High-speed atomic force microscopy of dental enamel dissolution in citric acid

Alice Pyne¹, Will Marks¹, Loren M. Picco¹, Peter G. Dunton, Arturas Ulcinas, Michele E. Barbour², Siân B. Jones², James Gimzewski^{3,4,5}, and Mervyn J. Miles¹

¹*H.H. Wills Physics Laboratory, ²Department of Oral & Dental Science, University of Bristol, Bristol, UK;*

³*Department of Chemistry & Biochemistry, and ⁴California Nano Systems Institute, University of California,*

Los Angeles, U.S.A.; and ⁵International Center for Materials Nanoarchitectonics Satellite (MANA), National Institute for Materials Science (NIMS), Tsukuba, Japan

Summary. High-speed atomic force microscopy (HS AFM) in 'contact' mode was used to image at video rate the surfaces of both calcium hydroxyapatite samples, often used as artificial dental enamel in such experiments, and polished actual bovine dental enamel in both neutral and acidic aqueous environments. The image in each frame of the video of the sample was a few micrometers square, and the high-speed scan window was panned across the sample in real time to examine larger areas. Conventional AFM images of the same regions of the sample were also recorded before and after high-speed imaging. The ability of HS AFM to follow processes occurring in liquid on the timescale of a few seconds was employed to study the dissolution process of both hydroxyapatite and bovine enamel under acidic conditions. Buffered citric acid at pH values between 3.0 and 4.0 was observed to dissolve the surface layers of these samples. The movies recorded showed rapid dissolution of the bovine enamel in particular, which proceeded until the relatively small amount of acid available had been exhausted. A comparison was made with enamel samples that had been treated in fluoride solution (1 h in 300 ppm

NaF, pH 7) prior to addition of the acid; the speed of dissolution for these samples was much less than that of the untreated samples. The HS AFM used an in-house designed and constructed high-speed flexure scan stage employing a push-pull piezo actuator arrangement. The HS AFM is able to follow the large changes in height (on the micrometer scale) that occur during the dissolution process.

Introduction

Dental studies by atomic force microscopy (AFM) can provide unique information on the dissolution, or "erosion", of dental enamel, owing to its ability to image at high resolution in liquid environments so that the process of dissolution or erosion of enamel can be followed *in situ* in the microscope. The imaging rate of conventional AFM, at around one frame every one to two minutes, means that such processes can only be followed if changes occur on the timescale of several minutes, but enamel dissolves much more quickly than this with changes in structure after <1s acid exposure (White *et al.*, 2008). Alternatively, the dissolution process can be performed externally to the AFM in a series of short exposures to the acidic environment interleaved with examination of the sample in the AFM (Finke *et al.*, 2000; Wang *et al.*, 2005). This has two major disadvantages: i) the same location on the sample can not be found reliably each time the sample is reloaded into the AFM; this is particularly the case in degradation as landmarks become eroded, and ii) the stop-start procedure means that a process is not accurately represented.

Received April 26, 2009

Address for Correspondence: Prof. Mervyn J. Miles, H. H. Wiles Physics Laboratory, University of Bristol, Tyndall Avenue, Bristol, BS8 1 TL, UK
Phone: +44-117-928-8707, Fax: +44-117-925-5624
E-mail: m.j.miles@bristol.ac.uk

The ability to follow the dissolution process in the microscope in real time clearly has major advantages over time-lapse based techniques. In order to achieve measurements of the dissolution, a high-speed AFM capable of both several frames per second and of imaging samples in which the topography is relatively high and rapidly changing is required. Various versions of high-speed AFMs have been developed over the past ten years or more. Most of these instruments have been based on the principle of increasing the imaging rate of intermittent-contact mode AFM by increasing the resonant frequency of the cantilever by decreasing its size (Walters *et al.*, 1996; Viani *et al.*, 1999; Viani *et al.*, 2000; Ando *et al.*, 2001; Ando *et al.*, 2006; Fantner *et al.*, 2006). Ando *et al.* have recently published a review of this field (Ando *et al.*, 2008). Cantilevers of less than 10 μm in length having fundamental resonant frequencies around 1 MHz have been used and video rate movies of biomolecular processes at the single molecule level have been reported (Ando *et al.*, 2002). Such small cantilevers are suitable for low topographic height sample surfaces such as single biomolecules on flat mica surfaces, but these are inappropriate for samples with height variations of 100s of nanometres. We have developed a different high-speed AFM (HS AFM) (Humphris *et al.*, 2005; Picco *et al.*, 2007, 2008; Vicary and Miles, 2009) which uses cantilevers with dimensions the same as those used in conventional AFM, i.e., with lengths around 100 μm . This type of HS AFM is a DC mode in which the tip is essentially in contact with the sample throughout the high-speed scanning. There is evidence that the super-lubrication effect may play a role in reduction of tip-sample damage. This HS AFM technique has been used to image biological specimens such as collagen at up to 1000 frames per second (Picco *et al.*, 2007) and human chromosomes in air and in an aqueous environment in which the swollen chromosomes were over 0.5 μm in height (Picco *et al.*, 2008). This technique has been demonstrated to cope with large topographical height differences and therefore is an appropriate technique to follow the dissolution of dental enamel.

