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Ultrastructural changes of bovine tooth surfaces under erosion in presence of biomimetic hydroxyapatite

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Enamel and dentin are susceptible to acids from food sources leading to dental erosion, a global problem affecting millions of individuals. Particulate hydroxyapatite (HAP) on the tooth surface can influence the effects of acid attacks. Standardized bovine enamel and dentin samples with artificial saliva are used in an in vitro cyclic demineralization–remineralization protocol to analyze the structural changes experienced by tooth surfaces using high-resolution scanning electron microscopy and to evaluate the potential of a HAP-based oral care gel in the protection of teeth from erosive attacks. The interfaces between HAP particle and enamel HAP crystallites are investigated using focused ion beam preparation and transmission electron microscopy. The results show that erosion with phosphoric acid severely affects enamel crystallites and dentin tubules, while artificial saliva leads to remineralization effects. The HAP-gel forms a microscopic layer on both enamel and dentin surfaces. Upon acid exposure, this layer is sacrificed before the native tooth tissues are affected, leading to significantly lower degrees of demineralization compared to the controls. This demonstrates that the use of particulate HAP as a biomaterial in oral care formulations can help protect enamel and dentin surfaces from erosive attacks during meals using a simple and effective protection principle.

Keywords: biomimetic materials/erosion/hydroxyapatite/remineralization/teeth

1. Introduction

Human teeth are a fascinating biological material, a constant concern regarding public health, and an an economically relevant factor of global importance for human society.[1,2] Structurally, they consist of a dentin core covered by an enamel layer in the crown area. About 95–97 wt% of dental enamel consists of 30–50 nm wide and several micrometers long crystallites consisting of carbonated hydroxyapatite (cHAP). These are densely packed in parallel bundles forming columnar prisms surrounded by interprismatic enamel.[3,4] A small amount (~1 wt%) of soft organic material forms thin sheaths around the prisms and a discontinuous organic matrix between the mineral crystallites.[5,6] This delicate hierarchical organization makes enamel remarkably resistant against crack propagation and resulting fracture.[7] The enamel cap surrounds a body of dentin, a nanocomposite consisting of hydroxyapatite (HAP) crystallites embedded in a hydrated fibrous collagen-rich matrix. The macroscopic architecture of dentin is characterized by the presence of numerous parallel dentin tubules with diameters of ~1 µm, which are surrounded by a thin layer of collagen-free peritubular dentin and embedded in radially oriented, mineralized collagen fibril bundles.[8] Both enamel and dentin do not heal or remodel after eruption of the tooth, and their structure is optimized to sustain lifelong cyclic mechanical stress induced by chewing activities without suffering critical damage.[9] In healthy teeth, the dentin is not exposed to the relatively harsh conditions prevailing in the oral cavity such as extreme fluctuations of temperature and pH. However, this can be the case under certain clinical conditions such as excessive gingival recession or local abrasion of the enamel layer.[10] The outer enamel layer is constantly exposed to abrasion and chemical erosion of its mineral phase for decades. Its remarkable resistance and longevity are due to its hierarchical structure and the fact that the HAP mineral phase is constantly rebuilt to a small extent by natural remineralization. Here, saliva plays a prominent role by providing both—large amounts of free calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions and a mix of different proteins that play multiple roles in maintaining the integrity of the hard tissues such as the formation of a thin protective pellicle layer.[11] Under physiological conditions, the supersaturation of saliva with Ca^{2+} and PO_4^{3-} ions enables the formation of new minerals not only on the surface but also in deeper layers of enamel.[12] However, this natural remineralization process is slow and not sufficient to

compensate extrinsic erosive effects teeth have to sustain during their lifetime.

In contemporary society, the phenomenon of tooth erosion gains ground due to global changes in dietary habits. Data collected on the incidence and prevalence of erosion indicate a steadily growing occurrence of the clinical picture in individuals of different ages starting already in preschool children.[13,14] Tooth erosion mainly affects the enamel and is caused by extrinsic acids of non-bacterial origin coming mainly from the sector of food and beverages. Not only popular soft drinks in particular but also fruit juices and wines have been shown to have very low pH values overall, ranging between 2.5 and 3.4.[15] In vitro, tooth enamel mineral already dissolves at pH values below 5.5.[16] In the oral cavity, this critical pH value may vary due to the presence of Ca^{2+} and PO_4^{3-} ions in saliva and in the plaque fluid, which can act as a buffer system.[16] Besides, the actual efficiency and damage of an acid attack also depends on the quantity and quality of the pellicle layer, the duration of exposure, and the actions taken after the consumption of acidic food items.[14]

Oral care products containing HAP as an active ingredient have been shown to act as an extrinsic source for Ca^{2+} and PO_4^{3-} ions that can support the natural remineralization process by increasing their availability beyond the amount provided by saliva.[17–22] Furthermore, externally supplied particulate HAP has been shown to firmly attach to enamel and dentin surfaces, thereby forming layers that can protect the tooth surfaces from erosion and also represent a reservoir for the continuous release of Ca^{2+} and PO_4^{3-} ions.[23] Its ability to attach to the tooth surface has been qualitatively and quantitatively investigated, but detailed knowledge of the attachment processes on the length scale of HAP crystallites is still lacking.[23,24] The effects of demineralization of tooth tissues have been extensively investigated on the macroscopic level,[13,14] in particular in connection with caries.[15] Equally, it has been macroscopically shown that externally supplied HAP has a beneficial effect on the remineralization of teeth facing erosive stress.[21,22,25–28] However, neither the effect of erosive attacks nor the impact of saliva and externally supplied HAP on enamel and dentin surfaces have been visualized on the nanoscopic structural level of individual crystallites yet.

In this study, we investigate the potential of an HAP-based oral care gel in protecting tooth surfaces from erosive attacks in vitro using high-resolution field-emission scanning electron microscopy (SEM) and analyze the interfaces formed between synthetic HAP crystallites and crystallites from the enamel surface using focused ion beam (FIB) milling and transmission electron microscopy (TEM). We used a standardized bovine enamel and dentin surface model system providing samples with reproducible surface quality[23] (Figure 1) and a high concentration-HAP-gel formulated with synthetic HAP particles as the main active ingredient. Our experimental protocol simulated an average daily dietary routine with erosive attacks that mimic the consumption of popular acidic soft drinks. Artificial saliva was used to mimic the oral cavity environment. We documented the ultrastructural changes occurring on enamel and dentin surfaces during erosive attacks on the microscopic level to elucidate the

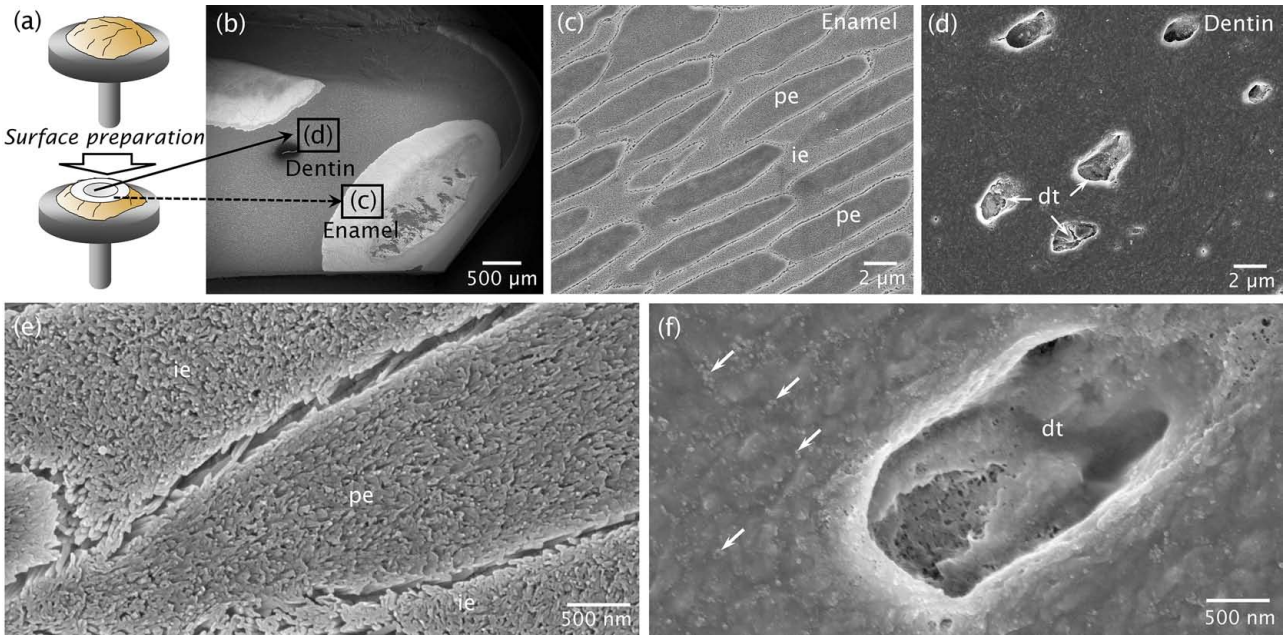

Figure 1. Standardized bovine tooth surfaces. (a) Schematic depiction of the performed preparation steps. (b) Each sample contains areas of artefact-free enamel (c) and dentin (d). (e) Detailed image of enamel crystallites in prismatic enamel (pe) and interprismatic enamel (ie). (f) Polished dentin surface with the exposed opening of a dentin tubule (dt). Arrows mark small spherical particles embedded in the surface, which also form agglomerations within the tubule opening

