ELSEVIER

Micron 36 (2005) 672–680

micron

www.elsevier.com/locate/micron

Ultrastructural examination of dentin using focused ion-beam cross-sectioning and transmission electron microscopy

R.K. Nalla[a], A.E. Porter[a,b,*], C. Daraio[c], A.M. Minor[b], V. Radmilovic[b,1], E.A. Stach[b], A.P. Tomsia[a], R.O. Ritchie[a]

[a]*Materials Sciences Division, Lawrence Berkeley National Laboratory, and Department of Materials Science and Engineering, University of California, Berkeley, CA 94720, USA*

[b]*National Center for Electron Microscopy, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA*

[c]*Department of Materials Science and Engineering, University of California, San Diego, CA 92093, USA*

Abstract

Focused ion-beam (FIB) milling is a commonly used technique for transmission electron microscopy (TEM) sample preparation of inorganic materials. In this study, we seek to evaluate the FIB as a TEM preparation tool for human dentin. Two particular problems involving dentin, a structural analog of bone that makes up the bulk of the human tooth, are examined. Firstly, the process of aging is studied through an investigation of the mineralization in 'transparent' dentin, which is formed naturally due to the filling up of dentinal tubules with large mineral crystals. Next, the process of fracture is examined to evaluate incipient events that occur at the collagen fiber level. For both these cases, FIB-milling was able to generate high-quality specimens that could be used for subsequent TEM examination. The changes in the mineralization suggested a simple mechanism of mineral 'dissolution and reprecipitation', while examination of the collagen revealed incipient damage in the form of voids within the collagen fibers. These studies help shed light on the process of aging and fracture of mineralized tissues and are useful steps in developing a framework for understanding such processes.

Keywords: Aging; Mineralized tissue; Dentin; Fracture; Microscopy; FIB; TEM

1. Introduction

Dentin, the most abundant mineralized tissue in the tooth, is composed largely of type-I collagen fibrils and nanocrystalline apatite mineral (Marshall et al., 1997), with composition similar to that of human bone. In comparison to bone, which has a complex hierarchical structure (Rho et al., 1998), dentin has a rather simple microstructure (Fig. 1). The most striking microstructural feature in dentin is the dentinal tubule, which are cylindrical channels, roughly 1–2 μm in diameter, formed during dentinogenesis, that course continuously from the dentin–enamel (DEJ) and the cementum–enamel (CEJ) junctions to the pulp with a thin, highly mineralized cuff of peritubular dentin surrounding each tubule. The mineralized collagen fibrils, usually 50–100 nm in diameter, constitute the structural backbone of dentin and are arranged orthogonal to these tubules, forming a planar, felt-like, intertubular dentin matrix (Jones and Boyde, 1984). Dentin thus offers a simple, ultrastructurally identical system to examine events associated with processes such as aging and fracture, without the added microstructural complexity of bone.

Transmission electron microscopy (TEM) has long been used for microstructural examinations at nanostructural size scales, with numerous studies on dentin being reported in archival literature over the last sixty years (e.g. Gerould, 1944; Shroff et al., 1954; Scott, 1955; Frank, 1959; Van Meerbeek et al., 1992; Inokoshi et al., 1993a,b; Van Meerbeek et al., 1995; Frank, 1999; Kwong et al., 2000; Hashimoto et al., 2003; Yoshida et al., 2004). The very

* Corresponding author. Address: Department of Materials Science, New Museums Site, Pembroke Street, University of Cambridge, CB2 3QZ, UK. Tel.: +44 1223 334302; fax: +44 1223 334366.

E-mail address: aep30@cam.ac.uk (A.E. Porter).

[1] Present address: School of Materials Engineering, Purdue University, West Lafayette, IN 47907, USA.

0968-4328/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.micron.2005.05.011

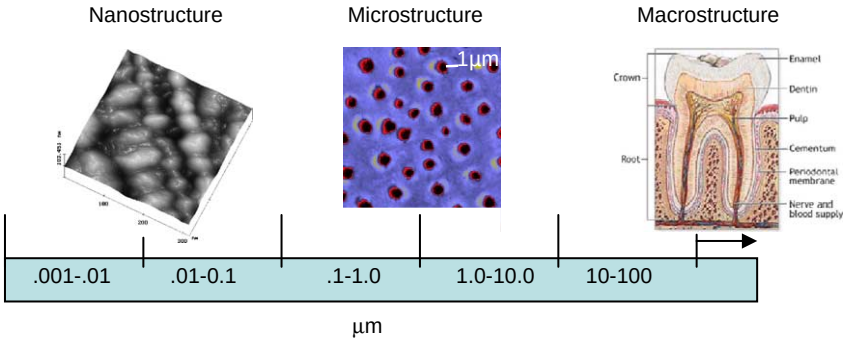

Fig. 1. The structural hierarchy of human dentin. The tubules running to the pulp chamber are shown in cross-section in the microstructure (Scanning Electron Micrograph. Courtesy: J. H. Kinney). The nanostructure (Atomic Force Microscope image, Courtesy: M. Balooch) reveals the regular banded appearance of type-I collagen.

early TEM work utilized specimens of pulverized dentin (e.g. Gerould, 1944), replicas (e.g. Shroff et al., 1954; Scott, 1955), and thin sections of decalcified dentin (e.g., Scott, 1955). However, the advent of diamond-knife microtomy in the 1950s (Fernandez-Moran, 1953) allowed for the imaging of non-decalcified thin sections of dentin (Frank, 1959), and has subsequently been extensively used in studies of such biological tissues at the nanostructural level (e.g., Kwong et al., 2000; Hashimoto et al., 2003; Yoshida et al., 2004). While microtomy has traditionally been the most successful way of obtaining electron-transparent slices of hard mineralized tissues, recently, the alternate technique of ion-beam milling has been explored for the same purpose. Such a technique involves specimen thinning using a beam of ions of sufficient energy to remove material, and has extensively been used for characterization of non-biological materials, such as composites (Cairney and Munroe, 2003), semiconductor (Morris et al., 1991; Basile et al., 1992; Overwijk et al., 1993), coatings (Saka et al., 1994; Kato et al., 1998), powders (Prenitzer et al., 1998; Cairney and Munroe, 2001) and oxidation products (Ford et al., 2000). There are several advantages of such a technique over microtomy; one is that focused ion-beam (FIB) preparation eliminates the need for time-consuming, multi-stage chemical fixation and embedding and also mechanical sectioning with a diamond knife, which potentially could introduce artificial damage. In addition, the use of dual beam FIB systems allows for very precise site specificity when making specimens.

