Real-time formation of salivary films onto polymeric materials for dental applications: Differences between unstimulated and stimulated saliva

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ABSTRACT

The formation of salivary films onto oral prostheses materials is of central importance for understanding their performance and interaction with oral tissue and flora. The aim of this work was to study and compare the salivary films formed from unstimulated and stimulated whole saliva on two common polymeric materials, polycarbonate and poly(methyl methacrylate). Irradiating these materials with UV light is a simple way to modify their wettability, roughness and ζ -potential. Therefore, the effect of UV exposure of polycarbonate and poly(methyl methacrylate) on saliva adsorption was also investigated. For this purpose a quartz crystal microbalance with dissipation and SDS-PAGE have been combined in order to associate the thicknesses and viscoelastic properties of the salivary films with their protein composition. SDS-PAGE results suggest that a larger diversity of proteins is involved in the formation of stimulated saliva pellicles. Furthermore, according to QCM-D, pellicles formed from stimulated saliva are thinner and stiffer than the ones formed from unstimulated saliva if the polymeric materials have not been exposed to UV light although both types of saliva form a biphasic layer. For UV-treated materials, the same is applied to polycarbonate but not to poly(methyl methacrylate) where stimulated saliva yields thicker and softer films than unstimulated saliva being the adsorption process of a multiphasic nature. These results highlight the importance of choosing the appropriate sample depending on the type of study to be performed.

Key words: Unstimulated saliva; stimulated saliva; polycarbonate; poly(methyl methacrylate); QCM-D; viscoelasticity.

1. Introduction

Saliva forms a complex protein layer on essentially all types of surfaces that come in contact with it [1-3]. The formation of the salivary film is a selective process where only certain proteins present in saliva adsorb to the exposed surface [1, 4-7]. Among other functions, saliva plays a key role in the oral cavity as a lubricating agent and also as an antibacterial barrier. However, the composition of the salivary conditioning film has been demonstrated to affect its lubricating properties [8], and since some proteins serve as anchoring sites for oral microorganisms [9] or exert an antibacterial activity [7], it will also affect bacterial [10, 11] and fungal [12] attachment, and the subsequent biofilm formation [12, 13]. Due to the physicochemical properties of the surfaces that govern protein adsorption, the proteinaceous film formed onto different materials will vary in composition [2, 5-7], structure [2, 3, 8, 10, 13-15] and properties [2, 3, 6, 8, 10, 13, 14]. However, the knowledge about the structure and composition of the salivary pellicle formed onto prosthetic devices, like orthodontic appliances and removable dentures, is limited. Polycarbonate, PC, and poly(methyl methacrylate), PMMA, are two synthetic polymers commonly used in the fabrication of such devices even though previous studies have shown that devices made of these materials are easily colonized by microorganisms leading to plaque related diseases [7, 16]. Therefore, many efforts have been done to modify their surface properties in order to prevent this issue, mainly by increasing the negative charge of the exposed surface in order to promote the adsorption of the positively charged antimicrobial components of saliva [7].

In most saliva adsorption studies, irrespective of the surface material, either unstimulated [2, 3, 5, 6, 11, 14] or stimulated saliva [1, 8, 10, 13, 15, 17-19] has been used. Both types of saliva differ in their protein composition and relative concentrations [20], and consequently it is expected that stimulation will affect the formation of the pellicle and its structural properties. Since the presence of foreign bodies inside the oral cavity such as removable dentures, stimulates the secretion of saliva it is important to confirm if such structural differences exist. Therefore, in this study, we have used the quartz crystal microbalance with dissipation technique (QCM-D) to study the formation of salivary films from both unstimulated and stimulated saliva onto PC and PMMA coated QCM-D sensors. By measuring the changes in frequency and energy dissipation, QCM-D enables to follow and to quantify in real time adsorption and desorption processes onto many types of surfaces. Additionally, it allows determining the viscoelastic and structural characteristics of the adsorbed layer [21].

