an important role in the interaction between the pellicle outer layer and external compounds. CAPB consists of a long hydrocarbon chain and a polar head group that contains a quaternary ammonium cation and a carboxylate. Therefore, it is reasonable to hypothesize that this surfactant would develop electrostatic interactions with the mucins in the pellicle outer layer, eventually resulting on its collapse and the subsequent release of coupled solvent. Indeed, SLD values from NR fits supported the presence of CAPB in the pellicle outer layer after being rinsed with surfactant-free buffer. However, one should be cautious with this interpretation, as it might also be the SLD change can be attributed to some specific salivary components being removed by the surfactant.

The gentler effect of $C_{12}E_5$ on salivary pellicles revealed by QCM-D and ellipsometry was also supported by force spectroscopy and NR data. Force spectroscopy showed that steric forces did not significantly change after exposure of pellicles to $C_{12}E_5$ solutions (Fig. 4a,b). This indicates a poor interaction between the nonionic $C_{12}E_5$ and the pellicle outer layer that was confirmed by NR data (Fig. 5b,c, Table 1). Effectively, NR indicated that that $C_{12}E_5$ had a very minor effect on the thickness or hydration of the pellicle outer layer. While the mucins in this layer also contain hydrophobic domains that could constitute potential interaction sites with $C_{12}E_5$,

these sites probably form complexes with additional salivary components that prevent inter/intra-chain mucin interactions¹², and therefore also prevent mucin- $C_{12}E_5$ interactions. This is in agreement with previous results suggesting a very limited interaction between $C_{12}E_5$ and mucins³³. NR also suggested an increase in the hydration of the pellicle inner layer after exposure to $C_{12}E_5$. Regardless of whether this originated through replacement or solubilization mechanisms, which cannot be concluded from our data, NR data supports that the $C_{12}E_5$ -induced partial pellicle removal observed in ellipsometry and QCM-D experiments would take place mainly in the inner layer. However, the fact that $C_{12}E_5$ had a very limited effect on the pellicle outer layer also indicated that this surfactant did not primarily affect the components of the pellicle inner layer that contribute to anchor the outer layer. Further studies would be required to identify the specific pellicle components that interact with $C_{12}E_5$ as well as the corresponding interaction mechanisms.

Overall, the presented experimental data show that the investigated nonionic and amphoteric surfactants interact with reconstituted salivary pellicles in a gentler way than SDS. However, the extent to which they modified the pellicles as well as the underlying mechanisms differed. The amphoteric CAPB surfactant would mainly interact, probably by means of attractive electrostatic forces, with the mucin components of pellicles, leading to their coiling/collapse and a subsequent release of trapped water. In this regard, it is worth to note that the lubricating properties of salivary pellicles and, therefore, their ability to protect from mechanical damage is often related to their highly hydrated nature^{24,34}. Thus, the use of CAPB in oral care product might be in detriment of this property. In contrast, $C_{12}E_5$ removed a lower amount of salivary pellicles than CAPB. Moreover, exposure to $C_{12}E_5$ did not affect significantly the pellicle outer layer. Instead, our data indicated that the effect of this nonionic surfactant on the adsorbed pellicle mass might instead be related to the replacement or solubilization of a small fraction of the pellicle inner layer.