potential of HAP-particle-based oral care formulations in protecting tooth surfaces from erosive damage. Moreover, we provide information on the nature of the interface forming between synthetic and natural enamel HAP crystallites. Our results are expected to extend the knowledge on HAP as a mineral phase of teeth and its modes of action as a biomaterial providing important data for the further development of HAP-based biomimetic oral care products.

2. Materials and methods

2.1 Bovine tooth enamel model

All experiments were carried out on bovine tooth samples with a consistently high and reproducible surface quality following a preparation protocol adapted from Fabritius-Vilpoux *et al*.[23] First, small tooth slices were sectioned from incisors extracted from the lower jaws of male cattle aged 24 months using a water-cooled disc cutter (Buehler, Iso Met 5000, Linear Precision Saw, 3000 rpm, 2 mm/min). The obtained pieces were glued to standard aluminum SEM holders using an instant adhesive. Thus, either the labial surface or the lingual surface was available for the surface preparation steps (Figure 1(a)). The sample surfaces were first ground parallel to the sample holder surface using abrasive papers with increasingly finer grit (600–4000) at 150 rpm on a water-cooled rotating laboratory grinding machine (Struehrs ROTOPOL 22; Figure 1(a)). Due to the convex (labial surface) or concave (lingual surface) geometry of the samples, the exposed surface of all samples included both enamel and dentin regions (Figures 1(b)–1(d)). The ground samples were cleaned with water and subsequently polished using a 1 µm diamond particle suspension on a textile-covered disc followed by 50 nm silica particles for 30 s. Thereafter, the samples were rinsed with 100% ethanol and sonicated for 30 s in ethanol. To remove all residual polishing artifacts, the sample surfaces were gently etched in 0.07% phosphoric acid for 5 s followed by a 5-s rinsing step in demineralized water and the removal of excess liquid with tissue. The enamel and dentin surfaces were inspected using SEM, and their quality was used as a baseline for evaluation of the remineralization and erosive effects of the following experiments.

2.2 In vitro experimental procedure

Our experimental protocol was designed to simulate an average daily routine spanning about 36 h that included three meals (“breakfast”, “lunch”, and “dinner”), two dental hygiene applications (post-breakfast

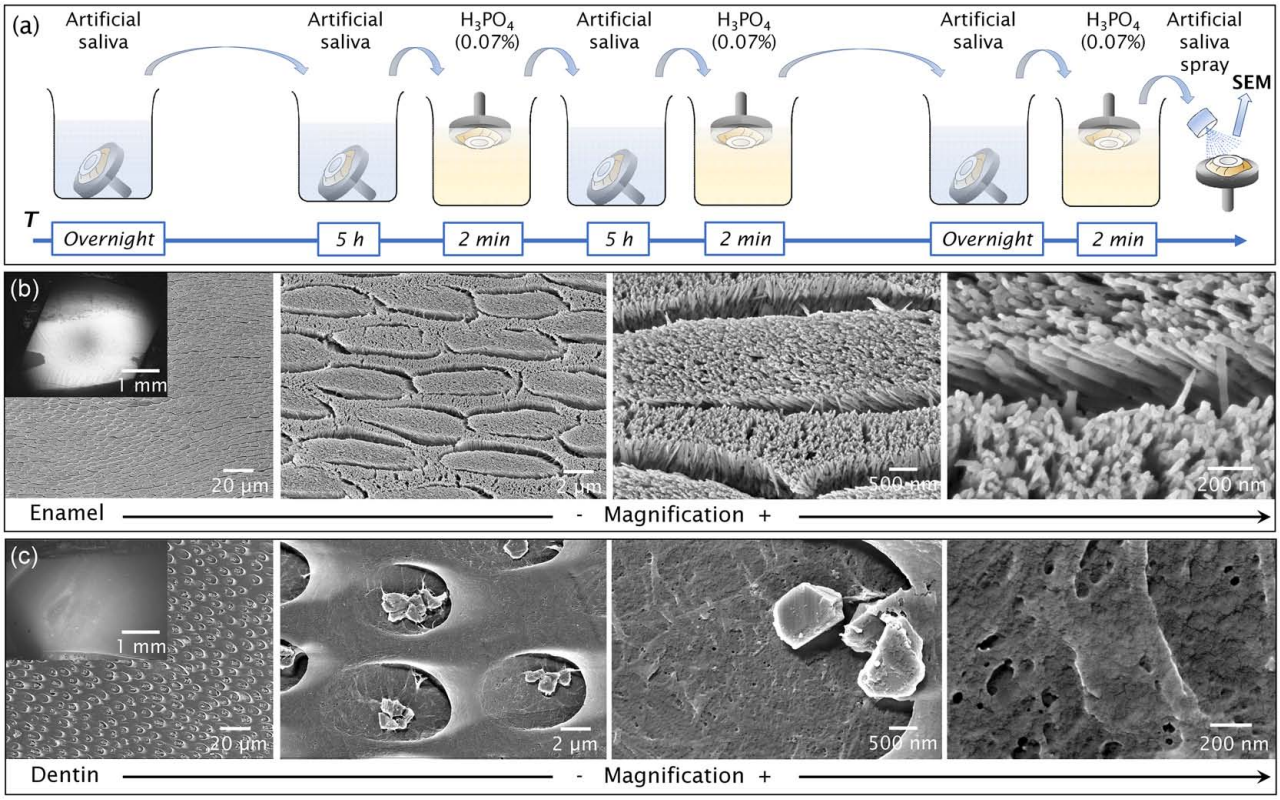

Figure 2. Effects of chemical erosion on enamel and dentin surfaces. (a) Schematic representation of the protocol used for erosive etching. (b, c) Scanning electron microscopy (SEM) micrographs of representative enamel (b) and dentin (c) surface areas including overviews of the inspected tooth surface (left) and detailed images with magnification increasing from left to right. (b) Both prismatic and interprismatic enamel consist of arrays of individual parallel crystallites with thinned tips separated by deep crevasses. (c) The dentin tubules are strongly enlarged and contain variable amounts of particulate debris. Both dentin and inner tubule surfaces have a fibrous texture

and post-dinner), and an overnight resting period. To exclude effects of mechanical abrasion, brushing steps were omitted. Each experiment was preceded by an overnight incubation in 100 ml artificial saliva. To mimic the oral cavity environment, we chose commercially available artificial saliva (Glandosane Spray, Stadapharm, Bad Vilbel, Germany) as a storage medium for the tooth samples. The viscosity and mineral content of Glandosane were all right in the range of natural values. Concerning the ionic saturation with regard to the tooth minerals, the formulation contains 0.0001 48 g/cm^{-3} calcium chloride dihydrate and 0.0003 48 g/cm^{-3} potassium monohydrogen phosphate. The experimental sequences for the chemical erosion, the remineralization in saliva, and the erosion in presence of an HAP-gel depicted in Figures 2(a), 3(a), and 6(a) were performed at room temperature. Each of the three experiments was carried out on three tooth samples. On every sample, five randomly chosen areas were inspected using SEM.