The aim of the present work was to find out if the stimulation of saliva secretion has an effect on the formation of the pellicle onto polymeric materials commonly used in dentistry (PC and PMMA) by following the real-time adsorption of both unstimulated human whole saliva (US) and stimulated human whole saliva (SS). Since the exposure of both PC and PMMA to UV light is a simple method to alter the surface properties of these materials by making them more hydrophilic and negatively charged [22] the effect of their UV light pre-treatment on the pellicle formation has also been studied. This, together with the analysis of the protein composition of the pellicles performed by means of SDS-PAGE, will provide a deeper understanding of the formation process. To the authors' knowledge this is the first time that QCM-D has been used to elucidate whether or not US and SS show different adsorption patterns, and if there are any differences in the structural and rheological properties of the pellicles.

2. Materials and Methods

2.1 Surfaces

PMMA and PC sheets were purchased from Goodfellow Cambridge Limited, UK. For ζ -potential determination the sheets were cut into 4 mm $\times$ 7 mm pieces and attached to the ZEN 3600 Zetasizer (Malvern Instruments Ltd., UK) nano cell holder. COOH-modified polystyrene particles, micromer® (micromod Partikeltechnologie GmbH, Germany), of 100 nm size were used as tracer particles in 5 mM Na-phosphate buffer, pH 7 at 37 °C. For water contact angle, AFM, and QCM-D measurements, gold coated quartz crystals (QSX-301) were spin-coated (6000 rpm) for 30 seconds with 0.5 wt% polymeric solutions in toluene for PMMA and dichloromethane for PC. Spin coated sensors were then placed on a heater for 1 h at 80 °C. An OCA Plus drop shape analyzer (Dataphysics Instruments GmbH, Filderstadt, Germany) was used to measure the water contact angle at room temperature. An atomic force microscope (Asylum MFP-3D-SA Santa Barbara, USA), AFM, operated in tapping mode in air was used for the quantitative analysis of the topography of the spin coated sensors. For all measurements AC 240 TS cantilevers (Olympus Corporation, Japan) with a nominal spring constant of 2 N/m was used. Untreated surfaces were used right after cleaning with water and ethanol and treated surfaces were UV irradiated in an ozone atmosphere at both wavelengths 185 nm and 254 nm (PSD-UV4, Novascan Technologies, IA, USA) for different time lengths.

2.2 Saliva Collection

Saliva samples from six healthy non-smoking donors, aged between 24 and 40 years, were collected into sterilized chilled tubes. Donors were not allowed any food intake at least two

hours prior to the saliva collection. Unstimulated saliva was collected first by drooling into a chilled tube. Stimulated saliva was collected right afterwards and stimulation was induced by chewing a piece of parafilm®. All samples were centrifuged at 7500xG (4°C) for 10 minutes. The supernatants were pooled together according to the saliva type and then aliquoted and stored at - 80°C until use. Since this study used anonymized biological material, according to the *Helseregisterloven* §2 from the Research Ethics Committee, there is no need for ethic approval.

2.3 Salivary Film Characterization

Real time formation of the pellicles was followed with QCM-D (Q-Sense E4, Biolin Scientific). Saliva solutions (25% (v/v) in 10 mM sodium phosphate buffer, pH 7, supplemented with 50 mM NaCl, from now on PB7) were added into the flow cell at 100 µl min⁻¹ for 2 min, and then the flow rate was reduced to 10 µl min⁻¹. The total adsorption time was 1 hour and the temperature was 37 °C. Then, the cell was rinsed with saliva free buffer at 100 µl min⁻¹ for 10 minutes and the pellicle was eluted by adding 0.5% (w/v) SDS in water at a flow rate of 100 µl min⁻¹ for 10 min. A final rinsing step of 10 min at 100 µl min⁻¹ with buffer was done. By fitting the measured changes in frequency and dissipation with the Voigt model [21] it was possible to determine the viscoelasticity of the salivary films as the ratio between the loss and storage modulus G''/G' .

