Supplementary Material

Pellicle modification with casein and mucin does not affect surface loss from erosion and abrasion

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Materials and Methods

Preparation of polished human enamel specimens

Enamel specimens were prepared from caries-free human molar teeth extracted by dental practitioners in Switzerland. Before the extraction, the patients were informed about the possible anonymous use of their teeth for research purposes and their consent was obtained. All teeth were stored in 1% chloramine T trihydrate solution after extraction. Because we were using teeth from a pool of extracted teeth, the local ethics committee (Kantonale Ethikkommission: KEK) categorized them as “irreversibly anonymized;” therefore no ethical approval was necessary. Flat ground enamel specimens with a 200 µm cut-off layer were prepared from the buccal sides of teeth as previously described [1]. Samples were stored in a mineral solution (1.5 mM $CaCl_2$, 1.0 mM KH_2PO_4, 50 mM NaCl, pH 7.0) [2] and underwent further polishing with a 1 µm diamond abrasive (60 s, LaboPol-6, DP-Mol Polishing, DP-Stick HQ, Struers) immediately before the experiment.

Protein aqueous solutions

The exact procedure for the preparation of aqueous dispersions (pH 7.0) of casein and mucin was adopted from Cheaib et al [3]. Briefly, 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.

Pooled human saliva

Paraffin wax-stimulated whole saliva was collected from 25 healthy donors with no signs of gingivitis or active caries. The donors provided their informed oral consent to use the saliva for research purposes in this study. The collected saliva was pooled and centrifuged at

4000 g for 20 min at 4°C. The supernatant was divided into small aliquots and stored at −80°C. Salivary aliquots were thawed immediately before the experiment. No ethical approval was necessary because the saliva pool was categorized as "irreversibly anonymized", and the experiment was carried out in accordance with the approved guidelines and regulations of the local ethics committee (Kantonale Ethikkommission: KEK).

Protein and salivary pellicle coatings on human enamel

Polished human enamel samples were incubated in pooled human saliva for 1 h at RT, followed by immersion in casein (0.5 wt.%), mucin (0.27 wt.%) or the casein-mucin mixture (1:1, v/v) for 1 h at RT. Samples were then dip-and-rinsed in three portions of fresh deionized water and left to dry at RT.

Atomic force microscopy (AFM)

Tapping-mode AFM imaging was performed using a PycoLE system, Molecular Imaging Inc., and silicon nitride cantilevers (k = 42 N/m, 1 line/s). Images were recorded in height, amplitude and phase mode with a size of 512 × 512 pixels. Height images were flattened and plane adjusted. Height (topography) images were further analyzed using Gwyddion software. Human enamel coated by the proteins and salivary pellicle was prepared as described above.

Results

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

The polished enamel had numerous scratches from the polishing procedure and a rough surface (Fig. 1A). Formation of the salivary pellicle resulted in a layer with a typical globular topography and still-visible scratches on the polished enamel tissue underneath (Fig. 1B). The incubation of the pellicle layer in mucin solution did not result in a visible change in

the pellicle topography, where a similar globular morphology and a few large particles were found (Fig. 1C). Modification of the salivary pellicle with casein solution resulted in the appearance of ring-like structures on the globular surface (Fig. 2A-C). These spherical structures were approximately 120-150 nm in diameter and 9-10 nm in height (Fig. 2D), and homogeneously distributed. Chain-like aggregates of these ring-like structures were also observed (Fig. 2B), which looked similar to TEM images of the casein solution found in an earlier study (suppl. figure of Kevin and Christoph study). Application of the casein-mucin mixture to the pellicle layer caused rather inhomogeneous deposition of large (2-3 µm) agglomerates of rods and particles in certain areas of the samples (Fig. 2E). The dimensions of the ring-like structures in these agglomerates were similar to those of the casein particles at the pellicle surface (Fig. 2C).

Discussion

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

The exposure of the *in vitro* salivary pellicle to a casein-mucin mixture has been shown to improve its erosion-inhibiting properties [3] and to suppress initial colonization by cariogenic bacteria [4]. In the current study, we were interested to elucidate the possible assembly of a casein-mucin complex at the pellicle surface to explain the earlier results. Because the salivary pellicle itself has an uneven knotted surface [5, 6] and it is naturally and easily formed on dental tissues, human enamel was used in the AFM experiments. The prepared enamel showed numerous scratches from the polishing procedure (Fig. 1A). Incubation in pooled human saliva led to the formation of a salivary pellicle, which covered most of the scratches (Fig. 1B). The globular topography of the salivary pellicle observed in this study was in agreement with the results of other studies in the field [7, 8]. No threads of mucin were found after it had been incubated in a mucin aqueous solution (Fig. 1C). Large aggregates appeared on the pellicle

surface; however, the overall morphology of the pellicle did not change much. It can be suggested that negatively charged mucin either did not adsorb onto the negatively charged salivary pellicle [5], or it was incorporated inside the layer through hydrophobic interactions, remaining undetectable in AFM images.