2.3 Chemical erosion and remineralization

The erosion experiments were carried out using phosphoric acid, H_3PO_4, diluted to a concentration of 0.07% (pH 2.2 at room temperature) to mimic the conditions present during the consumption of popular acidic soft drinks.[15] Each sample was stored in artificial saliva for 5 h, followed by an exposure to acid for 2 min. This procedure was repeated twice, with the second resting period lasting overnight (Figure 2(a)). The effect of artificial saliva on the tooth samples was tested with the same experimental procedure, omitting the etching steps (Figure 3(a)). After the final protocol step of both test series, each sample was rinsed for 5 s in demineralized water followed by 3 s in 100% methanol to remove residual water. After this, the samples were air-dried and prepared for inspection with SEM.

2.4 Effect of biomimetic HAP on chemical erosion

For studying the effect of synthetic HAP on chemical tooth erosion, a biomaterial-based oral care gel (Karex gelée, Dr. Kurt Wolff GmbH & Co. KG, Bielefeld, Germany) was used. This gel combines particulate HAP with ingredients that enable the formation of a gel matrix. The main active ingredient was present in the form of a chemically pure HAP powder whose structure and particle size distribution have been characterized in detail in an earlier study.[23] The secondary active ingredient was calcium lactate, a water-soluble calcium salt that was intended to act as a source for Ca^{2+} ions. During the entire experimental procedure, the tooth samples were stored in 100 ml artificial saliva. The experiments started with one application of the HAP-gel in the morning ("post-breakfast") to samples incubated in artificial saliva overnight, followed by erosive attacks after 5 h

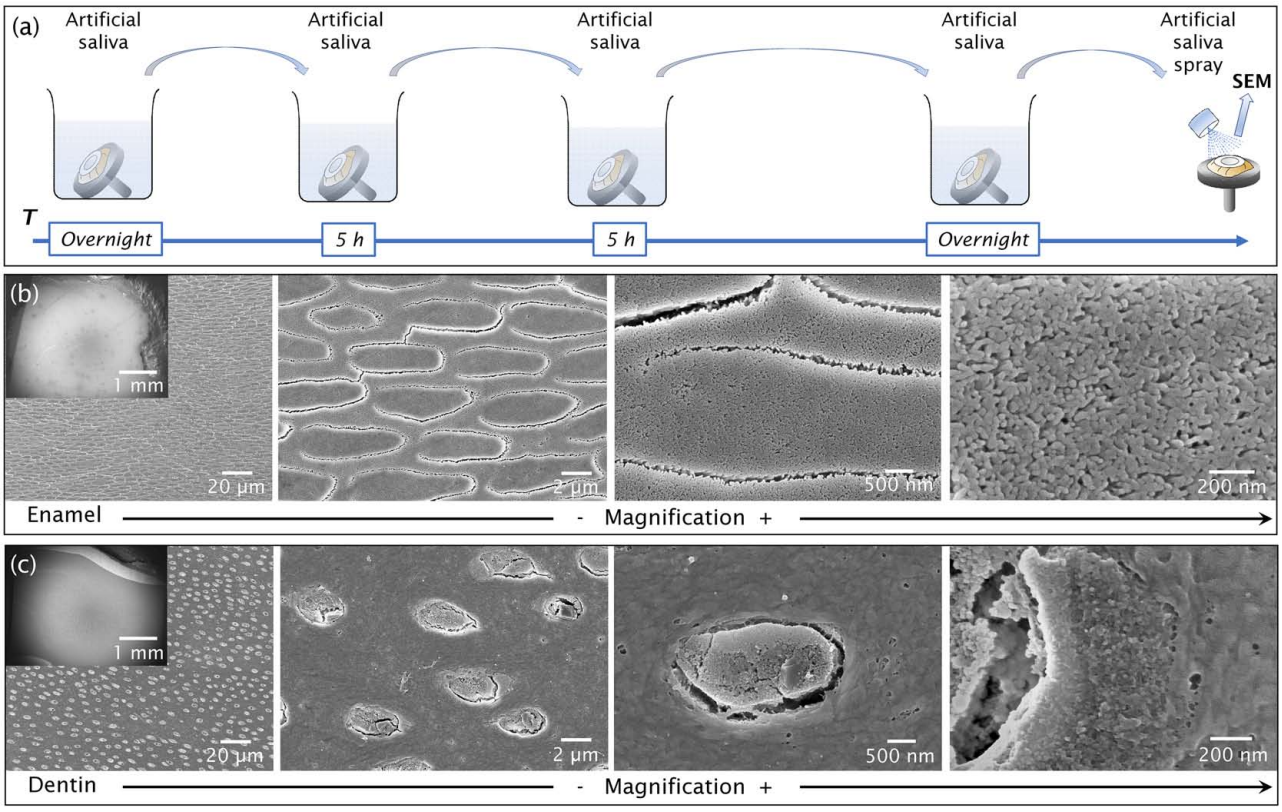

Figure 3. Effect of exposure to artificial saliva on enamel and dentin surfaces. (a) Schematic representation of the protocol used for remineralization. (b, c) SEM micrographs of representative enamel (b) and dentin (c) surface areas including overviews of the inspected tooth surface (left) and detailed images with magnification increasing from left to right. (b) Prismatic and interprismatic enamel are separated by narrow crevasses and show a compact surface made up by the rounded tips of enamel crystallites. (c) The dentin tubules have the same size as those without saliva treatment and are filled with a dense matrix of spherical particles

("lunch") and 10 h ("dinner"). After the last erosive attack, another application of the HAP-gel simulated the evening dental care procedure. The HAP-gel was applied by gently rubbing a small quantity onto the sample surfaces with a gloved finger. After storing the samples overnight in saliva, the experiment ended with a third erosive attack ("breakfast"). Each erosive attack was performed by dipping the sample in 100 ml 0.07% phosphoric acid for 2 min under gentle agitation. The exposure time was chosen under the assumption that it largely corresponds to the average time spent drinking during a meal. To remove residual acid, two samples were spray-cleaned with artificial saliva, and one sample was gently swiveled in 100 ml of artificial saliva (Figure 5(c)) to monitor the effect of spraying on particle adhesion. To define a baseline for the erosion experiments, three samples were treated with a HAP-gel application followed by spray-cleaning with artificial saliva (Figure 4). All experiments ended with rinsing each sample in demineralized water for 5 s followed by 3 s in 100% methanol. Subsequently, the samples were air-dried and prepared for SEM inspection.

2.5 TEM analysis of particle–tooth interfaces

To obtain enamel surfaces with adhering HAP particles, four polished tooth samples (see Figure 1) were dipped into 200 ml of a dispersion containing 10% of HAP powder in demineralized water (pH 7.0) for 1 min each under continuous stirring. Subsequently, the samples were washed for 5 s in demineralized water, submerged in 100% methanol for 2 s, and dried in a warm air stream. One sample was removed and prepared for SEM inspection. The surfaces of the other samples were covered with a very thin layer of embedding resin for TEM (EPON 812) that was cured in a vacuum desiccator to ensure that all particles are fully embedded without air inclusions between particle crystallites and enamel crystallites. For the preparation of lamellar slices through the particle–enamel interfaces using FIB, the entire samples were sputter-coated with a 4-nm-thick layer of platinum using a Gatan PECS 682 coating device and inspected in a FEI Helios Nanolab 600 FIB-SEM. Small bulges on the otherwise flat sample surfaces indicated the presence of particles on the enamel surfaces and were used to define the locations for the FIB sections. Following a standard protocol,[54] areas of 15 × 5 µm were coated with an about 3-µm-thick protective layer of platinum using deposition mode. Subsequently, the material on both sides of the protective strip was milled away (Figure 6(a)), exposing lamellae of about 10 µm in height. The lamellae were cut free and transferred to special TEM half-grids in an automated lift-out procedure with a micromanipulator needle. After being attached through deposition of platinum, the lamellae were carefully thinned on both sides until they became electron translucent. The half-grids were transferred to a JEOL JEM 2200FS TEM where they were examined at 200 kV in bright-field scanning mode (bright-field scanning transmission mode (STEM-BF), Figure 6(b)), high-angle annular dark field mode (HAADF; Figure 6(c)), and conventional imaging mode (TEM; Figures 6(d)–6(f)).