In order to assess the differences in the composition of the pellicles, one side of both untreated and UV-treated PC and PMMA coins was exposed to US and SS (25% in PB7), at 37 °C under constant agitation (300 rpm), in 96 well plates (Nunc, Denmark). After 1 h, the unbound remaining saliva (depleted samples from now on) were collected and frozen (-20 °C). Prior to the SDS-PAGE analysis all the samples, US and SS as well as the depleted ones, were freeze dried (Telstar LyoQuest, Spain) and resuspended in 0.02 ml miliQ water. Samples were diluted again in Laemmli sample buffer (Bio-Rad Laboratories, USA), 0.01 ml of saliva in 0.005 ml sample buffer, and heated at 95 °C for 5 minutes. Then, 0.015 ml of each sample were loaded in the wells and run at 200 V for 30-35 minutes. A molecular weight reference standard (Precision Plus Protein™ Dual Color Standards, Bio-Rad Laboratories, USA) was used. Gels were stained with Coomassie Brilliant Blue R-250, CBB R-250 (Bio-Rad Laboratories, USA) for 2 h, de-stained in 10% acetic acid and photographed. Then, gels were fixed in 25% methanol and 10% acetic acid for 1 h and rinsed with H₂O for 20 minutes. Right afterwards, gels were oxidized in 2% periodic acid (Sigma-Aldrich, USA) and rinsed twice with water. Finally, gels were immersed in Schiff's reagent (Schiff's fuchsin-sulphite reagent,

Sigma-Aldrich, USA) for 1 h in the dark under constant agitation and de-stained with 0.5% (w/v) potassium metabisulfite (Sigma-Aldrich, USA) and 3% acetic acid in water until background was eliminated. Differences in band intensities were assessed by means of ImageJ software [23].

2.4 Statistics

QCM-D results are presented as mean value from three independent measurements $\pm$ the standard deviation. Significance levels were calculated with R-Studio (Boston, MA, USA) using the ANOVA test. Under the condition of normality and homogeneity of variances, the Tukey multiple comparison of means was carried out ($p \leq 0.05$).

3. Results

3.1 SDS-PAGE

Figure 1. *Unstimulated and Stimulated saliva SDS-PAGE*. Odd lanes correspond to unstimulated saliva, US, and even lanes to stimulated saliva, SS. Lanes (1) and (2) correspond to saliva controls. For the remaining lanes saliva was incubated with the following surfaces prior to SDS-PAGE: (3) and (4) untreated PC; (5) and (6) UV-treated PC; (7) and (8) untreated PMMA; (9) and (10) UV-treated PMMA. The image presents a combination CBB stained (MW < 100 kDa) and CBB+PAS stained (MW > 100 kDa) gels after protein separation. Ellipses highlight the bands where differences were observed between the reference US and SS samples and square boxes mark the bands with lower intensities than the reference bands as detected by densitometry analysis.

Differences in protein composition between unstimulated and stimulated saliva (US and SS respectively) were assessed by means of SDS-PAGE (Fig.1). These differences correspond to the following CBB stained bands: ~ 70 kDa, ~ 55 kDa, 54 kDa, 44–48 kDa, ~ 28 kDa, ~ 24 kDa, 15–20 kDa, and 10 kDa. All these bands showed a higher intensity for SS suggesting that the amount of these proteins in SS is higher than in US as it was confirmed by the densitometry analysis (figure SM1-1). On the other hand, high molecular weight PAS stained bands and the CBB 80 kDa band were more intense for US compared to SS.

After incubating the saliva samples with the surfaces under study, some differences between them and the non-incubated saliva could also be observed. While SS exposed to untreated PC, PC-0, shows decreased intensities (indicating that proteins were adsorbed onto the substrate) in the 44–48 kDa, 28 kDa, 24 kDa, 15–20 kDa, and ~10 kDa bands, for US, only a contribution from the 28 kDa band could be appreciated. The analysis of the US and SS samples exposed to untreated PMMA, PMMA-0, shows equally or less intense bands as for PC-0, suggesting similar composition of the pellicles on both kind of surfaces.

UV-treatment of the surfaces induces changes in their topography as well as on their ζ -potential and wettability (SM2-1 & SM2-2). The evaluation of the samples exposed to UV-treated PC, PC-30, reveals almost no differences compared to PC-0 exposed US and SS except for the lack of contribution from the 24 kDa band for SS. Similarly, the exposure of both saliva types to UV-treated PMMA, PMMA-30, provokes an intensity decrease in the same bands as for PMMA-0 exposed saliva, indicating that the adsorbed salivary films were formed by the same type of proteins for both surfaces.

