Effect of calcium ions on in vitro pellicle formation from parotid and whole saliva

A. Ash [a]*, M.J. Ridout [a], R. Parker [a], A.R. Mackie [a], G.R. Burnett [b] and P.J. Wilde [a]

a Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

b GlaxoSmithKline Consumer Healthcare, Weybridge, Surrey, KT13 0DE, UK

* Corresponding author: a.ash@ifr.ac.uk

Abstract

The salivary pellicle is a protein-rich, bacteria-free, self-assembling film that adsorbs to all surfaces within the oral cavity. The pellicle has numerous functions that are vital for maintaining oral health. Currently however, there are no commercially available artificial salivas that accurately mimic the complex film forming properties (i.e. film thickness and viscoelasticity) of human saliva. To understand these properties further we have examined the *in vitro* formation of the salivary pellicle, by adsorbing stimulated parotid saliva (PS) and whole mouth saliva (WMS) from 14 healthy volunteers, onto oxidised silicon surfaces, using a quartz crystal microbalance with dissipation monitoring (QCMD) and a dual polarisation interferometer (DPI). A dramatic impact on the hydrated mass, polymer mass, thickness and polymer concentration of the pellicle for both WMS and PS was observed when the natural calcium concentration of the respective salivas was increased from 0 mM to 10 mM. In addition, QCMD data showed that on addition of 10 mM calcium the salivary pellicle formed by both PS and WMS became more predominantly elastic. The results presented here also suggest that calcium can easily diffuse in and out of the pellicle, permitting free calcium exchange between the saliva and the adsorbed pellicle under physiological conditions, which may potentially facilitate the mineralisation of enamel.

Key Words: in vitro pellicle, calcium ions, silicon, quartz crystal microbalance, dual polarisation interferometer.

1. Introduction

The salivary pellicle is a protein rich bacteria-free, adsorbed film that assembles on all surfaces within the oral cavity and is formed by the selective adsorption of salivary proteins derived from whole saliva [1-3]. The formation of this pellicle is considered to be a selective process, as only a limited number of the proteins that have been detected in whole saliva are found to reside in the pellicle [4]. Currently, phosphoproteins such as, acidic proline rich proteins (aPRPs), histatin and statherin are the main salivary proteins thought to play an important role as pellicle precursor proteins. As such, these proteins have been widely studied with respect to their ability to adsorb onto oral surfaces and thus influence early pellicle formation [2, 5-8]. Furthermore, these proteins contain calcium binding domains that may serve to provide a region of high calcium concentration close to the tooth surface, thus facilitating the mineralisation of teeth. The importance that the pellicle plays in preserving oral health becomes apparent in individuals who suffer from Xerostomia (i.e. dry mouth syndrome) [9-11]. Without adequate saliva in the mouth to produce an effective salivary pellicle, these individuals can suffer from increased dental caries and mucous membrane damage. Although artificial salivas are currently available as a treatment[12, 13], they only provide limited relief to the discomfort that Xerostomia inflicts, principally because current artificial salivas do not adequately mimic the complex interfacial film forming properties (i.e. thickness and viscoelasticity) of human saliva [14], which plays a key role in oral lubrication[15]. A number of studies have looked at the interplay between the structure of the pellicle and the ionic composition of the saliva [16-20]. Of particular interest has been to understand the role calcium ions play on pellicle structure, as calcium ions are thought to have a bearing on the attraction between pellicle proteins and the surface to which pellicle proteins adsorb [18, 21]. For example, Tanizawa et al [17] showed that calcium ions were able to enhance pellicle formation onto hydroxyapatite surfaces via calcium bridging of

proteins. Whilst Proctor *et al.* [15] showed that chelation of calcium from saliva caused a dramatic decrease in the mechanical properties of an adsorbed salivary film, consistent with the breakdown of pellicle structure. Thus, information regarding the physical structure of the pellicle under varying calcium conditions should help elucidate the mechanisms behind pellicle formation. Therefore, in this study we examine the *in vitro* formation of the salivary pellicle at different concentrations of calcium, from stimulated parotid saliva (PS) and stimulated whole mouth saliva (WMS) onto silicon oxide surfaces, using a quartz crystal microbalance with dissipation monitoring (QCMD) and a dual polarisation interferometer (DPI). Together these techniques yield information on changes in the pellicle structure in terms of adsorbed mass, thickness, polymer concentration, and density of the WMS and PS pellicles. Although there have been studies of the salivary pellicle formation using QCMD [22-24], we believe that this is the first time that both DPI and QCMD have been used in combination to observe the real-time adsorption of the salivary pellicle in this way. It is hoped that this approach will also help us to understand the mechanisms underlying the formation and breakdown of the salivary pellicle. This new knowledge not only augments our current understanding of the salivary pellicle, which is important for the development of more realistic salivary mimetics, but also demonstrates how different interfacial techniques can be used to complement ones findings.

2. Materials and methods

2.1 Saliva collection: Saliva collection was undertaken according to a protocol previously assessed by an independent ethics panel. The saliva was obtained from 14 apparently healthy non-smoking male and female volunteers, ranging in age from 20 to 50 years. The subjects had no history or current signs of oral conditions that could affect oral fluid composition. In order to collect stimulated WMS, volunteers rinsed their mouth with 10ml of bottled still water (Waitrose, Norwich, UK) twice. Volunteers then chewed on flavour-free gum (Gumlink, Dandyvej, Denmark) and expectorated the saliva into a small sterile collection bottle for up to 15 min, or until they had produced 10 mL of saliva. The stimulated PS was collected using a sterilised Lashley suction cup which was placed over the parotid duct [25]. The salivary secretion was stimulated by sucking 'Rosey Apples' boiled sweets (Asda, Norwich, UK). This continued for up to 45 minutes, or until 20-30 mls of saliva had been produced. Salivas were kept in ice upon expectoration, and were used immediately for study; and therefore it was deemed that no protease inhibitors were required. Moreover we aimed to mimic the behaviour of the *in vivo* pellicle as closely as possible, including potential proteolysis of the pellicle and pre-cursor proteins.

