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Structural and compositional changes in the salivary pellicle induced upon exposure to SDS and STP

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Sodium dodecyl sulphate (SDS) and sodium tripolyphosphate (STP) act to remove stained pellicle from dentition and loosen deposits on tooth surfaces that may become cariogenic over time. This study investigated how SDS and STP impact the salivary pellicle adsorbed onto hydroxyapatite and silica sensors using a dual polarisation interferometer and a quartz-crystal microbalance with dissipation. After the pellicle was exposed to SDS and STP the remaining pellicle, although weaker, due to the loss of material, became less dense but with a higher elastic component; suggesting that the viscous component of the pellicle was being removed. This would imply a structural transformation from a soft but dense structured pellicle, to a more diffuse pellicle. In addition, the majority of proteins displaced by both SDS and STP were identified as being acidic in nature; implying that the negatively charged groups of SDS and STP may be responsible for the displacement of the pellicle proteins observed.

Keywords: salivary pellicle; SDS; sodium tripolyphosphate; QCM-D; DPI; mass spectrometry

Introduction

The salivary pellicle is a protein rich film that forms on all surfaces within the mouth, and provides a barrier to dissolution of enamel by dietary acids, and concomitantly lubricates the mouth facilitating the consumption and processing of food (Carpenter 2012). However, the pellicle is ambivalent in nature as it also provides the primary sites for the attachment of bacteria, which in certain cases can be responsible for the development of plaque, a risk factor for caries (Hannig & Hannig 2009). The pellicle therefore is juxtaposed between protecting the oral cavity from acidic and abrasive damage, whilst concomitantly aiding the adsorption of plaque-forming bacteria close to the tooth surface (Wolff & Larson 2009). Widespread dentifrice products used in conjunction with tooth brushing act to reduce these plaque deposits, as well as tooth stain removal (Meyers et al. 2000). Certain ingredients, such as detergents (eg sodium dodecyl sulphate (SDS)) and abrasives (eg silica) are common to many dentifrices. These ingredients are used to remove stained pellicle from dentition and loosen deposits on tooth surfaces that may become cariogenic over time (Joiner 2010). In addition, polyphosphates (eg sodium tripolyphosphate (STP), sodium pyrophosphate and sodium hexametaphosphate), display strong reactivity to enamel surfaces (White 2002). These anionic polyphosphates have not only been shown to remove pellicle proteins that have become stained, but have also been shown to reduce plaque development (Shellis et al. 2005). The safety of polyphosphate salts and detergents found in dentifrice products (such as STP and SDS) is now well established and they are commonly found in many dentifrices worldwide (Fairbrother & Heasman 2000; Gerlach 2002). Whilst SDS is known to remove pellicle proteins (Hahn Berg et al. 2001; Hannig, Khanafer et al. 2005; Santos et al. 2010; Veeregowda et al. 2012), and STP has been shown to be effective in the *in vitro* removal of stain (Ayad et al. 2000), the impact of STP on salivary pellicle structure has not been investigated or characterised in detail. As the pellicle is the primary interface between the oral environment and the hard and soft tissue of the mouth it plays an important role in oral physiological and pathological processes (Hannig & Joiner 2006). Consequently, a deeper understanding of the structural changes that SDS and STP induce in the salivary pellicle is important to achieve. Therefore, this study investigated how SDS and STP impact the preformed salivary pellicle adsorbed onto silica and hydroxyapatite (HA) surfaces using dual polarisation interferometry (DPI) and a quartz crystal microbalance with dissipation (QCM-D), and identified the proteins which SDS and STP displace using a HA chromatography column. Changes in the physical structure of the salivary pellicle (surface mass, density, thickness and viscoelasticity) and the identification of the proteins that SDS and STP displace are reported to help understand not only the mechanism by which SDS and STP remove the pellicle from the surface, but also what

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effect exposure to these chemicals has on the structure of the remaining salivary pellicle.

Materials and methods

Saliva collection

Saliva collection was undertaken according to a protocol previously assessed by an independent ethics panel (reference number: 10/H0311/15 registered online at ClinicalTrials.gov ID: NCT01167504; Protocol ID: IFR03-2010). The saliva was obtained from 14 apparently healthy, non-smoking, male and female volunteers, ranging in age from 20 to 50 years. Volunteers refrained from eating 1 h prior to donation, and rinsed their mouths twice with 10 ml of bottled still water (Waitrose, Bracknell, UK). Volunteers then chewed on flavour-free gum (Gumlink, Vejle, Denmark) and expectorated whole mouth saliva (WMS) into a small sterile collection until they had produced 10 ml of saliva. The stimulated parotid saliva (PS) was collected using a sterilised Lashley suction cup (Lashley 1916) with salivary secretion stimulated by sucking citric acid containing 'Rosey-Apples' boiled sweets (ASDA, Leeds, UK). This continued until 20–30 ml of saliva had been produced. Samples were kept on ice upon expectoration, and were used within 10 min of collection. Ten saliva samples (five WMS and five PS) were exposed to QCM-D and DPI HA sensors and rinsed with 10 mM SDS; another 10 saliva samples (five WMS and five PS) were also exposed to QCM-D and DPI HA sensors but then rinsed with 10 mM STP. Thirdly, 10 saliva samples (five WMS and five PS) were exposed to QCM-D and DPI silica sensors and rinsed with 10 mM SDS; and finally 10 saliva samples (five WMS and five PS) were exposed to QCM-D and DPI silica sensors but then rinsed with 10 mM STP.

Solutions

The concentration of SDS and STP in oral hygiene products is ~ 1.5% w/w and 10% w/w, respectively. In order to account for the dilution of STP and SDS by the saliva during use in the mouth a concentration of 10 mM SDS (0.29% w/w) and 10 mM STP (0.36% w/w) was used. 0.1 M phosphate buffer (Sigma-Aldrich, Dorset, UK) was used, with ultra-pure water as the solvent (Nanopure Diamond, Barnstead Int., Dubuque, IA USA).

Quartz crystal microbalance with dissipation monitoring (QCM-D)

The measurements were performed using a D300 QCM-D (Q-Sense AB, Vastra Frolunda, Sweden) with a QAFC 302 axial flow measurement chamber maintained at 36.8°C. HA and silica coated AT-cut piezoelectric quartz crystals sandwiched between gold electrodes (QSX-303, Q-Sense AB, Vastra Frolunda) were used as the substrata. The sensor was excited to oscillate by applying an alternating current across the sensor electrodes at its fundamental resonant frequency (ie 5 MHz), and at the 3rd, 5th and 7th overtones. The frequency change (Δf) and the dissipation change (ΔD) at each of the four frequencies were measured as salivary proteins adsorbed onto the sensor. Changes in the frequency of the oscillating sensor were related to the changes in the hydrated mass adsorbing on to the quartz crystal sensor using the Sauerbrey model, in Equation 1 (Sauerbrey 1959). The Sauerbrey model was considered the most conservative model to use, as this gives the lowest value of hydrated mass that the pellicle could be, compared with other models that may overestimate the amount of pellicle present.

$$\Delta m = -\frac{\rho_0 v_0}{2f_n^2}\Delta f \tag{1}$$

where Δm represents a change in adsorbed areal mass (ng cm^{-2}), ρ_0 the density of the quartz crystal (2,650 kg m^{-3}), v_0 the shear velocity in quartz (3,340 m s^{-1}), f_n the resonant frequency, 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 1,000 kg m^{-3} as has been assumed in previous work (Veeregowda et al. 2011). Softer films dampen the sensor's frequency of oscillation which was calculated by a secondary parameter known as the dissipation (D). The change in D is inversely proportional to the decay time (τ) and resonant frequency (f) of the oscillating sensor as follows:

$$D = \frac{1}{\pi f \tau} \tag{2}$$

The D300 QCM-D 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, the faster the sensor will stop oscillating (reducing decay time), and thus increasing the dissipation.

