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Evaluation of bacteria-induced enamel demineralization using optical profilometry

Sarah E. Cross^{1,2,§}, Jens Kreth^{3,§}, R. Paul Wali^{1,2,6,§}, Richard Sullivan⁵, Wenyuan Shi^{2,4}, and James K. Gimzewski^{1,2,6,§}

¹UCLA Department of Chemistry and Biochemistry, Los Angeles, CA 90095, USA

²UCLA California NanoSystems Institute, Los Angeles, CA 90025, USA

³University of Oklahoma Health Sciences Center, Microbiology and Immunology, Oklahoma City, OK 73104

⁴UCLA Molecular Biology Institute, Los Angeles, CA 90095, USA

⁵Colgate-Palmolive, Piscataway, NJ 08855, USA

⁶International Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science (NIMS), 305-0044 Ibaraki, Japan

Abstract

Objectives—*Streptococcus mutans* is considered a major causative of tooth decay due to its ability to rapidly metabolize carbohydrates such as sucrose. One prominent excreted end product of sucrose metabolism is lactic acid. Lactic acid causes a decrease in the pH of the oral environment with subsequent demineralization of the tooth enamel. Biologically relevant bacteria-induced enamel demineralization was studied.

Methods—Optical profiling was used to measure tooth enamel decay with vertical resolution under one nanometer and lateral features with optical resolution as a result of *S. mutans* biofilm exposure. Comparison measurements were made using AFM.

Results—After 72 hr of biofilm exposure the enamel displayed an 8-fold increase in the observed roughness average, (R_a), as calculated over the entire measured array. Similarly, the average root mean square (RMS) roughness, R_{RMS} , of the enamel before and after biofilm exposure for 3 days displayed a 7-fold increase. Further, the direct effect of chemically induced enamel demineralization using biologically relevant organic acids was shown. Optical profiles of the enamel surface after addition of a 30% lactic acid solution showed a significant alteration in the surface topography with a corresponding increase in respective surface roughness statistics. Similar measurements with 10% citric acid over seconds and minutes give insight into the demineralization process by providing quantitative measures for erosion rates: comparing surface height and roughness as metrics.

Significance—The strengths of optical profilometry as an analytical tool for understanding and analyzing biologically relevant processes such as biofilm induced tooth enamel demineralization were demonstrated.

*Corresponding author: Mailing Address: Department of Chemistry and Biochemistry, University of California, Charles E. Young Drive East, Los Angeles, CA 90095. gim@chem.ucla.edu.

§authors contributed equally

Keywords

enamel erosion; optical profilometry; biofilm; *Streptococcus mutans*; enamel demineralization; citric acid; lactic acid; AFM

Introduction

Dental erosion, particularly in the case of bacteria-induced changes in dental materials, is an area where nanoscale surface analysis is of widespread interest. Dental caries is a widespread chronic disease which affects humans and certain animals (1). The etiology of caries is dependent on several factors, including host susceptibility, intake of carbohydrates and the presence of a bacterial flora capable of acid production. In combination with host behavior, genetic variations, cultural and social variables, caries can develop initiated by the acid induced demineralization of the enamel tooth surface (2, 3).

In this study non-contact optical profiling was used to characterize surface demineralization in tooth enamel due to mono-species bacterial biofilm activity. Although several studies have been conducted on the etching process of tooth enamel with demineralization agents using atomic force microscopy (4, 5), scanning electron microscopy (6-8), transmission electron microscopy (9), x-ray scanning analytical microscopy (10) and confocal laser scanning microscopy (11), these techniques often require significant sample preparation, imaging time or vacuum conditions and may cause problems such as charging when using SEM.; however, there has been relatively little analysis of tooth enamel using non-contact optical profilometry. To date one previous study evaluated the protective effect of fluoride components on tooth enamel treated with hydrochloric acid using optical profilometry (12); however, to our knowledge there is no literature available for analyses of *S. mutans* mono-species biofilm induced enamel demineralization using optical profilometry.