Ion beam techniques have long been used for material removal in biological materials to 'etch' away surface layers of the targeted material by bombarding it with ionized gas molecules. This has been utilized to enhance the underlying microstructure for highly detailed structural observations (e.g., Lewis et al., 1968; Kanaya et al., 1982; Yonehara et al., 1989) and also in studies of dentin–adhesive interfaces (e.g., Van Meerbeek et al., 1992; Inokoshi et al., 1993a,b) and dentin restorative systems (e.g, Inokoshi et al., 1993a,b). Most of these studies have employed an ion beam from a gas discharge of an inert gas (typically argon), though more recently, 'focused' ion beams (FIB) that utilize a liquid metal (typically gallium) source (Orloff et al., 2003) to produce a probe of small diameter, now close to 10 nm (Van Es et al., 2004), are increasingly being used in large number of applications such as metrology, inspection, cross sectioning, failure analysis, mask-less micromachining, and preparation of thin foils for TEM (Reyntjens and Puers, 2001). Such a system has been previously used for cross-sectioning dentin–resin interface specimens with limited success (Van Meerbeek et al., 1995); artifacts produced by the process hid the actual underlying nanostructure. Different kinds of artifacts, such as amorphization, gallium ion implantation, structural modification, etc. have been observed in other non-biological samples (Hutchinson et al., 2003; Wang et al., 2005; Miller et al., 2005) treated by the FIB. FIB preparation of human dentin was first reported in 2001 (Hoshi, et al., 2001) who used energy-filtered TEM analysis of FIB-prepared samples to study the organic-inorganic interface of apatite crystals. This work followed earlier work on FIB preparation of human tooth enamel (Hayashi, 1998; Giannuzzi, 1999). These studies concentrated on the microstructural characterization of the enamel and dentin samples, using FIB as a sample preparation technique in lieu of microtomy.

In the present work, we seek to explore the use of FIB-based milling for TEM sample preparation of dentin in two case studies. First, we will use the technique to examine the mineral phase in human dentin, specifically in the context of aging. As a natural consequence of aging, an altered dentin, termed transparent or sclerotic dentin, often forms gradually (Vasiliadis et al., 1983; Micheletti Cremasco, 1998). Transparency occurs when the tubule lumens become filled with mineral (Fig. 2), decreasing the amount of light scattered. These changes have previously been associated with a change in the mineralization and a marked reduction in the fracture resistance of dentin (Kinney et al., 2005). We will use FIB-based cross-sectioning to examine such changes in the biomineralization. Next, while the macroscopic fracture behavior of dentin has been discussed extensively (Rasmussen et al., 1976; Kahler et al., 2003; Kruzic et al., 2003; Nalla et al., 2003a,b,c; Kinney et al., 2005), little effort has been focused on imaging the damage

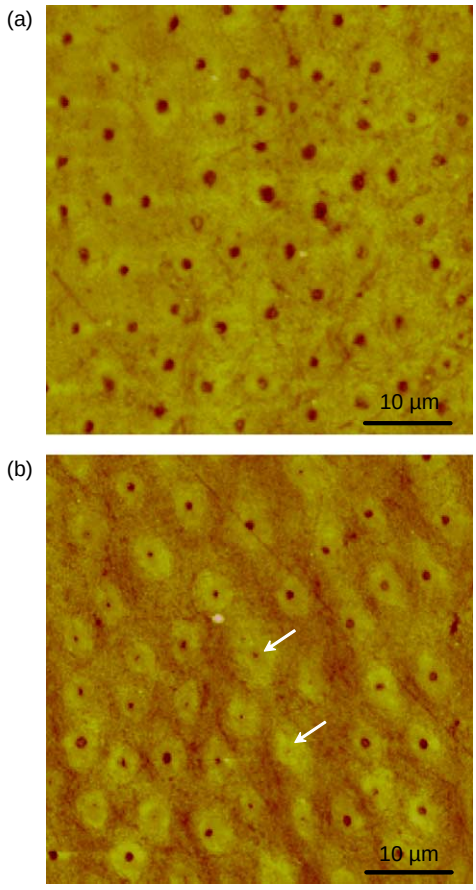

Fig. 2. Atomic force microscopic images of (a) normal (donor age = 25 years), and (b) transparent (donor age = 67 years) dentin. The dark contrast indicates the tubule sites and the white arrows in (b) show representative tubule lumens occluded with intratubular mineral. (Courtesy: M. Balooch).

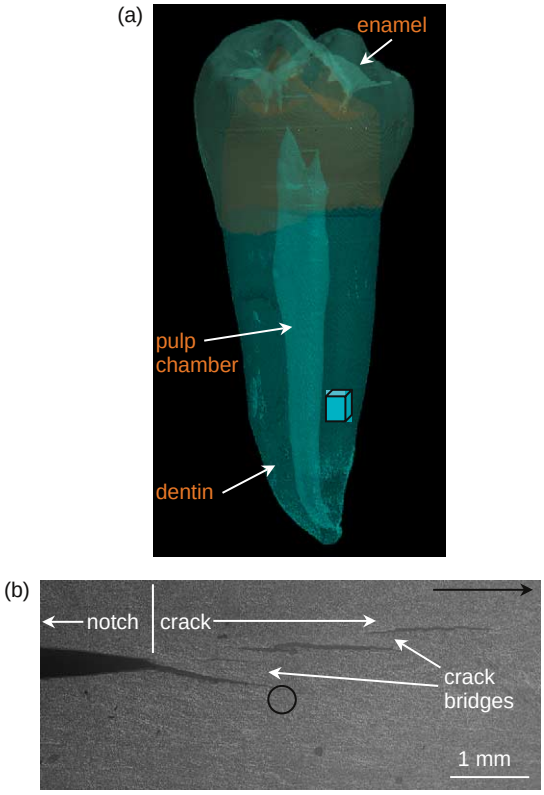

Fig. 3. (a) X-ray tomogram of a human tooth showing approximate locations from which the blocks for the TEM specimens were obtained the apex region in transparent human dentin. (b) Optical micrograph showing a typical crack emanating out of a notch in elephant dentin with the encircled area showing a typical site of interest close to the macroscopic crack. Note evidence of 'crack bridging', a potent mechanism of toughening in dentin (see results section). The black arrow at the top is the direction of nominal crack growth.

induced during the fracture event at the nanostructural level. We will seek to address this using dehydrated elephant dentin that we had previously tested (Kruzic et al., 2003). Such studies are intended to further our understanding of the aging, fracture and failure of dentin, with the specific aim of demonstrating the applicability of FIB sample preparation for hard mineralized tissues.