Dual polarisation interferometer (DPI)

Measurements of surface layer thickness and refractive indices (RI) were performed in real time using an AnaLight Bio200 DPI (Farfield Sensors, Manchester, UK). The sensor (silica or HA) was clamped in a temperature controlled enclosure allowing the temperature to be maintained at 36.8°C for all experiments. Polarised light from a helium neon laser (wavelength, 632.8 nm) passed through the sensor *via* two optical paths. One

light path close to the chip surface in contact with the sample through its fringe field, and the other independent of the surface was used as a reference signal. This light was oscillated between two polarisations: transverse magnetic (TM) and transverse electric (TE) at a frequency of 50 Hz (Cross et al. 2008). Essentially, TE and TM respond differently to protein adsorption/displacement and therefore provide two independent measurements of the adsorbing material, permitting determination of the mass, thickness and density of the adsorbing film. By solving Maxwell's equations simultaneously for the phase change of the TE and TM, values for the mean RI (n_f) and thickness (d_f) of the adsorbed film can be obtained (assuming a single homogeneous isotropic layer) using a model analogous to the 'three-phase model' used in ellipsometry (Arwin 2000). Because the RI 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 (Feijter et al. 1978); where n_{buffer} is the RI of the water and *dn/dc* the RI increment of the adsorbed pellicle:

$$\Gamma = d_f \frac{n_f - n_{buffer}}{dn/dc} \quad (3)$$

These calculations were carried out using the Analight Explorer software (version 1.5.4.18811, Farfield Scientific, Manchester, UK) (For more detail on DPI see Cross et al. (2008) and Swann et al. (2004)). The assumed RI increment *dn/dc* was 0.15 ml g^{-1}, a value typical for protein films (Westwood et al. 2010).

Sensor properties

The substrata used for the formation of the salivary pellicle were QCM-D and DPI HA (which constitutes the main mineral of dental enamel) and silica coated sensors. These surfaces differ in both their physical and chemical composition and were used in order to observe how the pellicle and the displacers (STP and SDS) behave on the two different surfaces. The root mean square surface roughness of the DPI sensors was higher for both silica (4.7 nm) and HA (18.7 nm) sensors when compared to the QCM-D silica (1.2 nm) and HA (1.4 nm) sensors measured by MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA, USA). The hydrophobicity of the DPI HA sensors (contact angle 42° ± 13°) and QCM-D HA sensors (43° ± 0.8°) were similar; whereas the silica DPI sensors were 68° ± 1° as opposed to 32° ± 1° for the QCMD silica sensors prior to cleaning (measured by Attension Theta optical tensiometer, Biolin Scientific, Stockholm, Sweden). The isoelectric point of silica was lower (≈ pH 2) (Vansant et al. 1995) than HA (≈ pH 8) (Bengtsson & Sjoberg 2009) so that under the neutral conditions (≈ pH7) used for the adsorption of the salivary pellicle in this study, the HA surface is likely to have carried a slight positive charge and the silica a strong negative charge. These sensor properties will affect the interaction of SDS and STP and will be discussed later. Finally, the surface areas of the sensors were both < 0.00005 m^2.

Pellicle formation protocol

Each respective saliva sample was then measured concomitantly on the QCMD and DPI (both static adsorption systems, ie not flow-cell). Upon injection of 0.5 ml of saliva, pellicle formation was monitored for 120 min. Subsequently the pellicle was then rinsed with 2 ml of phosphate buffer solution (pH 7.4) to remove loosely adsorbed material. After 10 min the remaining pellicle was rinsed with 0.5 ml of 10 mM SDS (pH 7.4) or 10 mM STP (pH 8.3). Again, 10 min was given before rinsing with a phosphate buffer solution (see Figures 1 and 2).

Sensor cleaning

After the completion of the experiment, QCMD and DPI surfaces were cleaned with 2% w/v SDS (Sigma-Aldrich), followed by 2% w/v Hellmenax, then copiously rinsed

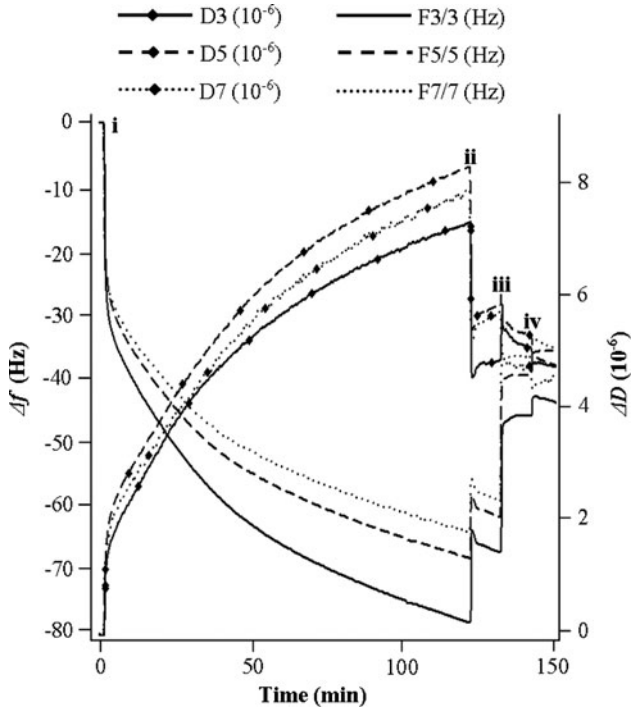

Figure 1. Salivary pellicle adsorption profile for a parotid saliva sample on a QCM-D HA sensor. (i) Addition of saliva; (ii) phosphate buffer rinse; (iii) 10 mM STP rinse; (iv) phosphate buffer rinse.

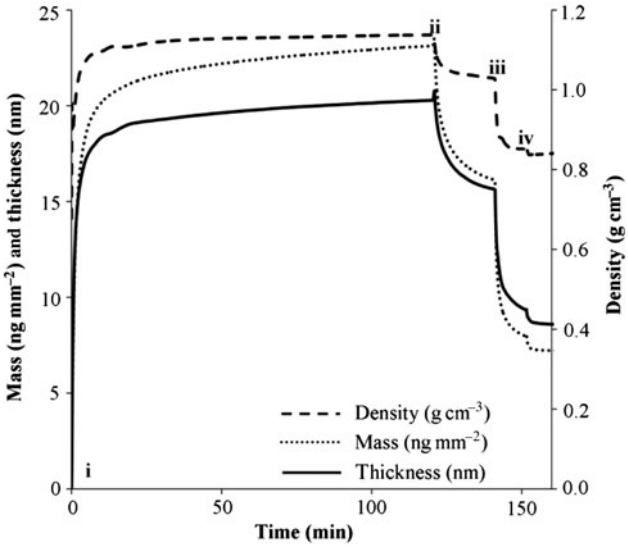

Figure 2. Salivary pellicle adsorption profile for parotid saliva sample on a DPI HA sensor. (i) Addition of saliva; (ii) phosphate buffer rinse; (iii) 10 mM STP rinse; (iv) phosphate buffer rinse.

with buffer followed by MiliQ water. The QCMD sensors were further dried with oxygen free nitrogen gas and exposed to UV-ozone (Bio-Force Nanosciences, Inc., Ames, IA, USA) for 20 min, whereas the DPI sensors were kept in 20% isopropanol until the next experiment.

Fast protein liquid chromatography (FPLC)

Salivary protein fractionation was performed *via* a Bio-CAD SPRINT Perfusion Chromatography workstation (PerSeptive Biosystems, Framingham, MA, USA); using CHT ceramic HA particles (40 µm diameter; density 0.63 g ml^{-1}) (Bio-Rad Laboratories, Hemel Hempstead, UK) as the column packing medium (surface area of HA in one column 0.095 m^2), which was replaced after each experiment. Saliva was injected into the column and allowed to adsorb for 2 h. Unadsorbed salivary proteins were then flushed out of the column (10 column volumes) with phosphate buffer. The remaining adsorbed proteins were then eluted using either 10 mM SDS or 10 mM STP (10 column volumes). Detection of proteins displaced by 10 mM SDS and 10 mM STP was performed at 220 nm; eluted proteins were collected (2 ml per fraction) using an Advantec SF-2120 super fraction collector (Advantec MFS, Inc., Dublin, CA, USA). Subsequently each 2 ml protein fraction was dialysed against de-ionised water using Spectra/Por 3 dialysis tubing with a MWCO of 3.5 kDa (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) and then concentrated down to 0.5 ml using a vacuum concentrator (Speed-vac SPD131DDA, Thermo Scientific, Basingstoke, UK).

SDS-PAGE

The pellicle protein fractions were further separated by molecular weight using SDS-polyacrylamide gel electrophoresis (PAGE). Protein samples (10 µl) were run on 8 cm × 9 cm, 4–12% NuPage MES BIS-TRIS pre-cast gels (Invitrogen Life Technologies Ltd, Paisley, UK) according to the manufacturer's instructions. The voltage was set at 200 V, 100 W and a current of 350 mA per gel for 35 min. The gel was then fixed in a solution containing 50% methanol, 10% acetic acid and 40% ultra-pure water, and stained with Coomassie Brilliant Blue R-250 (Thermo-Scientific). Protein standards (Mark 12^{TM} Unstained standard, Invitrogen Life Technologies Ltd) were used as molecular weight markers.