At this point, it is worth to discuss the limitations of this work and, subsequently, future directions to address them. As previously discussed, we chose silica as a substrate for this study as (i) it is a surface that can be used in all employed techniques that (ii) shares with both enamel and oral mucosal surfaces two physicochemical aspects known to play a key role in the adsorption of salivary components: wettability and ionic character. Thus, it is reasonable that the main observations of our work are of relevance for pellicles on oral surfaces i.e., enamel and mucosa. In this regard, it is of relevance that pellicles reconstituted *in situ* on enamel in the oral cavity show a similar structure than those reconstituted *in vitro* on silica¹⁵. A bigger difference might be expected between silica and mucosal surfaces. While mucosal surfaces are populated by highly hydrophilic anionic transmembrane mucins, properties shared by silica, it is also known that mucosal and enamel pellicles exhibit some differences with respect to structure and composition³⁵. 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 mucins¹⁸. Studies on e.g., MUC1 coated silica substrates could be approached by means of the techniques used in this work and would provide further insights into the interaction of pellicles with different types of surfactants. Another aspect to consider of this work is that we investigated pellicles reconstituted *in vitro*. The study of pellicles formed *in situ* might have given additional information, since they differ from those formed *in vitro*^{36,37}. In this regard, pellicles go through a maturation process when exposed to bulk saliva in the oral cavity for long periods. However, many studies have also shown that pellicles formed after short times e.g., minutes, already fulfill protective functions up to a high degree³⁸. We focused our study on pellicles reconstituted *in vitro* as reconstitution *in situ* was not an option for most of the techniques employed. Nevertheless, in order to minimize the limitations of *in vitro* studies, we always employed fresh saliva to reconstitute the pellicles. Pellicle-surfactant interactions would undoubtedly benefit from investigations by means of techniques that allow working with pellicles formed *in situ*. Among them, Transmission Electron Microscopy (TEM) has been proved as a powerful technique for structural investigations of pellicles. For instance, TEM has been successfully used to determine how the structure of pellicles reconstituted *in situ* are modified by a variety of mechanical and chemical challenges³⁹. Finally, a natural follow-up of the present study would be to investigate bacterial attachment on pellicles previously exposed to nonionic and amphoteric surfactants.

Methods

Chemicals. Cocamidopropyl betaine (CAPB) was purchased from Elemental SRL (Ref. M-1247-0.5). Pen-taethylene glycol monododecyl ether ($C_{12}E_5$) and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (Refs. 76437 and 436143). Chain deuterated $C_{12}E_5$ was provided by the ISIS deuteration facility. All water used was of ultrahigh quality (UHQ), processed in an Elgastat UHQ II apparatus (Elga Ltd., High Wycombe, Bucks, England). Phosphate buffered saline (PBS) buffer was prepared from tablets from Sigma Aldrich (Ref. P4417) 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). Unless otherwise specified, any other chemicals used were at least of analytical grade.

All surfactants were used at a concentration well above the CMC (Critical Micelle Concentration) in PBS. More information on the CMC of each surfactant is found in Supplementary Information Section S1. The concentrations used were 2.5 times the CMC in water: 21.25 mM for SDS, 0.16 mM for $C_{12}E_5$ and 7.3 mM for CAPB.

Saliva collection. Stimulated human whole saliva (HWS) was used for all experiments. The saliva was collected from two subjectively healthy adult donors, from whom informed consent was obtained, by chewing on parafilm⁴⁰ while drooling into a chilled tube. After collection, saliva from the two donors was pooled and immediately used. Ethical approval was obtained from the committee of research ethics at Lund University (2018/42). All methods carried out in this work were performed in accordance with the Declaration of Helsinki.

Silica surfaces. Silica was used as a substrate for salivary pellicles in all experiments. For force spectroscopy and ellipsometry experiments, p-Doped (boron) silicon wafers with a resistivity of 10–20 Ω -cm (Semiconductor

Wafer Inc., Taiwan) oxidized in an oxygen atmosphere were used. For Neutron Reflectivity (NR) measurements, single crystal silicon (100) blocks ($8 \times 5 \times 2$ cm, Siltronic, France) were used. AFM, ellipsometry and NR surfaces were cleaned by means of a standard RCA protocol, with 5:1:1 $\text{H}_2\text{O}:\text{NH}_3:\text{H}_2\text{O}_2$ at 80°C for 10 min⁴¹, followed by 10 min plasma (for surfaces used in AFM and ellipsometry experiments) or ozone (for surfaces used in NR experiments) treatment. Quartz Crystal Microbalance with Dissipation (QCM-D) silica sensors (Q-Sense, Sweden) were cleaned instead with a gentler protocol consisting of (1) 5 min plasma treatment, (2) immersion into a Hellmanex II solution (2% v/v) for 10 min, (3) extensive rinsing with UHQ water and (4) 10 min plasma treatment. All cleaning procedures yielded hydrophilic surfaces with $<5^\circ$ water contact angles.