2. Materials and methods

2.1. Biomineralization in transparent dentin

Recently extracted transparent (donor age: ≧65 years) human molars, obtained according to protocols approved by the University of California San Francisco Committee on Human Research, were used. Sections, ~0.9×0.9×1 mm, were cut with a regular slow-speed (~100 RPM) diamond saw (TechCut II, Allied High Tech Products, Inc., Rancho Dominguez, CA) from the apex region (Fig. 3a). Each section was mounted using AE15 (M-bond) adhesive kit on a 2×1 mm copper slot grids grid (SPI Supplies, West Chester, PA). Following this step, the samples were pre-thinned to about 50–60 μm using a conventional grinder (Minimet 1000, Buehler, Inc., Lake Bluff, IL) and dimpler (VCR Dimpler 296 D500i, South Bay Technology, Inc., San Clemente, CA) and then carbon coated to minimize specimen charging during the ion-beam milling.

The area of interest in each coated sample were then reduced to a thickness of ~100 nm to ensure sufficient electron transparency for TEM. This was achieved using a dual-beam FIB system (Strata 235, FEI Company, Hillsboro, OR), which contained both a focused gallium ion beam and a conventional field-emission scanning electron (FESEM) column. The ion beam was operated at 30 kV with varying beam current ranging from 20,000 pA down to 100 pA as the electron-transparent portion of the sample became thinner. Successive steps of 20,000, 1000, 500, 300 and 100 pA were employed, each lasting ~15 min. The initial high current (20,000 pA) was necessary to eliminate smear layers from the pre-thinning procedures and for rapid early-stage milling. After the milling, TEM studies, specifically bright-field imaging, selected area electron diffraction (SAED), were performed

on the thinned areas using a JEOL 3010 TEM (JEOL USA Inc., Peabody, MA), operated at 300 kV.

2.2. Fractured dentin

Dentin from fractured shards of elephant tusk taken from an adult male elephant (*Loxodonta africana*) was used for this section of the study; elephant tusk dentin was chosen as it is very similar microstructurally to normal/healthy human dentin (Raubenheimer et al., 1990; Nalla et al., 2003a,b,c), without strict limitations on possible sample sizes. Sections, $\sim 2 \times 1 \times 2$ mm, were cut with a slow-speed saw from an area very near the macroscopic crack in specimens of dehydrated elephant dentin (Fig. 3b) that had previously been used for fracture experiments (Kruzic et al., 2003), and also from a block of undamaged (untested) dentin to provide a baseline. The choice of specimens was made with the intention of observing any incipient damage on the nanostucture. Each section was then mounted, pre-thinned and carbon-coated as described in the previous sections. The area of interest in each coated sample was then reduced to a thickness of ~ 100 nm to ensure sufficient electron transparency for TEM using the ion-beam milling procedures described above. After the initial milling using relatively high currents (from 20,000 down to 100 pA), consecutive thinning steps using currents of 50, 10 and 5 pA and a final 'polishing' step using a 2 pA current were subsequently performed and was needed to allow clearer observation of the collagen as such steps were observed to remove the surrounding interfibrillar mineral. The choice of such relatively low current levels for the last few steps entailed much longer milling times (the entire milling process took ~ 8 h). The TEM used for the actual imaging was a JEOL 3010 TEM, operated at 300 kV; imaging was performed under bright-field conditions.

3. Results and discussion

As an alternative approach to microtomy, FIB milling was seen to be advantageous in the thinning of particularly fragile specimens such as mineralized tissue, which face the risk of additional damage when mechanical techniques such as ultramicrotomy are used. In addition, FIB-milling substantially speeds up the specimen preparation process as ultramicrotomy often takes 2 weeks due to the required fixation and embedding. We have investigated two particular cases of (typically) fragile specimens here:

- transparent (aged) dentin, where large mineral crystals loosely fill up the tubules and could be lost during ultramicrotomy-based sectioning, and
- fractured dentin, where such sectioning could artificially induce further damage.

For the transparent dentin, the objective was to examine the mineral phase; conversely, for the case of fractured dentin, our objective was to focus on the collagen phase, as detailed below.

3.1. Biomineralization in transparent dentin

Fig. 4 shows a typical scanning electron micrograph showing the thinning of a sample of transparent dentin during the ion-beam milling process. Progressive thinning was used to reduce the sample thickness to allow for electron transparency. Examination of the thinned sections using TEM clearly revealed differences in the morphology of the mineral phase with location (Fig. 5a–d). The intertubular mineral had a fine, needle-like morphology (Fig. 5a), while the dentinal tubules ~ 1 μm in diameter, were observed to be partially filled with much coarser mineral crystals (Fig. 5c). Selected area electron diffraction (SAED) studies were also performed to examine the chemical nature of these crystals; typical results are included in Fig. 6. Indexing of the principal rings and spots in the corresponding diffraction patterns (as shown in Fig. 6) revealed the presence of hydroxyapatite, the major constituent of the inorganic phase in all mineralized tissues, in both the intra- and inter- tubular cases. Such observations are consistent with our previous work where we used ultramicrotomy to examine changes in the mineralization with aging in dentin (Porter et al., 2005), making FIB-milling a good complementary process to traditional microtomy.

The same sample was sectioned to electron transparency with an ultramicrotome for comparison, and the cross-section of a tubule is shown in Fig. 5d. As compared to the tubule sectioned with the FIB (Fig. 5b), the tubule sectioned with the microtome seems to have only very partially been filled; it is plausible that some mineral was lost during the microtoming process.

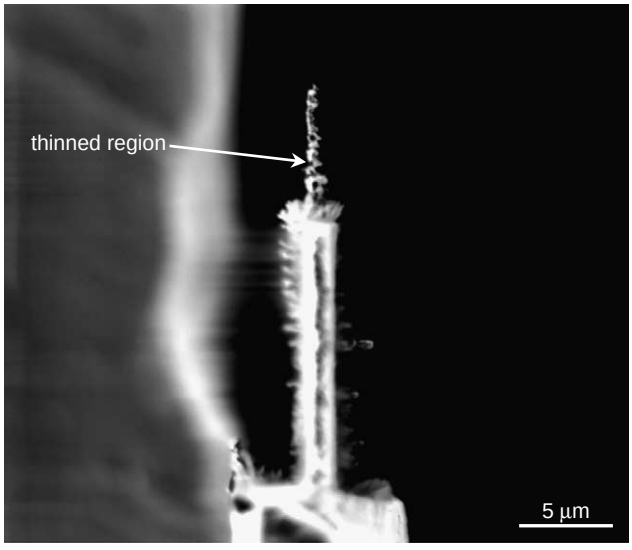

Fig. 4. Scanning electron micrograph showing the progressive thinning of a sample of transparent dentin. The thinned sections were then observed in a TEM.