In situ *trypsin hydrolysis of protein bands*

Gel pieces of interest were excised using a 5 ml diamond pipette tip (Gilson Scientific Ltd, Luton, UK) and then washed with two 15 min incubations in 200 mM ammonium bicarbonate (ABC) in 50% (v/v) acetonitrile (200 µl) to equilibrate the gel to pH 8 and remove the stain, followed by 10 min incubations with acetonitrile (Fisher Scientific UK Ltd, Loughborough, UK) (200 µl). Any cysteine thiol side chains were then reduced by incubation with 10 mM dithiothreitol in 50 mM ABC (200 µl) for 30 min at 60°C before being alkylated with 100 mM iodoacetamide in 50 mM ABC (200 µl) for 30 min in the dark at room temperature. The gel pieces were then washed with two 15 min incubations in 200 mM ABC in 50% (v/v) acetonitrile (200 µl) followed by 10 min in acetonitrile (200 µl) to dehydrate and shrink the gel pieces before air drying. The protein was digested by the addition of 100 ng of trypsin in 10 µl of 10 mM ABC (modified porcine trypsin; Promega UK Ltd, Southampton, UK), or a mixture of 100 ng of trypsin and 100 ng of endoproteinase GluC (Roche Diagnostics Ltd, West Sussex, UK) in 10 µl of 10 mM ABC before incubation overnight at 37°C. Following digestion the samples were acidified by incubating with 10 µl of 1% (v/v) formic acid for 10 min. The digest solution removed from the tube into an Eppendorf tube and the gel pieces were then washed with 50% acetonitrile (20 µl) for 10 min to recover more digest proteins from the gel. The combined extracted digest samples were then dried down at the low drying setting (some heat) on a Speed Vac SC110 (Savant Instruments, Holbrook, NY, USA) fitted with a Refrigerated Condensation Trap and a Vac V-500 (Buchi, Flawil, Switzerland). The samples were then frozen at –80°C until ready for mass spectrometry analysis.

Tandem mass spectrometry (LC-MS/MS)

LC-MS/MS analysis was performed using a LTQ-Orbitrap mass spectrometer (Thermo Scientific) and a

nanoflow-HPLC system (nanoACQUITY: Waters, Elstree, UK). Peptides were trapped on line to a Symmetry C18 Trap (5 µm, 180 µm × 20 mm) which was then switched in-line to a UPLC BEH C18 Column (1.7 µm, 75 µm × 250 mm) held at 45°C. Peptides were eluted by a gradient of 0–80% acetonitrile in 0.1% formic acid over 50 min at a flow rate of 250 ml min^{-1}. The mass spectrometer was operated in positive ion mode with a nano-spray source at a capillary temperature of 200°C. The Orbitrap was run with a resolution of 60,000 over the mass range m/z 300–2,000 and an MS target of 10^6 and 1 s maximum scan time. The MS/MS was triggered by a minimal signal of 2,000 with an Automatic Gain Control target of 30,000 ions and maximum scan time of 150 ms. For MS/MS events selection of 2+ and 3+ charge states selection were used. Dynamic exclusion was set to one count and 30 s exclusion time with an exclusion mass window of ± 20 ppm. Proteins were identified by searching the Thermo RAW files converted to Mascot generic format by Proteome Discover 1.1 (Thermo-Scientific) and proteins were identified by interrogating the Sprot_trembl20121031 proteome database (taxonomy Homo Sapiens) using the MASCOT v2.4.1 search engine (Perkins et al. 1999).

Results

QCM-D

Figure 1 shows typical adsorption profiles for saliva obtained by QCM-D, whilst Figure 3 shows a box plot

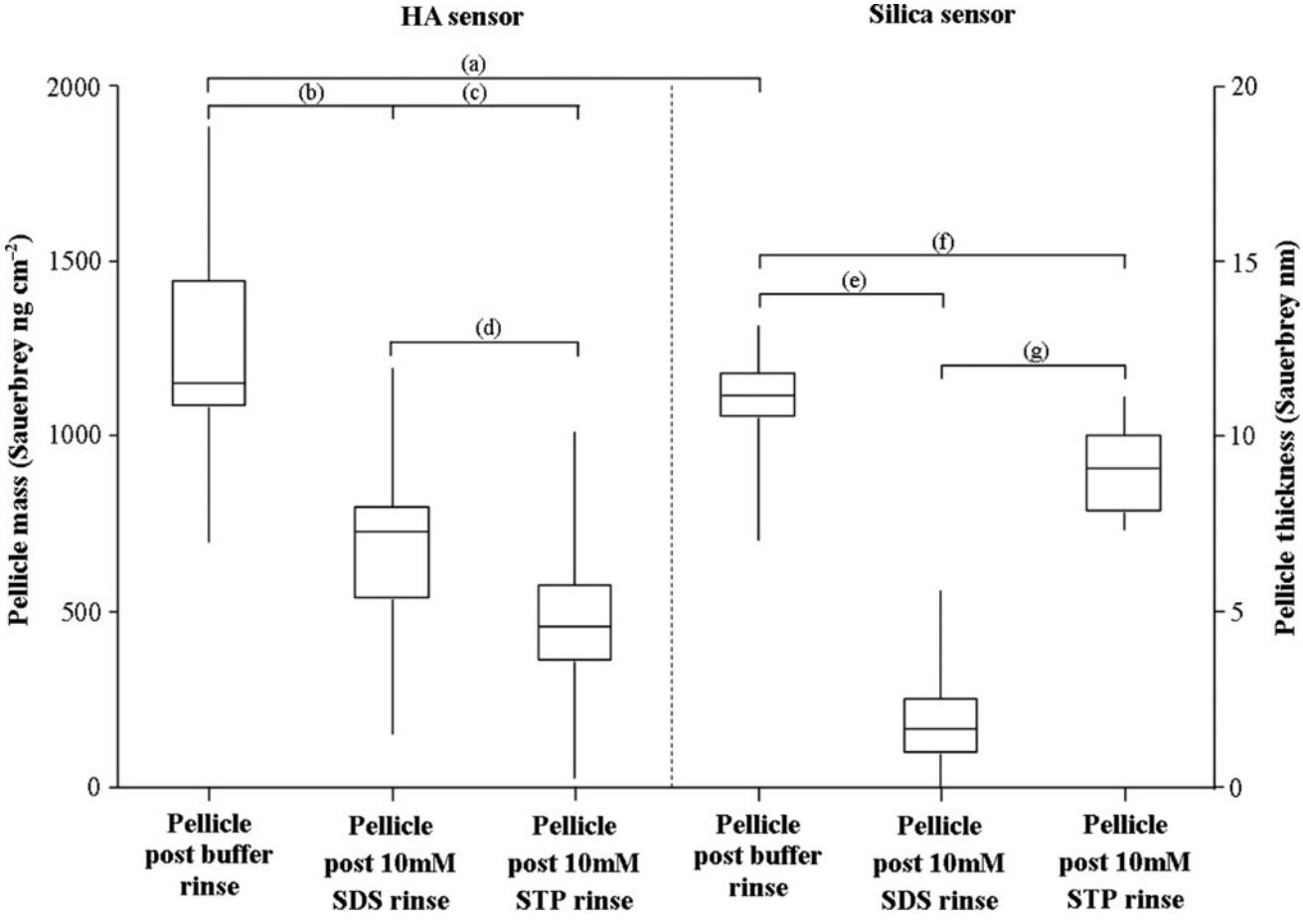

Figure 3. Box plot displaying the Sauerbrey mass (primary axis) and thickness (secondary axis) of the combined WMS and PS salivary pellicles on HA and silica sensors before and after rinsing with 10 mM STP and 10 mM SDS; and the statistical differences between them. (a) No significant difference ($p = 0.113$) between pellicle adsorbed to HA and silica sensors; (b), (c) significant difference ($p < 0.001$) between pellicle before and after rinsing with 10mM SDS and 10mM STP; (d) no significant difference ($p = 0.18$) between pellicles after exposure to 10mM SDS or 10mM STP; (e) significant difference ($p < 0.001$) between pellicle before and after rinsing with 10mM SDS; (f) significant difference ($p = 0.001$) between pellicle before and after rinsing with 10mM STP; (g) significant difference ($p < 0.001$) between pellicles after exposure to 10mM SDS and 10mM STP.

