Supplementary Material

Acquired enamel pellicle modification with Casein and Mucin in different concentrations and its impact on initial dental erosion

Sieber K.R.[a], Schmidt C.[a], Rakhmatullina E.[a], Baumann T.[a*], Lussi A[a], Carvalho T.S.[a]

Department of Preventive, Restorative and Pediatric Dentistry, University of Bern, Freiburgstrasse 7, CH-3010, Bern, Switzerland.

* Corresponding Author

Tommy Baumann (✉)

Department of Restorative, Preventive and Pediatric Dentistry,

University of Bern, Freiburgstrasse 7, CH-3010, Bern, Switzerland.

Tel.: +41 31 632 86 02

Fax: +41 31 632 98 75

E-mail: tommy.baumann@zmk.unibe.ch

Materials and Methods

Protein aqueous solutions

Stock solutions of pig gastric mucin (MUC5AC) and whole casein were prepared in ultrapure water and the pH set to 7.0. The solutions were centrifuged 3 times for 5 min at 5000 g (10°C). The supernatants were collected and stored at 2–8°C. The protein concentration in the supernatants was measured using a NanoDrop spectrophotometer (ND-1000; Thermo Scientific, USA). The final casein, mucin, and casein-mucin solutions were prepared by mixing and/or diluting these stock solutions to the desired concentrations.

Dynamic light scattering (DLS)

DLS analysis was carried out using a Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern, UK) at a scattering angle of 90°.The experiment was performed at 25°C.

Negative stain transmission electron microscopy (NS-TEM)

NS-TEM images were recorded with a Philipps CM-10 transmission electron microscope at 80 kV using a 2 k × 2 k CCD camera (Olympus SIS, Münster, Germany). A total of 3.5 µl of a 0.5 wt.% aqueous solution of casein, a 0.27 wt.% aqueous solution of mucin and a casein-mucin mixture (1:1, v:v) was adsorbed for 1 min on glow discharged (20 s) carbon film-coated copper grids, washed once with distilled water and stained twice with 2% uranyl acetate for 12 s. Excess liquid was removed with filter paper between all preparation steps.

Reconstituted human saliva

Reconstituted human saliva was used in QCM-D experiments. A total of 40 mg of lyophilized human pooled saliva was dispersed in 20 ml of adhesion buffer (50 mM KCl, 50 mM K_2HPO_4, 50 mM KH_2PO_4 and 50 mM $CaCl_2$ in ultrapure water at pH 6.8) and kept at RT under constant stirring (450 rpm) for 30 min. The solution was centrifuged once at 6500 rpm for 5 min at 10°C. The protein content in the supernatant was analyzed using a NanoDrop spectrophotometer (ND-1000). The freshly prepared reconstituted saliva was immediately used for the QCM-D experiment.

Quartz crystal microbalance with dissipation mode (QCM-D)

The QCM-D module (E1; Q-sense, Gothenburg, Sweden) was run at constant flow rates of 500 µl/min, resulting in estimated shear rates of 4.7 s^{-1}. This setup operated at a fundamental

frequency of 5 MHz, and the 3rd, 5th, 7th, 9th, 11th and 13th overtones were used, which corresponded to resonance frequencies of 15, 25, 35, 45, 55 and 65 MHz, respectively. QCM sensors with gold coating (QSX301) were purchased from LOT-Oriel AG (Romanel-sur-Morge, Switzerland). Adhesion buffer (50 mM KCl, 50 mM K_2HPO_4, 50 mM KH_2PO_4 and 50 mM $CaCl_2$ in ultrapure water at pH 6.8) was pumped over the sensors until the QCM yielded a constant baseline for ΔD and Δf. The sensor surfaces were then exposed to protein solutions (30 min) or reconstituted saliva (1 h). Next, the sensors were again perfused with adhesion buffer until constant ΔD and Δf values were obtained. Subsequently, protein solutions were pumped over the protein or pellicle coated sensors. Once again, adhesion buffer was pumped through the chambers containing sensors until the values of ΔD and Δf were constant. All experiments were carried out at 25°C. The adsorbed mass and viscoelasticity of the salivary pellicle before and after incubation with proteins was determined using the Voigt model provided in the software program QTools 3.0 (Q-sense, Gothenburg, Sweden). The QCM-D results for the adsorption of casein, mucin and the casein-mucin mixture were fitted using a Sauerbrey model.