2.6 SEM analysis

All air-dried tooth samples from the in vitro experiments and the reference sample from the TEM analysis were sputter-coated with

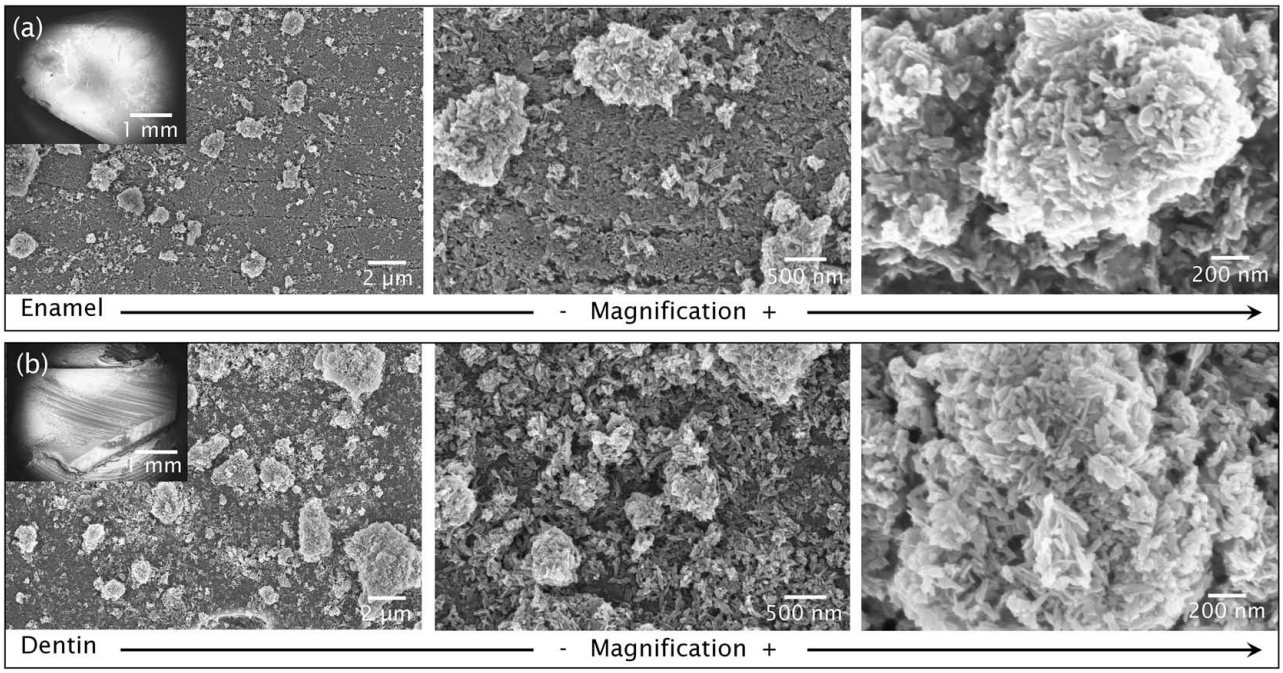

Figure 4. SEM micrographs of the baseline state of tooth samples after application of the HAP-gel. (a) Overview (left) and detailed image (middle) of the enamel surface covered with HAP particles of different sizes together with a detailed image (right) of the particle microstructure at high magnification. (b) Overview (left) and detailed image (middle) of the dentin surface covered with HAP particles and a detailed image (right) of the particle microstructure showing the constituting HAP crystallites

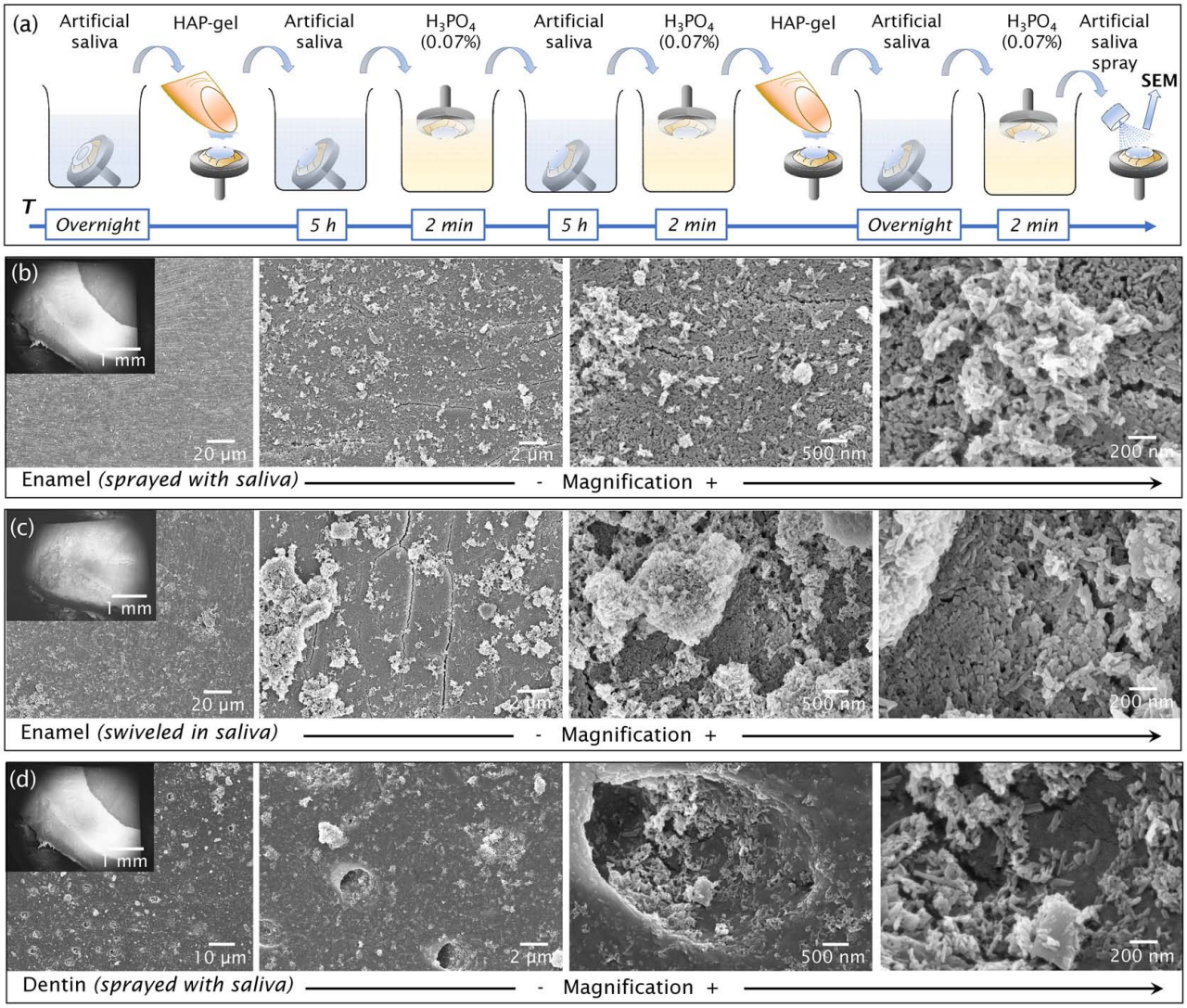

Figure 5. Effects of the erosion experiments on enamel and dentin surfaces repeatedly treated with HAP-gel. (a) Schematic representation of the protocol used for the erosion experiments. (b)–(d) SEM micrographs of representative enamel (b, c) and dentin (d) surface areas including overviews of the inspected tooth surface (left) and detailed images with magnification increasing from left to right. (b) Enamel surfaces cleaned by spraying them with saliva show well-preserved crystallites in both prismatic and interprismatic enamel. The surfaces are covered with HAP particles of relatively small sizes that show signs of structural disintegration. (c) Enamel surfaces cleaned by swiveling them in saliva show a similar microstructure but are more densely covered with HAP particles that also have larger sizes. (d) The dentin areas are also covered with numerous HAP particles, which are also frequently filling the normal-sized dentin tubules

4 nm of platinum using a Gatan PECS 682 coating device. Images of the sample surfaces were recorded using a Zeiss Crossbeam 1540 XB FIB-SEM and a Zeiss Merlin SEM (Gemini 2) at an acceleration voltage of 5 kV using a 30 µm aperture. The inspected regions were randomly chosen from low magnification views of the sample surfaces.