2.2 Saliva adsorption: In addition to the calcium already present in saliva, the calcium concentration of both WMS and PS were increased by 1 mM Calcium chloride ($CaCl_2$(Sigma-Aldrich, UK)) and 10mM $CaCl_2$ respectively. Furthermore, to test the adsorption of the salivary pellicle in the absence of free calcium, a 2 mM EDTA solution (VWR International Ltd., UK) was used to sequester all calcium-ions from WMS and PS ($CaCl_2$ and NaEDTA solutions were prepared in deionised and filtered ultra-pure water Nanopure Diamond, Barnstead Int., USA). Sewon *et al.* [26] showed that the average calcium concentration of stimulated WMS was 1.3 ±0.4 mM. Therefore 2 mM EDTA was used to

sequester calcium from both parotid and whole mouth salivas. This was performed so that the salivas could be reduced to a known calcium concentration starting point. Thus the salivas containing EDTA were used as the control saliva sample. The WMS and PS samples collected from each volunteer were divided into three aliquots respectively:

(i) 0 mM calcium (saliva + 2 mM EDTA)

(ii) The natural concentration of the saliva plus 1 mM $CaCl_2$

(iii) The natural concentration of the saliva plus 10 mM $CaCl_2$

Each respective saliva sample was then measured concomitantly on the QCMD and DPI (both static adsorption systems i.e. not flow-cell). Upon injection of 0.5 ml of saliva, pellicle formation was monitored for 20 minutes (early stage pellicle formation begins to plateau after 20 minutes adsorption, see figure 1.). Subsequently the pellicle was rinsed with a 0.5ml calcium solution, which had a concentration equal to the calcium concentration of the saliva being used (i.e 0 mM, 1 mM or 10 mM $CaCl_2$). At this point the data was recorded as the post calcium rinse value. Finally the pellicle was rinsed twice with deionised water, and upon the second rinsing, the data was recorded as the post water rinse value.

2.3Dual polarisation interferometer (DPI): Measurements of surface layer thickness and refractive indices (R.I.) were performed in real time using an AnaLight Bio200 DPI (Farfield Sensors Ltd., Manchester, UK). The device used a silicon oxynitride sensor chip that was clamped in a temperature controlled enclosure allowing the temperature to be maintained at 20^{o}C for all experiments. The two optical paths present within the sensor allowed polarised light from a helium neon laser (wavelength, 632.8 nm) to pass through the sensor. One light path was in contact with the sample, and the other acted as a reference signal. This light was switched between two polarisations: transverse magnetic (TM) and transverse electric (TE) by a polariser switch that oscillated at 50 Hz. After traversing the chip length the emergent

light beams combined to form an interference fringe pattern that was detected by a 1024 x 1024 pixel CMOS detector, located at the far field and passed the output to a digital signal processor that was linked to the polariser switch where the type of polarised light was linked to the fringe pattern being produced [27]. Essentially, TE and TM respond differently to protein adsorption/displacement and therefore provide two independent measurements of the adsorbing material which permits one to uncover the polymer mass, thickness and density of the adsorbing film (see figure 1). However, in order to retrieve this structural information from these two phase changes some basic assumptions were used: the adsorbed film behaved as a single homogeneous layer (e.g. uniform composition & density along the chip length) and was isotropic (e.g. R.I. of TM = R.I. of TE). By solving Maxwell's equations simultaneously for the phase change of the TE and TM, one can obtain values for the mean R.I. (n_f) and thickness (d_f) of the adsorbed film. Because the R.I. is a linear function of the concentration over a wide range of concentrations, the absolute amount of the adsorbed molecules (Γ) (referred to as pellicle 'polymer' mass) can be obtained via the de Feijter formula [28]; where n_{water} is the R.I. of the water and dn/dc the R.I. increment of the adsorbed pellicle:

$$\Gamma = d_f \frac{n_{f-} n_{water}}{dn/dc}$$

These calculations were carried out using the Analight Explorer software (version 1.5.4.18811, Farfield Scientific, Manchester, UK). The assumed refractive index increment dn / dc was 0.15, a value typical for protein films [29].

2.4 DPI sensor cleaning: After the completion of the experiment, surfaces were rinsed with 2% v/v Hellmanex for 5 minutes, rinsed with water, followed by 2% w/v SDS, then rinsed with 50% v/v IPA (Sigma-Aldrich, UK).

2.5 Quartz crystal microbalance with dissipation monitoring (QCMD): The measurements were performed using a D300 QCMD (Q-Sense AB, Vastra Frolunda, Sweden) with a QAFC 302 axial flow measurement chamber maintained at 20^{o}C. Silicon dioxide coated AT-cut piezoelectric quartz crystals sandwiched between gold electrodes (QSX-303, Q-Sense AB, Vastra Frolunda, Sweden) were used as the substrate. The sensor was excited to oscillate by applying an alternating current across the sensor electrodes at its fundamental resonant frequency (i.e 5 MHz), and at the 3rd, 5th and 7th overtones (i.e. 15 MHz, 25 MHz and 35 MHz, respectively). The QCMD then measured the frequency change (Δf) and the dissipation change (ΔD), at each of the four frequencies, as salivary proteins adsorbed onto the sensor.

2.6 Measuring change in frequency to calculate pellicle 'hydrated' mass: Changes in the frequency of the oscillating sensor were related to the changes in the hydrated mass adsorbing on to the quartz crystal sensor via the Sauerbrey model [30]:

$$\Delta m = -\frac{\rho_0 v_0}{2 f_n^{\,2}} \Delta f$$

where Δm represents a change in adsorbed areal mass (ng cm^{-2}), ρ_0 the density of the quartz crystal (2650 kg m^{-3}), v_0 the shear velocity in quartz (3340 m s^{-1}), f_n the resonant frequency (5 MHz), and Δf is the actual change in frequency recorded by the instrument. In addition, pellicle thickness was calculated from the hydrated mass by assuming a value for the density of 1000 kg m^{-3}.

The viscous properties of softer films dampen the sensor's frequency of oscillation, and in such cases the Sauerbrey model underestimates the mass, whereupon another method of analysis is needed to fully characterise the film. Therefore, in addition to recording frequency changes, the QCMD measures a second parameter known as dissipation (D) (see Figure 2).