Results and discussion

Casein, mucin and casein-mucin mixtures in aqueous solution

A high prevalence of large assemblies (>80%) was found in both casein and mucin aqueous dispersions using DLS analysis (Table 1). Mucin samples contained aggregates of 900 nm on average, whereas casein samples contained aggregates of 300 nm. The mixture of casein and mucin resulted in aggregate sizes of 400 nm, which was closer to the sizes of the casein assemblies alone (Table 1). The reduction of the total protein concentration in the casein-mucin mixtures from 0.77 wt.% to 0.50 wt.% and to 0.27 wt.% (at the same casein-to-mucin ratio) did not affect protein assembly significantly. Hydrophobic interactions and formation of hydrogen bonds between casein and mucin could induce formation of a casein-mucin complex and, as a result, the loss of macromolecular hydration, which, in turn, could affect the sizes determined by DLS.

TEM images of the casein solution revealed chain-like structures with a length of 100-600 nm and a diameter of the individual particles of 50-70 nm (Figure 1A, arrows), which correlates well with published results for casein micelles [de Kruif, 1998; Marchin et al., 2007; Trejo et al., 2011]. These structures were branched or folded. Images of aqueous mucin showed loose polymer coils and kinks of varied sizes, although the diameters of the observed structures were at the resolution limit of negative stain preparations (2 nm, Figure 1B,

arrows). When casein and mucin were mixed, dense globular, but heterogeneous, nano-particles were formed with typical diameters of 25 nm (Figure 1C).

Casein, mucin and casein-mucin mixture on model gold substrates

QCM-D was used to monitor dissipation and frequency change in the sensor during protein adsorption. Changes in the resonance frequency (Δf) show mass uptake or release at the QCM sensor surface, with shifts to lower values indicating more proteins adsorbed to its surface. Changes in the dissipation factor (ΔD) reflect the viscoelasticity of the adsorbed layer. The injection of casein, mucin and the casein-mucin mixture resulted in the adsorption of 858±83, 435±40 and 662±13 ng/cm^2 of protein, respectively (Table 2). Adsorption of casein micelles showed rapid surface saturation (Figure 2) and larger adsorbed masses than in the case of mucin (Table 2). We suggest that the fast adsorption plateau indicated formation of a "monolayer" of casein aggregates with a reversibly bound second layer that could be desorbed by subsequent washing. The remaining "monolayer" of casein was less fluffy than the mucin coating (Table 2). The frequency change in the case of mucin did not reach a saturation plateau and showed slow adsorption kinetics (Figure 2). Slow continuous adsorption of mucin has previously been observed on hydrophobic and hydrophilic surfaces [Lindh et al., 2002]. While showing the least adsorbed mass, mucin had the highest ΔD/Δf value among the coatings (Table 2), indicating formation of a viscous soft layer.

Interestingly, if mucin was adsorbed first and was followed by casein, a mass increase of over 70% was detected. At the same time, in the viscoelastic properties (ΔD/Δf) decreased, indicating a denser, more rigid coating structure than the one seen before casein injection (Table 2). Because casein micelles are small, show rapid adsorption and seem to have a higher affinity for the gold sensor surface, they could potentially be adsorbed on the vacant surface sites between bulky mucin macromolecules, as well as become entrapped in the interior of the mucin layer or adsorbed on its surface.

When the adsorption sequence was changed, i.e. casein was adsorbed first and then followed by the injection of mucin, completely different observations were made. Approximately 15% of the coating mass was lost relative to the original casein mass (Table 2). It seemed that the interaction of mucin with the casein layer was not favorable and the adsorption onto the gold surface became impossible because of the presence of the previously adsorbed casein.