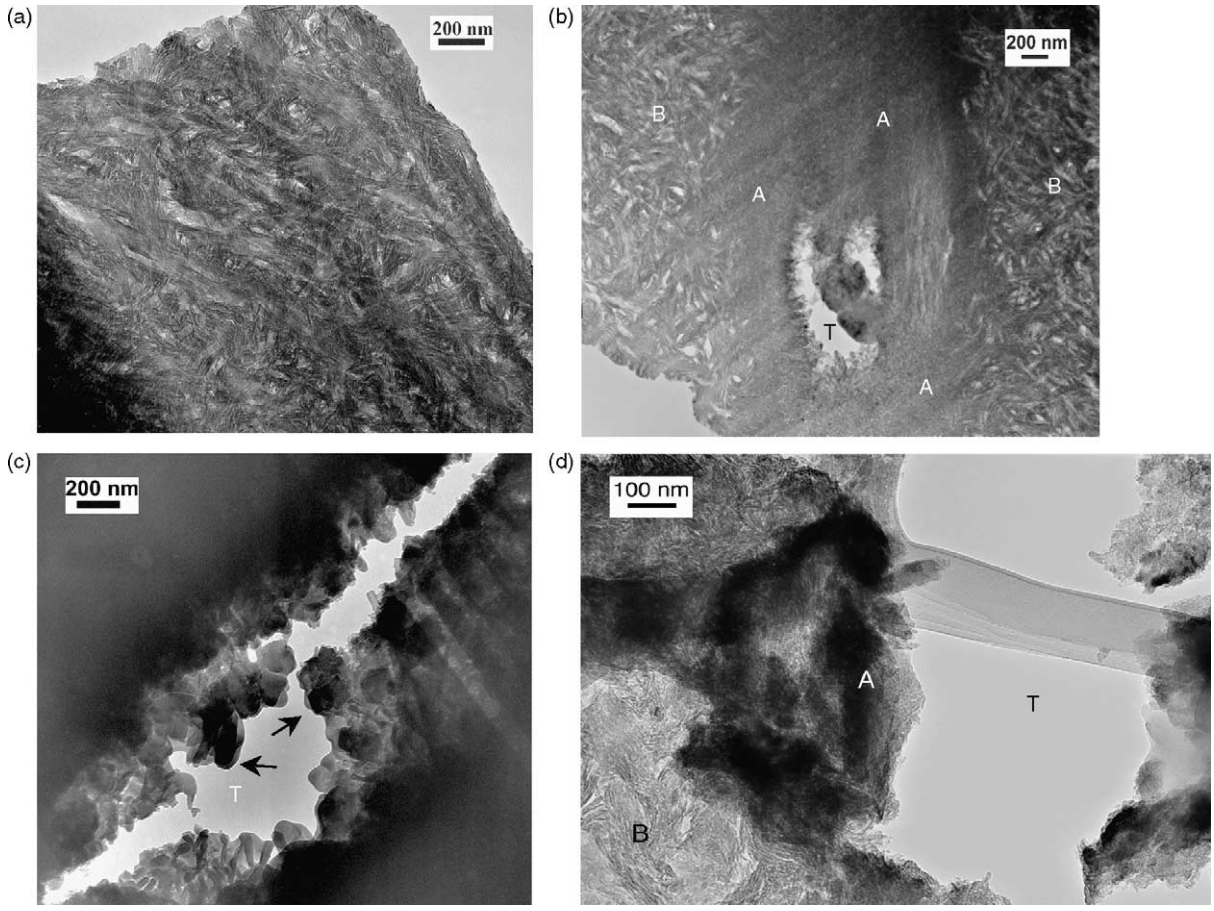

Fig. 5. Transmission electron micrographs of transparent dentin thinned using FIB-milling showing (a) intertubular dentin (note the fine, needle-like crystals), (b) a partially filled tubule (c) a tubule along its long axis partially filled with large mineral crystals (indicated by arrows), (d) The same sample as (a–c) prepared with an ultramicrotome instead of a FIB. Note the cross-section of the microtomed-tubule seems to have been torn, whereas the tubule in (b) sectioned with the FIB is still fully intact. (T, tubule; A, intratubular mineral; B, intertubular mineral).

These observations are important from the perspective of understanding aging related changes and corresponding structure–property relationships in dentin. With age, we have observed, based on the results reported herein and our previous studies, two distinct changes in the mineralization patterns in human dentin:

- the tubules are filled up with large mineral crystals, which are chemically identical to the surrounding, much finer intertubular mineral (Fig. 5), and
- the average intertubular crystal size shows a significant decrease with aging, as reported in our previous work, and its chemical composition is invariant with age (Kinney et al., 2005; Porter et al., 2005).

These observations suggest that a process involving dissolution of intertubular mineral, followed by re-deposition into the tubules could be occurring during the natural process of aging of these tissues. While the biological and chemical imperatives that drive such a mechanism to occur with aging are yet unclear, the consequences on the mechanical properties of the tissue, particularly fracture resistance, are quite dramatic (Kinney et al., 2005). In normal dentin, the tubules are often the sites of microcrack nucleation in the vicinity of an advancing macroscopic crack tip (Nalla et al., 2003a,b,c). We believe that such microcracking spawns so-called ‘crack bridges’ or ‘uncracked ligaments’, which are tracts of unbroken dentin (roughly 50–250 μm thick, through optical and X-ray tomographic observations; see Nalla et al., 2003a,b,c) that span the crack and reduce the driving stress at the crack tip (see schematic in Fig. 7; also see Fig. 3b). Our previous work has demonstrated that this bridging is a potent extrinsic mechanism of toughening[2] in dentin (Kruzic et al., 2003; Nalla et al., 2004). With aging-induced transparency, however, a good fraction of the tubules are filled with mineral, greatly reducing the number of potential

[2] Subcritical crack extension can be considered as the mutual competition between two classes of mechanisms: (i) intrinsic toughening mechanisms that operate ahead of the crack tip and act to enhance the material’s inherent resistance to microstructural damage and cracking, and (ii) extrinsic toughening mechanisms that operate primarily behind the crack tip by promoting crack-tip shielding, which reduces the local stress intensity actually experienced at the crack tip (Ritchie, 1988; Evans, 1990; Ritchie, 1999).