3.2 Saliva adsorption onto untreated surfaces

The changes in frequency and dissipation (3rd, 5th, and 7th harmonics) produced by the adsorption of human whole unstimulated and stimulated saliva (US and SS respectively) onto both PC-0 and PMMA-0, surfaces is presented in Fig. 2. It can be observed that the maximum change in frequency (3rd harmonic) is obtained for the adsorption of US onto PC-0 while the minimum change results from the adsorption of SS onto PMMA-0. Furthermore, when US and SS pellicles are compared, ΔF (US) > ΔF (SS) for both types of surfaces. On the other hand, the changes in dissipation, ΔD , are very similar for most of the experimental conditions, $\Delta D \sim 15 \times 10^{-6}$, except for SS adsorption onto PMMA-0 which yields the lowest change, $\Delta D \sim 12 \times 10^{-6}$. These results can also be appreciated in Fig. 3, where ΔD is plotted against ΔF . It

1 is worth noting that after the adsorption step, all salivary films show a clear splitting among
 2 the different harmonics for both ΔF and ΔD .

3
 4 Figure 2. *Real-time formation of salivary films onto untreated PC and PMMA surfaces.* The
 5 curves represent the mean values from three independent measurements for the changes
 6 produced on the 3rd 5th and 7th overtones. (i) Adsorption of saliva; (ii) phosphate buffer rinse;
 7 (iii) 0.5 % (w/v) SDS rinse; (iv) phosphate buffer rinse. Panels a and c correspond to the
 8 measurements performed on polycarbonate sensors with unstimulated and stimulated saliva
 9 respectively and panels b and d represent the measurement done on PMMA sensors with
 10 unstimulated and stimulated saliva respectively. Saliva samples were diluted, 25% (v/v), in 10
 11 mM Na-phosphate buffer supplemented with 50 mM NaCl at pH 7 and all measurements
 12 were performed at 37 °C.

13 After 60 minutes of saliva adsorption, the surfaces were rinsed with buffer, exposed to 0.5%
 14 (w/v) SDS solution and rinsed again with buffer labeled as steps i, ii, iii and iv respectively in
 15 Fig. 2. The first rinsing step resulted in a small increase in ΔF (became less negative) and a
 16 small decrease in ΔD (became less positive) irrespective of the type of saliva and polymeric
 17 surface, suggesting the displacement of the loosely attached proteins. Also, the exposure to
 18 SDS produced changes in the frequency and dissipation signals. While when the substrate was
 19 PC-0 it resulted in similar trends in ΔF and ΔD for both US and SS, the interaction of SDS
 20 with the pellicles formed onto PMMA-0 substrates was markedly dependent on the type of
 21 saliva: i) For both pellicles formed onto PC-0 ΔF initially increased but, while for the US
 22 films the signal stabilized for the SS ones it kept increasing. On the other hand, ΔD increased

for both saliva types, although for SS an initial sharp decrease resulted in a smaller final signal change. ii) For the US film adsorbed onto PMMA-0 ΔF sharply rose by approximately 60% and ΔD went down by approximately 65%, whereas for SS films SDS provoked a small but sharp decrease on ΔF ($\sim 4\%$), followed by an increment of approximately the same amount and a rise on ΔD of $\sim 40\%$. After removing SDS from the flow chamber all frequencies increased indicating that the surfactant and some protein-surfactant complexes were removed from the surface. The opposite trend, a signal decrease was observed for ΔD . The combined elution (steps ii, iii and iv) had the largest effect on the pellicle formed by US onto PMMA-0, ΔF went up by $\sim 75\%$ of its value after 60 minutes adsorption and ΔD was reduced by $\sim 90\%$.

Figure 3. Changes in dissipation as a function of frequency. Dissipation vs Frequency plots for the adsorption of unstimulated (black) and stimulated (grey) whole saliva onto untreated polycarbonate (a) and poly(methyl methacrylate) (b) spin coated sensors. Saliva samples were diluted, 25% (v/v), in 10 mM Na-phosphate buffer supplemented with 50 mM NaCl at pH 7 and all measurements were performed at 37 °C. The curves represent the mean values from three independent measurements for the changes produced on the 3rd overtone.