The flow of premixed casein-mucin solution over the sensor resulted in an adsorbed mass and viscoelastic properties comparable to the results of sequential adsorption of individual proteins, although the ΔD/Δf value was even lower than that when the proteins were injected sequentially, indicating an even stiffer layer (Table 2). Because the adsorption curve of the

casein-mucin mixture was similar to that of casein alone (Figure 2), we assume that the adsorption process was mostly determined by the interaction of casein with the sensor surface. Casein micelles adsorbed fast, while casein bound to mucin would probably also promote the adsorption of the entire complex owing to the interaction of casein with the gold surface. The anchoring of the unbound free mucins from the casein-mucin mixture is thought to be unlikely, taking into account poor mucin adsorption on the sensor surface and the fact that there is nearly no adsorption on the casein layer.

Interaction of casein, mucin and casein-mucin mixture with in vitro salivary pellicle

To test the hypothesis that protein is incorporated into the salivary pellicle, QCM-D experiments were carried out. The injection of reconstituted human saliva led to a gradual mass adsorption up to an average of 504 ± 42 ng/cm^2 and greater ΔD/Δf values than were found in the analysis of single proteins (Table 3). Subsequent injection of casein and mucin resulted in a mass loss (-9% and -18%, respectively) at approximately the same ΔD/Δf of the total layer as found before the addition of the protein. The flow of the casein-mucin mixture over the pellicle layer induced an increase in mass of 2% and an increase in the viscoelastic properties of the layer (Table 3).

The mass loss in the case of mucin addition is most likely to have resulted from interactions with mucin macromolecules, as some salivary proteins such as proline-rich proteins, histatin, statherin and lactoferrin interact with human mucins [Senapati et al., 2010]. These proteins are components of the salivary pellicle and can be exchanged by the mucins over a certain period of time [Svendsen et al., 2008]. Thus, QCM-D experiments did not confirm the incorporation of mucin into the salivary pellicle. The lack of interactions between mucins and the salivary pellicle explains the lack of effect of this protein on the erosion-inhibiting potential of the *in vitro* salivary pellicle as found in a previous study [Cheaib and Lussi, 2011].

The minor decrease in the adsorbed mass after the addition of casein (Table 3) could be related to the desorption of some salivary components during the continuous flow.

The increase in pellicle viscoelasticity after the injection of the casein-mucin mixture (Table 3) could be due to the deposition of casein-mucin complexes. While their limited number at the pellicle surface and simultaneous desorption of salivary proteins by non-complexed casein and mucin would not lead to significant changes in the adsorbed mass, the adsorption of these clusters could cause the observed increase in the ΔD/Δf value and an overall increase in the layer thickness and thus provide a better barrier between dental tissue and the acidic environment.

Due to technical reasons, the QCM-D measurements had to be performed on a gold surface and could not be done on enamel. Although these two materials have different surface properties, and the pellicles formed on them would not be completely similar, we belief that the results can be used as a proxy of what would happen at the enamel surface and pellicle.

Summary and Conclusions

The results suggested the formation of a complex between casein and Mucin. Casein adsorbed readily on gold and salivary pellicle surfaces, showing fast adsorption kinetics. Casein structures with sizes of 50-70 nm were found in TEM. The assembly of mucin and its interaction with surfaces was affected by the presence of casein. In particular, the addition of casein promoted the adsorption of mucin on the salivary pellicle and gold surface. The findings confirmed the hypothesis that casein promotes the interaction of mucin with the salivary pellicle. This could lead to the previously observed improvement of the erosion-inhibiting property of the salivary pellicle [Cheaib and Lussi, 2011]. Furthermore, the adsorption of casein-mucin aggregates could also obstruct some of the specific binding sites for the adhesion of oral bacteria at the pellicle surface, contributing to less colonization by cariogenic bacteria [Cheaib et al., 2015].

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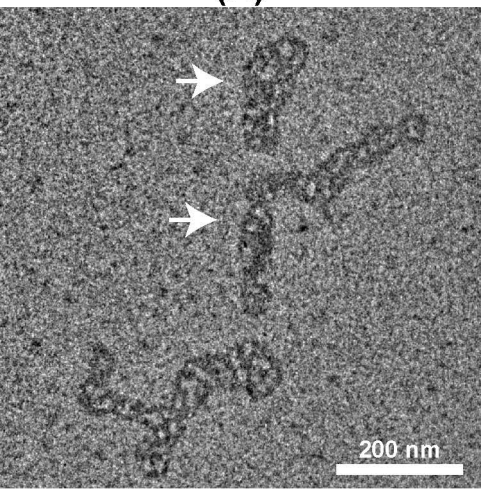
Figure captions