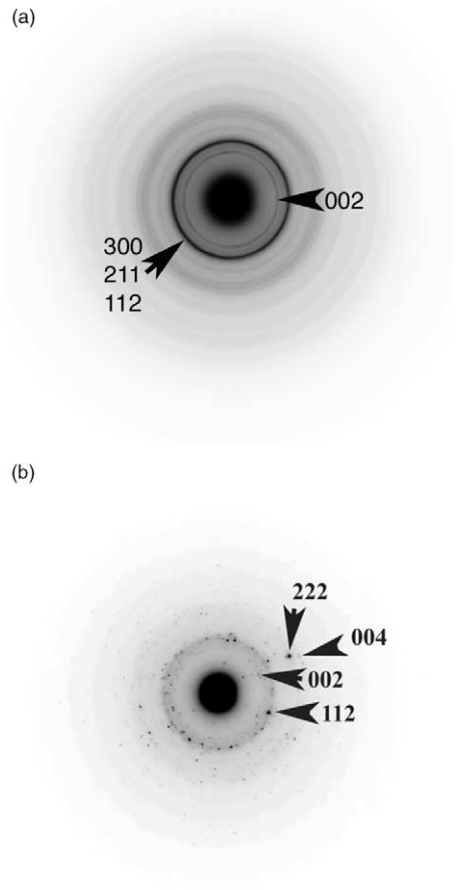

Fig. 6. Typical SAED patterns obtained for the (a) inter- and (b) intratubular mineral in transparent dentin; the indexing confirmed the presence of hydroxyapatite in both cases. Note that the spots are more clearly delineated in the intratubular case, supporting the observation of a much larger intratubular crystal size.

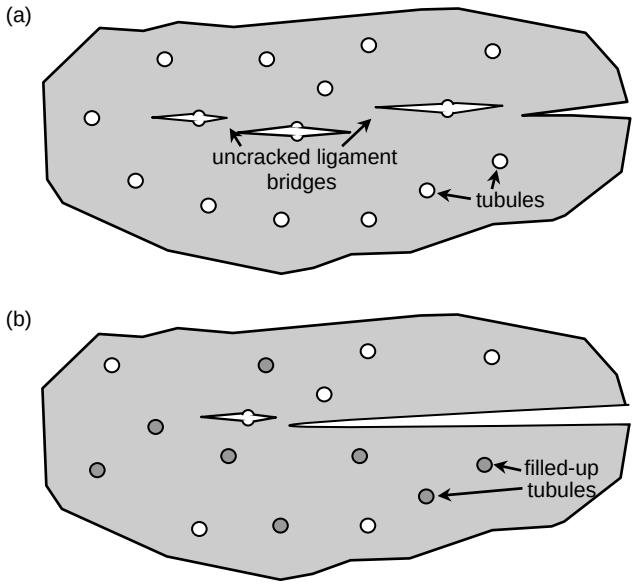

Fig. 7. Schematic illustrations of the differences in fracture mechanisms in (a) normal dentin, and (b) transparent dentin. In normal dentin, microcrack formation ahead of the crack tip leads to the formation of uncracked ligaments due to imperfect link-ups between the microcracks and the main crack tip. With transparent dentin, mineral accretion seals up some of the tubules, leading to fewer (if any) uncracked-ligament bridges are formed. The absence of such bridges, which normally increase the toughness by sustaining part of the applied loads that would otherwise be used for crack extension, results in the diminished fracture toughness of transparent dentin.

nucleation sites for microcracks. This reduces the contribution to the toughness from crack bridging, leading to an overall reduction in the fracture resistance by ~33% (Kinney et al., 2005). While this would explain, at least partially,[3] the increased incidences of tooth fracture among the elderly, it also provides for an excellent model of how changing the structure of a biological tissue has consequences on its properties.

3.2. Fractured dentin

Figs. 8–10 show typical TEM micrographs obtained for areas taken from the vicinity of a macroscopic crack in fractured elephant dentin. In Fig. 8, the 'banded' nanostructure of the collagen network that forms the basic network is clearly evident for undamaged dentin, without the need for any chemical staining as is commonly employed for ultramicrotomed sections. Note the characteristically spaced gap zones (dark bands) arising from the Hodge–Petruska structure (Weiner and Wagner, 1998) in Fig. 8b. The milling procedure that we have employed thus appears to have removed most of the surrounding interfibrillar mineral and the ground matter exposing the collagen with the intrafibrillar mineral in the gap zones, thus revealing the banding pattern much more clearly than is often seen in microtomed slices after chemical staining. The observation of this structure confirms that the FIB can be utilized for ion milling TEM samples even when the organic component is of interest. Indeed, we believe that the technique can thus be used quite effectively when it is necessary to examine the collagen structure in highly localized areas of interest, without the need for chemical staining.

Fig. 9 shows corresponding TEM micrographs for damaged dentin. Note the microcrack at the site of the tubule (Fig. 9a), and the nano-scale bridges that form across such a crack (Fig. 9b). While the exact nature of these bridges (typically 50–100 nm in diameter) is as yet unclear, they are clearly remnants of the organic matrix, possibly even non-collageneous proteins, as has been suggested by other investigators (P. Hansma, private communication,

[3] It is plausible that there are also changes in the organic collagen component, particularly the chemical cross-linking, that contribute to the lowered toughness through a reduction in the strength of the bridges formed; such changes are currently being examined.

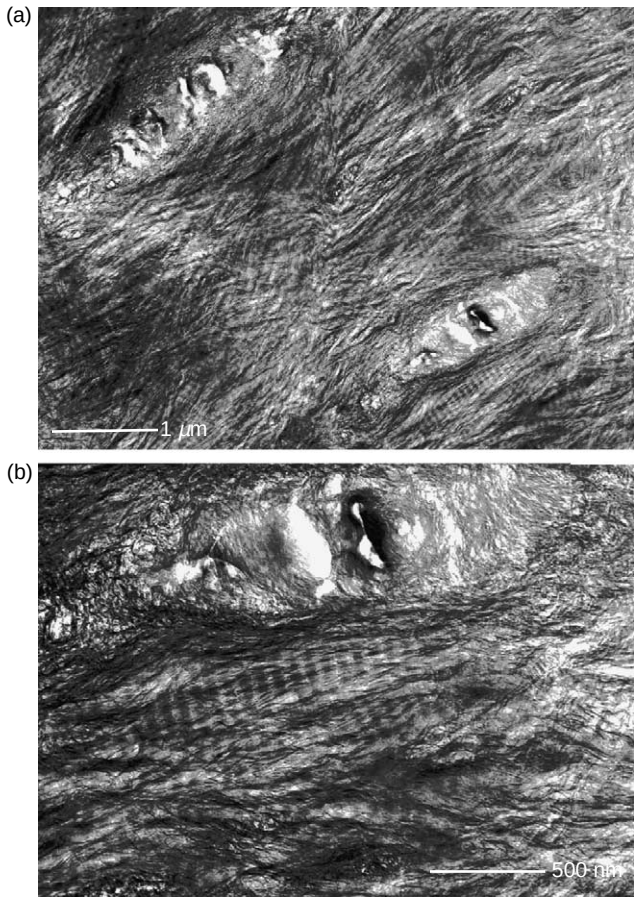

Fig. 8. Transmission electron micrographs showing the microstructure of undamaged elephant dentin. Note the structure of the collagen fibers, with the characteristic 'banded' structure, which was clearly revealed.