3. Results

3.1 Analysis of enamel and dentin surfaces (baseline)

The baseline state to evaluate microscopical structural changes of enamel and dentin induced by our experiments are our standardized bovine tooth surface samples (Figures 1(a) and 1(b)). In enamel areas, prismatic and interprismatic enamel can be well distinguished by the clearly visible prism boundaries consisting of around 100–200 nm wide crevasses where crystallites are missing. The crevasses are not deeper than the thickness of a few crystallites. Prism outlines vary from round to oblong oval depending on the orientation of the prism with respect to the exposed surface plane (Figure 1(c)). Individual crystallites are about 30 nm thick and several hundreds of nanometers long. The crystallites forming the superficial layer are rarely thinned, but their tips appear mostly rounded off. Overall, they form a dense layer with relatively few voids where crystallites are missing

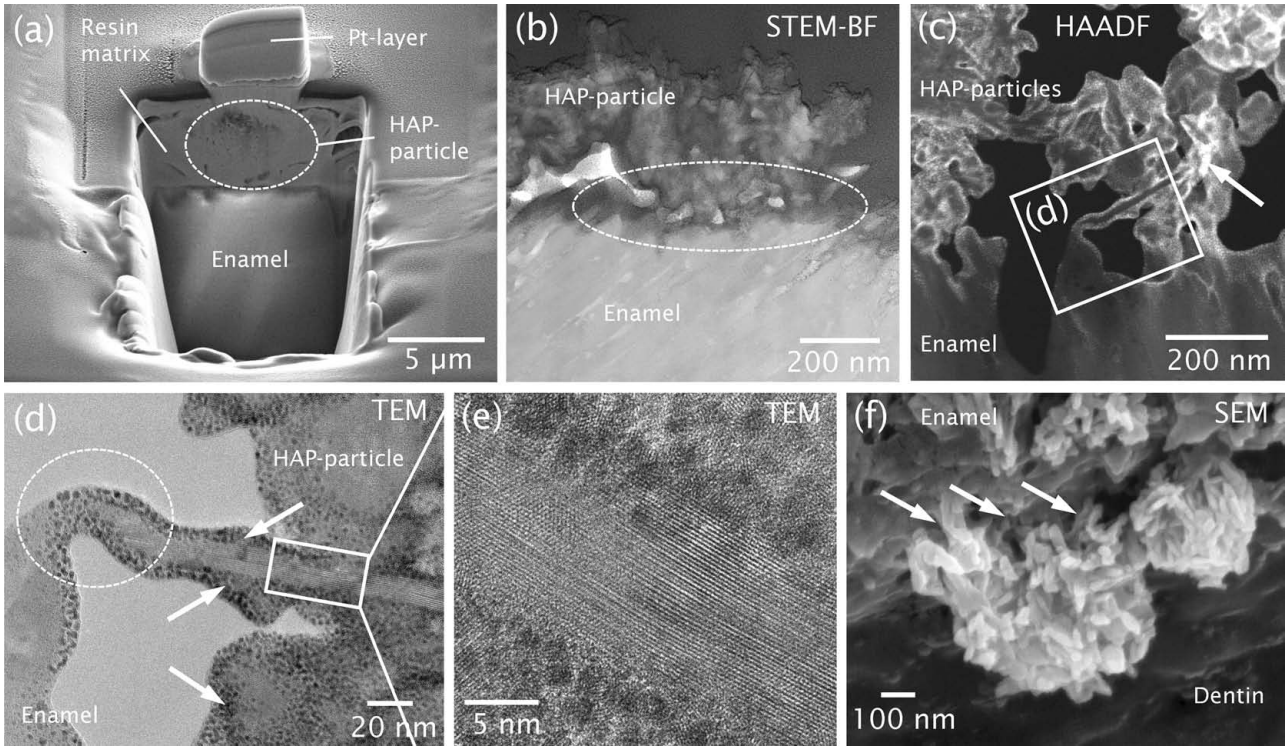

Figure 6. TEM investigation of the interface between crystallites from the HAP particles and the bovine enamel sample surface. (a) Overview of the HAP particle–enamel interface exposed perpendicular to the sample surface using FIB-SEM during the preparation of thin TEM lamellae. (b) Bright field STEM image of a HAP particle attached to the enamel surface (encircled area). (c) HAP particles connecting to the enamel surface imaged using HAADF. Bright spots (arrow) indicate the presence of higher atomic number material, probably gallium from the milling process. (d) TEM detail images show that HAP crystallites from particles (right) and enamel (left) in close vicinity (≤50 nm) are connected by way of bridges of a solid material (encircled area). Both crystallite types show areas with visible atomic lattice planes indicative for highly crystalline material, which are absent in the connecting amorphous material. The darker spots (arrows) are presumably gallium particles originating from the FIB preparation. (e) Detail of the crystal lattice. (f) SEM image of a reference sample showing the presence of bridge connections between crystallites from HAP particles and enamel (arrows). Pt layer, platinum layer

(Figures 1(e) and 7(a)). The surface of dentin areas is generally flat and smooth with very few small particles present. Exposed dentin tubules show round-to-oval cross-sectional outlines depending on their angle with respect to the surface plane. Their diameters vary between 1.0 and 1.5 µm (Figure 1(d)). Elongated HAP crystallites are rarely observed embedded in dentin surfaces; however, there are numerous spherical particles with 30–50 nm dia. embedded in the superficial layer (Figure 1(f)). Dense agglomerations of these particles are also present in the openings of tubules where they form irregularly shaped lumps (Figures 1(d) and 1(f)).

3.2 Effect of erosive attack

The chemical erosion experiment (Figure 2(a)) caused severe superficial changes to both enamel (Figure 2(b)) and dentin (Figure 2(c)). In enamel, the crevasses separating prismatic and interprismatic areas are now up to 1 µm broad and deep enough for their bottoms being no longer visible. The HAP crystallites are present as dense arrays of individual parallel rods with occasional void spaces between them. Their overall thickness appears slightly decreased, and their ends are mostly thinned out to sharp tips (Figures 2(b) and 7(b)). The crevasses reveal crystallite lengths of up to 1 µm (Figure 2(b)). The most obvious effect in the dentin areas is that the diameters of exposed tubules have increased to 4.5–5 µm. In almost all cases, particulate debris is located within their openings. This debris consists of either up to 2-µm-large blocky particles with straight faces and edges or more irregularly shaped particles that have rounded edges and display a granular substructure. The dentin surface shows a fibrous texture, which is even more pronounced in the visible lumina of the tubules. The fiber strands below the surface form irregularly oriented networks. Very rarely, nanoscopic particles can be observed on or immediately below the dentin surface (Figure 2(c)).

3.3 Effect of storage in artificial saliva

Control experiments investigating the effect of saliva were only performed using the same exposure intervals as shown in Figure 2, but without etching steps (Figure 3(a)). This treatment resulted in a compaction of the surfaces, particularly in the enamel areas (Figures 3(b) and 7(c)). The dimensions of the crevasses separating prismatic and interprismatic enamel were unchanged with respect to the baseline state (compare Figures 1(c) and 1(e) and 3(b)). The superficially visible enamel crystallites appear slightly thickened

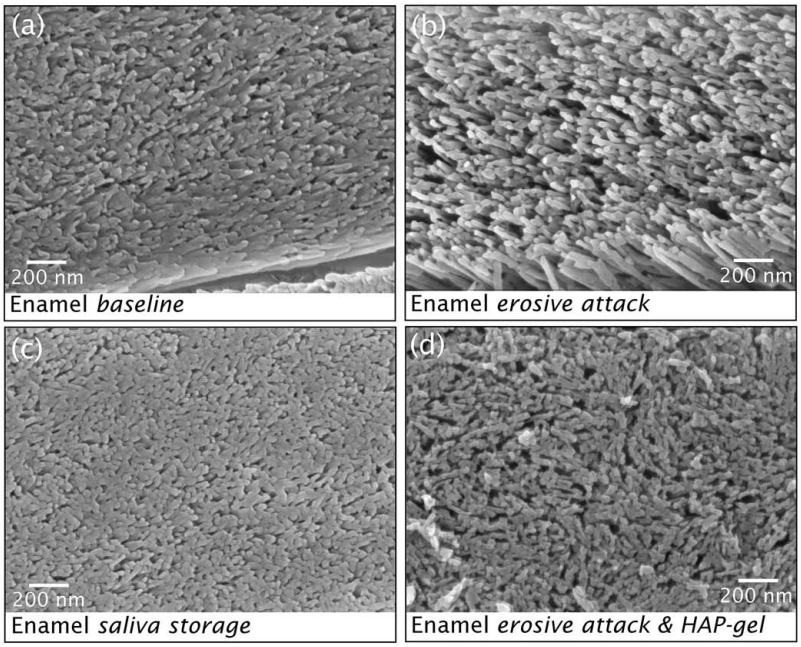

Figure 7. (a) Comparison of representative SEM images depicting hydroxyapatite crystallites in prismatic enamel areas of differently treated samples. (a) Bovine enamel model prepared using the standardized protocol serving as baseline state without any further experimental treatment. (b) Enamel surface after erosive attack with using 0.07% phosphoric acid. (c) Enamel surface after storage in artificial saliva. (d) Enamel surface after 36 h of simulated daily routine in artificial saliva including two applications of HAP-gel and three erosive attacks. For detailed depictions of the experimental procedures, please see Figures 2, 3, and 6 as well as Section 2