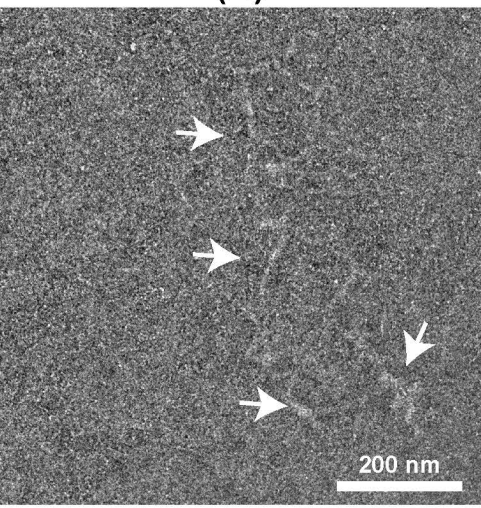
Figure 1. Negative stain TEM images of (A) casein, (B) mucin and (C) a casein-mucin mixture prepared from buffer solutions and adsorbed on hydrophilic carbon films. Note that the casein-mucin composite nanoparticles were not homogeneous.

Figure 2. Change in resonance frequency (n=5) during adsorption of casein (0.5 wt.%), mucin (0.27 wt.%) and casein (0.5 wt.%)-mucin (0.27 wt.%) mixture. Buffer rinse was applied after the adsorption of each of the components.

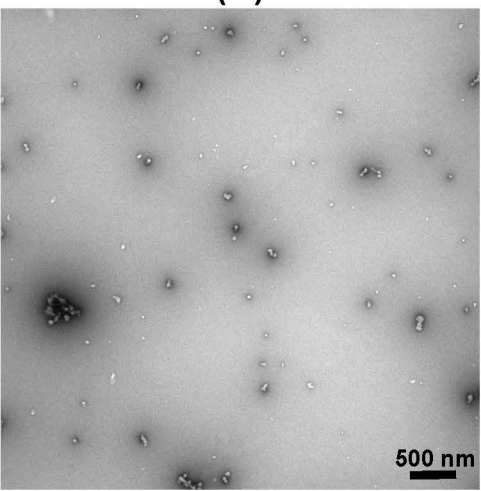
(A)



(B)



(C)