Methods and Materials

Dental samples

Two basic types of dental sample were used in these studies. One was a polished, sintered hydroxyapatite disc (Hitemco, Old Bethpage, NY, USA and Dr. Richard Sullivan, Colgate-Palmolive Co., Piscataway, NJ, USA.), which is generally used as a model sample, and, although

it does not reproduce the structure of natural mammalian tooth enamel, it provides a reproducible surface which is often used as an analogue for dental enamel. The second type of sample was bovine enamel embedded in polymer resin and polished to a finish using alumina powder. Specimens were ultrasonicated in deionised water prior to use.

Citric acid solutions buffered to various pH values were prepared for the dissolution experiments and could be injected into the sample liquid cell during scanning. The buffer solution used in the experiments reported here was of pH 3 and made up using 0.1 M citric acid (pKa 4.77) and 0.1M sodium citrate with respective volumes of 8.545 ml and 1.455 ml. No further deionised water was added. This gives an acid concentration of 85.5 mM. Typically, around 150 μl of acid were added to 30 μl of pure water in the liquid cell to give a typical final concentration of 70 mM on the sample. The typical volume of acid injected was 65 μL .

Some bovine enamel samples were soaked in fluoride solutions for one hour (300 ppm NaF, pH 7) to simulate the effect of treatment with fluoride toothpaste or mouthwash.

Fig. 1. Diagram of the high-speed AFM flexure stage

Fig. 2. HS AFM images recorded in water of (a) a hydroxyapatite surface and (b) a bovine enamel surface.

HS AFM

The high-speed scanning system used for video-rate imaging in both air and in liquid was based on a flexure stage, constructed in-house, and has been described previously (see, for example, Picco *et al.*, 2008). A diagram of the flexure stage can be seen in Figure 1. The high-speed system was mounted on a Veeco Dimension 3100 for conventional tapping and contact mode imaging in air and in liquid such that samples mounted on the high-speed flexure scanner could also be imaged at conventional imaging speeds. With the flexure stage enabled to scan at 5000 lines per second, and with slow scan (y) of 20 frames per second, provided by the Dimension scan tube, taken over by the in-house constructed high-speed controller and data capture system high speed AFM was possible on the same scan area as the conventional imaging. The cantilever was mounted on the scan tube with its long axis aligned with the slow scan direction. The scan tube also provided the z-direction control used to maintain on average a constant preset cantilever deflection. A sinusoidal voltage waveform was used to drive the scan (a correction is applied to each frame in real-time to linearise the data) while a triangular waveform was used for the frame scan during high-speed imaging. Cantilever bending was measured by the usual optical lever method. Deflection data were captured and image construction performed in real time. Cantilever bending was measured by the usual optical lever and quadrant photodiode. Deflection data were captured and image construction performed in real time.

Results and Discussion

The surfaces of both hydroxyapatite samples and bovine enamel samples both as received and fluoride-treated were imaged with conventional AFM in contact mode and with the high-speed AFM in liquid. Figure 2a shows a typical still image taken from a high-speed AFM movie of the hydroxyapatite. The hydroxyapatite surface consists of clusters of more spherical in shape and more monodisperse in size crystallites (around 200 nm) than the bovine enamel surface shown in Figure 2b. The bovine enamel structure consists of a wider range of crystallites size a few hundred nanometres in lateral dimensions. In some regions, polishing grooves can be seen. Neither structure appeared to change significantly between imaging in air and in an aqueous environment. Figure 3a shows a sequence of images taken from a high-speed movie of a hydroxyapatite sample in water; this is a control experiment for comparison with the addition of citric acid to the environment, which is shown in Figure 3b. In water, the surface of the hydroxyapatite sample does not noticeably change over a period of several minutes. However, with the addition of citric acid, changes were observed in the surface structure. Individual crystallites were seen to dissolve and suddenly disappear in an apparently random sequence on the surface, as careful study of the movie frames in Figure 3b will show. Similar experiments were performed on bovine enamel. Firstly, a control experiment was performed with HS AFM of the enamel surface in water. Figure 4a shows a