Quartz crystal microbalance with dissipation. Quartz Crystal Microbalance with Dissipation (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 elsewhere⁴². Briefly, an alternating-current voltage is applied through a gold-coated quartz chip to stimulate the shear mode oscillation of the quartz crystal. In our experiments, we used QCM-D surfaces where the gold was further coated with a silica layer (ref QSX 303, Q-sense AB, Sweden) that were further cleaned according to the protocol provided above.

Experiments consisted in the continuous monitoring of frequency and dissipation shifts for the different overtones of the sensors over the following experimental steps: (1) First, PBS buffer was flowed into the sensor chamber and allowed to stabilize until a stable baseline was achieved for both monitored signals. (2) Then, fresh HWS was flowed into the chambers for ~ 5 min and allowed to adsorb for additional ~ 55 min under non flow conditions. (3) This was followed by ~ 5 min of rinsing with PBS buffer followed by ~ 55 min of stabilization in the same conditions. (4) Next, surfactant solutions were flowed through the chamber for ~ 5 min before the flow is again stopped allowing the surfactants to interact with salivary pellicles a further ~ 55 min. (5) This was followed by a PBS rinsing step. Solutions were supplied into the QCM-D chamber using an Ismatec peristaltic pump IPC-N 4 at a flow rate of $0.1 \text{ mL} \cdot \text{min}^{-1}$.

The Q-Tools software (Q-Sense AB, Sweden) was employed for fitting data to the Voigt model (details on the fits are provided in Supplementary Information Section S2). Fitting to the Voigt model provides the layer thickness. In order to provide an areal mass value, we assumed a value for the pellicle density of 1.08 g/cm^{343} .

Ellipsometry. Ellipsometry investigations were performed with a Rudolph thin film ellipsometer (type 43603-200E, Rudolph Research, USA) equipped with a Xenon light source filtered to 442.9 nm , with a setup based on null ellipsometry according to the principles of Cuypers⁴⁴ and automated according to the concept of Landgren and Jönsson⁴⁵. Theoretical principles can be found elsewhere⁴⁶. Samples were placed in a trapezoid cuvette made of optical glass (Hellma, Germany) equipped with a magnetic stirrer and temperature set to 25°C . The determination of the silicon complex refractive index, and of the thickness and refractive index of the silicon oxide layer was performed using air and water as ambient media⁴⁵. Four zone measurements were conducted to minimize systematic errors. Ellipsometry experiments were performed by means of similar steps to those followed in QCM-D investigations. However, in this case a flow velocity of $15 \text{ mL} \cdot \text{min}^{-1}$ was used. The adsorbed amount, Γ , was then calculated using de Feijter's equation⁴⁷:

$$\Gamma = \frac{(n_f - n_o)d_f}{dn/dc} \quad (1)$$

where n_f is the refractive index of the adsorbed film, d_f its thickness, n_o the refractive index of the bulk solution and dn/dc is the refractive index increment as a function of bulk concentration for which a value of 0.18 mL/g was assumed⁴⁷.

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 utilized for the acquisition of force ramps. Rectangular silicon nitride levers with a nominal normal spring constant of $0.1 \text{ N} \cdot \text{m}^{-1}$ were employed in all the experiments (OMLC-RC800PSA, Olympus, Japan). Before every experiment, tips were rubbed against a clean freshly cleaved mica surface in PBS buffer, a procedure that leads to the removal of asperities and hence achievement of a smooth tip surface⁴⁸.

Force ramps were obtained at different lateral positions by operating the AFM in the force volume (FV) mode⁴⁹ and analyzed with the FSAS software (<https://git.io/JmEOS>). Specifically, FV measurements consisted on 64×64 force ramps obtained at a speed of $1 \mu\text{m} \cdot \text{s}^{-1}$ over an area of $2 \mu\text{m} \times 2 \mu\text{m}$. Analysis of force ramps was done following a process detailed in a previous work¹², Supplementary Information Section S3). Briefly, raw force ramps were first transformed into a deflection vs sample position representation by scaling a position sensitive photodetector signal by the slope of the contact region of force ramps obtained on a clean mica surface. Then, the contact point was obtained for each ramp by fitting its contact region with the Hertz contact model for a sphere-plane geometry and used as an offset for sample vertical position. Then, deflection was transformed into tip-sample interaction force by scaling the cantilever deflection by its spring constant (which was calculated for each cantilever by means of the Sader method⁵⁰). Force ramps were then converted into a force vs tip-sample distance representation. Finally, for analysis of steric interactions the non-contact region of the ramps (forces for a tip-sample distance $d_{ts} > 5 \text{ nm}$) were fitted to an exponential function:

$$F_{exp} = F_0 e^{-\frac{d_{ts}}{c_{exp}}} \quad (2)$$

from where an exponential amplitude, F_0 , and a characteristic length, λ_{exp} , indicative of the thickness of the steric repulsive layer^{30,31} could be calculated.

For force spectroscopy experiments, human whole saliva (100 μl) was dropped onto clean hydrophilic silica surfaces and left to adsorb for 1 h. Then, surfaces were extensively rinsed with PBS buffer and placed immediately in the AFM. Subsequent treatment of the salivary pellicles with surfactants and corresponding rinsing steps with PBS buffer were performed in-situ following the same order as for QCM-D investigations.

Neutron reflectivity. Neutron reflectivity (NR) measurements were performed on INTER (<https://doi.org/10.5286/ISIS.E.RB1820559>) and SURF (<https://doi.org/10.5286/ISIS.E.RB1720420>), horizontal time-of-flight reflectometers at the ISIS neutron source (UK) and at SuperAdam (<https://doi.org/10.5291/ILL-DATA.CRG-2539>), a monochromatic machine ($\lambda = 5.21 \text{ \AA}$) with a horizontal scattering plane at Institut Laue-Langevin (France)³². A Q -range of $0.01 \text{ \AA}^{-1}$ to $0.3 \text{ \AA}^{-1}$, where $Q = \frac{4\pi}{\lambda} \sin(\theta)$ was achieved using two angles of incidence; 0.7 and 2.3 on INTER. To achieve a similar Q -range, incidence angles of were 0.35, 0.65 and 1.5 were used on SURF whereas on SuperAdam this was achieved by measuring the reflected beam over a range of sample angles. The measured reflected intensity, $I(Q)$, was normalised by the direct beam, I_0 , to achieve the absolute reflectivity, $R(Q)$. PEEK solid liquid flow cells were used. The cells were cleaned by bath sonication in ethanol, in 1:1 ethanol/UHQ mixture and finally in UHQ. All experiments were performed at room temperature. Solutions were exchanged using an HPLC pump at flow rate of 2 mL/min. In all experiments, the footprint was controlled to only illuminate the region within the area of the surface covered in liquid.

The silicon blocks were initially characterized in PBS buffer prepared with both H_2O (hPBS, $\text{SLD} = -0.56 \times 10^{-6} \text{ \AA}^{-2}$), silicon matched water (0.38:0.62 $\text{D}_2\text{O}:\text{H}_2\text{O}$, smwPBS, $\text{SLD} = 2.07 \times 10^{-6} \text{ \AA}^{-2}$) and D_2O (dPBS, $\text{SLD} = 6.36 \times 10^{-6} \text{ \AA}^{-2}$). Human Whole Saliva (HWS) was then injected into the cell, allowing it to adsorb for 60 min. After adsorption, the pellicles were rinsed with dPBS, and then characterized in dPBS, smwPBS and hPBS. Salivary pellicles were then exposed for one hour to surfactant solutions: CAPB ($\text{SLD} = 0.36 \times 10^{-6} \text{ \AA}^{-2}$), chain deuterated C_{12}E_5 (dC_{12}E_5 , $\text{SLD} = 3.76 \times 10^{-6} \text{ \AA}^{-2}$) and fully hydrogenated C_{12}E_5 (hC_{12}E_5 , $\text{SLD} = 0.129 \times 10^{-6} \text{ \AA}^{-2}$). In all cases, the surfactants were dissolved in PBS at a concentration of 2.5 times the value of the Critical Micelle Concentration (CMC) in water (Supplementary Information Section S1). The surfactant-treated pellicles were then rinsed with dPBS and characterized in dPBS, smwPBS and hPBS (except for CAPB, for which only dPBS and hPBS characterizations were performed). All measurements were carried out at room temperature.