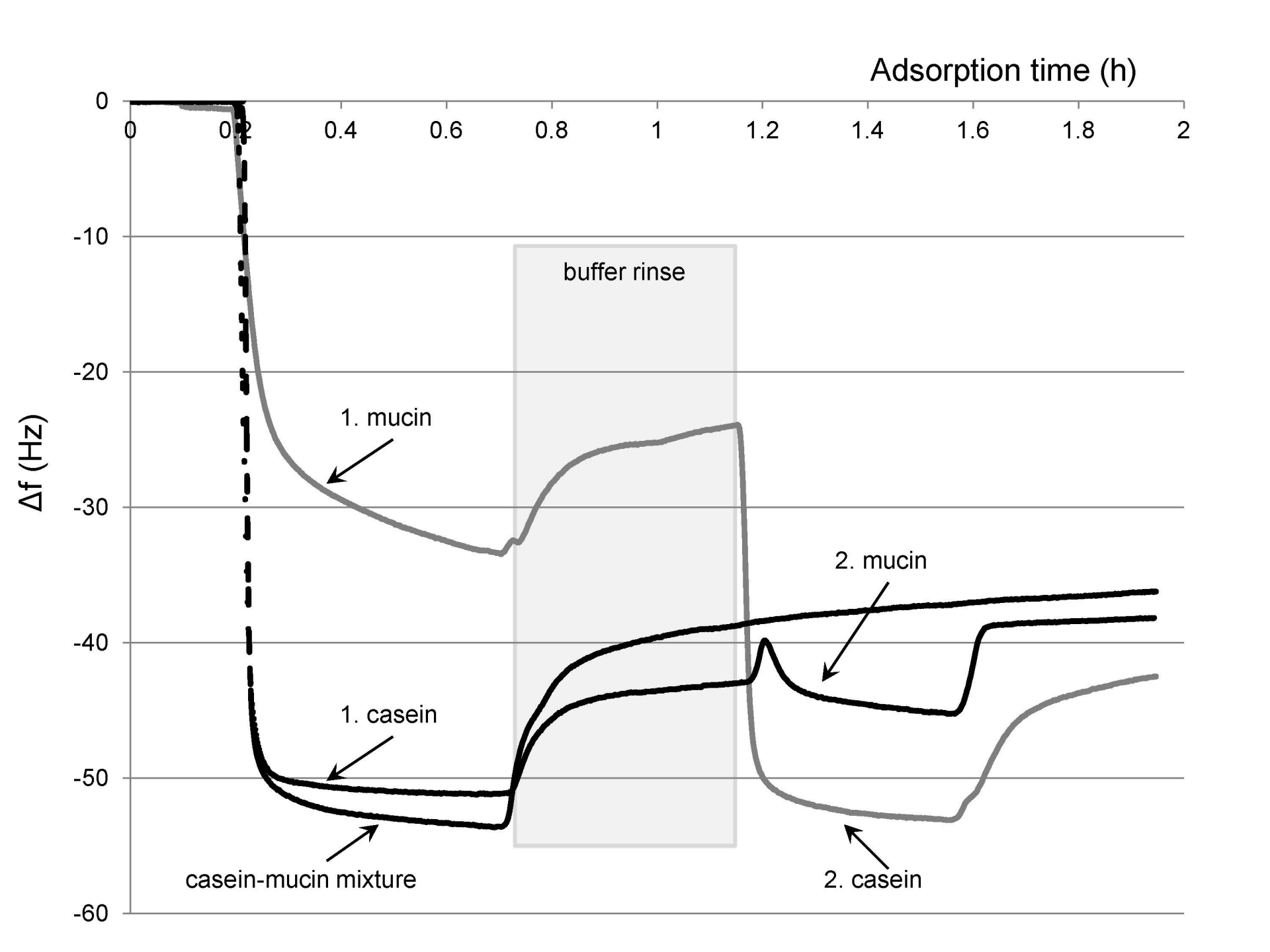

Adsorption time (h)
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
-10
-20
-30
-40
-50
-60
Δf (Hz)
buffer rinse
1. mucin
2. mucin
1. casein
casein-mucin mixture
2. casein

Table 1. Sizes of the self-assembled aggregates in casein, mucin and casein-mucin aqueous solutions (DLS).

Sample	Peak 1 (nm)	Peak 2 (nm)	Intensity (%)	
			Peak 1	Peak 2
Casein (0.50 wt.%)	20	300	16	84
Mucin (0.27 wt.%)	50	900	11	89
Casein-mucin (0.77 wt.% total)	40	400	23	77
Casein-mucin (0.50 wt.% total)	35	390	28	72
Casein-mucin (0.27 wt.% total)	35	370	31	69

Table 2. Adsorbed mass of proteins at the surface of the QCM-crystal and change in ΔD/Δf values. *Numbers correspond to the sequence in which proteins were injected.

QC modification	ΔD/Δf	Mass at the surface (ng/cm^2)
Casein (layer 1)	4.40 ± 0.06E-8	858 ± 83
Mucin (ad-layer 2)	3.43 ± 0.42E-8	721 ± 30
		Δm = -129 (-15%)*
Mucin (layer 1)	6.80 ± 0.64E-8	435 ± 40
Casein (ad-layer 2)	4.60 ± 0.35E-8	745 ± 44
		Δm = 310 (+71%)*
Casein-mucin mixture	3.66 ± 0.66E-8	662 ± 13

Table 3. Change in mass and ΔD/Δf of the salivary pellicle upon addition of casein, mucin and a casein-mucin mixture. Salivary pellicle was formed by the injection of reconstituted human saliva. Averages from 3 experiments are shown.

QC modification	ΔD/Δf	Mass at the surface (ng/cm^2)
In vitro salivary pellicle	1.56 ± 0.2E-07	504 ± 42
Salivary pellicle + casein	1.54 ± 0.10E-07	460 ± 12
Salivary pellicle + mucin	1.50 ± 0.10E-07	412 ± 21
Salivary pellicle + casein-mucin mixture	1.81 ± 0.11E-07	513 ± 75