2.7 Measuring change in dissipation to determine pellicle viscoelasticity:

Dissipation is inversely proportional to the decay time (τ) and resonant frequency (f) of the oscillating sensor as follows:

$$D = \frac{1}{\pi f \tau}$$

The D300 QCMD measures the decay time (τ) by stopping the current to the sensor and allowing the sensor to freely oscillate to a standstill. The decrease in the amplitude of the oscillation with time is dependent on the viscoelasticity of the adsorbed layer. The softer the adsorbed layer (higher viscous component), the faster the sensor will stop oscillating (reducing decay time), and thus increasing dissipation. For the Sauerbrey model to be valid, the dissipation values have to be low (i.e. $< 1\times10^{-6}$ per 10 Hz), which implies rigid, evenly distributed, and thinly adsorbed layers. For the cases where dissipation values were above 1×10^{-6} per 10 Hz, (i.e. for soft or viscoelastic films that do not fully couple to the oscillating crystal) Sauerbrey will underestimate the mass. In these cases the Voigt model can be used, which combines both dissipation and frequency measurements at different overtones (3, 5 and 7) [See 31 for more details]. By applying the data from these measurements into the Voigt model included in Q-Sense's QTools software (Q-Sense AB, Vastra Frolunda, Sweden) it was possible to extract estimations of the thickness and mass of the salivary pellicle whilst taking into consideration the viscoelasticity of the salivary pellicle.

2.8 QCMD sensor cleaning: After the completion of the experiment, surfaces were cleaned with 2% v/v Hellmanex (Müllheim, Germany), rinsed with water, followed by 2% w/v SDS (Sigma-Aldrich, UK), then rinsed with water and dried with nitrogen. Finally sensors were exposed to UV-ozone (Bio-Force Nanosciences Inc., Iowa, USA) for 30 minutes. Prior to next experiment, sensors were re-exposed to UV ozone for 10 minutes.

2.9 Statistics: Significant differences in pellicle mass, thickness and density in relation to calcium concentration of WMS and PS was determined by one-way ANOVA and Tukey post hoc analysis at three points of the experiment (i.e. peak, post calcium rinse and post water rinse values) using GenStat (14^{th} Edition, VSN International Ltd, Hemel Hempstead, UK). Level of significance was set at $p < 0.05$. Linear regression functions were fitted and R^2 values calculated using Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

3. DPI Results:

3.1 Pellicle 'polymer' mass: The mean pellicle polymer mass for both WMS and PS did not change significantly with the addition of 1mM $CaCl_2$ or with the removal of calcium (using 2 mM EDTA) from saliva (See Fig. 3). However, the mass for WMS increased significantly when 10 mM $CaCl_2$ was added to WMS and PS respectively. Upon rinsing each pellicle with a $CaCl_2$ solution (at a concentration equal to the $CaCl_2$ concentration present in the saliva) the pellicle formed maintained its additional mass. However, upon rinsing pellicles with de-ionised water any increase in mass that occurred in both WMS and PS was completely removed.

3.2 Pellicle Thickness: Increasing the natural concentration of calcium in WMS and PS by an additional 1 mM $CaCl_2$ had no effect on the thickness of the pellicle (see Fig. 3). However, increasing the natural calcium concentration of saliva by 10 mM resulted in a significant increase in the thickness of the resultant pellicle. Upon rinsing the pellicle with a calcium solution (at a concentration equal to the $CaCl_2$ concentration present in the saliva) the mean pellicle thickness for all three salivas (i.e. 0 mM $CaCl_2$ (natural saliva +2 mM EDTA),

natural saliva + 1 mM $CaCl_2$ and natural saliva + 10 mM $CaCl_2$) remained similar. Upon rinsing with de-ionised water, a dramatic loss in pellicle thickness was again observed. In particular, WMS and PS containing an extra 10 mM $CaCl_2$ showed large losses in pellicle thickness when rinsed with de-ionised water.

3.3 Pellicle Density: The addition of 1 mM $CaCl_2$ or the removal of calcium from saliva had no significant effect on the density of PS or WMS pellicles. However, a large drop in the density of the pellicles formed from both WMS and PS occurred when the natural calcium concentration of saliva was increased by 10 mM $CaCl_2$ (see Fig.3). Subsequent rinsing of the pellicles with de-ionised water returned the density of all salivary pellicles to statistically similar values.

4. QCMD Results:

4.1 Pellicle hydrated mass & thickness: Hydrated mass measured via QCMD takes into consideration the hydration, or water, contained within salivary pellicle. The addition of 1 mM $CaCl_2$ to natural saliva or the removal of calcium (using 2 mM EDTA) from saliva had no significant effect on the hydrated mass of PS or WMS pellicles. However, a notable increase in the hydrated mass for pellicles formed from both WMS and PS occurred when the natural calcium concentration of these salivas was increased by 10 mM $CaCl_2$ (see Table 1). For example, the frequency change observed at the 3rd overtone, which applying the Sauerbrey equation is inversely proportional to the hydrated mass of the pellicle, showed a decrease in frequency from -65 ±15 Hz at 0 mM $CaCl_2$ down to -104 ±42 Hz when the natural calcium concentration of saliva was increased by 10mM $CaCl_2$ (See Figure 4 (b) and (e)). When the dissipation of the oscillations was also considered alongside the frequency of oscillation changes, the significant difference between pellicle thickness and mass was also

observed with the addition of 1 mM $CaCl_2$ to saliva. This was reflected in an increase in peak values for Voigt fitted thickness and mass respectively (see Table 1).

4.2 Pellicle viscoelasticity: Dissipation values in this study were used to improve modelling of hydrated mass and thickness from Sauerbrey model to the Voigt model. Overall the mean thicknesses and hydrated masses calculated using the Voigt model are a factor of 1.6 – 3.1 higher than the Sauerbrey values. This shows that all the films are viscoelastic. However, the dissipation value observed at the 3rd overtone also gave an independent qualitative insight into the viscoelasticity of the pellicle. Fig 4 (c) and (f) show that the decrease in frequency is highly associated ($p< 0.02$) to an increase in dissipation for both PS and WMS at all three concentrations of calcium in saliva. This correlation is predicted by the Voigt model as both negative frequency shift and dissipation are proportional to the thickness for thin films (ref 28). However, the slope of the correlation indicates differences in the viscoelasticity of the films. This phenomenon was more pronounced in the saliva that had 0 and 1 mM $CaCl_2$ added to it. For example, the gradient of the curve for PS and WMS pellicle dissipation, as a function of frequency, was twice as high in WMS and PS that contained an extra 10 mM $CaCl_2$ as opposed to saliva containing 0 mM $CaCl_2$, or saliva with an added 1 mM $CaCl_2$. For a given value of Δf the 0 mM and 1 mM $CaCl_2$ films have higher dissipations (ΔD) indicating they are relatively more viscous whereas the 10 mM $CaCl_2$ films are relatively elastic.