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Author contributions

H.B. and J.E.G.M. performed most of the experimental work and data analysis. R.J.L.W., P.G., A.K. and R.B. participated in NR experiments and corresponding data analysis. K.M. and P.L. synthesized deuterated surfactants. T.A., R.B. and J.S. designed the study. H.B. and J.S. wrote the first version of the manuscript. All authors reviewed and edited the final version of the manuscript.

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The authors declare no competing interests.

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Effect of nonionic and amphoteric surfactants on salivary pellicles reconstituted in vitro

- Supplementary Information -

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S1. Critical micellar concentration (CMC) of surfactants

The CMC in water (at 25°C) for each of the surfactants used in this work has been reported in the literature: 8.5 mM for SDS [1], 0.065 mM for C₁₂E₅ [2] and 2.92 mM for CAPB [3]. In our work, we exposed salivary pellicles to solutions in PBS buffer of these surfactants, in all cases with a surfactant concentration 2.5 time higher than the CMC in water. The CMC of the surfactants is known to lower with increasing ionic strength. Therefore, choosing these values ensured that the surfactant concentration was well above the CMC in PBS buffer i.e., the solvent used in this study.

Indeed, the CMC of SDS in PBS buffer was shown in literature to be 2mM [4]. However, we did not find this information for the CMC in PBS buffer of C₁₂E₅ or CAPB. In order to make sure that we worked at concentrations above the CMC in PBS buffer, we used the drop volume method for determining this quantity for both C₁₂E₅ and CAPB.

The method follows Tate's law;

$$W = 2\pi r \gamma \quad (\text{Eq. S1})$$

where W is the drop weight, r is the capillary radius and γ is the surface tension. When the weight, W , is equal to the right hand side of the equation, a drop falls from a small capillary. This can then be exploited to determine the surface tension of solutions. Above the CMC the surfactant will no longer cause the surface tension to lower. Therefore, by determining the concentration at which the surface tension is no longer affected, the CMC can be found.

The drop volume method is carried out on the Lauda drop volume tensiometer TVT, which consists of a 1ml syringe, with a plunger moved by a stepper motor so that the expelled volume (and mass) of the droplet can be accurately known. These droplets detach when the force of gravity exceeds that of the surface tension and knowing the drop volume (weight), the radius of the tip and the density of the liquid the surface tension can be calculated.

The surface tension for each concentration of surfactant was measured three times and averaged. The results are shown in Figure S1. The CMC in PBS buffer of C₁₂E₅ was found to be 0.022 mM and that of CAPB was 1.1 mM. These are both below the CMC in water.

Figure S1. Surface tension measurements for solutions of a) C₁₂E₅ and b) CAPB in PBS buffer

S2. Voigt modelling of QCM-D data

The experimental outputs of QCM-D experiments are the shifts in the frequency of the resonance, f_n , and the dissipation factor, D_n , of each of the overtones of the sensor surface. If the adsorbed layer is rigid enough, the adsorbed amount and the shift in f_n are linearly related [5]. However, protein films may not be considered rigid, but viscoelastic instead. For viscoelastic films, where noticeable shifts in the dissipation factor, ΔD_n , are observed, more trustable data are obtained by applying the Voigt model. The expressions obtained by applying the Voigt model for a viscoelastic film adsorbed onto a solid substrate and immersed into a Newtonian fluid are [6]:

$$\frac{\Delta f_n}{f_n} = -\frac{d_p \rho_p}{d_Q \rho_Q} \left[1 - \eta_l \rho_l \frac{\left(\frac{\eta_p}{\rho_p} \right) \omega_n}{\mu_p^2 + \omega_n^2 \eta_p^2} \right] = -\frac{\Gamma_p}{d_Q \rho_Q} \left[1 - \eta_l \rho_l \frac{\left(\frac{\eta_p}{\rho_p} \right) \omega_n}{\mu_p^2 + \omega_n^2 \eta_p^2} \right] \quad (\text{Eq. S2a})$$

$$\Delta D_n = \frac{1}{d_Q \rho_Q} \left[\eta_l \rho_l \frac{d_p \mu_p \omega_n^2}{\mu_p^2 + \omega_n^2 \eta_p^2} \right] \quad (\text{Eq. S2b})$$

In these equations d , ρ , and ω_n stand for thickness, density, and $2\pi f_n$ respectively. The subscripts Q , p , and l stand for quartz crystal, protein film, and liquid medium respectively. By a numerical fit of the frequency and the dissipation values obtained at three different overtones ($n = 5, 7$ and 9) not only the adsorbed amount, Γ , can be obtained, but also the viscoelastic properties of the film, like the shear elastic modulus, μ , and the shear viscosity η [7].