Surface metrology using non-contact, optical profilometry has been widely used in recent years for quality control in industry with applications such as data storage, semiconductors, optical telecommunication and MicroElectroMechanical Systems (MEMS) (13, 14). Optical profiling, or white light interferometry, is a non-destructive, highly sensitive technique typically used for surface characterization and dynamic measurements. With the capability to resolve, repeatedly, large surface areas with sub-nanometer resolution this technique was shown to be a novel complementary technique for applications in the fields of biology and biotechnology.

S. mutans can be isolated from the healthy human dental bacterial flora (15, 16). However, under certain circumstances which have not been fully understood, *S. mutans* can increase in numbers initiating an ecological shift in the bacterial biofilm composition towards a decrease in diversity (15, 17). This could lead to the development of dental caries (demineralization and tooth decay). *S. mutans* is known to express several virulence traits, including the synthesis of glucan-polymers from dietary sucrose, promoting a relatively firm attachment to the tooth surface (1). In addition, *S. mutans* encodes specific high and low affinity transport systems for carbohydrates, which are used almost exclusively as energy and carbon sources (18, 19). These transport systems enable *S. mutans* to take up several different carbohydrates even if they are present in low concentrations. Subsequently, these carbohydrates are metabolized and one of the end-products is lactic acid, which is excreted into the environment (1). The lactic acid can cause a decrease in the pH as low as 4 which causes tooth enamel demineralization (20). Thus, optical profilometry was used to measure nanometer decay in tooth enamel as a result of *S. mutans* biofilm exposure and this outcome was compared to chemically induced enamel demineralization by biologically relevant

organic acids, lactic acid and citric acid. Data for both surface roughness and surface height from a single study is provided, allowing comparison of the two metrics for surface loss.

Materials and Methods

Enamel Samples

Polished round bovine enamel chips (diameter 5 mm) were generously donated from Colgate-Palmolive (Piscataway, NJ). The chips were cleaned with 70% ethanol prior to inoculation for biofilm growth and rinsed with sterile deionized water.

Biofilm Culture

S. mutans strain UA140 was used to grow mono-species biofilms. The enamel chip was placed into a plastic Petri dish which was filled with 5 ml Brain Heart Infusion medium (BHI, Difco, Sparks, MD) supplemented with 1% fresh prepared sucrose from a filter-sterilized 20% stock solution. The Petri dish was incubated for 72 hours at 37° C as static culture. Every 24 hours, the medium was replaced with pre-warmed fresh BHI plus 1% sucrose. The removed media was microscopically inspected for obvious contamination. After 72 hours, the enamel chip was retrieved and the biofilm removed with a cotton tip and vigorous rinsing with deionized water.

Lactic Acid Treatment

Enamel samples were mounted on a glass slide to allow positioning of the sample for imaging. Keeping constant alignment of the enamel sample, 30% lactic acid (prepared from 85% DL-lactic acid syrup, Sigma, St. Louis, MO) was applied drop-wise to the top of the enamel surface to ensure complete coverage. The sample was monitored continuously to ensure that evaporation of the lactic acid did not occur during the etching intervals between imaging. The enamel surface was imaged sequentially after 45, 90 and 225 min of lactic acid exposure. Between time intervals the surface was rinsed with deionized water, air dried, analyzed using the optical profiler and then subjected to further lactic acid exposure as necessary.

Citric Acid Treatment

An experimental schematic for the citric acid erosion experiments is included in Figure 5. Enamel samples were covered with polymer-backed lab tape. An X was scratched through the center of the tape with a razor blade. One quadrant of the tape was removed and the sample was gently placed with tweezers face up in a beaker of citric acid (10% w/w NOW citric acid powder in Millipore DI H₂O). After the exposure time the sample was removed from the bath with tweezers, rinsed with DI water, air dried, checked using the optical profiler, and then subjected to further citric acid exposure as necessary. Samples were acid exposed between 0-45 sec and 0-25 min. All reported data come from a single measurement after the final acid exposure and rinse were completed.