5. Discussion

The formation of the salivary pellicle is a dynamic process that begins almost instantly (see Figs. 1 and 2) with the adsorption of low molecular weight proteins, followed by the adsorption of larger salivary proteins and protein aggregates with time [2]. Some evidence suggests that there is potential for Vroman-like [32] effects to take place, for example; between mucin MUC 5B with statherin and PRP1 [33]. However, Svendesen et al.[34] found that this exchange may be quite limited during the first hour of pellicle formation. Therefore under the experimental conditions used in this study the Vroman effect is unlikely to apply to the same extent in salivary proteins as is exhibited by blood serum proteins. Nevertheless, pellicles formed in this study showed striking similarities with work carried out by Hannig *et al.* [35]. They showed that after 1 minute the salivary adsorption process was able to form an electron dense pellicle layer, 10–20 nm thick, on an enamel surface; a thickness that was not dissimilar from our own results, despite the differences in the substrates being used. This not only augments confidence in the other physical parameters (i.e. mass and density) reported in this study, but also demonstrates that proteins in saliva are flexible with regard to the surfaces

they adsorb to; thus permitting a salivary pellicle to form on diverse surfaces such as foods and dental implants[21].

The physical structure of the salivary pellicle has been thought to be influenced by calcium ions in saliva, as these divalent cations can permit the cross-linking of proteins [36]. Cross-linking of proteins has been shown to be important in maintaining micelle-like protein aggregates present in saliva, which may play an important role in the formation of the salivary pellicle [37, 38]. However, in this study the DPI data showed little difference in the polymer mass, thickness and density of salivary pellicles formed from WMS and PS when calcium was sequestered using EDTA (i.e. 0 mM calcium) or when saliva was supplemented with 1 mM $CaCl_2$. Not until the natural calcium concentration of WMS and PS was increased by 10 mM $CaCl_2$ that a significant effect on the pellicle polymer mass, thickness and density was observed. The addition of 10 mM $CaCl_2$ to saliva resulted in a pellicle that was thicker, with a higher surface polymer mass; but more diffuse than pellicles formed from saliva containing no calcium or saliva with 1mM $CaCl_2$ added. This would suggest that the pellicle formed in the presence of an extra 10 mM $CaCl_2$ is much more nebulous than when a natural calcium concentration was used. This phenomenon was also more pronounced in pellicles formed from PS, where the increase in pellicle thickness and mass, and the concomitant decrease in density was higher than WMS pellicles. This was likely a consequence of the higher proportion of proteins in PS that contain calcium binding domains (i.e. statherin, histatins) [39], and therefore PS was more sensitive to the presence of calcium ions[40].

Further differences between PS and WMS adsorption also impact the physical nature of the respective salivas. For example, PS is a serous fluid that contains a number of low molecular weight proteins ($\leq$ 15 kDa), such as cystatins, and acidic proline-rich proteins that have

properties which make them suitable for forming the primary layer of the pellicle [41, 42]. These properties include being able to diffuse rapidly to the solid/liquid interface, re-arrange at the surface, and interact with neighbouring molecules [43]. In contrast, WMS contains high molecular weight proteins (≥ 180 kDa) such as mucins, which do not diffuse rapidly to the surface because of their size and highly glycated side chains. Moreover, mucins increase the viscosity of saliva and thus concomitantly slow down the diffusion of other proteins present in saliva. These physical differences between PS and WMS also influence the conversion of raw data (i.e. Δf and ΔD) to modelled data (i.e. Sauerbrey and Voigt) when analysing QCMD data[44]. Consequently the qualitative changes in Δf and ΔD of the respective salivas, when observing differences in pellicle structure at different concentrations of calcium, where highlighted in this study.

The QCMD data showed that by increasing the natural calcium concentration of WMS and PS by 10 mM $CaCl_2$ one could generate a statistically significant ($p< 0.05$) increase in hydrated mass of the pellicle. This, however, was reciprocated by a decrease in pellicle viscoelasticity which was observed by a slower increase in dissipation with increasing negative frequency change (see Figs. 4 (c) and (f)). All salivary pellicles, regardless of calcium concentration had a high association between the dissipation of the pellicle as a function of frequency. This correlates well with the work carried out by Voinova et al. [31], who show that dissipation increases with film thickness; so that as the salivary pellicle gets thicker, both the negative change in frequency, and the positive change in dissipation increase. This phenomenon was amplified by the addition of 10mM $CaCl_2$ to both PS and WMS, manifesting itself as the change in the gradient of the slope observed in Figure 4(c) and (f). At 10 mM calcium the dissipation was increasing less with variation of frequency. This means that the salivary pellicles were

becoming less dissipative and more elastic (closer to Sauerbrey behaviour) when 10 mM $CaCl_2$ was added to the saliva.

We also observed little difference in the hydrated mass and thickness of salivary pellicles formed from WMS and PS when calcium was sequestered using EDTA (i.e. 0 mM calcium) or when 1 mM $CaCl_2$ was added to the calcium already present in the saliva. This raised an interesting question: why did adding 1 mM $CaCl_2$ to the natural calcium concentration of saliva show no response in terms of pellicle structure; but increasing the natural calcium concentration of saliva by an extra 10 mM $CaCl_2$ triggered a significant increase in mass, thickness and concomitant decrease in density and pellicle viscoelasticity. It appears that at low concentrations of calcium in saliva (i.e. 0 mM $CaCl_2$ and natural calcium concentration of saliva + 1 mM $CaCl_2$) there was no impact on the mass, thickness density and viscoelasticity of the pellicle, and that the salivary pellicle could readily form in the absence of calcium. This correlates well with the observations found by Proctor *et al.* [15] where they demonstrate that the surface tension of saliva at the air-water interface was unaffected by removal of calcium from saliva using 2 mM EDTA; thus also concluding that the passage of proteins to a surface is unaffected by the absence of calcium from saliva. They also showed that the elastic modulus of the adsorbed salivary film was significantly reduced upon removal of $CaCl_2$ by EDTA. However, this was measured at very low frequencies (0.2 Hz), which would detect changes in intermolecular interactions, whereas the high frequencies used by QCMD (>5MHz) were not as sensitive to these changes. This suggests that the change in viscoelasticity observed by Proctor *et al.* [15] was not due to a total breakdown of the adsorbed pellicle in the absence of $CaCl_2$ but more likely connected to the overall elasticity of the protein film through calcium mediated intermolecular interactions. We hypothesise that increasing the natural calcium concentration of saliva by 10 mM $CaCl_2$ increases the