We have used the Voigt model to fit our experimental data and for this purpose we employed the Q-Tools software (Q-Sense AB, Sweden). Initial input values given for fluid density and viscosity, and for the density of the adsorbed protein films are specified below.

Fixed Parameters		Parameters to fit	
Fluid density (kg/m ³)	1000	Layer η (Pa·s)	0.0001 – 0.01
Fluid η (Pa·s)	0.001	Layer μ (Pa)	$10^3 - 10^7$
Layer density (kg/m ³)	1080	Layer d (m)	$10^{-10} - 10^{-6}$

S3. Analysis of force spectroscopy data

Raw force ramps consisted in a set of photodetector signals for different sample vertical positions values (Fig. S3a). The offset for the photodetector signal was first found out as the average of values far from the contact. Then, this signal was converted into cantilever deflection values by multiplying by a factor obtained from a linear fit of the contact region of force ramps obtained on clean mica surfaces.

The contact point i.e., offset for the sample vertical values was found by fitting the contact region of the force ramp to the Hertz model as detailed in [8] (Fig. S3b). Finally, the cantilever deflection signal was converted into tip-sample interaction force by multiplying by the cantilever elastic force constant determined by means of the Sader method [9], and the sample vertical positions transformed into tip-sample distance values (d_{ts}) by adding to the former the cantilever deflection (Fig. S3c). Finally, the noncontact region of this representation ($d_{ts} > 5\text{nm}$) was fitted to an exponential function (Fig. S3c).

Figure S3. **a)** Raw force ramp obtained on a salivary pellicle in PBS buffer. **b)** Same force ramp in a deflection vs sample vertical displacement representation where the contact point by fitting the contact region to the Hertz model. **c)** Same force ramp in a force vs probe-sample distance representation where the non-contact region was fitted to an exponential function.

S4. QCM-D experiments for the salivary pellicle treated with SDS

QCM-D was used to confirm previous ellipsometry studies which have shown SDS to cause a complete desorption of the pellicle. Figure S4 shows the adsorption of Human Whole Saliva (HWS) causes a frequency shift from the baseline (0Hz) to -65Hz (7th overtone) in the bulk and -60Hz after rinsing with PBS. This clearly shows an adsorbed protein layer, however, after SDS is introduced into the cell, the frequency immediately returns to zero indicating the entire film has been removed.

Figure S4. Frequency shifts monitored using QCM-D (5th, 7th and 9th overtones) for the acquired pellicle before and after SDS/PBS rinsing cycles as described in the main text.

S5. Thickness and refractive index values from ellipsometry

Figure S5 shows thickness and refractive indices for the experiment represented in Fig. 2 of the manuscript. Overall, these data support that exposure of salivary pellicles to CAPB resulted in lower thickness and higher density than exposure to $C_{12}E_5$.

Figure S5. a) Thickness and **b)** refractive index values for the same experiments as those represented in Fig. 2 of the main manuscript where salivary pellicles on hydrophilic silica surfaces were exposed to $C_{12}E_5$ (blue) and CAPB (yellow) solutions.

S6. Atomic Force Microscopy of salivary pellicles before and after exposure to surfactants

Salivary Pellicles before and after treatment with $C_{12}E_5$ and CAPB surfactants were visualized by means of an Atomic Force Microscope (AFM) operated in the Peak Force Tapping Mode (Fig. S6). It can be observed that salivary pellicles and salivary pellicles treated with $C_{12}E_5$ exhibited planar homogeneous surfaces. However, pellicles treated with CAPB exhibited features with lateral sizes in the order of $\sim\mu\text{m}$ and heights in the order of $\sim 20\text{nm}$ occupying $\sim 5\%$ of the surface area.