Atomic Force Microscopy

Atomic force microscopy (AFM) (21) imaging was performed as previously described by Cross *et al.* (22, 23). *S. mutans* UA140 biofilms were cultured on enamel chips as described above. The biofilms were air dried and immediately imaged using AFM. All images were obtained using a Nanoscope IV Bioscope (Veeco Digital Instruments, Santa Barbara, CA). Images were collected in contact mode using sharpened silicon nitride cantilevers with experimentally determined (24) spring constants of 0.02 N/m and a tip radius of <20 nm. Height and deflection images were simultaneously acquired at a scan rate of 1 Hz. The images presented in this study are deflection mode images.

Optical Profilometry

To allow positioning of the enamel for imaging, the enamel samples were prepared as described above, air dried and mounted on a glass slide. Surface measurements of the enamel chips were obtained using a WYKO NT-1100 white light interferometer (Veeco, Tucson, AZ). In principle, the NT 1100 interferometer is an optical device equipped with a Mirau interferometric objective which allows for precise examination of lateral features with optical resolution, as well as height changes below one nanometer in surface topography (25-27). Figure 1 shows a schematic of an optical profiler set-up. All images were collected in Vertical Scanning Interferometry (VSI) mode. Data analyses, including surface roughness measurements, were calculated according to parameters in the Veeco, Wyko Optical Profilometry software and described in further detail below. For the citric acid measurements, the optical profiler computed average parameters for identically sized regions selected by the operator as representative of each quadrant.

Surface Roughness Statistics

Surface measurements of the enamel samples were calculated using parameters in the Wyko Optical Profiler software. The calculated surface roughness average, R_a , and root mean square (RMS) roughness, R_{RMS} , were defined as the main height as calculated over the entire measured length or area and as the average between the height deviations and the mean surface taken over the evaluation area, respectively [as defined in the software parameters for the Veeco, Wyko Optical Profiler]. R_a is calculated per the ANSI B46.1 standard according to equation [1]

$$R_a = \frac{1}{MN} \sum_{j=1}^N \sum_{i=1}^M |Z_{ij}| \quad (1)$$

for a three-dimensional measurement where M and N are the number of data points in X and Y , and Z is the surface height relative to the mean plane. Similarly, the root mean square roughness, R_{RMS} , was calculated according to equation [2]

$$R_{rms} = \sqrt{\frac{1}{MN} \sum_{j=1}^N \sum_{i=1}^M Z^2(x_i, y_j)} \quad (2)$$

for a three-dimensional surface where M and N are once again the number of data points in X and Y , and Z is the surface height relative to the mean plane. The RMS roughness is representative of the standard deviation of the profile heights of the measured surface.

Surface Loss Measurements

Surface loss was calculated from a single optical profile of a single sample that contained multiple regions, including a reliable reference point. The reference point was needed to establish a reference height to which eroded regions may be compared. It was essential that the reference point and all relevant erosion regions be included in a single image as optical profilometry data are highly processed and processing may vary from image to image. Subregions of identical size located centrally in each erosion region were selected for computation of average surface height and average surface roughness. The selection of subregions avoids noise due to incomplete spatial separation of erosion regions. Surface heights and roughnesses were calculated using the Wyko Optical Profiler software.

Statistical Analysis

Results are summarized as both mean $\pm$ s.d. as well as median values. Measurements were analyzed using a two sample independent Student's *t*-test to assess the statistical significance of the mean values at the 95% confidence level. Differences among mean values are reported using exact *P* values.

