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Original Research Article

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.

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 forma-

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tion 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 × 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 s 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 min. 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 h and the temperature was 37 °C. Then, the cell was rinsed with saliva free buffer at 100 μl min^{-1} for 10 min 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 min. Then, 0.015 ml of each sample were loaded in the wells and run at 200 V for 30–35 min. A molecular weight reference standard (Precision Plus ProteinTM 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 H_2O for 20 min. 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 ± 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

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,