Fig. 3 shows the ΔD vs ΔF plots for the adsorption of US and SS onto the untreated polymers, and enables obtaining additional information about the formation of the pellicles. The low density of the data points at the beginning of the curves indicates an initial fast adsorption step, and as the adsorption kinetics gradually slow down, the data points get closer. The initial fast step yields the largest changes in frequency and dissipation for US on PC-0, $\Delta F = -58.00 \pm 1.98$ Hz and $\Delta D (10^{-6}) = 6.73 \pm 0.53$, while the lowest were obtained for SS adsorption on PMMA-0, $\Delta F = -30.46 \pm 11.51$ Hz and $\Delta D (10^{-6}) = 3.12 \pm 0.76$. Furthermore, the changes in the slope can be related to different processes occurring during the formation of the pellicles affecting their rheological and structural properties. Both types of saliva show similar adsorption patterns with a marked biphasic nature for both PC and PMMA. According to

figure SM3-1, the acoustic ratios ($\Delta D/\Delta F$) grow with time for all the tested samples, and although the values obtained for US and SS films lay within the experimental error, after 60 minutes adsorption, these ratios are higher for SS films.

3.2 Saliva adsorption onto UV-treated surfaces

When the surfaces have been pretreated with UV-light before exposing them to saliva the resulting changes in frequency are larger than the ones obtained for the untreated surfaces. Also, differences were observed when comparing US and SS pellicles, ΔF (US) and ΔD (US) are larger than ΔF (SS) and ΔD (SS) for PC-30, while for PMMA-30 ΔF (US) $\approx$ ΔF (SS) and ΔD (US) $<$ ΔD (SS) (Fig. 4). Once again, after 60 minutes adsorption, the different harmonics for both ΔF and ΔD are clearly separated. Rinsing with buffer resulted in small changes in ΔF and ΔD for all the tested conditions except for the US pellicle formed on PMMA-30, where a large increase in frequency and a large decrease in dissipation were obtained. Also, when the salivary films were exposed to SDS they showed similar trends in ΔF and ΔD , both increased, for most cases except for US films formed onto PC-30 where an initial decrease was followed by a slight increase. The total effect of all the rinsing steps, similarly to the behavior observed for the untreated materials, resulted in a ΔF increase and a ΔD decrease compared to the 60 minutes adsorption level being the change maximum for the US pellicle adsorbed onto PMMA-30.

Figure 4. *Real-time formation of salivary films onto UV-treated PC and PMMA.* The curves represent the mean values from three independent measurements for the changes produced on the 3rd 5th and 7th overtones. (i) Adsorption of saliva; (ii) phosphate buffer rinse; (iii) 0.5 % (w/v) SDS rinse; (iv) phosphate buffer rinse. Panels a and c correspond to the measurements performed on polycarbonate sensors with unstimulated and stimulated saliva respectively and panels b and d represent the measurement done on PMMA sensors with unstimulated and stimulated saliva respectively. Saliva samples were diluted, 25% (v/v), in 10 mM Na-phosphate buffer supplemented with 50 mM NaCl at pH 7 and all measurements were performed at 37 °C.

The ΔD vs ΔF plots for the adsorption of US and SS onto the UV-treated polymeric substrates are presented Fig. 5. The main differences that can be appreciated when these are compared to the curves obtained for the untreated substrates come from the shape of the curves. Whereas in Fig. 3 ΔD grows continuously, as ΔF becomes more negative in Fig.5 the curves adopt a characteristic ‘S’ shape, which indicates a multiphasic nature where the last phase shows a stabilization of the $\Delta D/\Delta F$ ratio. However, an initial fast adsorption step is observed again and it yields similar values for both ΔF and ΔD regardless the kind of substrate.

Figure 5. *Changes in dissipation as a function of frequency.* Dissipation vs Frequency plots for the adsorption of unstimulated (black) and stimulated (grey) whole saliva onto UV-treated polycarbonate (a) and poly(methyl methacrylate) (b) spin coated sensors. Saliva samples were diluted, 25% (v/v), in 10 mM Na-phosphate buffer supplemented with 50 mM NaCl at pH 7 and all measurements were performed at 37 °C. The curves represent the mean values from three independent measurements for the changes produced on the 3rd overtone.