of the calculated change in pellicle mass and thickness on silica and HA sensors before and after exposure to 10 mM SDS and 10 mM STP. The mean mass for the combined PS and WMS salivary pellicles was 1,215 ± 289 ng cm^{-2} (12 nm ± 3 nm thick) and this was slightly higher on the HA sensor than on the silica sensor at 1,103 ± 139 ng cm^{-2} (11 nm ± 1 nm thick) but the difference was not statistically significant (p = 0.11). After exposure to 10 mM SDS the pellicle adsorbed onto the HA sensor was reduced to 663 ± 297 ng cm^{-2} (7 ± 3 nm thick); and was reduced to 491 ± 293 ng cm^{-2} (5 ± 3 nm thick) after exposure to 10 mM STP. This showed that the remaining pellicle adsorbed onto the HA substratum was larger in mean thickness and mass when exposed to 10 mM SDS compared with 10 mM STP. However, a significant reversal of this result took place when SDS and STP were exposed to the pellicle adsorbed on the silica surface. This showed a large reduction in the remaining pellicle mass and thickness after exposure to 10 mM SDS (mass: 194 ± 171 ng cm^{-2}; thickness: 2 ± 2 nm thick); but only a small change when the pellicle was exposed to 10 mM STP (911 ± 142 ng cm^{-2}; 9 ± 1 nm thick).

By comparing the ratio between Δf and ΔD (see Figure 4) the viscoelastic properties of the pellicle with respect to the induced energy dissipation of the sensor per coupled unit mass was observed. The results showed that the salivary pellicle before exposure to SDS and STP on both HA and silica substrata had similar viscoelastic properties (–2.2 MHz and –3.4 MHz respectively). When the pellicle was exposed to 10 mM SDS, it became predominantly more elastic relative to the untreated pellicle on both the HA (–7.2 MHz) and silica (–7.8 MHz) sensors. However, when the pellicle was exposed to 10 mM STP, it only became predominantly more elastic relative to the untreated pellicle on the HA (–6.8 MHz) sensor; and not on the silica (–2.9 MHz) sensor.

DPI

In Figure 5 a box plot displays the remaining pellicle structure (eg mass, thickness and density) on the HA and silica sensors before and after exposure to 10 mM SDS and 10 mM STP. Unlike the QCM-D results, a significant difference in the structure of the pellicle on the DPI HA sensor (mean mass: 1,390 ± 731 ng cm^{-2}) compared to the DPI silica sensor (mean mass: 366 ± 52 ng cm^{-2}) was observed. After displacing the pellicle adsorbed on the HA sensors with 10 mM SDS for 10 min, the remaining pellicle had a mean mass of 354 ± 228 ng cm^{-2}, a mean thickness of ≤6 ± 3 nm, and a

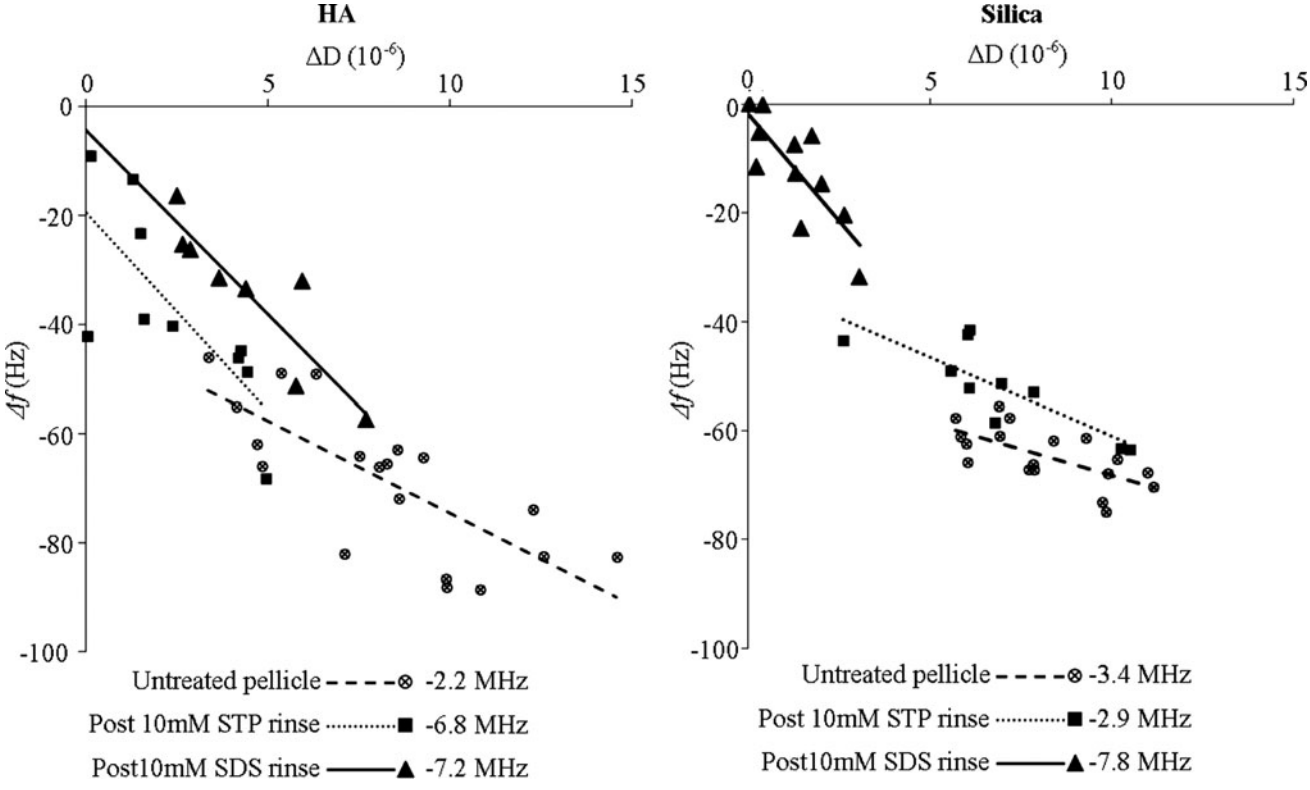

Figure 4. $\Delta f/\Delta D$ plot displaying the different elastic properties of the combined WMS and PS salivary pellicles before and after rinsing with SDS and STP on both HA and silica surfaces. (A test for outliers was performed using the 'outlier test' function in the R statistical package and these were removed from the plots; see Fox & Weisberg 2010 for more details.)