Results

Biofilm development

Previous results demonstrated that *S. mutans* is able to form a biofilm on hydroxyapatite *in vitro* (28). Hydroxyapatite is composed of a complex calcium phosphate mineral and is a principal component found in tooth enamel. We used bovine enamel to study the effect of biofilm initiated demineralization of enamel surfaces as a model for the human tooth. *S. mutans* biofilms were grown on bovine enamel in the presence of 1% sucrose, which was previously shown to be a saturating amount for *S. mutans* single-species biofilm formation (29). Incubation over a 72 h time period, with replacing the media every 24 hours, resulted in biofilm formation which was visible by eye as a thick layer of cells covering the upward side of the bovine enamel chip. An optical profile and an atomic force microscopy (AFM) image of a single-species *S. mutans* biofilms grown overnight on hydroxyapatite in the presence of 1% sucrose are shown in Figure 2. The images show colonies of *S. mutans* cells embedded in secreted extracellular matrix material, composed of glucan-polymers, which allow firm attachment of these cells to the enamel surface. Although the lateral resolution is not as good as that of AFM, the biofilm is readily visible using optical profilometry. Typically biofilms are challenging to image using other techniques; however this is not the case with optical profilometry, even when imaging under liquid conditions.

Lactic acid induced demineralization

The synthesis of glucan-polymers from dietary sucrose allows a relatively firm attachment of *S. mutans* biofilms to the tooth surface and leads to retention of lactic acid produced during fermentation. A drop in pH is associated with an increased acidic environment and the demineralization of tooth enamel can occur when alteration in the environment results in a pH of 5.5 or lower (1). Nanometer decay in tooth enamel as a result of chemically induced demineralization by lactic acid was measured using optical profilometry.

Exposure of the enamel surface to 30% lactic acid resulted in a significantly altered surface topography with a large increase in surface roughness. The initial polished surface displayed a relatively smooth topography consistent with that described above and shown elsewhere (5). With progressive exposure to lactic acid (45, 90 and 225 min) the surface topography shifted significantly. Typical R_a and R_{RMS} values before etching with lactic acid were 21.3 and 28.6 nm, respectively. After 45 min and 90 min of exposure, the surface roughness increased significantly. Representative R_a and R_{RMS} values after 45 min were 115.1 and 141.9 nm, respectively. After 90 min the calculated values were 211.2 and 261.7 nm, respectively. Typical R_a and R_{RMS} values of the enamel surfaces after 225 min of exposure were 325.2 and 411.1 nm, respectively. Optical profiler images of a typical enamel sample with 30% lactic acid treatment for progressive time intervals are shown in Figure 3(a-d). The images show a significant increase in apparent surface roughness with prolonged treatment, as reflected by the representative roughness values described above.

Change in surface morphology of bovine enamel caused by *S. mutans* single species biofilms

Analysis of tooth enamel before and after biofilm exposure showed a significant change in surface morphology when examined using optical profilometry. Prior to biofilm inoculation

the enamel surface appeared relatively smooth with characteristic topography consistent with that of polished enamel in previous studies (5) as shown in Figure 3e. With exposure to bacteria for 72 hr the surface morphology changed significantly. Figure 3f shows an optical profilometry image of an enamel surface after etching due to biofilm colonization of the surface. These images clearly reveal a large shift in the topography of the surface when exposed to the bacterial biofilm. After biofilm colonization, the enamel specimens appear coarse with significantly increased height variation (Fig. 3f, z range = 4.25 μm) and no longer exhibit the typical flat, smooth topography of untreated enamel (Fig. 3e, z range = 1.2 μm).

Surface roughness of bovine enamel before and after biofilm exposure

To quantify the observed change in topography of the enamel samples, surface roughness analyses of the enamel before and after biofilm inoculation were performed. Table 1 shows the measured surface roughness average, R_a , and root mean square roughness, R_{RMS} , as taken over the entire measured array. Values for R_a , the main height as calculated over the entire measured area of enamel, before and after biofilm growth were determined to be 42.8 ± 26.5 nm (mean $\pm$ s.d.; median=30.3 nm) and 346.9 ± 253.6 nm (mean $\pm$ s.d.; median=282.6 nm), respectively (Table 1 and Fig. 4d; n=7). Roughness average values, R_a , before and after biofilm inoculation were determined to be statistically different using a two sample independent *t*-test at the 95% confidence level ($p < 0.009$). In addition, measured R_{RMS} values, the RMS average between the height deviations and the mean surface taken over the evaluation area of the enamel, for the surface before and after biofilm growth were determined to be 59.4 ± 34.4 nm (mean $\pm$ s.d.; median=42.7 nm) and 423.0 ± 271.4 nm (mean $\pm$ s.d.; median=370.5 nm), respectively (Table 1 and Fig. 4e; n=7). A two sample independent *t*-test indicated that the population means were significantly different from each other at the 95% confidence level ($p < 0.005$). Histograms of the measured surface roughness average, R_a , and root mean square roughness values, R_{RMS} , taken on enamel before (light grey) and after (dark grey) biofilm degradation are shown in Figure 4.