with rounded tips. Especially in voids where adjacent crystallites are missing, these tips appear broader than the rest of the crystallites. Patches with crystallites that appear to be fused are frequently observed on the sample surface (Figure 3(b)), giving it a denser appearance compared with the baseline state (Figure 1(e)). The shape and dimensions of the dentin tubules exposed on the otherwise flat and smooth dentin surfaces correspond to those measured in the baseline state (Figure 1(d)). Their lumina are almost always filled with densely agglomerated, 30–50 nm wide spherical particles that form a dense matrix in which sometimes larger blocks with straight edges and flat surfaces are embedded (Figure 3(c)). The superficial layer of dentin is almost completely free of particles or debris and has a very smooth texture without signs of embedded fibers (Figure 3(c)).

3.4 Effect of HAP-gel application on surface erosion

Figure 4 shows the surfaces of enamel and dentin after application of the HAP-gel before the erosion experiment. The enamel surface is covered by numerous, mostly spherical HAP particles with a broad range of sizes (Figure 4(a)). The largest particles observed were about 2 µm in diameter. The majority of the particles were between 100 and 300 nm wide and provide dense coverage with an even distribution on the surface. The structure of the visible prismatic and interprismatic enamel areas as well as the width and depths of the crevasses separating them (Figure 4(a)) correspond to those seen in the untreated enamel samples (Figures 1(c) and 1(e)). Preferential adhesion of particles to the crevasses was not observed. The dentin surface areas are almost completely covered with HAP particles, which often pile up on top of each other (Figure 4(b)). Open dentin tubules are only very rarely visible. Besides the particles in the size range observed on enamel, larger particles exceeding 4 µm in diameter also adhered to dentin. The particles were stable nanometer-sized agglomerations of uniform, about 30-nm-thick and 80-nm-long HAP crystallites that were randomly oriented (Figures 4(a) and 4(b)). Compared to a detailed analysis of their native structure,[23] they have not been altered while being in the gel. Dried-up residues of the other, particularly the organic ingredients of the gel, were observed neither on the particles themselves nor on the exposed tooth surfaces. The described state of enamel and dentin (Figure 4) serves as a baseline to evaluate the effect of the gel during the erosion experiments (Figure 6(a)).

3.4.1 Effect of the erosion experiments on enamel regions

After the erosion experiments, both prismatic and interprismatic enamel could still be well distinguished (Figures 5(b) and 5(c)), but the crevasses separating them appeared less obvious than in the samples that were not treated with HAP-gel (Figures 2 and 3). The sizes of the superficially visible enamel crystallites corresponded to those described for the baseline state, and their tips appeared more roundish than thinned (Figures 5(b) and 5(c) and 7(d)). Overall, they form a dense surface with few voids. The HAP-gel particles that still reside on the surface have undergone much more severe structural changes. The majority of them show a high degree of disintegration,

resulting in numerous individual and small agglomerations of few crystallites being dispersed on the enamel surface (Figures 5(b) and 5(c)). Larger clusters remaining attached lack the compact appearance of the particles observed before the experiment (see Figure 4). In contrast to the spray-cleaned samples (Figure 5(b)), the swiveled sample shows a much denser coverage with HAP particles, and the observed sizes are also significantly larger. In some areas, particles were observed piling up in multiple layers, resembling the baseline state (Figures 4(a) and 5(c)). In general, particles directly adhering to the enamel surface appeared more severely eroded than those further away (Figure 5(c)).

3.4.2 Effect of the erosion experiments on dentin regions

The surface of dentin areas is covered with many but generally rather small residual HAP particles. They are often only a few crystallites large and appear to be at least partially embedded in the otherwise smooth dentin surface (Figure 5(d)). Occasionally, larger particles up to 2 μm in diameter and sometimes, small clusters of them are also present. Exposed dentin tubules have the same diameters of 1–1.5 μm as seen in the baseline state (Figure 1(d)). Their margins are sharp and do not show signs of erosion. Roughly half of the exposed tubules are filled with HAP particles and their debris that completely plug their openings (Figure 5(d)). The open tubules always have HAP particles attached to their walls; their sizes range from less than 500 nm in diameter down to a few fused crystallites. Occasionally, small compact and boulder-shaped particles can also be found attached to tubule walls (Figure 5(d)). These resemble the particles observed in tubules after erosive attack only (see Figure 2(c)).

3.5 Interaction of HAP particles with tooth surface

HAP particles are not washed away by rinsing in water and must thus tightly adhere to the surface. Investigation of their bonding state to the enamel surface required the preparation of TEM cross sections. The nature of the connections that form between HAP crystallites from the particles and those constituting the exposed enamel surface is revealed by STEM and TEM images obtained from thin lamellae prepared through the enamel–particle interface using FIB preparation (Figure 6(a)). The images confirm that the enamel consists of parallel, very densely packed HAP crystallites (Figure 6(b)). The enamel surface profile line reveals a significant roughness on the nanometer scale, with local height differences in the range of several hundreds of nanometers (Figures 6(b) and 6(c)). The crystallites constituting the particles have generally smaller diameters than those forming the enamel and are randomly oriented in space (Figures 6(b) and 6(c)). Individual highly crystalline regions, where atomic lattice planes are clearly distinguishable, are always surrounded by regions of amorphous material without visible lattice planes (Figures 6(d) and 6(e)). Direct contact between crystallites from particles and enamel was not observed. Instead, crystallites located in close vicinity (less than 50 nm) were connected by bridges of unstructured amorphous material that never display atomic lattice plane patterns (Figures 6(b)–6(d)). HAP-particle and enamel crystallites at distances above 50 nm from each other were not observed forming such connections. All amorphous material including the bridges are frequently decorated with small, electron-dense granules, particularly in peripheral areas (Figures 6(d) and 6(e)). Randomly distributed bright areas visible in HAADF mode (Figure 6(c)) also indicate the presence of higher atomic number material. A reference sample prepared for and inspected with SEM confirms the presence of bridges between enamel and HAP-particle crystallites (Figure 6(f)).

4. Discussion

The goals of this study require well-defined clean and artifact-free enamel and dentin surfaces to evaluate the experimentally induced structural changes on the micro- and nanoscale. Since sufficient numbers of human incisor teeth with a similar age, anamnesis, and wear status are hard to acquire, we used bovine incisor teeth for our experiments. The structure and properties of bovine incisors closely resemble those of human teeth,[29] and they are an established model material for both in vitro[26] and in situ[30] studies. These studies have shown that particularly proteins and other organic molecules from the pellicle layer and natural saliva influence the adhesion and binding affinity of HAP particles to tooth surfaces.[31] To avoid such phenomena, we used samples that were cleaned of any contaminations in the form of organic residues and polishing particles by a final cleaning step in 0.07% phosphoric acid for 2 s (Figures 1 and 7(a)). This ensured obtaining results purely based on the effects of our experimental substances and, in the case of the interphase formation between HAP crystallites from particles and enamel only, the mineral–mineral interaction.

Etching tooth enamel with mild acids has a long tradition in the investigation of its microstructure, in particular the spatial organization of the enamel rods (prisms).[1] More recently, it has also been employed to investigate the crystallographic properties of enamel crystallites.[4] Owing to its relevance to health and economy, erosive tooth wear has been extensively studied by the dentistry community focusing both on mechanical wear and abrasion as well as chemical erosion.[14] However, the majority of available studies focus on the length scale of the complete denture, individual teeth, or the enamel prisms, respectively. To investigate the processes involved in chemical erosion and its prevention on the micro- and nanoscopic level, we designed an in vitro approach mimicking the commonly assumed in vivo situation[14] as accurately as possible. Our erosion experiments (Figures 2, 3 and 4) recreate a situation where the tooth sample is stored in artificial saliva (representing times between meals), which is periodically interrupted by erosive attacks (representing uptake of food/drink) or applications of HAP-gel alternating with erosive attacks at time intervals simulating a daily routine of about 36 h. Brushing was omitted from the routine to exclude abrasive effects from the analysis. The erosive attacks were performed using 0.07% phosphoric acid (pH 2.2), which corresponded to the effect of commonly consumed soft drinks.[15] The exposure time of 2 min was assumed to be a typical time spent drinking a usual quantity of liquid.