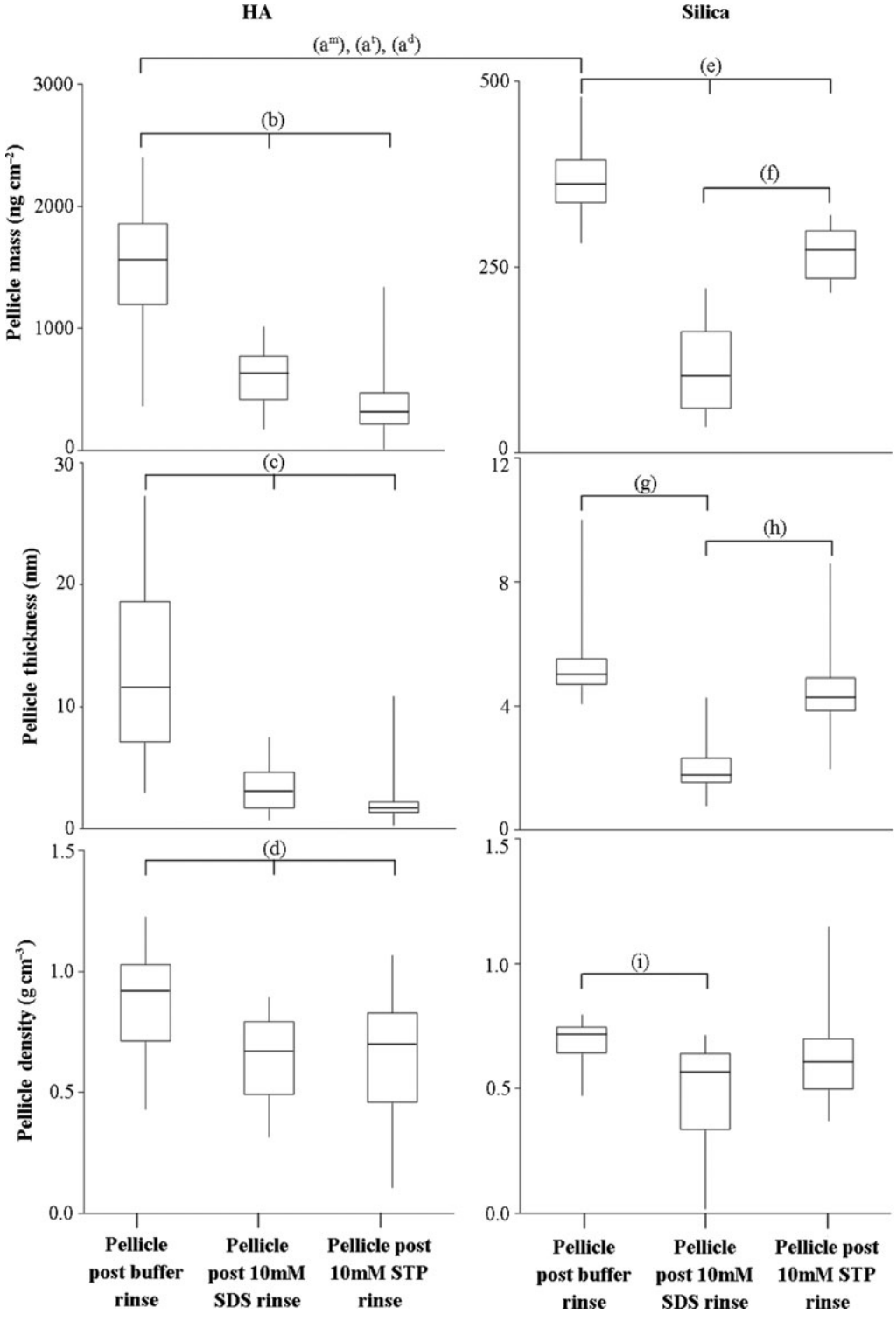

Figure 5. Box plot displaying the changes in thickness, mass and density of combined WMS and PS salivary pellicles adsorbed to a DPI HA and silica sensor before and after rinsing with 10 mM STP and 10 mM SDS. Significant difference ($p < 0.05$) between pellicle (a^m) mass, (a^t) thickness, and (a^d) density adsorbed to HA and silica. Significant difference ($p < 0.05$) in (b) pellicle mass, (c) thickness, and (d) density after rinsing with 10 mM SDS or 10 mM STP on a HA coated sensor. (e) Significant difference ($p < 0.05$) in pellicle mass, after rinsing with 10 mM SDS or 10 mM STP on a silica coated sensor. (f) Significant difference ($p < 0.05$) between remaining pellicle mass rinsed with 10 mM SDS and pellicles rinsed with 10 mM STP. (g) Significant difference ($p < 0.05$) in pellicle thickness, after rinsing with SDS and STP. (h) Significant difference ($p < 0.05$) between SDS and STP. (i) Significant difference ($p < 0.05$) in pellicle mass, after rinsing with SDS.

Table 1. Differences and similarities between SDS and STP eluted proteins displaced from HA.

	Protein name	Cleaning agent	Score	Protein ID mass	Sig sequences	PI
P01011	Alpha-1-antichymotrypsin	SDS	288	47,792	11	5.33
Q5EFE6	Anti-RhD monoclonal T125 kappa light chain	SDS	313	26,024	4	8.7
P23280	Carbonic anhydrase 6	SDS	346	35,459	8	6.51
P06744	Glucose-6-phosphate isomerase	SDS	145	63,335	6	8.43
P04406	Glyceraldehyde-3-phosphate dehydrogenase	SDS	256	36,201	7	8.57
P01591	Immunoglobulin J chain	SDS	172	18,543	4	5.12
P13645	Keratin, type I cytoskeletal 10	SDS	764	59,020	12	5.13
P35527	Keratin, type I cytoskeletal 9	SDS	300	62,255	7	5.14
P04264	Keratin, type II cytoskeletal 1	SDS	1,223	59,020	24	8.15
P35908	Keratin, type II cytoskeletal 2 epidermal	SDS	1,339	65,678	27	8.07
P35908	Keratin, type II cytoskeletal 2 epidermal	SDS	943	65,678	20	8.07
C3PTT6	Pancreatic adenocarcinoma upregulated factor	SDS	975	21,553	8	6.74
Q96DR5	Parotid secretory protein	SDS	262	27,166	6	5.35
P81605	Preproteolysin	SDS	70	11,391	2	6.08
P20061	Transcobalamin-1	SDS	206	48,689	4	4.96
P52209	6-Phosphogluconate dehydrogenase	STP	1,292	53,619	13	6.8
P60709	Actin, cytoplasmic 1	STP	236	42,052	8	5.29
P01023	Alpha-2-macroglobulin	STP	1,290	164,613	36	6.03
P12814	Alpha-actinin-1	STP	1,747	103,563	37	5.25
P06733	Alpha-enolase	STP	848	47,481	17	7.01
P04083	Annexin A1	STP	1856	38,918	27	6.57
P12429	Annexin A3	STP	760	36,524	14	5.63
P13928	Annexin A8	STP	321	37,086	7	5.56
P06703	Calcyclin	STP	186	10,230	7	5.33
P06702	Calgranulin-B	STP	319	13,291	3	5.71
P04040	Catalase	STP	959	59,947	24	6.9
P00450	Ceruloplasmin	STP	577	122,983	18	5.44
P01024	Complement C3	STP	476	188,569	17	6.02
P01040	Cystatin-A	STP	240	11,000	6	5.38
P04080	Cystatin-B	STP	193	11,190	2	6.96
P54108	Cysteine-rich secretory protein 3	STP	226	28,524	11	8.09
Q01469	Fatty acid-binding protein	STP	353	15,497	6	6.6
P06396	Gelsolin	STP	541	86,043	14	5.9
P00738	Haptoglobin	STP	845	45,861	17	6.13
P69905	Haemoglobin subunit alpha	STP	1,049	15,305	5	8.72
P68871	Haemoglobin subunit beta	STP	981	16,102	9	6.75
P62805	Histone H4	STP	257	11,360	4	11.36
P01876	Ig alpha-1 chain C region	STP	319	38,486	4	6.08
P01860	Ig gamma-3 chain C region	STP	133	42,287	5	8.23
P01834	Ig kappa chain C region	STP	401	11,773	4	5.58
Q9Y6R7	IgGFc-binding protein	STP	990	596,443	25	5.14
Q6NS95	IGL@ protein	STP	287	25,475	6	6.19
Q0KKI6	Immunoglobulin light chain	STP	775	24,300	9	8.29
Q9NPP6	Immunoglobulin heavy chain variant	STP	5,177	45,613	15	5.75
Q2TUW9	Lactoferrin	STP	713	79,812	22	8.51
P30740	Leukocyte elastase inhibitor	STP	1,384	42,829	14	5.9
P31025	Lipocalin-1	STP	1,470	19,409	15	5.39
P23141	Liver carboxylesterase	STP	298	62,766	9	6.15
P40926	Malate dehydrogenase	STP	393	35,937	5	8.92
Q9HC84	Mucin-5B	STP	535	611,584	14	6.2
P59666	Neutrophil defensin 3	STP	273	10,580	3	5.71
P80188	Neutrophil gelatinase-associated lipocalin	STP	296	22,745	8	9.02
P62937	Peptidyl-prolyl cis-trans isomerase A	STP	238	18,229	8	7.68
P30044	Peroxiredoxin-5	STP	260	22,301	6	8.93
P18669	Phosphoglycerate mutase 1	STP	394	28,900	10	6.67
P13796	Plastin-2	STP	229	70,814	8	5.29
P12273	Prolactin-inducible protein	STP	2,847	16,847	11	8.26
P07237	Protein disulphide-isomerase	STP	525	57,480	16	4.76

(*Continued*)

Table 1. (*Continued*).