In contrast to mucin, casein micelles and their aggregates were found throughout the pellicle surface (Fig. 2A, 2B). The ring-like appearance of the casein structures and the shapes of their aggregates corroborated well with TEM images of the casein dispersion reported earlier (suppl. figure of Kevin and Christoph study). The 120-150 nm diameter of the micelles was in good agreement with other AFM investigations of dry casein samples [9], whereas a high diameter-to-height ratio (Fig. 2C, 2D) was typical for the soft particles after drying [10]. The source of the ring-like, rather than spherical, shape of the structures remained unclear unless inversion of the micelles occurred at the relatively hydrophobic pellicle surface or drying caused collapse of water channels inside the casein micelle [11, 12] and a change in the density distribution within the particle. The large size of the aggregates (3–4 μm, Fig. 2E), which were observed on the pellicle after its exposure to the casein-mucin mixture, was found in a dry state where disintegration of the protein complex or flattening could take place. These clusters looked similar to the AFM images of a dry mucin-chitosan complex on mica [13].

References

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Figures

Figure captions

Figure 1. Examples of surface topography (dry state) imaged by AFM: (A) polished human enamel; (B) *in vitro* salivary pellicle prepared at the enamel surface and (C) salivary pellicle surface after incubation in a mucin dispersion (0.27 wt.%).

Figure 2. Topographies of the *in vitro* salivary pellicle (dry state) after incubation (1 h) in: (A-C) casein (0.5 wt.%) and (E) a casein (0.5 wt.%)-mucin (0.27 wt.%) mixture. Two scanned areas of the salivary pellicle exposed to a casein dispersion are shown (A and B) demonstrating ring-like structures (A) as well as their large aggregates (B). Magnification of the ring-like casein structures (C) and a cross-sectional analysis of the particle's width and height (D). Arrows indicate ring-like structures.