4.1 Structural changes resulting from acid attack–erosion

Given the relatively weak acid and the short exposure of three times 2 min, the samples exposed to erosive attacks show remarkably severe ultrastructural changes of both enamel and dentin. The enamel HAP crystallites, despite being densely packed without interstitial spaces,[5,6] do not dissolve uniformly at the surface plane of the samples. Instead, individual crystallites become thinned along their long axis over nanometers down into the subsurface layer, resulting in the enamel appearing as a lawn-like layer of densely spaced needles with pointed tips (Figures 2(b) and 7(b)). This etching image indicates a material loss at the interfaces of the crystallites. The dissolution models for crystalline HAP in acidic environments discussed in literature indicate that crystalline HAP is more susceptible to dissolution on surfaces parallel to their longitudinal axis than on transverse faces, although the exact dissolution mechanism of crystallites in size ranges occurring in enamel is still under debate.[32] However, this tendency for anisotropic dissolution is not enough to explain how closely packed individual enamel crystallites with initial widths of 50 nm and less get exposed and separated over depths of 1 µm and more without disintegrating. Moreover, individual crystallites appear to be only insignificantly thinned along their long axes, and significantly pointed tips are only observed in the most distal few nanometers of the crystallites. Since HAP is the calcium phosphate phase with the lowest solubility in water,[33] the observed etching picture indicates the presence of a less chemically stable phase at the interfaces between enamel crystallites. The small amount of organic matrix in enamel (about 1 wt%) is mostly concentrated around the prism superstructures, and only very little of it is interspersed between crystallites.[34] A successive washout of organic matrix components could possibly contribute to the observed etching picture, but their low concentration would provide only very few sites where a dissolution front can penetrate into deeper regions. Recently, it was shown that human enamel crystallites are surrounded by an around 2-nm-thin layer identified as amorphous, magnesium-rich phosphate using atom probe tomography correlated with TEM.[35] Amorphous mineral phases are known to have a much higher solubility than crystalline ones.[33] Assuming that bovine enamel crystallites have a similar composition, the amorphous phase between individual crystallites will dissolve much faster upon acidic attack than the less soluble bulk of the crystallites, leading to erosion as we observe it in our bovine enamel surfaces (Figures 2(b) and 7(b)). The dissolution behavior described above is observed in both prismatic and interprismatic regions of enamel and is particularly pronounced at their interfaces. In native enamel, there is no void space between prismatic and interprismatic enamel. The interfacial crevasses observed in our baseline samples must be considered as the result of the desiccation process necessary for SEM inspection (Figures 1(c) and 1(e)). The increase in width observed in these crevasses after erosive attack may however be also attributed to the loss of the thin organic sheath surrounding the prisms.[6,34] Its components become exposed and washed out during the erosive attack, allowing the acid to access successively deeper regions, thereby further increasing the chemical erosion in these areas.

The surface of dentin subjected to the chemical erosion protocol (Figure 2(c)) appears much smoother compared to the baseline state (Figures 1(d) and 1(f)). The small, 30–50-nm-wide spherical particles embedded in the surface correspond in size and shape with the silica particles used for the final polishing step and were pressed into the soft organic phase on the dentin surface during sample preparation. Upon etching, these particles have disappeared together with the superficial layers of dentin. The few elongated larger particles, whose dimensions qualify them as dentin HAP crystallites interspersed in the organic matrix, also disappeared, indicating that they were dissolved during the experiment. The irregularly oriented networks of fiber strands that appear below the dentin surface are the collapsed residues of the collagen-rich organic matrix from at least a small volume of dentin that was demineralized. A limitation of our experimental setup was not allowing to quantify the effective amount of material removed by erosion. The most severe effect we observed is that the tubules increase in width from about 1–1.5 µm to 4.5–5 µm over the entire visible depth. This can only be explained by the complete dissolution of the peritubular dentin, which is described to be free of organic matrix and whose thickness[9] corresponds to the observed increase of tubule width. The tubule walls are now devoid of mineral, as indicated by their fibrous surface texture. Within the tubules, the agglomerations of spherical polishing particles have been washed out completely. The residual larger blocky particles (Figure 2(c)) may be broken-out particles from the abrasive papers used for sample preparation. However, on the basis of our investigative method, we cannot exclude that these are mineral particles that precipitated and grew from the dissolved peritubular dentin during the storage periods in artificial saliva. In healthy teeth under intraoral conditions, the effects we observed would be by far less pronounced, since all exposed surfaces would be covered by a pellicle layer that consists mainly of salivary molecules such as proteins and glycoproteins.[31] This pellicle layer protects the enamel from erosive attacks by providing a diffusion barrier and influencing the remineralization process.[31,36] The intermediate storage in artificial saliva did not result in a noticeable protective effect, which is most probably due to the absence of proteins in the formulation. While Ca^{2+} and PO_4^{3-} ion sources are present in the artificial saliva, possible remineralization effects could not be identified due to the efficiency of the acid in eroding enamel and dentin.

4.2 Structural changes resulting from storage in artificial saliva—remineralization

The reason to choose artificial saliva as storage medium during our experiments was to recreate an environment that provided an ionic composition resembling the intraoral conditions[31] and at the same time exclude organic components such as proteins and other macromolecules. This enables to study the influence of the inorganic components on the mineral phase and their buildup and

breakdown, respectively. Consequently, we do not observe the formation of an acquired pellicle neither on enamel nor on dentin surfaces even after exposure to artificial saliva for the entire 36 h, including three changes of the storage medium. Compared to the baseline state (Figures 1(c) and 1(e) and 7(a)), the enamel surface undergoes a densification in both prismatic (Figures 3(b) and 7(c)) and interprismatic (Figure 3) regions. The observed fusion and thickening of superficial HAP crystallite tips and the reduction of void spaces indicate a uniform growth on the existing surfaces. Since dissolution and recrystallization of the enamel crystallites can be ruled out under the applied experimental conditions, the deposited material is most likely a compound based on the various salt ions [sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}), Ca^{2+}, chlorine (Cl^-), and PO_4^{3-} ions] present in the artificial saliva. Surface structures that are newly precipitated are not observed the increase in material thickness is restricted to crystallites that are already present. It is therefore safe to assume that we observe calcium phosphate remineralization on crystallite surfaces. This is surprising since some studies report the artificial saliva we used (Glandosane spray) to demineralize both enamel and dentin.[37,38] However, the erosive effects reported were measured after exposure times in the range of weeks. Due to our short 36 h protocol, it is unlikely that such effects affect our results. The exact composition and crystallographic phase of the newly formed material is not yet known. The only organic compounds present in the formulation of the artificial saliva are the cellulose derivative carmellose and the sugar alcohol sorbitol. The fact that the superficial enamel crystallites do not show any typical signs of irradiation damage under the electron beam indicates that little-to-no organic material is incorporated into the material that formed on the crystallites.

Structurally, the dentin surfaces incubated in artificial saliva only (Figure 3(c)) do not differ from the baseline state (Figures 1(d) and 1(f)). Neither mineral precipitates nor any kind of layer or structure formation was observed, indicating that the components of the artificial saliva did not interact with the dentin or the effects were too small to be observed. The tubule diameters remained unchanged, and their filling of compacted abrasive and polishing particles was not notably affected or washed out by the storage medium (Figures 1(d) and 1(f) and 3(c)).

These results show that although HAP is present in both enamel and dentin, a noticeable remineralization from salivary ions only affects the enamel HAP crystallites. Their exposed surfaces probably provide nucleation sites where mineral can then grow, forming a thin layer. Due to the large amount of organic matrix in dentin, HAP crystallites are much too rarely exposed for this process to happen.