3.3 Viscoelastic modelling of the pellicles

In order to calculate the thickness and the viscoelasticity of the pellicles, the Voigt-based viscoelastic model has been used and the obtained results are presented in Fig. 6. Both types of saliva render films with similar thicknesses when adsorbed onto the untreated PC surfaces, 23.9 ± 2.6 nm and 23 ± 1.1 nm (US and SS respectively), whereas when untreated PMMA is used the salivary films adsorbed from US is thicker than the one obtained from the SS sample,

21.7 $\pm$ 1.4 nm for US and 16.4 $\pm$ 3.5 nm for SS. A different behavior is obtained when the surfaces were pre-treated with UV light: while for PC the US films were thicker than the SS ones, 30.4 $\pm$ 0.9 nm and 27.85 $\pm$ 0.03 nm respectively, US pellicles adsorbed onto UV-treated PMMA were thinner than the SS ones, 24.5 $\pm$ 0.9 nm and 26.4 $\pm$ 0.4 nm for US and SS respectively.

While the analysis of ΔD vs ΔF plots provides qualitative information about the viscoelastic properties of the salivary pellicle, quantitative information can be obtained when the ratio between the storage modulus, G' and the loss modulus, G'' , is calculated. These parameters can be obtained by fitting ΔF and ΔD by means of the Voigt model. For the salivary films formed onto the untreated surfaces, the ones adsorbed from US were more viscoelastic than for SS (G''/G' (US) $>$ G''/G' (SS)). Nevertheless, for the pellicles formed onto UV-treated surfaces the viscoelastic properties depended on the polymer type: i) For treated PC a slightly softer pellicle (larger G''/G' ratio) was obtained for US films, however, the difference lays within the experimental error; ii) for treated PMMA, US films were stiffer compared to SS films.

Figure 6. *Salivary film thickness and viscoelasticity.* Salivary film thicknesses (a) and viscoelasticity (b) after 60 minutes adsorption time onto untreated and UV-treated polycarbonate and poly(methyl methacrylate) surfaces. The values were obtained by fitting the changes in frequency and dissipation with the Voigt model. Saliva samples (unstimulated-dark grey and stimulated-light grey) were diluted, 25% (v/v), in 10 mM Na-phosphate buffer supplemented with 50 mM NaCl at pH 7 and all measurements were performed at 37 °C. The results represent the mean values from three independent measurements $\pm$ the standard deviation. Significance levels are in agreement with the SD bars.

4. Discussion

The main differences observed between US and SS in Fig. 1 can be attributed to a higher content of proline rich proteins, which might be positively and negatively charged, in SS [24-26]. This is in good agreement with previous studies where it was reported that stimulation

1 increased the secretion of parotid saliva and therefore the presence of PRPs [20, 24],
2 especially bPRPs and gPRPs [27]. Thus, it is expected that the exposure of US and SS to any
3 substrate will result in pellicles with at least different amounts of these proteins.

4 As it is shown in SM2-2, the untreated surfaces, PC-0 and PMMA-0, are negatively charged
5 and highly hydrophobic. On the other hand, the UV-treated surfaces PC-30 and PMMA-30
6 have a stronger negative charge and a hydrophilic character. This fact would explain the
7 similarities in protein composition obtained for the pellicles formed onto the different
8 substrates. The adsorption of saliva onto PC-0 and PMMA-0 would be mainly driven by both
9 electrostatic and hydrophobic interactions [26, 28] while for PC-30 and PMMA-30 adsorption
10 will be ruled by electrostatic forces. Therefore, electrostatic forces will promote the
11 adsorption of positively charged proteins, such as bPRPs, and these will enable the binding of
12 negatively charged proteins, e.g. acidic and glycosylated PRPs. However, the unstructured
13 conformation of PRPs [29] may also facilitate their direct interaction with both untreated and
14 UV-treated surfaces, either through the positively charged residues or through the
15 hydrophobic prolines [26].