Typical line profiles for the two enamel surface-types, before and after biofilm growth, are also shown in Figure 4. Line profiles of enamel before biofilm growth (Fig. 4a light grey, 4b) show relatively flat traces compared to those after 72 hr biofilm activation (Fig. 4a black, 4c). Line scans of the enamel before treatment generally have z-heights under ~ 0.01 μm , whereas those on taken on the treated enamel display much larger deviation in z-height, with peak to valley values typically 0.5 μm or greater, as expected from the significant increase in corresponding surface roughness measurements.

Citric acid induced demineralization

Citric acid is a common ingredient in soft drinks and other popular foods and beverages. The pH associated with this acidic environment can affect demineralization rates of tooth enamel. Nanometer decay of tooth enamel as a result of chemically induced demineralization by citric acid was measured using optical profilometry.

Exposure of the enamel surface to 10% citric acid resulted in a significantly altered surface topography with a large increase in surface roughness initially, that eventually leveled off at around 1 μm . The initial polished surface displayed a relatively smooth topography consistent with that described above and shown elsewhere (5). With progressive exposure to citric acid (10, 15 and 45 s or 10, 20, 25 min) the surface topography shifted significantly. Optical profiler images of a typical enamel sample with 10% citric acid treatment for progressive time intervals are shown in Figure 5. Within each image a large representative region was chosen for height and roughness analysis. The images show a significant increase in apparent surface roughness especially after short times. Typical R_a and R_{RMS}

values before etching with citric acid were 21.3 and 28.6 nm, respectively. Those jumped to 200, 300, and 900 nm after 10, 15, and 45 s respectively (see Table 2). The images after 10, 20, 25 min show the roughness value plateaus at nearly 1 micron. In addition, height measurements were taken at each interval to quantify demineralization. The height drops a few microns in the first 10-15 s and in some cases fell by more than 10 μm after 45 s as shown in Figure 6. The rate of loss then slows and after 25 min the average rate is about 1 $\mu\text{m}/\text{min}$. It is difficult to compare data across samples because of significant variation among identically prepared samples that leads to widely different demineralization rates under identical conditions.

Discussion

In this study, high resolution height variation analysis of tooth enamel demineralization as a result of mono-species *S. mutans* biofilm exposure was shown. In addition, chemically induced enamel demineralization by lactic acid and citric acid was measured using optical profilometry. Images of polished enamel surfaces before and after 72 hr exposure to mono-species *S. mutans* biofilm inoculation clearly revealed a significant increase in height variation and apparent surface roughness. Average R_a and R_{RMS} values before and after biofilm growth increased from 42.8 to 346.9 nm and 59.4 to 423.0 nm, respectively. Subsequent quantitative analysis of the enamel surface topography after biofilm colonization revealed an 8- and 7-fold increase in the corresponding surface roughness values R_a and R_{RMS} , respectively, as illustrated in both the optical profiler images as well as in the histograms of the measured surface roughness statistics. Similarly, optical profiling of chemically induced enamel etching using 30% lactic acid or 10% citric acid showed easily measurable shifts in the surface topography and related surface roughness values with progressive acid exposure. Relatively high concentrations of acid were used to accelerate the demineralization effect. The *in vitro* biofilm model used during this study was composed of a single bacterial species, *S. mutans*. Dental plaque is composed of several hundred different species. So far over 500 different phylotypes have been identified (30, 31). Although not all of them are involved in the *in vivo* demineralization of enamel, certain species are known to produce a low pH after administration of glucose (20). Among these species are streptococci, which in fact can constitute up to 80% of early dental plaque (32). Our study demonstrates the potential to investigate the demineralization process of plaque samples compared to defined multi-species mixtures of streptococci in a biofilm model. The availability of fluorescent labeled *S. mutans* (33) makes it possible to combine optical profilometry with confocal laser scanning microscopy, thus defining positions of specific bacteria in a mixed bacterial species biofilm with regard to the demineralization process.