	Protein name	Cleaning agent	Score	Protein ID mass	Sig sequences	PI
P63104	Protein kinase C inhibitor protein 1	STP	436	27,899	10	4.73
P50395	Rab GDP dissociation inhibitor beta	STP	375	51,087	14	6.11
Q9HD89	Resistin	STP	348	12,096	2	6.51
P10599	Thioredoxin	STP	256	12,015	7	4.82
O60603	Toll-like receptor 2	STP	845	90,920	3	6.17
P37837	Transaldolase	STP	986	37,688	21	6.36
P29401	Transketolase	STP	682	68,519	10	7.58
Q6P5S2	UPF0762 protein C6orf58	STP	793	38,244	15	5.78
P18206	Vinculin	STP	927	124,292	27	5.5
P02774	Vitamin D-binding protein	STP	477	54,526	9	5.4
P30838	Aldehyde dehydrogenase	STP or SDS	617(326)	50,762	18(7)	6.11
P01009	Alpha-1-antitrypsin	STP or SDS	853(573)	46,878	15(14)	5.37
P04745	Alpha-amylase 1	STP or SDS	1,098(1,230)	58,415	12(25)	6.47
P05109	Calgranulin-A	STP or SDS	220(81)	10,885	4(2)	6.51
P01036	Cystatin-S	STP or SDS	250(117)	16,489	2(3)	4.95
P01877	Ig alpha-2 chain C region	STP or SDS	469 (127)	37,301	8 (3)	5.71
P01859	Ig gamma-2 chain C region	STP or SDS	759 (330)	36,505	11 (5)	7.66
Q6PIL8	IGK@ protein	STP or SDS	2,920 (745)	26,103	14 (8)	6.15
P22079	Lactoperoxidase	STP or SDS	673 (105)	81,149	18 (6)	8.89
P09960	Leukotriene A-4 hydrolase	STP or SDS	803 (547)	69,868	15 (14)	5.8
P61626	Lysozyme C	STP or SDS	841 (102)	16,982	7 (2)	9.38
P59665	Neutrophil defensin 1;	STP or SDS	2,229 (355)	10,536	6 (6)	6.54
P00558	Phosphoglycerate kinase 1	STP or SDS	963 (457)	44,985	24 (11)	8.3
P01833	Polymeric immunoglobulin receptor	STP or SDS	1,705 (648)	84,429	23 (17)	5.58
P07737	Profilin-1	STP or SDS	384 (261)	15,216	7(7)	8.44
P02787	Serotransferrin	STP or SDS	419 (337)	79,294	12 (10)	6.81
P02768	Serum albumin	STP or SDS	1831 (146)	71,317	33 (5)	5.92
P60174	Triosephosphate isomerase	STP or SDS	363 (286)	31,057	9 (6)	5.65
P25311	Zinc-alpha-2-glycoprotein	STP or SDS	977 (822)	34,465	17 (19)	5.71
Q96DA0	Zymogen granule protein 16 homologue B	STP or SDS	510(467)	22,725	8(6)	6.74

Note: Scores in brackets represent significant sequences derived from SDS rinse.

mean density of $\leq 0.6 \pm 0.2$ g cm^{-3}. Similarly, after exposing the pellicle to 10 mM STP the remaining pellicle had a mean mass of 246 ± 289 ng cm^{-2}, a mean thickness of $\leq 4 \pm 4$ nm, and a mean density of $\leq 0.6 \pm 0.3$ g cm^{-3}. However, when observing the remaining pellicle on the silica sensor after the application of 10 mM SDS and 10 mM STP, a major difference ($p < 0.05$) between the two was observed. After the application of 10 mM SDS the remaining pellicle structure had a mean mass of 113 ± 63 ng cm^{-2}, a mean thickness of 2 ± 1 nm, and a mean density of 0.5 ± 0.2 g cm^{-3}. Whilst after the application of 10 mM STP the remaining pellicle structure had a much higher mean mass (268 ± 38 ng cm^{-2}), mean thickness (5 ± 2 nm), and mean density (0.6 ± 0.2 g cm^{-3}).

FPLC

Whole mouth salivary proteins displaced from a HA column were collected to establish which proteins 10 mM SDS and 10 mM STP were displacing. Typical chromatograms are shown in Figure 6, showing that STP appears to be much more effective at displacing proteins from the HA column than SDS based on the relative areas under the curves. Further STP solution removed proteins as soon as contact was made with the column, whereas the SDS did not displace proteins until after exposure for ~ 5 min. SDS-PAGE of the proteins desorbed from the HA column are also shown in Figure 6 and clearly demonstrate more protein bands from the STP-desorbed fractions than SDS-desorbed fractions, suggesting a greater variety of different proteins are desorbed using STP than SDS.

LC-MS/MS

Analysis of *in vitro* formed pellicle was performed by a combination of chromatography, electrophoretic separation and tandem mass spectrometry. The displacement of salivary proteins from HA using 10 mM STP (see Table 1) showed the presence of 74 proteins from the major bands selected and 35 proteins using 10 mM SDS (see Table 1). Salivary proteins that are commonly found in the *in vivo* pellicle, such as α-amylase, and cystatins, appear to be displaced by both STP and SDS. In addition, the identification of keratin (type II cytoskeletal 2

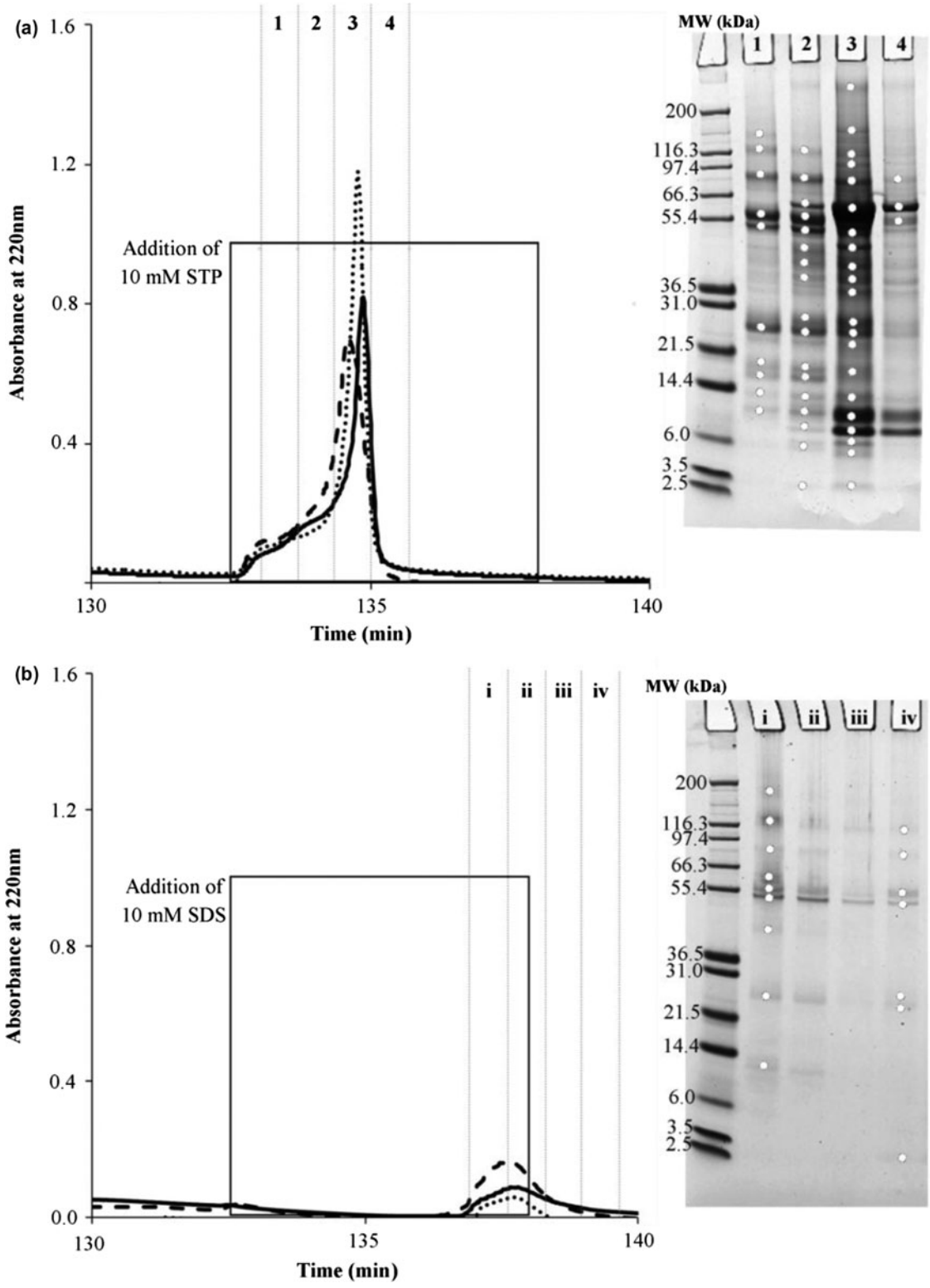

Figure 6. (a) Chromatogram showing the displacement of attached proteins to HA by 10 mM STP (three repeats) and the fractions collected (labelled 1–4); and the accompanying typical electrophoretic profile observed of those fractions separated by SDSPAGE (lanes 1–4) alongside Mark12™ Unstained Standard. (b) Chromatogram showing the displacement of attached proteins to HA by 10 mM SDS (three repeats) and the fractions collected (labelled i–iv); and the accompanying typical electrophoretic profile observed of those fractions separated by SDSPAGE (labelled i–iv).