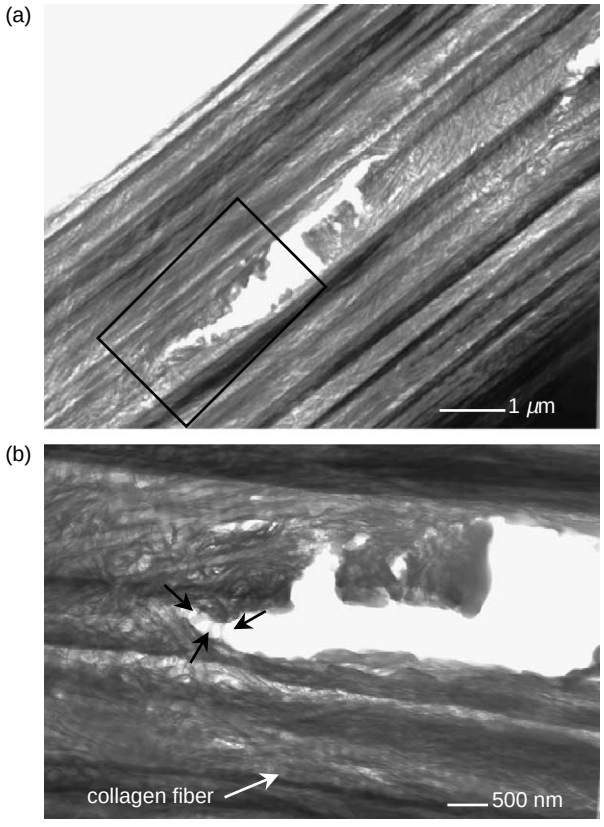

Fig. 9. Transmission electron micrographs showing a microcrack emanating from a tubule in damaged elephant dentin. The boxed region in (a) is shown at a higher magnification in (b). There appears to be evidence of bridging across the microcrack at the ultrastructural size scale as indicated by black arrows in (b); note also a collagen fiber in the foreground.

2005). Further work is needed in order to assess these observations. Thus, the concept of bridging at much larger, *macroscopic* length scales (see Fig. 3b) that has been discussed extensively for dentin (Kruzic et al., 2003) and bone (Nalla et al., 2003a,b,c; Nalla et al., 2005) is seen to extend to the *nanoscopic* level. While such bridging may be of minimal importance to the toughening for large, macroscopic cracks (Nalla et al., 2004), it is conceivable that it might be significant for smaller cracks.

Further examination of the collagen fibers in the vicinity of the microdamaged region revealed some evidence of the mode of incipient observable damage. Fig. 10 shows TEM images of a section of such a collagen fiber. There is clear evidence of void formation/cavitation (indicated in Fig. 10b) that appears to be how observable damage initiates in these materials. No such voids were seen in the undamaged dentin; further investigation of this phenomenon is definitely warranted and is currently being undertaken. Fratzl and co-workers (Fratzl et al., 1998), based on X-ray scattering experiments on tendon collagen, suggested that at small strain collagen deforms by straightening of kinking in the fibers and then in the molecules, with higher strains leading to molecular gliding and disruption of the fibrillar structure. It is conceivable that the collagen close to the fracture event experiences very high strains and that the void formation within the fibers that we are observing is a result of the disruption of the structure suggested previously.

Thus, for both of the cases investigated in this study, FIB-milling was able to generate high-quality specimens that could be used for subsequent TEM examination. Both the mineral and collagen phases could be imaged quite clearly after the milling process, and useful insight was gained by the use of the FIB to section the specimens.

4. Concluding remarks

The FIB-based milling procedure used here is able to expose details both of the organic and inorganic components of mineralized biological tissues. We were able to utilize this technique to examine very fragile specimens, which could be easily damaged through the use of traditional ultramicrotomy. Another advantage of this technique is site specificity possible with the dual-beam FIB. It is possible to choose very particular areas of interest, for

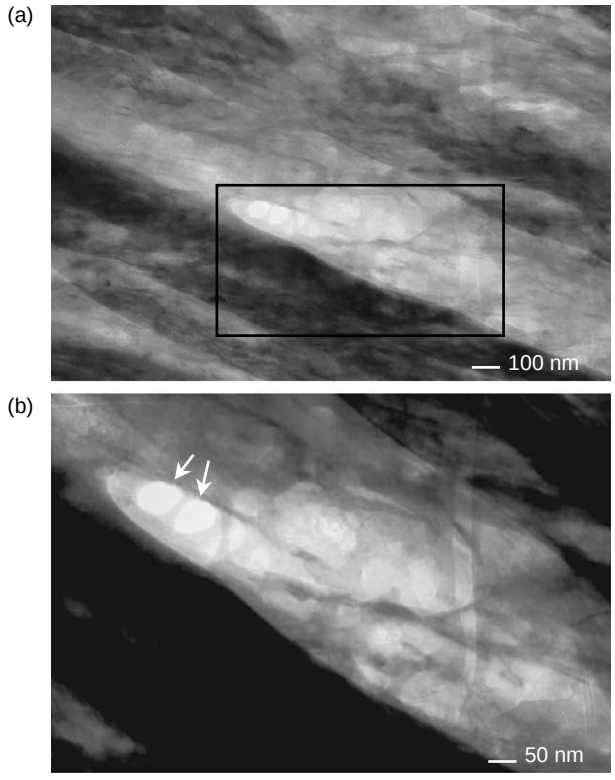

Fig. 10. Transmission electron micrographs showing incipient 'damage' within a single collagen fiber in damaged elephant dentin. The boxed region in (a) is shown at a higher magnification in (b). Note the evidence of voids being formed within the fiber as indicated by white arrows in (b).

example, 1–2 μm diameter dentinal tubules in the present study, and selectively thin them. While our study has helped elucidate the mechanisms involved in aging and fracture of mineralized tissues, many problems remain that deserve further investigation. Dentin provides a simple system to study such processes; the necessities of in vivo remodeling and repair imply that there is an added dimension of complexity added by the consequent secondary osteons (in cortical bone) and hemi-osteons (in trabecular bone) to understanding such processes in human bone. The exact nature of the interfaces between different tissues is still under debate and detailed ultrastructural examinations would be useful, particularly in the context of processes of aging and fracture. A better understanding of the initial formation and changes in the mineralization patterns, both local and global, and the collagen structure is known to occur with age and disease is also needed. Finally, work directed at understanding the nanoscale mechanisms of failure along the lines of this study should also be pursued.