Furthermore, the technique described here is also able to address questions about the susceptibility of dental materials to erosion induced by the lactic acid from oral bacteria. Earlier work demonstrated that certain dental materials like resin composites can accelerate *S. mutans* growth, eventually leading to lactic acid exposure. This demineralization effect is likely more prominent in the *in vitro* system used, since there is no balance between demineralization and re-mineralization and no buffer capacity as seen with saliva.

The citric acid study reveals how the polished surface is eroded in the first few seconds. First small pits form where structurally weaker enamel is dissolved by the acid. These smaller pits grow vertically and laterally, coalescing to form larger pits. Within one minute there are pits microns deep and wide and spires of tough undissolved enamel rising precipitously from the surface like plateaus. This increase in surface area of contact between solvent and surface increases the erosion rate. After about 1 minute the spires of uneroded enamel become weak and possibly break off, leaving a new flatter uneroded surface beneath. At this time the system reaches a sort of equilibrium where erosion continues at a roughly

constant rate and roughness settles to about 1 micron. It must be noted, however, that for the particular application of dental caries study this technique is limited to the initial stage of caries lesion as further caries development is a subsurface phenomenon.

There is significant variation in erosion rates among different samples subjected to identical conditions. This apparent variation makes it difficult to compile data from several samples (see figures 6e, 6f). Height and roughness values can only be compared across samples as an order of magnitude. The erosion rates are determined with a single sample so the confidence in a reported erosion rate is high, however, a comparison of erosion rates is again only meaningful as an order of magnitude until the variation is understood and controlled for.

Conclusion

Nanoscale topology and analysis of biotic and abiotic surfaces are of significant interest. The unique ability of optical profilometry to measure nanometer scale surface properties non-destructively, demonstrates the capacity of this technique to study processes causing morphological changes to the tooth surface itself and dental materials. Moreover, this technique has the ability to operate real-time under liquid conditions (25). Conceivably, optical profilometry may even find itself in a dental setting as a profilometer that has the capability to be modified for diagnostic purposes.

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Figure 1.

Schematic representation of an optical profiler. Light from a halogen lamp is collimated using a series of objectives. The collimated beam is split and half goes down to the surface, reflects back up, and recombines with the original beam giving a characteristic interference pattern that is translated into a topographical image by computer software.

Figure 2. *S. mutans* single-species biofilm images. **(a)** Optical profile of an *S. mutans* biofilm on hydroxyapatite; scale bar = 30 μm . **(b)** Atomic force microscopy (AFM) deflection mode image of an *S. mutans* single-species biofilm on hydroxyapatite; scale bar = 2.5 μm .

Figure 3. 3-Dimensional optical profilometry images showing lactic acid demineralization of tooth enamel. **(a)** the relatively smooth surface of a polished enamel sample. Scale bar = 0.89 μm . **(b)**, **(c)**, **(d)** the same area of enamel after 45 min, 90 min, 225 min of 30% lactic acid exposure. Scale bars = 1.07 μm , 2.25 μm and 3.97 μm , respectively. **(e)** the smooth polished enamel surface before and **(f)** after biofilm inoculation for 3 days (72 hr). Note the significant shift in the topography of the surface after biofilm colonization. Scan size = 124 $\times$ 94 μm .

Figure 4.