Figures

Figure 1

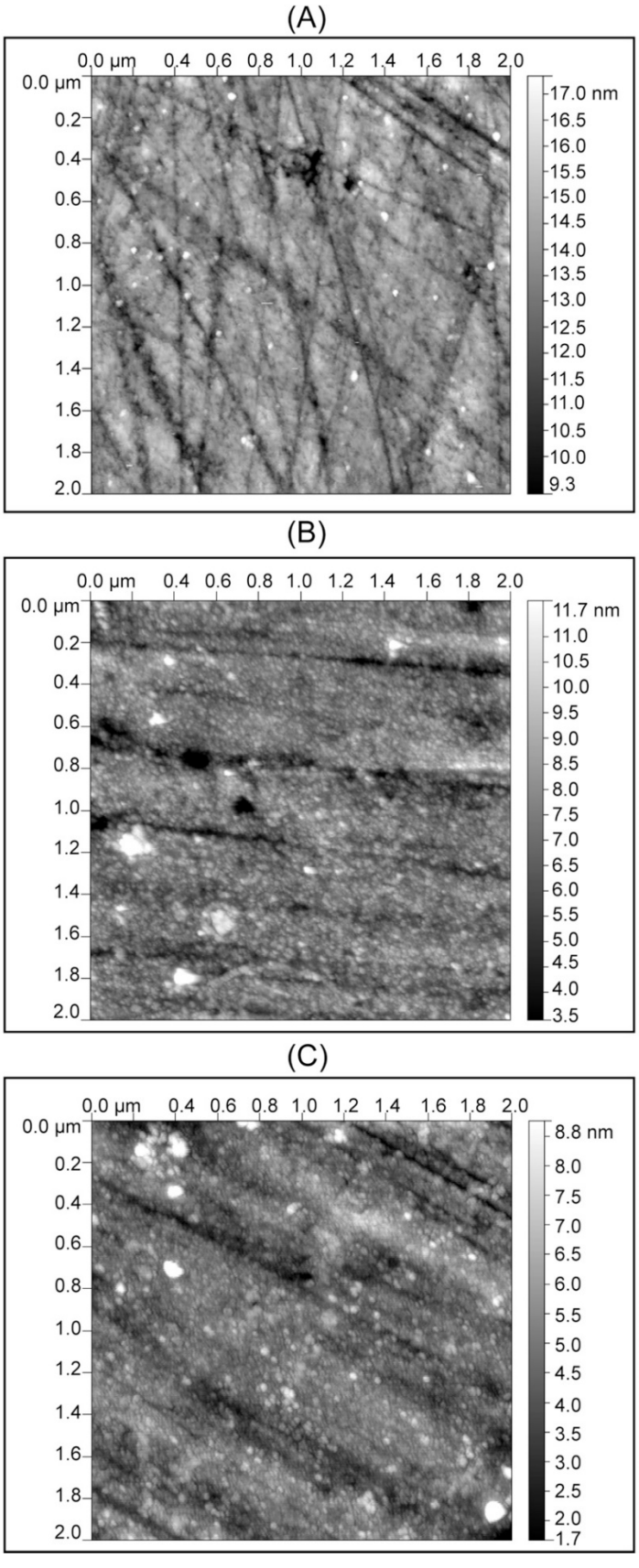
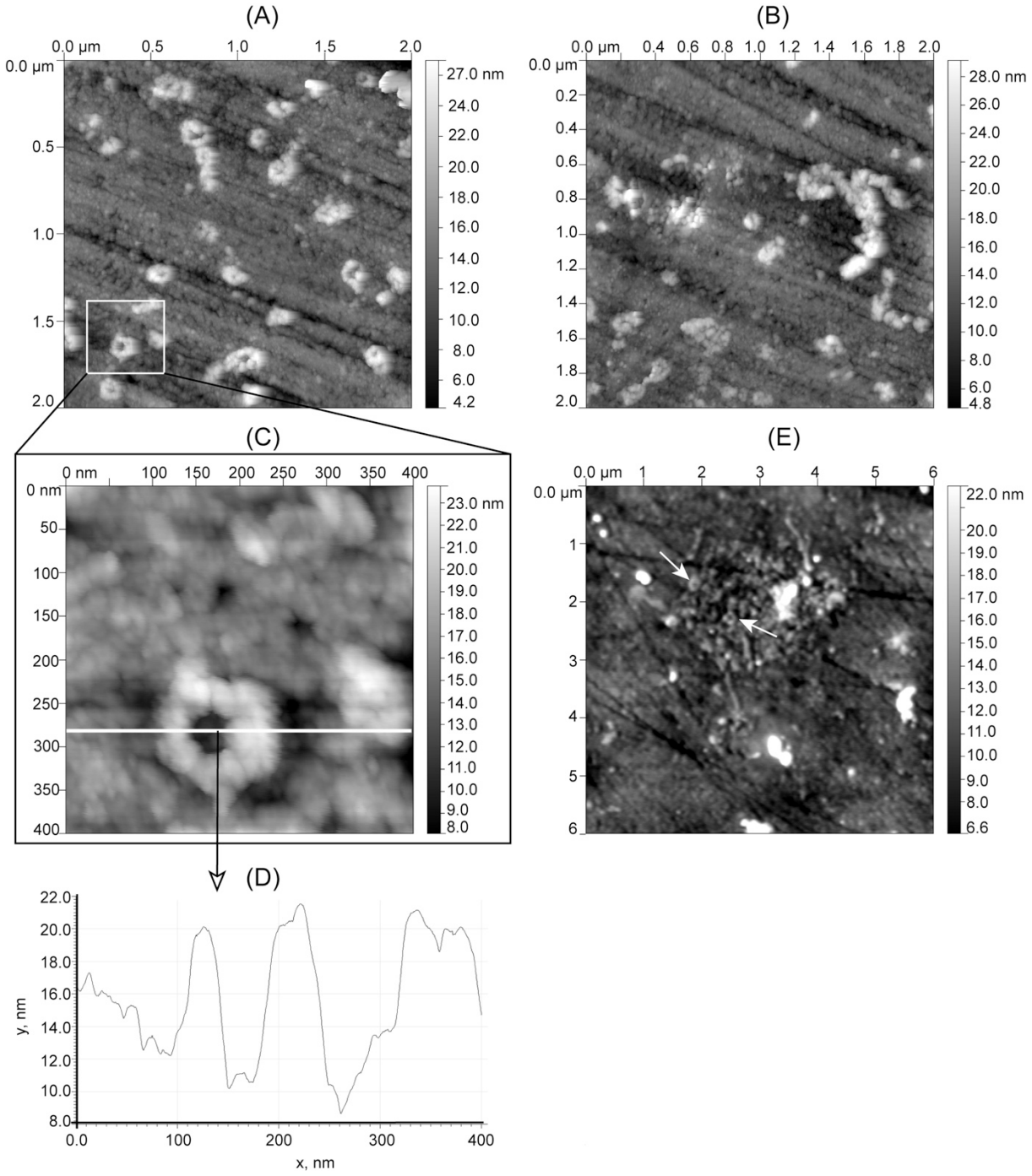


Figure 2


(A)
(B)
(C)
(D)
(E)
0.0 µm
0.5
1.0
1.5
2.0
27.0 nm
24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.2
0.4
0.6
0.8
1.2
1.4
1.6
1.8
28.0 nm
26.0
4.8
0 nm
100
150
200
250
300
350
400
50
23.0 nm
21.0
19.0
17.0
15.0
13.0
11.0
9.0
y, nm
x, nm
1
2
3
4
5
6
22.0 nm
6.6