aggregation of proteins in saliva. Consequently, rather than small individual proteins packing to the surface forming densely packed salivary films, they form large protein aggregates in the saliva, prior to pellicle adsorption, and subsequently deposit onto the sensor surface forming thicker more diffuse films. This explicates the increase in mass, thickness and decrease in density observed by the DPI measurements upon increasing the natural calcium concentration of saliva by 10 mM $CaCl_2$.

It was also interesting to note that upon washing with de-ionised water the density of the pellicles return to that observed for saliva with no added calcium, suggesting that the nebulous protein adsorbed was easily removed, leaving a more dense and robust basal layer. The rapid loss of protein from the pellicle on rinsing with de-ionised water highlights the importance of maintaining the pH and ionic balance of saliva in order to preserve the stability of the pellicle. It is likely that upon introduction of de-ionised water to the pellicle, ions and proteins, leached out into the water, primarily due to the hypotonicity of the de-ionised water. This rapid decrease in the ionic concentration of the pellicle would have increased the electrostatic repulsion between anionic moieties within proteins and reduced intermolecular interactions [18, 45, 46]. This destabilised the pellicle facilitating its displacement, which explains the observed dramatic decrease in pellicle thickness and mass with concomitant increase in density (see Fig. 3).

To conclude, this study lays the foundations on how regulating the calcium concentration of saliva provides a mechanism that can control the physical properties of the salivary pellicle. In addition, we also observed how the overall structure of the pellicle, in terms of mass and thickness is relatively insensitive to calcium at low concentrations, allowing a flexibility to

adapt to changing physiological and environmental conditions. it is important to recognise that these in vitro results do not necessarily represent the true nature of how the salivary pellicle forms in the oral cavity. However, they do provide some evidence as to how the pellicle may behave on oral surfaces with the addition of calcium; although further research is required to develop a more realistic representation of pellicle formation in the oral cavity. For example, the use of hydroxyapatite or hydrophobic sensors may be more representative of conditions in the mouth, for which work is currently being carried out.

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Fig. 1: Adsorption profile of WMS forming a pellicle over time on a DPI sensor. **(a)** Real time TM and TE phase changes that show: **I** the baseline recorded in deionised water, **II** the peak value of the adsorbed pellicle, **III** the phase shift post calcium rinse. **IV** & **V** the phase shifts post water rinse. **(b)** The derived thickness, polymer mass and density changes derived from TE and TM phase changes **(a)** using Maxwell's equations. Thickness, mass and density of the pellicle increase rapidly as the saliva rapidly adsorbs to the sensor surface. Following 20 minutes adsorption a calcium rinse removed loosely adsorbed material from the pellicle. Upon rinsing the pellicle with water, the thickness and mass was reduced with a concomitant increase in pellicle density.

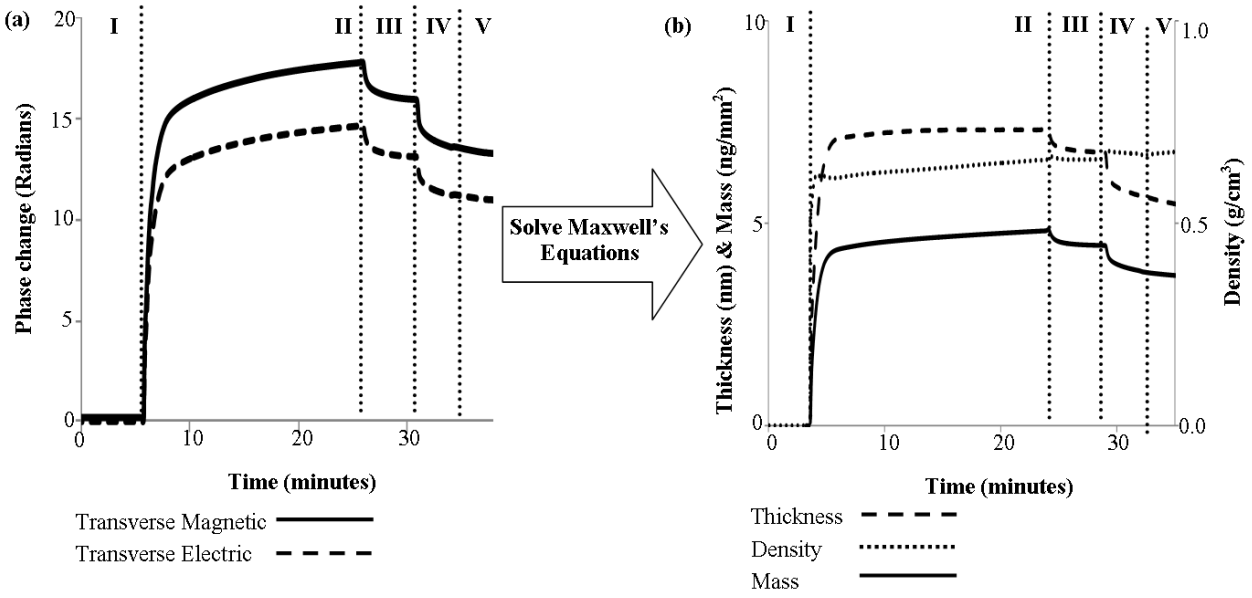

Fig. 2: Frequency (a) and dissipation (b) changes versus time measured for the 3rd overtone (15 MHz) by QCMD for the adsorption of WMS at different calcium concentrations of saliva. **I** baseline recorded in deionised water. **II** Peak value. **III** post calcium rinse. **IV** & **V** water rinse. Increasing concentrations of calcium in saliva display lower frequency oscillations with respective higher dissipation values.