Acknowledgements

This work was supported by the National Institutes of Health under Grant No. 5R01 DE015633 (for RKN and APT), by the Director, Office of Science, Office of Basic Energy Science, Division of Materials Sciences and Engineering, Department of Energy under No. DE-AC03-76SF00098, and the University of Maine (for AEP). The authors also wish to thank Drs J.R. Jinschek, C.F. Kisielowski, J.J. Kruzic, J.H. Kinney, M. Balooch, D. Fyhrie, P. Hansma, S.J. and G.W. Marshall for many helpful discussions. The authors acknowledge the use of the facilities of the National Center for Electron Microscopy at the Lawrence Berkeley National Laboratory, also funded by the Department of Energy.

References

Basile, D.P., Boylan, R., Baker, B., Hayes, K., Soza, D., 1992. FIBXTEM-focused ion beam milling for TEM sample preparation. MRS Symn Proc. 254, 23–41.

Cairney, J.M., Munroe, P.R., 2001. Preparation of transmission electron microscope specimens from FeAl and WC powders using focused ion beam milling. Mater Character 46, 297–304.

Cairney, J.M., Munroe, P.R., 2003. Redeposition effects in transmission electron microscope specimens of FeAl-WC composites prepared using a focused ion beam. Micron 34, 97–107.

Evans, A.G., 1990. Perspective on the development of high toughness ceramics. J. Am. Ceramic Soc. 73, 187–206.

Fernandez-Moran, H., 1953. A diamond knife for ultra-thin sectioning. Exp. Cell Res. 5, 255–256.

Ford, S., Munroe, P.R., Young, D.J., 2000. The development of aligned precipitates during internal carbonitridation of Fe-Ni-Cr alloys. Mater High Temp. 17, 279–286.

Frank, R.M., 1959. Electron microscopy of undecalcied sections of human adult dentine. Arch. Oral Biol. 1, 29–32.

Frank, R.M., 1999. Ultrastructure of human dentine 40 years ago- Progress and perspectives. Arch. Oral Biol. 44, 979–984.

Fratzl, P., Misof, K., Zizak, I., Rapp, G., Amenitsch, H., Bernstorff, S., 1998. Fibrillar structure and mechanical properties of collagen. J. Struct. Biol. 122, 119–122.

Gerould, C.H., 1944. Ultramicrostructure of human tooth as revealed by the electron microscope. J. Dent. Res. 23, 239–245.

Giannuzzi, L.A., Prenitzer, B.I., Drown-MacDonald, J.L., Shofner, T.L., Brown, S.R., Irwin, R.B., Stevie, F.A., 1999. Electron microscopy sample preparation for the biological and physical sciences using focused ion beams. J. Process Anal. Com. 4, 162–167.

Hashimoto, M., Ohno, H., Sano, H., Kaga, M., Oguchi, H., 2003. In vitro degradation of resin-dentin bonds analyzed by microtensile bond test, scanning and transmission electron microscopy. Biomaterials 24, 3795–3803.

Hayashi, Y., Yaguchi, T., Ito, K., Kamino, T., 1998. High resolution electron microscopy of human enamel reactions prepared with focused ion beam system. Proc. Scanning 20, 234–235.

Hoshi, K., Ejiri, S., Probst, W., Seybold, V., Kamino, T., Yaguchi, T., Yamahira, N., Ozawa, H., 2001. Observation of human dentine by focused ion beam and energy-filtering transmission electron microscopy. J. Microscopy 201, 44–49.

Hutchinson, C.R., Hackenberg, R.E., Shiflet, G.J., 2003. A comparison of EDS microanalysis in FIB-prepared and electropolished TEM thin foils. Ultramicroscopy 94, 37–48.

Inokoshi, S., Hosoda, H., Harnirattisai, C., Shimada, Y., 1993a. Interfacial structure between dentin and seven dentin bonding systems revealed using argon ion beam etching. Oper Dent. 18, 8–16.

Inokoshi, S., Willems, G., Van Meerbeek, B., Lambrechts, P., Braem, M., Vanherle, G., 1993b. Dual-cure luting composites: Part I: filler particle distribution. J. Oral Rehabil. 20, 133–146.

Jones, S.J., Boyde, A., 1984. Ultrastructure of dentin and dentinogenesis, Dentin and Dentinogenesis. CRC Press, Linde. Boca Raton pp. 81–134.

Kahler, B., Swain, M.V., Moule, A., 2003. Fracture-toughening mechanisms responsible for differences in work to fracture of hydrated and dehydrated dentine. J. Biomech. 36, 229–237.

Kanaya, K., Muranaka, Y., Fujita, H., 1982. Uncoated observation and etching of non-conductive materials by ion beam bombardment in scanning electron microscopy. Scan Electron Microsc. 4, 1379–1394.

Kato, T., Hong, M.H., Nunome, K., Sasaki, K., Kuroda, K., Saka, H., 1998. Cross-sectional TEM observation of multilayer structure of a galvannealed steel. Thin. Solid Films 319, 132–139.

Kinney, J.H., Nalla, R.K., Pople, J.A., Breunig, T., Ritchie, R.O., 2005. Transparent root dentin: mineral concentration, crystallite size, and mechanical properties. Biomaterials 26, 3363–3376.

Kruzic, J.J., Nalla, R.K., Kinney, J.H., Ritchie, R.O., 2003. Crack blunting, crack bridging and resistance-curve fracture mechanics in dentin: effect of hydration. Biomaterials 24, 5209–5221.

Kwong, S.-M., Tay, F.R., Yip, H.-K., Kei, L.-H., Pashley, D.H., 2000. An ultrastructural study of the application of dentine adhesives to acid-conditioned sclerotic dentine. J. Dent. 28, 515–528.

Lewis, S.M., Osborn, J.S., Stuart, P.R., 1968. Demonstration of an internal structure within the red blood cell by ion etching and scanning electron microscopy. Nature 220, 614–616.