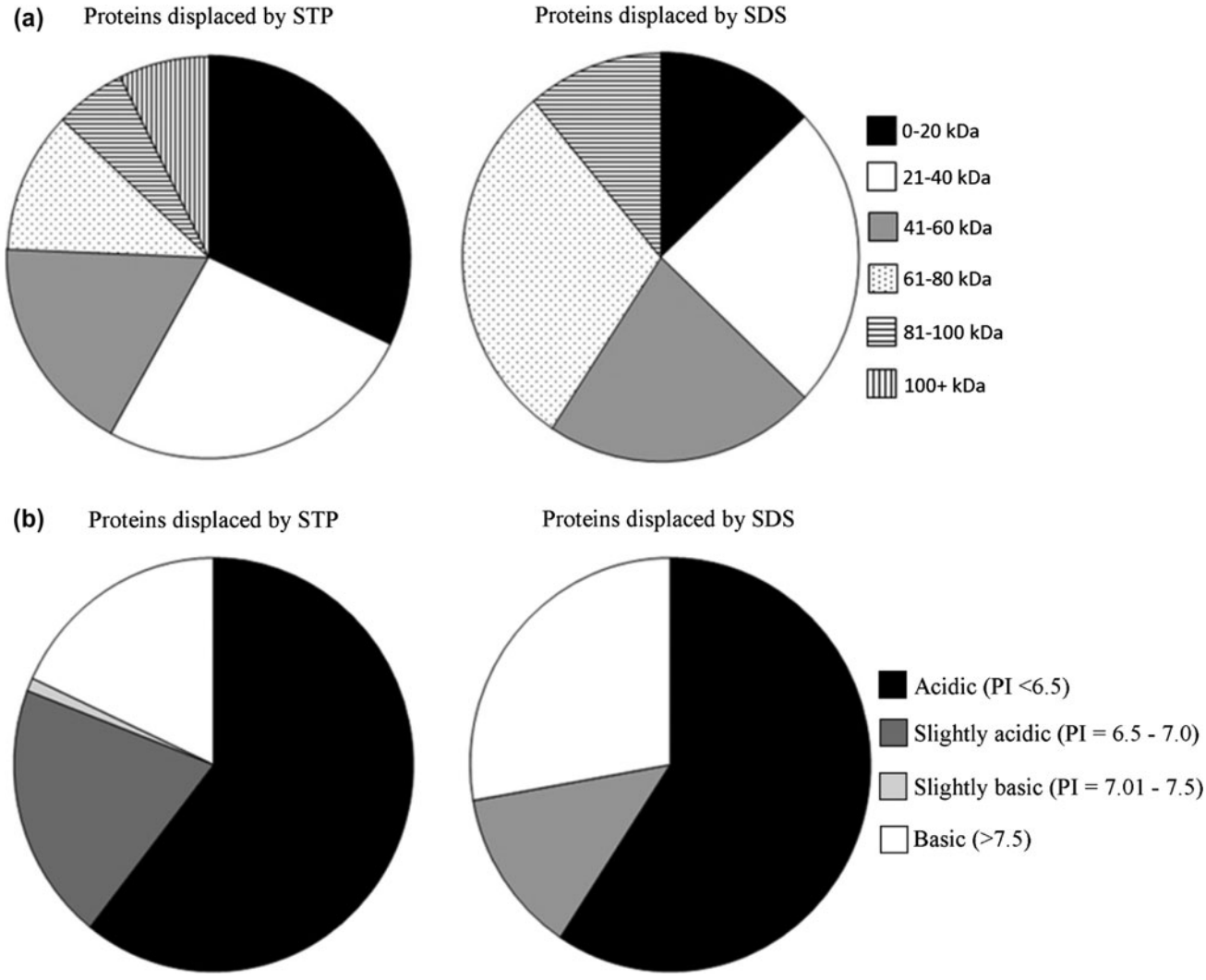

Figure 7. Qualitative classification of the *in vitro* pellicle proteins displaced by 10 mM STP and 10 mM SDS according to (a) molecular weight and (b) isoelectric point. STP displaced significantly more proteins than SDS. The pie charts only show the relative contributions of displaced proteins detected by LC-MS/MS out of 100% and do not represent the total amount of protein displaced.

epidermal) in the *in vitro* pellicle suggests that the oral epithelium may also be a source of pellicle proteins. The presence of a number of enzymes (eg α-amylase, carbonic anhydrase and lactoperoxidase), some of which have been shown to be immobilised in an active conformation in the pellicle layer (Deimling et al. 2004; Hannig, Hannig et al. 2005), highlight the dynamic nature of the salivary pellicle.

The pellicle proteins identified were grouped according to their molecular weight (MW) to distinguish which types of proteins were being displaced when the pellicle was exposed to SDS and STP (See Figure 7a). The STP displaced significantly more protein compared to SDS; and across a wider range of MWs, including a lot of high MW (≥ 100 kDa) proteins. Meanwhile, the SDS displaced fewer proteins, of which most were above 55 kDa. In addition, the proteins were also grouped according to their theoretical isoelectric points (Figure 7b). Considering that the pH value of stimulated saliva can increase up to pH 8 (Fábián et al. 2007), and that most of the proteins identified had isoelectric points below 7, would suggest that the majority of proteins displaced by STP and SDS were acidic in nature.

Discussion

The first part of the present study used QCM-D and the DPI to investigate the physical structure of the salivary pellicle at a solid surface. These techniques made it possible to quantify the adsorption/desorption processes in real time, and observe the response of the pellicle

structure upon exposure to SDS and STP (Dixon 2008; Moore et al. 2011). The initial mass and thickness of the salivary pellicle is much greater (up to 10-fold higher) than would be expected from a monolayer of protein. This agrees with previous work where the adsorbed layer thickness (Barrantes et al. 2014) and adsorbed mass (Macakova et al. 2010) were both indicative of multilayer formation. Proctor et al. (2005) showed that the strength of the pellicle was very sensitive to calcium, yet Ash et al. (2013) showed that calcium does not significantly affect film mass or thickness. This leads to the now widely accepted conclusion that the salivary pellicle is formed initially from a strongly adsorbed primary layer of protein, followed by adsorption of multilayers of protein strengthened by bridging interactions with calcium. Understanding this pellicle structure is important for understanding how additives such as STP and SDS affect the structure and properties of the salivary pellicle. In both instruments 10 mM STP had a more substantial impact on the salivary pellicles adsorbed on the HA coated sensors compared with salivary pellicles adsorbed on to silica sensors. This difference in response was likely to be due to the electrostatic differences between the two surfaces, one negative, ie silica, and one positive, ie HA. It is likely that STP was sequestering cationic calcium ions of the HA coated sensors, and in doing so, was strongly adsorbing to the HA (Kandori et al. 2008). In this way, STP may displace the salivary pellicle indirectly *via* competitive adsorption for the HA surface. However, relying solely on competitive adsorption to explain the displacement of pellicle from the HA surface using STP does not explain the small amount of pellicle displacement from the anionic silica surface that was observed when using STP.

As the STP molecule is polyanionic and the surface of the silica sensor (under the conditions of this experiment) was also anionic, if competitive displacement was the only mode of action, no displacement of pellicle adsorbed to the silica surface would be observed, as STP would be repelled from the negative silica surface. One explanation for this phenomenon could be that STP was interacting with the pellicle directly by sequestering, and thus removing calcium ions that cross-link proteins within the secondary layers of the pellicle. Previously calcium ions have been shown to increase the strength of salivary films (Proctor et al. 2005), and therefore logically their removal from the pellicle *via* STP sequestration may result in a pellicle that is more loosely bound and therefore easier to displace.