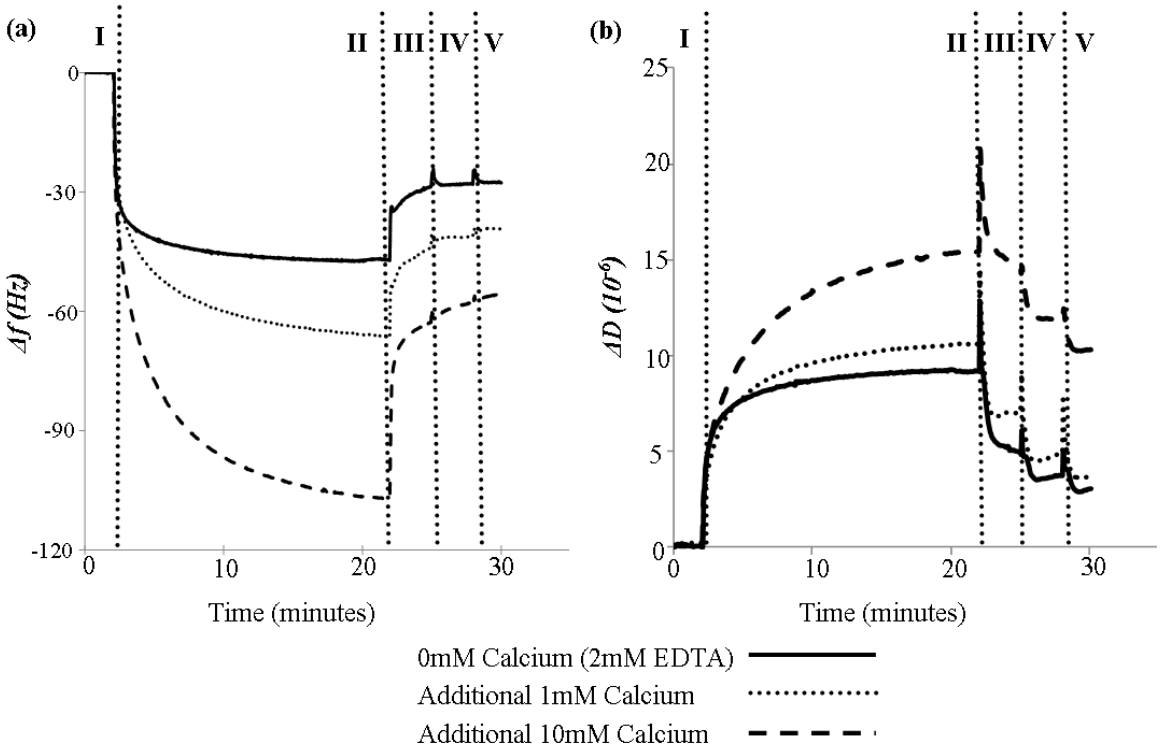

Fig. 3 (a) Box plot of DPI measured polymer mass, thickness and density changes formed from **(a)**WMS containing 0 mM Calcium (2 mM EDTA) (n=10); the natural concentration of WMS + 1 mM $CaCl_2$ (n=10); and natural concentration of WMS +10 mM $CaCl_2$ (n=10).