16 However, the formation of all pellicles is characterized by initial fast adsorption kinetics that
17 correspond to the adsorption of low molecular weight proteins [30] such as PRPs. Although
18 these were found in lower amounts in US than in SS, the fact that this step yields larger ΔF for
19 US might indicate that either other proteins not detected by us were adsorbed on the surfaces
20 [5, 6, 26] or that more hydrated films were formed by US [15]. It is also worth highlighting
21 that this initial layer is not affected by the UV treatment, as can be deduced from the similar
22 values obtained for the acoustic ratios at $\Delta t = 1$ min and $\Delta t = 5$ min (SM3-1). Apart from
23 providing information about the kinetics of protein adsorption, ΔD vs ΔF plots are also useful
24 in elucidating the structural properties of proteinaceous films [31-33] as well as in providing
25 qualitative information about their viscoelastic properties [3, 31]. ΔD vs ΔF show that for
26 each type of surface US and SS have a similar profile: i) for the untreated surfaces (Fig. 3) the
27 curves could be roughly divided into two regions with the second one having a steeper slope
28 than the first one reflecting the formation of an initial rigid phase and a more dissipative
29 second one [3, 14, 31]; ii) the curves obtained for the UV-treated surfaces (Fig. 5) have an “S-
30 shape” indicating a multiphasic adsorption that suggests that the initial adsorption is followed
31 by further adsorption on the bare surface or on top of the first layer and by conformational
32 changes and/or reorganization of the adsorbed proteins [2, 3, 34]. These results would indicate

1 that the forces that govern the surface-pellicle interaction would also affect protein-protein
2 interactions and, therefore the structure of the pellicle.

3 Additionally, different mechanism of interaction between the SDS and the pellicles can be
4 deduced from Fig. 2 and Fig. 4, suggesting either different protein composition or structure
5 [35]. For the untreated surfaces SDS interaction with the pellicles depends on both the type of
6 saliva and the type of surface: i) While for US onto PC-0, SDS adsorbed and complexed with
7 the proteins partially displacing the pellicle, as indicated by the peak in ΔF and the large
8 increase in ΔD , for SS, apparently a protein displacement was taking place as suggested by
9 the large change in ΔF after the last buffer rinse. ii) For the films adsorbed onto PMMA-0, the
10 US pellicle was largely displaced by SDS whereas for SS, mainly surfactant adsorption and
11 complexation with the pellicle was observed. In the case of the more hydrophilic UV-treated
12 surfaces, mainly a displacement of the pellicle occurred except for the US pellicle adsorbed
13 onto PC-30 where protein-SDS complexation led to little removal of the pellicle.

14 The thickness and viscoelasticity values obtained after fitting ΔF and ΔD with the Voigt
15 model (Fig. 6) corresponded well with the ones previously reported by other authors [3, 14,
16 15, 19, 36]. The fact that thicker pellicles were formed onto UV-treated materials would be
17 explained by a more expanded conformation adopted by the outer fraction of the
18 proteinaceous film as it is also reflected by the higher dissipation values obtained. This would
19 also apply to the thicker and more viscoelastic pellicles formed from US onto most of the
20 surfaces when the effect of stimulation is analyzed. Another reason could be the higher
21 content of mucins in US. Even though they have been proposed to be part of salivary films
22 formed on surfaces with similar properties [26], from our results it is difficult to say whether
23 or not they contribute to the formation of the pellicles.

24 5. Summary of results

25 Our results suggest that among the proteins resolved by SDS-PAGE, more types of proteins
26 are involved in the formation of stimulated saliva pellicles. However, this can be due to the
27 limitations of the technique. For untreated materials, polycarbonate and PMMA, unstimulated
28 saliva produces bigger changes in frequency than stimulated saliva. Although the differences
29 in thickness and viscoelasticity lay within the experimental error for polycarbonate, these
30 parameters are also larger for unstimulated saliva. The differences could also be appreciated
31 when the stability of the pellicles was tested against SDS elution; SS was more easily
32 removed for PC and US for PMMA. Larger changes in frequency and dissipation for the
33 adsorption of saliva were obtained when the surfaces had been UV-pretreated suggesting a

1 more expanded conformation of the proteinaceous layer as it was confirmed by the thickness
2 values. Again, the different effect produced by SDS, much larger frequency changes for SS on
3 PC and for US on PMMA, suggests differences in the structure of the pellicles due to the
4 mechanism of interaction between the proteins and the substrate and between the proteins
5 themselves.

6 6. Conclusions

7 The results of this study highlight the importance of choosing the appropriate type of saliva
8 depending on the kind of study to be performed. Unstimulated and stimulated saliva render
9 pellicles with different protein content. By means of QCM-D it was possible to detect
10 structural differences between them as suggested by the variations in thickness and
11 viscoelasticity and especially by the effect of SDS on the removal of the films.

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