SDS on the other hand was shown to displace significant quantities of pellicle from both silica and HA surfaces but, as has been observed in other studies (Hahn Berg et al. 2001; Santos et al. 2010) the displacement was more pronounced on the silica sensor. Although the exact mechanism of protein displacement *via* surfactants is still unclear, a previous study (Mackie et al. 1999) suggested that protein films can be displaced by surfactants by entering defects within the protein network and expanding these areas until the protein network breaks and is displaced, a process termed 'orogenic displacement'. It is well known that SDS binds extensively with proteins (Turro et al. 1995) and thereby provides an exclusively anionic SDS/protein complex, and that this complex would be expected to be strongly repelled from the anionic surface of silica at neutral pH. Therefore it may be hypothesised that the SDS displaced the proteins from the pellicle by interacting directly with the protein rather than the silica surface. Nonetheless, a significant amount of pellicle remained attached to both HA and silica sensors after exposure of the pellicle to 10 mM SDS and 10 mM STP. The remaining pellicle adsorbed onto the HA substratum was larger in mean thickness and mass after exposure to 10 mM SDS than when exposed to 10 mM STP. This agrees with Veeregowda et al. (2012) who also observed that the effects of a phosphate group (sodium hexametaphosphate) had a greater impact on the pellicle adsorbed to HA than SDS. The increase in elasticity (observed in the $\Delta f/\Delta D$ plots) and the concomitant reduction in pellicle density (see Figure 5) after exposure to SDS and STP, would suggest that the viscous component of the pellicle was being removed, whilst the elastic component of the pellicle remained present. This would imply a curious structural transformation from a soft but dense structured pellicle, to a more diffuse pellicle after exposure to SDS and STP; which may be significant given that structural changes in the salivary pellicle have been reported to be an important factor when trying to understand mouthfeel changes observed in people consuming foods that interact with pellicle proteins (Rossetti et al. 2008; Gibbins & Carpenter 2013).

When SDS and STP were exposed to the pellicle adsorbed on the silica surface, a large reduction in the remaining pellicle mass and thickness only took place after exposure to 10 mM SDS (only a small change occurred when the pellicle was exposed to 10 mM STP). It appears that SDS was able to reduce the viscous nature of the pellicle on both the silica and HA surfaces, whereas the STP appeared to leave a more robust pellicle on the silica surface. Unlike SDS, STP is not amphiphilic and cannot interact with pellicle proteins *via* hydrophobic interactions. It is also unlikely to adsorb onto the silica owing to the anionic nature of silica repelling the anionic STP. SDS, on the other hand, is an amphiphilic anionic detergent that although negatively charged has a much lower charge density than the STP molecule, and can therefore directly solubilise proteins within the pellicle regardless of the charge of the sensor the pellicle had been adsorbed to. This was reflected in the results by significant displacement of pellicle from

all sensors when exposed to SDS. However, contrary to this was the lack of protein displacement observed in the second part of this study, where pellicle proteins were exposed to 10 mM SDS and 10 mM STP *via* HA chromatography.

Rykke et al. (1990) also observed a lack of protein (albumin) displacement from HA when exposed to SDS, suggesting that the hydrocarbon tail of SDS lowered the desorbing potential of the molecule. If this was indeed the case, it would suggest that the interaction between pellicle proteins adsorbed onto the HA coating of the QCM-D and DPI sensors is more susceptible to protein displacement than that for the HA of the FPLC column. Another issue is the substantial difference in the surface area to volume ratio of the HA column compared to the DPI/QCMD sensors that could account for the differences observed. That is, the relatively constrained surface area of the sensors (< 0.00005 m^2) may have resulted in multilayer formation of protein on the sensors, due to the large amount of protein available to adsorb onto a small surface area. However, with a surface area of 0.095 m^2 in the HA packed column (>1000 times greater than that of the sensors), much less protein is available to adsorb per unit area compared with the DPI/QCMD sensors, therefore less multilayer formation could have occurred. This would result in a greater proportion of protein being directly and strongly adsorbed to the HA substratum. The differences in the amount of protein desorbed from powder and sensors could then be rationalised in terms of multilayer protein being relatively easier to desorb. Other studies (Hahn Berg et al. 2001; Santos et al. 2010) have also shown that SDS was less effective at protein displacement on HA surfaces when compared to silica. This compliments the observation of minute quantities of pellicle displacement when the SDS was exposed to the pellicle adsorbed onto HA *via* FPLC. STP, on the other hand was very effective in displacing proteins from all HA surfaces and this was reflected particularly well in the ion exchange chromatogram Figure 6a.

The observation that the majority of proteins displaced by SDS and STP were acidic in nature implies that the negatively charged sulphate group of SDS, and the negatively charged phosphate groups of STP, were where the key interactions between the cationic calcium ions of the HA surface and the adsorbed proteins were taking place. This interaction was much stronger for STP than it was for SDS. However, the high concentration of acidic proteins identified could be a consequence of the high concentration of acidic proteins throughout the pellicle (Siqueira & Oppenheim 2009) as opposed to any anionic (SDS) cationic (Ca^{2+}) interaction.

In addition, histatin and statherin, two proteins commonly found in the pellicle, did not appear to be displaced by either SDS or STP. It may be that these proteins are more tightly bound to HA compared to other pellicle proteins. However, it is also possible that these proteins were not detected by the mass spectrometer, as the high number of arginine and lysine residues in the N-terminal region of these proteins will produce small sized peptides with m/z values below 300, rendering them undetectable with a trypsin digest regime, a problem also observed by Siqueira et al. (2007). The use of a different digest enzyme, such as endoproteinase Lys-C may have allowed detection of these unidentified proteins.

It should also be noted that the presence of keratin in MS analysis is often an indicator of sample contamination. However, in this case it may be that intra-oral keratin from desquamated oral epithelial cells has incorporated into the pellicle, as was also observed by Yao et al. (2003). However, confirmation of the source of the keratins lies beyond the scope of this work.

As with most biological samples, complications in the analysis of data occurred as a consequence of the high variability in the adsorbed mass of pellicle observed between individuals; as has also been recorded in other studies (Ash et al. 2014). This was highlighted by the high standard deviation observed for the mean WMS pellicle that adsorbed onto both QCM-D and DPI HA sensors ($1{,}215 \pm 289$ ng cm^{-2} and $1{,}390 \pm 731$ ng cm^{-2} respectively). These differences are likely to be a consequence of the variable composition of the volunteers' saliva, a common difficulty encountered in salivary research (Jehmlich et al. 2013). The variability in measurements of biological samples such as saliva can conceal real trends in datasets when sample sizes or numbers of data points are relatively few. To increase the overall power of the system under study, the data from PS formed pellicles was combined with that of WMS formed pellicles. Increasing the number of datasets in this way confirmed whether trends in the data were statistically significant (Bausell & Li 2002). This was considered valid as the main aim of the study was to understand the impact of STP and SDS on the adsorbed pellicle as opposed to differences in PS and WMS pellicle.

Other differences in pellicle structure arose due to the physical and chemical properties of the sensors used in this study. For example, it has been shown that the protein profiles of the salivary pellicle adsorbed onto HA *in vitro* can differ from the salivary pellicle adsorbed onto enamel *in vivo*, even when formed from the same saliva (Carlén et al. 1998; Yao et al. 2001). However, proteins identified *in vivo* (Lendenmann et al. 2000; Yao et al. 2001) were also shown to be present *in vitro*, thus validating the system used in the current study.

It is also known that protein adsorption to a solid surface is affected by a number of parameters, such as the surface roughness of the sensor, or the hydrophobicity of the sensor (Rabe et al. 2011). As the DPI HA and silica sensors used in this study varied in a number of

physical parameters, it was not possible to determine with certainty whether the saliva sample, or the substratum of the sensor, had the greater effect on the amount of pellicle proteins adsorbing to the senor surface. However, and importantly for the interpretation of the results in this study, the chemical properties of the sensors remained consistent. That is to say that both DPI and QCM-D HA sensors would have remained positively charged and the silica sensors would have remained negatively charged at the pH used in these experiments. It was this phenomenon that was exploited to distinguish the modes of action by which the polyanionic STP and amphiphilic SDS displaced pellicle from the surface of the HA and silica sensors. In conclusion, the interaction of the polyanionic molecule STP with the salivary pellicle is strongly influenced by the electrical charge of the surface that the pellicle has adsorbed to. For example, STP removes pellicle from HA *via* competitive adsorption for the cationic calcium ions on the HA surface, and by sequestering calcium ions that cross link proteins within the pellicle. However, STP is less effective when removing pellicle from silica surface mainly due to electrical repulsion from the silica. Conversely, SDS was affected less by the surface that the pellicle had adsorbed onto and was able to displace significant quantities of pellicle adsorbed on both the HA and silica surfaces *via* a direct interaction with the pellicle. However, the desorbing potential of SDS was shown to be less effective on pure HA compared to HA-coated QCM-D and DPI sensors, and may be due to differences in the surface area to volume ratio of the HA powder compared to the constrained surface area of the HA sensors.

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