Figure S6. AFM images in PBS buffer of representative salivary pellicles before and after treatment with $C_{12}E_5$ and CAPB.

S7. Detailed neutron reflectivity analysis

We used the refnx software [10] for the analysis of NR data. In all cases, the interface between the silicon support and the bulk solvent was considered as a stratified medium composed by different slabs. For fitting the clean silicon blocks, we used a Si/SiO₂/Solvent structure. For salivary pellicles, we considered a two-layer structure successfully used in previous works [8, 11]. Thus, for fitting their NR profiles we used a Si/SiO₂/InnerLayer/OuterLayer/Solvent structure.

For fitting the pellicles treated with surfactants (dC₁₂E₅, hC₁₂E₅ and CAPB) we used a similar model as for non-treated salivary pellicles. However, in these cases we considered that the treated pellicles might include a percentage of surfactant:

$$SLD_{treated-layer} = (SLD_{nontreated-layer} \times Coverage_{salivary\ components}) + (SLD_{surfactant} \times Coverage_{surfactant})$$

Fits for the experimental data and parameters found from these fits follow in the pages below.

Effect of dC₁₂E₅ on salivary pellicles

Reflectivity data in different contrasts and corresponding fits for a clean silicon block, a salivary pellicle adsorbed on the same block and the same salivary pellicle after being treated with a dC₁₂E₅ solution are shown below.

Fit Parameters for the non-treated salivary pellicle:

Layer	SLD ($10^{-6} \text{ \AA}^{-2}$)		Thickness ($\text{\AA}$)	Roughness ($\text{\AA}$)	Hydration (%)
Si	2.07		-	-	-
SiO ₂	3.47		11 $\pm$ 1	2 $\pm$ 1	22.4 $\pm$ 1.2
Pellicle Inner Layer	dPBS	2.53 $\pm$ 0.05	46 $\pm$ 1	4 $\pm$ 1	62.0 $\pm$ 0.8
	smwPBS	2.51 $\pm$ 0.07			
	hPBS	1.26 $\pm$ 0.06			
Pellicle Outer Layer	dPBS	5.12 $\pm$ 0.18	286 $\pm$ 12	15 $\pm$ 1	97.1 $\pm$ 0.2
	smwPBS	3.10 $\pm$ 0.60			
	hPBS	2.20 $\pm$ 1.30			
Solvent	dPBS	6.36	-	52 $\pm$ 13	-
	smwPBS	2.07			
	hPBS	-0.56			

Fit Parameters for the salivary pellicle treated with a 2.5 CMC dC₁₂E₅ in PBS solution:

Layer		Proportion (%)	SLD ($10^{-6} \text{ \AA}^{-2}$)		Thickness ($\text{\AA}$)	Roughness ($\text{\AA}$)	Hydration (%)
Si		-	2.07		-	-	-
SiO ₂		-	3.47		11±1	2±1	25.0±9.0
Pellicle Inner Layer	Salivary Content	92±2	dPBS	2.53±0.05	45±1	4±1	70.1±0.5
			smwPBS	2.51±0.07			
			hPBS	1.26±0.06			
	dC ₁₂ E ₅	8±2	3.76				
Pellicle Outer Layer	Salivary Content	96±5	dPBS	5.12±0.18	277±16	15±1	98.0±0.1
			smwPBS	3.10±0.60			
			hPBS	2.20±1.30			
	dC ₁₂ E ₅	4±5	3.76				
Solvent		-	dPBS	6.36	-	54±15	-
			smwPBS	2.07			
			hPBS	-0.56			

Effect of $hC_{12}E_5$ on salivary pellicles

Reflectivity data in different contrasts and corresponding fits for a clean silicon block, a salivary pellicle adsorbed on the same block and the same salivary pellicle after being treated with a $hC_{12}E_5$ solution are shown below.