Fig. 3. Sequences of HS AFM images of an hydroxyapatite surface taken (a) in water ($3\ \mu\text{m} \times 3\ \mu\text{m}$), and (b) in citric acid at pH 3 ($1.5\ \mu\text{m} \times 1.5\ \mu\text{m}$). Time is indicated in seconds under each image.

series of frames from such an HS AFM movie. Except for some minor mechanical drift of the scan stages, there is very little discernible change in the images of the surface of the 20 s period. Most importantly, the HS AFM does not appear to cause significant damage to either the bovine enamel or the hydroxyapatite surface shown in Figure 2. Figure 4b is a series of frames taken from an HS AFM movie following the effect of adding citric acid buffered to Ph 3 to the sample environment. The first two frames (at times -4 s and -2 s) are recorded with the enamel in water before the acid was added at time 0 s, the third frame. Instability was immediately apparent in this image, and indeed in the subsequent images. There followed a rapid and extensive dissolution of dental enamel from the sample surface. As the surface structure was removed, sub layers were revealed and similarly dissolved for a total of about ten seconds at which time the sample surface became more stable with just a few individual crystallites being dissolved in the remaining time. The initial dissolution process of the bovine enamel surface was considerably more extensive and violent than in the case of the hydroxyapatite surface. That the dissolution process is greatly decreased after about ten seconds probably reflects the small volume of acid available within the liquid cell of the AFM rather than a different type of structure in the enamel being exposed by the dissolution process. It is probable that the acid solution in contact with the enamel rapidly becomes saturated, or nearly saturated, owing to the accumulation of calcium and phosphate ions from the dissolved

hydroxyapatite. With the current HS AFM system, it is difficult to obtain an accurate measurement of the depth of material dissolved in this process, but is between 500 nm and $1\ \mu\text{m}$. It is probably worth noting that such a large and rapid change in height would prove very difficult for high-speed AFM based on the small cantilever method.

For comparison with the HS AFM images shown in Figure 4, Figure 5 shows two larger images ($20\ \mu\text{m} \times 20\ \mu\text{m}$; height range: 100 nm) of the enamel surface recorded with conventional AFM and centred on the region scanned by the HS AFM. Both images were captured in deflection mode. This is an appropriate mode for comparison as the operation of the HS AFM in these experiments was essentially also in deflection mode. Figure 5a shows the enamel in water before the addition of the acid. With the exception of some polishing lines, the surface is mostly smooth with a few randomly located pits and structures of about 40 nm in diameter, similar to those seen in the HS AFM. Following the addition of the acid and HS AFM imaging of the dissolution process, the surface (Fig. 5b) has become much rougher with individual nodular crystallites more visible. There is no evidence in this image that the $1.5\ \mu\text{m}$ square HS AFM imaging has either affected the dissolution process or caused additional damage to the surface.

For comparison with the HS AFM images shown in Figure 4, Figure 5 shows two larger images ($20\ \mu\text{m} \times 20\ \mu\text{m}$; height range: 100 nm) of the enamel surface recorded with conventional AFM and centred on the region scanned by the HS AFM. Both images were

Fig. 4. Frames ($1.5 \mu\text{m} \times 1.5 \mu\text{m}$) taken from HS AFM movies of the surface of polished bovine enamel. Film strip (a) is a sequence of images recorded in water; the film strips in (b) show a sequence of images recorded before, during, and after the addition of citric acid at pH 3. The time of the addition of the acid is marked as 0 s.

captured in deflection mode. This is an appropriate mode for comparison as the operation of the HS AFM in these experiments was essentially also in deflection mode. Figure 5a shows the enamel in water before the addition of the acid. With the exception of some polishing lines, the surface is mostly smooth with a few randomly located pits and structures of about 40 nm in diameter, similar to those seen in the HS AFM. Following the addition of the acid and HS AFM imaging of the dissolution process, the surface (Fig. 5b) has become much rougher with individual nodular crystallites more visible. There is no

evidence in this image that the $1.5 \mu\text{m}$ square HS AFM imaging has either affected the dissolution process or caused additional damage to the surface.

It is well known that fluoride treatment of enamel surfaces protects against acid degradation. In order to investigate any short timescale effects of acid on fluoride treated enamel, HS AFM was performed and the results are shown in Figure 6. The amount of dissolution observed was very small and the extent varied for different regions of the enamel sample. The frames shown in Figure 6, taken from the movie of this experiment,

Fig. 5. Conventional speed deflection-mode AFM images of the bovine enamel surface (a) before and (b) after treatment with citric acid; scan area: $20\ \mu\text{m} \times 20\ \mu\text{m}$.

Fig. 6. Frames from HS AFM movie of fluoride-treated bovine enamel exposed to citric acid. No major changes in the surface structure were observed.

show very little change; the occasional disappearance of an individual crystallite can be observed. This is in marked contrast to the major erosion seen for the unprotected enamel surface in Figure 4b.