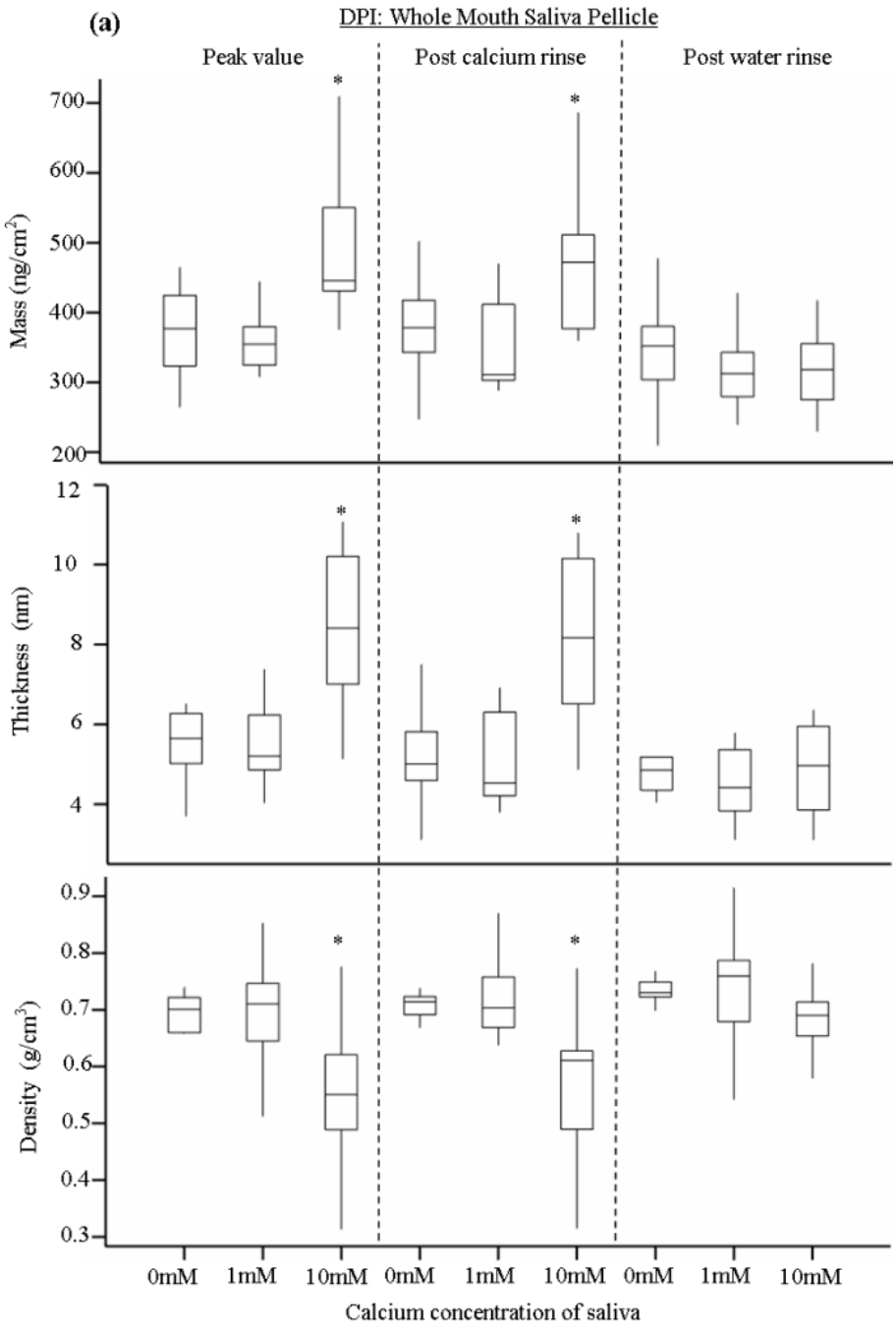

Fig. 3 (b) PS containing 0 mM Calcium (2 mM EDTA) (n=12); the natural concentration of PS + 1 mM $CaCl_2$ (n=12); and natural concentration of PS +10 mM $CaCl_2$ (n=12). Values reported are peak, post calcium rinse and post water rinse values (* = Significant difference ($p \leq 0.01$)).

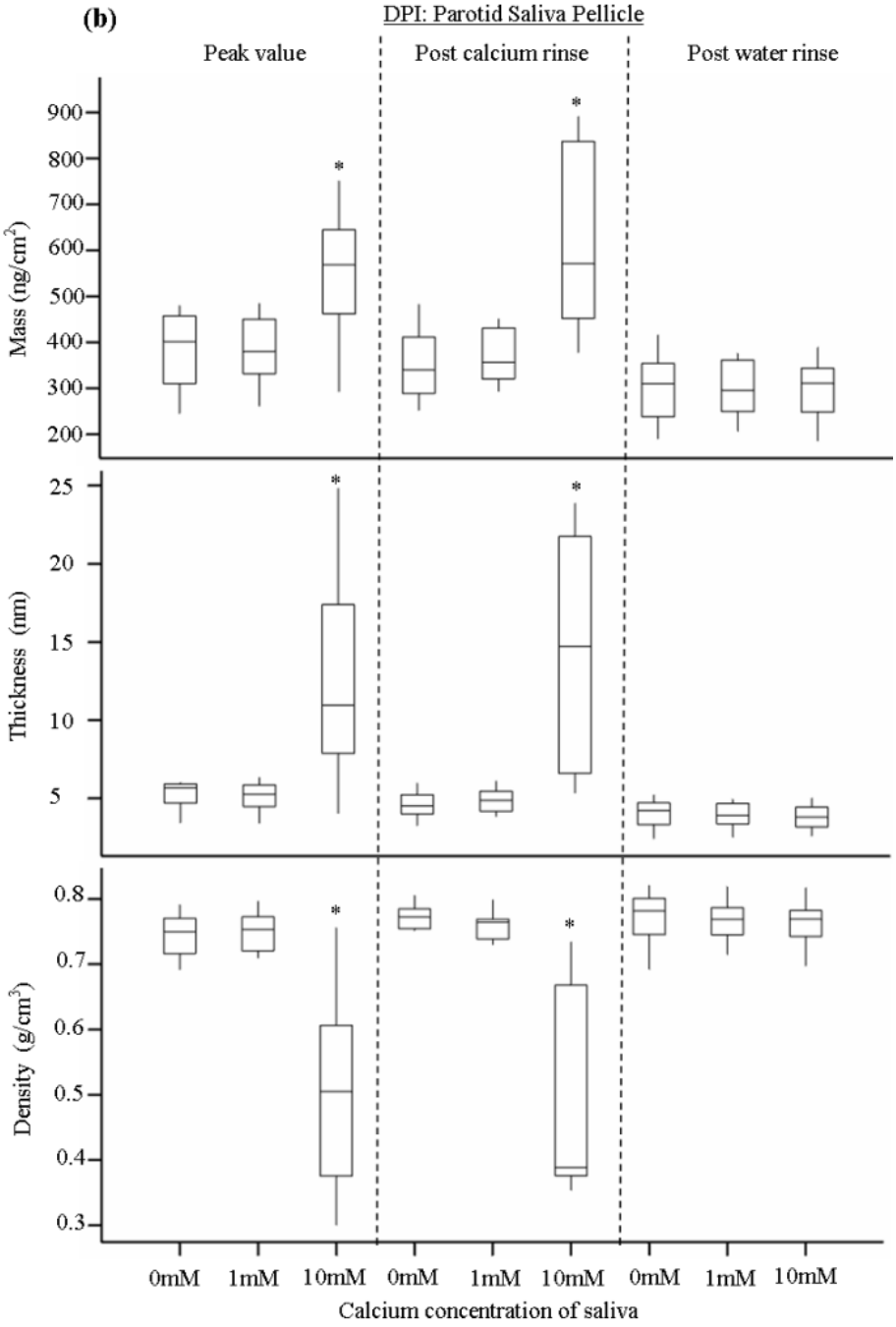

Fig. 4 (a) and **(b)** Comparison of frequency and dissipation changes to pellicle formed from PS containing 0 mM Calcium (2mM EDTA) (n=12), natural concentration of PS + 1mM $CaCl_2$ (n=12), and natural concentration of PS + 10 mM $CaCl_2$ (n=12). **(c)** ΔD as a function of Δf measured for the 3rd overtone by QCMD for the adsorption of PS pellicle at the above salivary concentrations. **(d)** and **(e)** Comparison of frequency and dissipation changes to pellicle formed from WMS containing 0 mM Calcium(2 mM EDTA)(n=10); natural concentration of WMS + 1 mM $CaCl_2$ (n=10); and natural concentration of WMS +10 mM $CaCl_2$ (n=10). **(f)** ΔD as a function of Δf measured for the 3rd overtone by QCMD for the adsorption of WMS pellicle.