Marshall Jr., G.W., Marshall, S.J., Kinney, J.H., Balooch, M., 1997. The dentin substrate: structure and properties related to bonding. J. Dent. 25, 441–458.

Micheletti Cremasco, M., 1998. Dental histology: study of aging processes in root dentine. Boll Soc. Ital Biol. Sper. 74, 19–28.

Miller, M.K., Russell, K.F., Thompson, G.B., 2005. Strategies for fabricating atom probe specimens with a dual beam FIB. Ultramicroscopy 102, 287–298.

Morris, S., Tatti, S., Black, E., Dickson, N., Mendez, H., Schweisow, B., Pyle, R., 1991. A technique for preparing TEM cross sections to a specific area using the FIB, ISTFA '91: The Proceedings of the 17th International Symposium for Testing and Failure Analysis. Los Angeles, CA.

Nalla, R.K., Kinney, J.H., Ritchie, R.O., 2003a. Effect of orientation on the in vitro fracture toughness of dentin: the role of toughening mechanisms. Biomaterials 24, 3955–3968.

Nalla, R.K., Kinney, J.H., Ritchie, R.O., 2003b. Mechanistic fracture criteria for the failure of human cortical bone. Nature Materials 2, 164–168.

Nalla, R.K., Kinney, J.H., Ritchie, R.O., 2003c. On the fracture of human dentin: is it stress- or strain-controlled? J. Biomed. Mater Res. 67A, 484–495.

Nalla, R.K., Kruzic, J.J., Ritchie, R.O., 2004. On the origin of the toughness of mineralized tissue: microcracking or crack bridging? Bone 34, 790–798.

Nalla, R.K., Kruzic, J.J., Kinney, J.H., Ritchie, R.O., 2005. Mechanistic aspects of fracture and R-curve behavior of human cortical bone. Biomaterials 26, 217–231.

Orloff, J., Utlaut, M., Swanson, L.W., 2003. High Resolution Focused Ion Beams: FIB and its Applications. Kluwer Academic/Plenum, New York, NY.

Overwijk, M.H.F., van den Heuvel, F.C., Sulle-Lieuwma, C.W.T., 1993. Novel scheme for the preparation of transmission electron microscopy specimens with a focused ion beam. J. Vac. Sci. Technol. B. 11, 2021–2024.

Porter, A.E., Nalla, R.K., Minor, A., Jinschek, J.R., Kisielowski, C.F., Radmilovic, V., Kinney, J.H., Tomsia, A.P., Ritchie, R.O., 2005. A transmission electron microscopy study of mineralization in age-induced transparent dentin. Biomaterials 26, 7525–7686.

Prenitzer, B.I., Giannuzzi, L.A., Newman, K., Brown, S.R., Irwin, R.B., Shofner, T.L., Stevie, F.A., 1998. Transmission electron microscope specimen preparation of Zn powders using the focused ion beam lift-out technique. Metall. Mater. Trans. A 29A, 2399–2406.

Rasmussen, S.T., Patchin, R.E., Scott, D.B., Heuer, A.H., 1976. Fracture properties of human enamel and dentin. J. Dent. Res. 55, 154–164.

Raubenheimer, E.J., Dauth, J., Dreyer, M.J., Smith, P.D., Turner, M.L., 1990. Structure and composition of ivory of the African elephant (Loxodonta africana). S. Afr. J. Sci 86, 192–193.

Reyntjens, S., Puers, R., 2001. A review of focused ion beam applications in microsystem technology. J. Micromech. Microeng. 11, 287–300.

Rho, J.-Y., Kuhn-Spearing, L., Zioupos, P., 1998. Mechanical properties and the hierarchical structure of bone. Med. Eng. Phys. 20, 92–102.

Ritchie, R.O., 1988. Mechanisms of fatigue crack propagation in metals, ceramics and composites: role of crack-tip shielding. Mater. Sci. Engng. 103, 15–28.

Ritchie, R.O., 1999. Mechanisms of fatigue-crack propagation in ductile and brittle solids. Int. J. Fract. 100, 55–83.

Saka, H., Kuroda, K., Hong, M.H., Kamino, T., Yaguchi, T., Tsuboi, H., Ishitani, T., Koike, H., Shibuya, A., Adachi, Y., 1994. Fabrication of cross sectional TEM specimens of metallic materials using a focused ion beam. HREM of interfaces in a hot-dip galvanised steel. ICEM 13, 1009–1010.

Scott, D.B., 1955. The electron microscopy of enamel and dentine. Ann. New York Acad. Sci. 60, 575–585.

Shroff, F.R., Williamson, K.I., Bertaud, W.S., 1954. Electron microscope study of dentine: the true nature of the dentinal canals. J. Oral Surg. (Chic) 7, 662–667.

Van Es, J.J., Gierak, J., Forbes, R.G., Suvorov, V.G., Van den Berghe, T., Dubuisson, P., Septier, A., 2004. An improved gallium liquid metal ion source geometry for nanotechnology. Microelectron Eng. 73–74, 132–138.

Van Meerbeek, B., Inokoshi, S., Braem, M., Lambrechts, P., Vanherle, G., 1992. Morphological aspects of the resin-dentin interdiffusion zone with different dentin adhesive systems. J. Dent. Res. 71, 1530–1540.

Van Meerbeek, B., Conn, L.J.J., Duke, E.S., Schraub, D., Ghafghaichi, F., 1995. Demonstration of a focused ion-beam cross-sectioning technique for ultrastructural examination of resin-dentin interfaces. Dent. Mater. 11, 87–92.

Vasiliadis, L., Darling, A.I., Levers, B.G., 1983. The amount and distribution of sclerotic human root dentine. Arch. Oral Biol. 28, 645–649.

Wang, Z., Kato, T., Hirayama, T., Kato, N., Sasaki, K., Saka, H., 2005. Surface damage induced by focused-ion-beam milling in a Si/Si p-n junction cross-sectional specimen. Appl. Surf. Sci. 241, 80–86.

Weiner, S., Wagner, H.D., 1998. The material bone: structure-mechanical function relations. Annu. Rev. Mater. Sci. 28, 271–298.

Yonehara, K., Baba, N., Kanaya, K., 1989. Application of ion-beam etching techniques to the fine structure of biological specimens as examined with a field emission SEM at low voltage. J. Electron. Microsc. Tech. 12, 71–77.

Yoshida, E., Hashimoto, M., Hori, M., Kaga, M., Sano, H., Oguchi, H., 2004. Deproteinizing effects on resin-tooth bond structures. J. Biomed. Mater. Res. 68B, 29–35.