Conclusions

These experiments have shown the value of the HS AFM in following processes at video rate in liquid environments. This particular HS AFM technique can accommodate relatively large and rapid changes in surface height and roughness, making it suitable for these dental enamel erosion studies. A striking observation from both the hydroxyapatite sample and the fluoride-protected enamel sample is that the dissolution proceeded by a 'quantized' disappearance of individual crystallites, suggesting that once dissolution of a crystallite was

initiated, the crystal became thermodynamically unstable and dissolved instantaneously. Alternatively this could be interpreted as a loosening of the bonds binding the crystallite to the surface, followed by a tip-mediated rapid displacement of the now-free crystallite. Further studies should examine the variation with location on the surface structure in dissolution for both the enamel surface and the fluoride protected enamel surface. The special nature of the nucleation sites for dissolution could provide valuable information for understanding the mechanisms and further protection strategies.

Acknowledgments

We are grateful for the support of the EPSRC IRC in Nanotechnology in this work and to the Bristol University for a Benjamin Meaker Professorship, which allowed the visit of Professor Gimzewski.

References

- Ando T, Kodera N, Takai E, Maruyama D, Saito K, Toda A: High-speed atomic force microscope for studying biological macromolecules. *Proc Natl Acad Sci* 98: 12468-12472 (2001).
- Ando T, Kodera N, Maruyama D, Takai E, Saito K, Toda A: A high-speed atomic force microscope for studying biological macromolecules in action. *Jpn J Appl Phys* 41: 4851-4856 (2002).
- Ando T, Uchihashi T, Kodera N, Miyagi A, Nakakita R, Yamashita H, Sakashita M: High-speed atomic force microscopy for studying the dynamic behavior of protein molecules at work. *Jpn J Appl Phys* 45: 1897-1903 (2006).
- Ando T, Uchihashi T, Kodera N, Yamamoto D, Miyagi A, Taniguchi M, Yamashita H: High-speed AFM and nano-visualization of biomolecular processes. *Pflugers Arch - Eur J Physiol* 456:211-225 (2008).
- Fantner GE, Schitter G, Kindt JH, Ivanov T, Ivanova K, Patel R, Holten-Andersen N, Adams J, Thurner PJ, Rangelow IW, Hansma PK: Components for high speed atomic force microscopy. *Ultramicroscopy* 106: 881-887 (2006).
- Finke M, Jandt KD, Parker DM: The early stages of native enamel dissolution studied with atomic force microscopy. *J Colloid Interface Sci*: 232:156-164 (2000).
- Humphris ADL, Miles MJ, Hobbs JK: A mechanical microscope: High-speed atomic force microscopy. *Appl Phys Lett* 86: Art. No.: 034106 (2005).
- Picco LM, Bozec L, Ulcinas A, Engledew DJ, Antognozzi M, Horton MA and Miles MJ: Breaking the speed limit with atomic force microscopy. *Nanotechnology* 18: Art. No.: 044030 (2007).
- Picco LM, Dunton PG, Ulcinas A, Engledew DJ, Hoshi O, Ushiki T, Miles MJ: High-speed AFM of human chromosomes in liquid. *Nanotechnology* 19: Art. No.: 384018 (2008).
- Viani MB, Schaeffer TE, Palocz GT, Pietrasanta LI, Smith BL, Thompson JB, Richter M, Rief M, Gaub HE, Plaxco KW, Cleland AN, Hansma HG, Hansma PK: Fast imaging and fast force spectroscopy of single biopolymers with a new atomic force microscope designed for small cantilevers. *Rev Sci Instrum* 70: 4300-4303 (1999).
- Viani MB, Pietrasanta LI, Thompson JB, Chand A, Gebeshuber IC, Kindt JH, Richter M, Hansma HG and Hansma PK: Probing protein-protein interactions in real time. *Nat Struct Biol* 7: 644-647 (2000).
- Vicary JA, Miles MJ: Real-time nanofabrication with high-speed atomic force microscopy. *Nanotechnology* 20: Art. No. 095302 (2009).
- Walters D, Cleveland JP, Thomson NH, Hansma PK, Wendman MA, Gurley G, Elings V: Short cantilevers for atomic force microscopy. *Rev Sci Instrum* 67: 3583-3590 (1996).
- Wang L, Tang R, Bonstein T, Orme CA, Bush PJ, Nancollas GH: A New model for nanoscale enamel dissolution. *J Phys Chem B* 109:999-1005 (2005).
- White AJ, Yorath C, ten Hengel V, Jones SB, Huysmans M-CDNJM, Barbour ME: Acid-mediated softening of human and bovine enamel at ultra-short exposure times. *Caries Research* 42:185 (2008).