4.3 Effect of HAP-gel application on tooth surface structure during erosive attack

Enamel and dentin samples imaged immediately after the application of our HAP-gel (Figure 4) were used as a baseline to evaluate its effect on the structural changes of the tooth tissues during the erosion experiment. We applied the HAP-gel by gently rubbing a small portion onto the sample surface, to achieve the best possible adhesion to the substrate as well as to mimic the in vivo application. The SEM analysis shows that both in enamel (Figure 4(a)) and dentin (Figure 4(b)), a thin layer of particles remains on the sample surface after preparation for SEM. The microstructure of the particles resembles those used in other studies,[23,31] where they were used in aqueous dispersions without additional substances.[23] We therefore assume that they are not structurally altered by residing in the gel formulation. On enamel, the HAP particles are quite evenly distributed, leaving enough uncovered area that the enamel crystallites and surface features can be well distinguished (Figure 4(a)). In dentin areas, the coverage is almost complete with almost all tubules occluded. The sizes of adhering particles are generally larger (Figure 4(b)), confirming observations that the organic matrix in dentin supports the adhesion of particulate HAP.[26,39,40] Dried-up residues of the other, particularly the organic ingredients of the gel, were observed neither on the particles themselves nor on the exposed tooth surfaces (Figure 4). Therefore, it must be assumed that they were washed off during the preparation for SEM. During the experiments, we performed two applications of the HAP-gel, post-breakfast after overnight storage in artificial saliva and post-dinner immediately after an erosive attack. The second application was followed by an overnight resting period. Lunch was simulated by an erosive attack without following HAP-gel application as is common practice in western societies. Each experiment was ended with an erosive attack and subsequent cleaning and preparation of samples for SEM. This way, the effect of the HAP-gel on erosion could be assessed best.

The most obvious effect observed on enamel surfaces after treatment is a significant reduction of the HAP-particle number and size (Figures 5(b) and 5(c)). Also, larger residual particles have a strongly corroded appearance and smaller ones are reduced to irregularly shaped agglomerations of crystallites. This shows that the particulate HAP from the gel underwent strong dissolution during the acid attacks. Unexpectedly, the structure of the enamel below the particle layer is practically unharmed (Figures 5(b) and 5(c) and 7(d)) and resembles the sample baseline state (Figures 1(c) and 1(e) and 7(a)). Moreover, the superficial HAP crystallites show in places rounded, broadened tips reminiscent of the situation observed in the samples treated with artificial saliva only (Figures 3(b) and 7(c)). This suggests that the acid treatment in the presence of the HAP-gel mainly affects the applied particulate HAP, while the enamel crystallites below are not or only very little affected. A possible reason is that the acidity of the solution is reduced by the dissolution of the applied HAP layer, which buffers the solution too strongly to affect the mineral underneath. HAP has been shown to act as a buffer in the oral cavity,[17] thereby reducing the erosive potential.[15,26,41,42] Additionally, the released Ca^{2+} and PO_4^{3-} ions could precipitate on the enamel crystallites, leading to a weak remineralization explaining the slightly thickened enamel crystallites.[12] Further, less prominent protective effects may be

also expected from the secondary active ingredients of the gel, namely, calcium lactate and calcium carbonate, which provide additional Ca^{2+} ions and can act as buffers, too.[43] In summary, our results indicate that the HAP-gel layer on the enamel acts as a sacrificial layer that absorbs the erosive effect of mild phosphoric acid, thereby protecting the enamel. In an intraoral environment, this effect can be expected to be even more pronounced due to the presence of a pellicle layer that has a buffering effect of its own and enhances the adhesion of particulate HAP.[31,44–46]

The protective effect of the HAP-gel is also observed on dentin surfaces, where the number and sizes of the HAP particles adhering to the surface are drastically reduced after the experiment (Figure 5(d)). The dentin surface structure (Figure 5(d)) does not show significant differences compared to the baseline state (Figures 1(d) and 1(f)), but we cannot exclude that mineral was lost during the erosive attacks. However, since we did not observe accumulated filamentous organic matrix as in the samples treated with acid only (Figure 2(c)), this loss must be much smaller. The most outstanding observation was that the peritubular dentin was intact and unaffected by dissolution (Figure 5(d)), as indicated by the tubules retaining the same diameters as in the baseline state (Figure 1(f)). Furthermore, their majority is still filled with particulate HAP originating from the gel (Figure 5(d)). The occlusion of dentin tubules is one of the main beneficial clinical effects described for HAP.[47–49] Our results show that this persists even under erosive attack, whereby the particles prevent the access of the acid into the lumina of the tubules.

4.4 Adhesion mechanism between enamel and HAP particle crystallites

The adhesion of HAP particles from oral care formulations to tooth tissues is indispensable to unfold their numerous clinical effects.[2,12,15,21,22,24,26,28,31,39,48–52] In our experiments, the HAP-gel forms a microscopic, yet dense layer of particles adhering to both enamel and dentin surfaces (Figure 4). The particles apparently adhere strong enough to resist the forces acting upon manipulation of the samples and various washing steps. However, the particle coverage observed in samples sprayed with saliva after the erosion experiment was significantly lower than in samples that were swiveled in saliva (Figures 5(b) and 5(c)). This finding indicates that the bonds formed between HAP particle crystallites and enamel crystallites are not very stable, which could however also be partly attributed to weakening effects by the final acid erosion step. In vitro and in situ studies investigating the microscopic adhesion mechanisms without[23] and with pellicle layer[31] have both shown that mineral bridges form between crystallites from HAP particles and the enamel. We confirm the presence of this interphase using TEM on thin slices through the interface between HAP particles and the polished enamel surface (Figures 6(b)–6(d)). The images reveal that the surface of the enamel has a significant elevation profile at this level of magnitude, which is mainly caused by missing parts of crystallites. In consequence, only very few contact points between crystallites from the even more corrugated HAP particles were observed (Figures 6(b) and 6(c)). In fact, bridges were only observed between the HAP particle and enamel crystallites that were located less than 50 nm apart. This indicates that the bridges arise from a local precipitation and growth process, possibly from both sides. In TEM, both crystallites from enamel and HAP particles show highly crystalline areas (Figures 6(d) and 6(e)). Signs of atomic lattice planes were never observed in the mineral bridges and in the superficial regions of crystallites, indicating that the constituting material is amorphous. The small, electron-dense particles observed implemented within these areas (Figures 6(e) and 6(f)) are typical for gallium and platinum deposition inherently caused by the milling and thinning process during sample preparation. The fact that mineral bridges between enamel and HAP-particle crystallites are also observed on samples that were not treated with FIB makes it very improbable that the bridge material originates from redeposition during the milling process. Since this experiment was carried out in deionized water of neutral pH in the absence of any organic contaminants, the material of the bridges is most probably an amorphous mineral phase whose constituents originate from local dissolution of the enamel and/or the HAP particles. As it has been shown that enamel crystallites are surrounded by a thin, amorphous magnesium-rich layer,[35] it is reasonable to assume that this material is the source of the ions forming the bridge material. Dissolution and successive phase transformation of amorphous precursor particles has been shown to induce the formation of low-crystalline HAP phases in vitro.[53] Here, further work is required to analyze both the exact chemical composition and crystallographic properties of the material with appropriate high-resolution techniques. Similar bridges between HAP crystallites were also found in situ—that is, in the presence of an acquired pellicle,[31] indicating that the mineral–mineral interaction between particulate HAP and enamel also occurs and plays a role in the intraoral environment.

5. Conclusion

Using an *in vitro* approach simulating erosive processes in the oral cavity, we show for the first time how acidic erosion and storage in artificial saliva affects the ultrastructure of bovine enamel and dentin surfaces on the crystallite level and how this is influenced by the application of biomimetic HAP particles in an oral care gel formulation. Acidic erosion leads to significant removal of material in enamel and dentin; especially affected are enamel crystallite interfaces and the peritubular dentin. This erosion behavior exposes large volumes deep into the tooth tissues, thereby promoting further damage and weakening the structural integrity of enamel and dentin. Storage in artificial saliva only leads to little mineral deposition on enamel crystallites; dentin is not or much less affected. We show that the regular application of HAP-gel as part of daily dental care leads to an almost-complete protection of enamel and dentin surfaces from erosive attacks such as those caused by acidic soft drinks using a convenient and effective principle of action. The particulate HAP forms a microscopic layer that is sacrificed during erosive attacks. The process is likely supported by remineralization induced from artificial saliva. Since synthetic biomimetic HAP gains increasing importance as biomaterial and

an active ingredient in dentistry and preventive oral care, it is important to understand its potential in preventing chemical erosion on the microscopic level for optimization and refinement of HAP-based oral care formulations that can help to reduce the global problem of dental erosion.

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