Typical line profiles of an enamel surface and histograms of roughness values showing change in surface topography due to biofilm inoculation. **(a)** Line profiles of surface before (light grey) and after (black) 72 hr of biofilm growth. **(b)** Optical profiler image of the enamel surface before biofilm inoculation with dashed-line corresponding to the light grey line scan in (a); image is $124 \times 94 \mu\text{m}$. **(c)** Optical profiler image showing etching of the enamel surface after 3 days of biofilm activation. Dashed-line corresponds to the black line profile in (a); image is $124 \times 94 \mu\text{m}$. **(d), (e)** Histograms of surface measurements, R_a and R_{RMS} , taken on enamel before (light grey) and after (dark grey) biofilm inoculation/degradation. **(d)** Measured roughness average, R_a , of the enamel. The R_a values obtained before (light grey) and after (dark grey) biofilm inoculation were 42.8 ± 26.5 and 346.9 ± 253.6 nm, respectively (mean $\pm$ s.d.; $n=7$). Statistical comparison concluded that these values were significantly different ($p < 0.009$). **(e)** The root mean square (RMS) roughness, R_{RMS} , for the enamel. Average R_{RMS} values before and after biofilm inoculation for 3 days were found to be 59.4 ± 34.4 and 423.0 ± 271.4 nm, respectively (mean $\pm$ s.d.; $n=7$). A two sample independent Student's t-test showed these values were statistically different ($p < 0.005$).

Figure 5.

Experimental schematic and data for citric acid experiments. First an enamel disk is covered with lab tape. Next an X is drawn through the tape with a razor and a quadrant of tape is removed. The disk then rests in an acid bath until it is removed for imaging. After an optical profile is taken, another tape quadrant is removed and the sample is placed back into the acid bath. An optical profile of the smooth polished sample surface (a), an image of four quadrants eroded for various times less than one minute (b), and an image of four quadrants eroded for various times less than 30 min (c). Image size $1240 \times 940 \mu\text{m}$

Figure 6.

Surface height and roughness as metrics to quantify mineral loss. Variation in surface height over one minute (a) and 30 minutes (b). Variation in roughness over one minute (c) and 30 minutes (d). Data from several samples supports a 2 region understanding with erosion of the initially flat surface proceeding at a different rate than erosion of the later roughened surface. After about one minute the RMS roughness plateaus, as does the erosion rate. Significant variation is seen among identically prepared samples (e) and (f). Perhaps this is a result of varying inclusions and grain boundaries stemming from different genetics and/or environments of the parent animals.

Table 1

Surface Roughness after inoculation

Enamel	R_a (nm)		R_{RMS} (nm)	
	Mean ± s.d.	Median	Mean ± s.d.	Median
Before biofilm inoculation	42.8 ± 26.5	30.3	59.4 ± 34.4	42.7
After biofilm inoculation	346.9 ± 253.6	282.6	423.0 ± 271.4	370.5

* Ra = roughness average (main height as calculated over the entire measured area);

RRMS = root mean square roughness (RMS average between the height deviations and the mean surface taken over the evaluation area);

s.d. = standard deviation; n = 7. Measurements before and after biofilm inoculation were statistically different ($p < 0.009$).

Table 2

Surface roughness and height after citric acid erosion for several samples

Time (s)	RRMS (nm)	σ RRMS (nm)*	Height (nm)	σ Height (nm)
0.00	126.96	52.53	0.02	0.02
10.00	243.30	44.59	-837.54	289.42
15.00	340.32	10.38	-1458.45	107.95
20.00	454.89		-3154.82	
30.00	531.37	30.08	-3066.56	88.25
45.00	737.54	142.69	-3986.32	1076.48
300.00	857.73		-446.55	
600.00	1254.26		-11390.43	
900.00	1438.20		-5893.55	
1200.00	949.85		-22917.90	
1500.00	1006.07	133.11	-20764.33	4325.78

* σ indicates the standard deviation of a set of measurements on bovine enamel that were nominally identically prepared but in fact varied widely perhaps due to the differing genetics and environments of the parent cows or perhaps due to processing techniques. The standard deviation of several measurements on the same sample was 1-2 orders of magnitude lower as the resolution of this optical measurement technique is roughly 1-10 nm.