*= significant difference ($p < 0.05$)

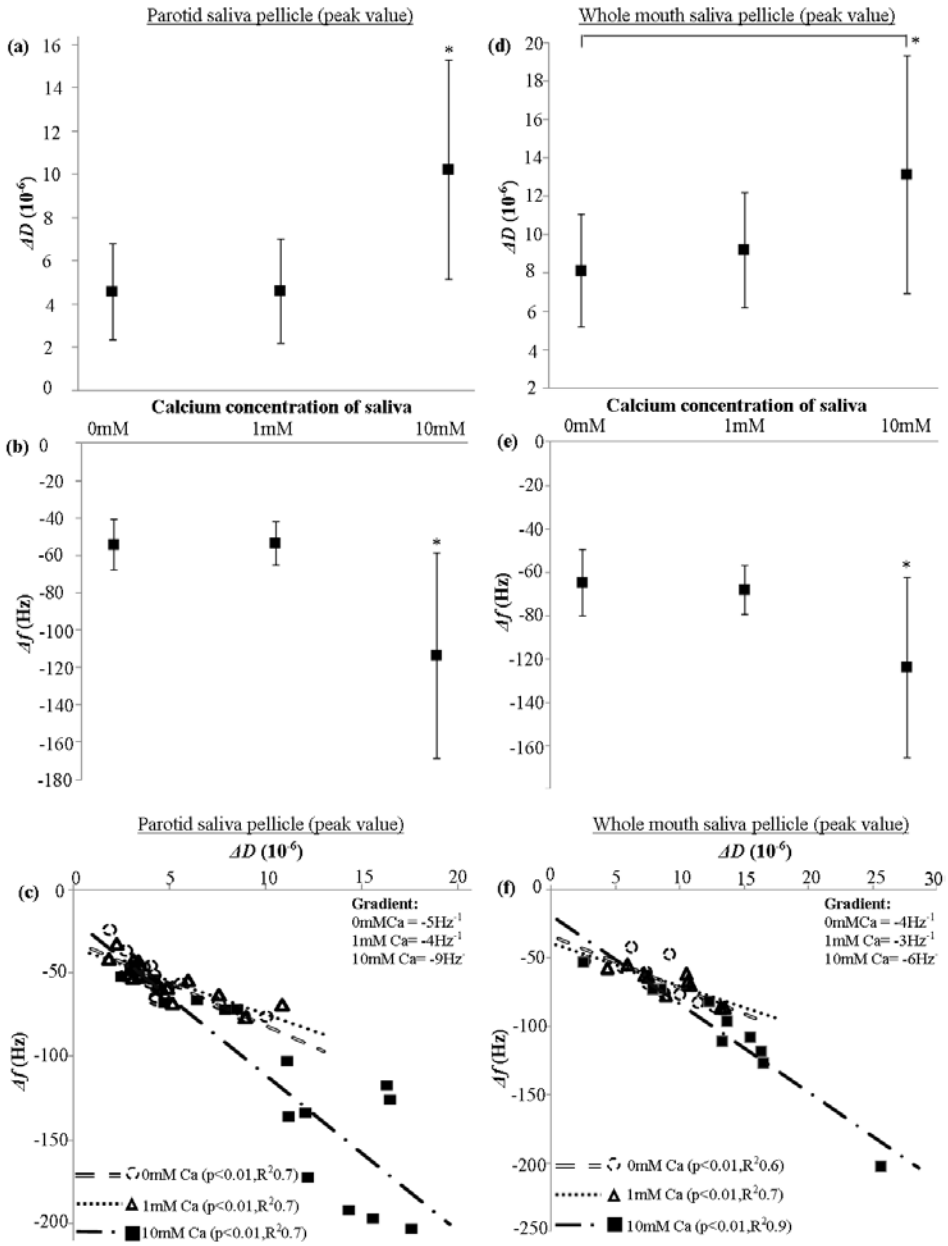

Table 1: Effect of the calcium concentration of WMS and PS on Sauerbrey and Voigt modelled thickness and hydrated mass changes to pellicle formed from **(a)** WMS containing 0 mM Calcium (2 mM EDTA) (n=10); natural concentration of WMS + 1 mM $CaCl_2$ (n=10); and natural concentration of WMS +10 mM $CaCl_2$ (n=10). And pellicle formed from **(b)** PS containing 0 mM Calcium (2mM EDTA) (n=12), natural concentration of PS + 1mM $CaCl_2$ (n=12), and natural concentration of PS + 10 mM $CaCl_2$ (n=12).

Whole mouth saliva pellicle

	Sauerbrey model		*Voigt model*	
Calcium con-centration (mM)	**Thickness (nm)**	**Mass (ng/cm^2)**	**Thickness (nm)**	**Mass (ng/cm^2)**
Peak values				
0	11.3(±2.7)^a	1132(±266)^a	25.7(8.9)^a	2568(891)^a
1	11.9(±2.0)^a	1190(±196)^a	37.2(10.6)^b	3718(1063)^b
10	18.2(±7.3)^b	1815(±729)^b	37.4(10.4)^b	3742(1040)^b
post calcium rinse values				
0	11.9(±2.1)	1186(±205)^a	24.3(5.9)^a	2428(588)^a
1	11.8(±2.2)	1175(±221)^a	32.2(5.3)^{ab}	3217(533)^{ab}
10	17.2(±8.2)	1718(±815)^a	37.2(10.5)^b	3724(1047)^b
post water rinse values				
0	9.2(±2.7)	923(±271)	17.1(5.6)	1705(560)
1	9.3(±2.0)	929(±199)	25.0(11.2)	2500(1117)
10	9.5(±1.5)	955(±151)	26.3(11.0)	2630(1097)

Parotid saliva pellicle

Peak values				
0	8.683(2.0)^a	868(203)^a	18.8(4.0)^a	1882(398)^a
1	8.6(1.7)^a	864(169)^a	15.3(6.5)^a	1526(648)^a
10	22.4(10.2)^b	2239(1021)^b	36.2(11.4)^b	3617(1139)^b
post calcium rinse values				
0	8.0(1.0)^a	807 (95)^a	16.3(5.1)^a	1631(508)^a
1	7.5(1.3)^a	747 (131)^a	12.4(6.6)^a	1244(655)^a
10	18.1(8.7)^b	1807 (867)^b	31.0(8.4)^b	3101(836)^b
post water rinse values				
0	6.2(1.5)	624.2(151)	14.6(4.4)	1464(443)
1	6.3(1.3)	633.2(134)	11.2(6.2)	1119(617)
10	5.1(1.6)	514.4(158)	12.8(6.5)	1280(647)

^{a} and ^{b} represent significant differences ($p \leq 0.05$) between respective means. ^{ab} is not significantly different between either ^{a} or ^{b}. **N.B.** Modelled mass differs from the modelled thickness due to the assumed density of the adsorbed pellicle and therefore the two parameters are statistically identical.