Fit Parameters for the non-treated salivary pellicle:

Layer	SLD ($10^{-6} \text{ \AA}^{-2}$)		Thickness ($\text{\AA}$)	Roughness ($\text{\AA}$)	Hydration (%)
Si	2.07		-	-	-
SiO ₂	3.47		10 $\pm$ 1	2 $\pm$ 1	25 $\pm$ 9
Pellicle Inner Layer	dPBS	2.90 $\pm$ 0.40	48 $\pm$ 1	2 $\pm$ 1	57.6 $\pm$ 0.3
	smwPBS	2.70 $\pm$ 0.04			
	hPBS	1.59 $\pm$ 0.13			
Pellicle Outer Layer	dPBS	5.70 $\pm$ 0.50	297 $\pm$ 10	23 $\pm$ 1	92.7 $\pm$ 0.5
	smwPBS	3.60 $\pm$ 0.20			
	hPBS	2.80 $\pm$ 1.60			
Solvent	dPBS	6.36	-	85 $\pm$ 9	-
	smwPBS	2.07			
	hPBS	-0.56			

Fit Parameters for the salivary pellicle treated with a 2.5 CMC $hC_{12}E_5$ in PBS solution:

Layer		Proporti on (%)	SLD ($10^{-6} \text{ \AA}^{-2}$)		Thickness (\AA)	Roughness (\AA)	Hydration (%)
Si		-	2.07		-	-	-
SiO ₂		-	3.47		10±1	2±1	25.0±9.0
Pellicle Inner Layer	Salivary Content	93±4	dPBS	2.90±0.40	42±1	2±1	63.2±1.2
			smwPBS	2.70±0.04			
			hPBS	1.59±0.13			
	hC ₁₂ E ₅	7±4	0.13				
Pellicle Outer Layer	Salivary Content	94±5	dPBS	5.70±0.50	280±9	23±1	96.7±0.3
			smwPBS	3.60±0.20			
			hPBS	2.80±1.60			
	hC ₁₂ E ₅	6±5	0.13				
Solvent		-	dPBS	6.36	-	46±12	-
			smwPBS	2.07			
			hPBS	-0.56			

Effect of CAPB on salivary pellicles

Reflectivity data in different contrasts and corresponding fits for a clean silicon block, a salivary pellicle adsorbed on the same block and the same salivary pellicle after being treated with a CAPB solution are shown below.

Fit Parameters for the non-treated salivary pellicle:

Layer	SLD ($10^{-6} \text{ \AA}^{-2}$)		Thickness ($\text{\AA}$)	Roughness ($\text{\AA}$)	Hydration (%)
Si	2.07		-	-	-
SiO ₂	3.47		11 $\pm$ 1	4 $\pm$ 1	45.0 $\pm$ 3.0
Pellicle Inner Layer	dPBS	2.55 $\pm$ 0.03	46 $\pm$ 1	2 $\pm$ 1	55.7 $\pm$ 0.3
	smwPBS	2.48 $\pm$ 0.02			
	hPBS	1.59 $\pm$ 0.03			
Pellicle Outer Layer	dPBS	4.84 $\pm$ 0.13	325 $\pm$ 11	21 $\pm$ 1	97.5 $\pm$ 0.2
	smwPBS	2.70 $\pm$ 0.40			
	hPBS	1.70 $\pm$ 0.70			
Solvent	dPBS	6.36	-	19 $\pm$ 14	-
	smwPBS	2.07			
	hPBS	-0.56			

Fit Parameters for the salivary pellicle treated with a 2.5 CMC CAPB in PBS solution:

Layer		Proporti on (%)	SLD (10 ⁻⁶ Å ⁻²)		Thickness (Å)	Roughness (Å)	Hydration (%)
Si		-	2.07		-	-	-
SiO ₂		-	3.47		11±1	4±1	45.0±3.0
Pellicle Inner Layer	Salivary Content	99.4±0.7	dPBS	2.55±0.03	35±1	2±1	54.4±0.9
			hPBS	1.59±0.03			
	CAPB	0.6±0.7	0.38				
Pellicle Outer Layer	Salivary Content	92.0±2.0	dPBS	4.84±0.13	117±8	20±1	95.5±0.5
			hPBS	1.70±0.70			
	CAPB	8.0±2.0	0.38				
Solvent		-	dPBS	6.36	-	24±3	-
			hPBS	-0.56			

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