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Near tubule and intertubular bovine dentin mapped at the 250 nm level

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Abstract

In this study, simultaneous diffraction and fluorescence mapping with a (250 nm)², 10.1 keV synchrotron x-ray beam investigated the spatial distribution of carbonated apatite (cAp) mineral and elemental Ca (and other cations including Zn) around dentin tubules. In 1 μ m thick sections of near-pulp root dentin, where peritubular dentin (PTD) is newly forming, high concentrations of Zn, relative to those in intertubular dentin (ITD), were observed adjacent to and surrounding the tubule lumens. Some but not all tubules exhibited hypercalcified collars (high Ca signal relative to the surrounding ITD), and, when present, the zone of high Ca did not extend around the tubule. Diffraction rings from cAp 00.2 and 11.2+21.1+30.0 reflections were observed, and cAp was the only crystal phase detected. Profiles of Ca, Zn and cAp diffracted intensities showed the same transitions from solid to tubule lumen, indicating the same cAp content and organization in ITD far from the tubules and adjacent to them. Further, the matching Ca and diffraction profiles demonstrated that all of the Ca is in cAp or that any noncrystalline Ca was uniformly distributed throughout the dentin. Variation of 00.2 and 11.2+21.1+30.0 diffracted intensity was consistent with the expected biaxial crystallographic texture. Extension of x-ray mapping from near 1 μ m resolution to the 250 nm level, performed here for dentin and its tubules, will provide new understanding of other mineralized tissues.

Keywords

dentin; bovine; peritubular dentin; intertubular dentin; synchrotron; x-ray diffraction; x-ray fluorescence; zinc; calcium; carbonated apatite; crystallographic texture

Introduction

Dentin is the internal mineralized tissue of teeth and toughens the structure. Mature dentin contains an array of more-or-less parallel tubules, each of which is surrounded by a collar of peritubular dentin (PTD) and is embedded in a matrix of intertubular dentin (ITD), a

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composite of collagen and nanocrystals of carbonated hydroxyapatite (cAp) with a composition similar to bone (Nanci 2008). Each dentin tubule contains the principal cytoplasmic process of an odontoblast. Like osteocytes in bone, dentin tubules have canaliculi radiating from them, canaliculi whose diameters are somewhat smaller than in bone (Mjör and Nordahl 1996; Okada et al. 2002; You et al. 2004; Kubek et al. 2010). The PTD, through which the canaliculi run, is hypercalcified with respect to ITD and contains little or no collagen (Takuma and Eda 1966; Weber 1968; Scott et al. 1974; Weiner et al. 1999; Gotliv et al. 2006; Gotliv and Veis 2007); instead, PTD is enriched in non-collagenous matrix proteins (DSP, DMP-1), phospholipids (Gotliv and Veis 2007; Nanci 2008) and the elements Na, Mg and K (Gotliv et al. 2006). Although considerable work has been done on dentin and on ITD (see (Kinney et al. 2003)), gaps remain in what is known quantitatively about the volume of mineralized tissue adjacent to the tubule lumen, e.g. the form of calcium immediately adjacent to the tubule. In part, this is because of the small dimensions (tubule dia. ~ 1–2 μm , PTD collar thickness one-half to two tubule diameters, tubule spacing 5–10 μm (Weber 1968; Scott et al. 1974)) and of the difficulty physically separating PTD from ITD (Weiner et al. 1999).

Tubules and associated PTD are one level of the hierarchical structure of tooth and its constituent phases (enamel, dentin and cementum). Figure 1 shows a simplified schematic of several levels: entire tooth (left); ITD, PTD and tubules in dentin (center); mineralized collagen fibrils surrounding a tubule (upper right); collagen triple helices and cAp platelets of each fibril (lower right). Tubules run from the dentinoenamel or dentinocementum junction (DEJ and DCJ, respectively) to the pulp cavity, and it is unclear whether the ITD-PTD-tubule structure is a fossil remaining from development of dentin, is important for ion/fluid exchange conduit in dentin, provides an important toughening mechanism in dentin or a combination of some or all of these.

Once the odontoblasts have laid down the collagen matrix, the mineralizing collagen surrounds a fairly wide lumen. With time, the lumen slowly narrows by addition of non-collagen-associated, mineralized tissue, the PTD (Avery 2002; Nanci 2008). Tubules have thicker PTD collars in crown compared to root dentin (Takuma and Eda 1966). Near the DEJ or DCJ, PTD is also thicker than in the vicinity of the pulp (Takuma and Eda 1966). In root dentin, and particularly in the region closest to the mineralization front, the subject of the present paper, PTD may be irregularly distributed around a tubule (Weber 1968) or even absent entirely (Takuma and Eda 1966).

The 250 nm x-ray mapping reported below focuses on the crystallographic structure and elemental composition (and gradients) of the volume immediately adjacent to the tubule lumen and out to the ITD midway between tubules. In ITD, collagen fibrils lie tangent to the nearest tubule (Scott et al. 1974), and, like in bone (Stühler 1937), the associated cAp nanoplatelets have their conventional orientations with *c*-axes parallel to the collagen triple helix axes. In materials science terms, the relationship between cAp and collagen means that cAp in PTD and ITD should exhibit biaxial crystallographic texture; a concept widely employed in metallurgical engineering (Cullity and Stock 2001). Crystallographic texture in materials like the composite dentin affects mechanical properties enormously. Preferred cAp orientation observed in PTD (isolated from ITD via density gradient fractionation) is similar to that in ITD despite their very different matrices (Weiner et al. 1999). Nondestructive diffraction mapping from intact specimens spanning multiple tubules is required, however, to demonstrate conclusively that cAp organization in ITD continues unchanged into the near tubule volumes or PTD.

Here, novel 250 nm x-ray diffraction mapping with simultaneous x-ray fluorescence mapping is applied to ~1 μm thick specimens of bovine root dentin taken from a position

near the pulp cavity. Bovine dentin (as opposed to that of humans or other species) was studied because it has plentiful PTD and the authors' prior experience with this tissue (Weiner et al. 1999; Gotliv et al. 2006; Gotliv and Veis 2007; Stock et al. 2008; Almer and Stock 2010). Very few studies of any type have mapped diffraction in polycrystalline samples with beam diameters of 250 nm, and the authors do not know of any such studies on mineralized tissue. Although species such as Hg and Zn have been mapped with x-ray fluorescence microprobes near carious lesions in root dentin (Anderson et al. 1996), near dental amalgam at $>1\ \mu\text{m}$ level (Harris et al. 2008) or at the boundary between unmineralized and mineralized articular cartilage (Zoeger et al. 2006; Zoeger et al. 2008), 250 nm mapping around tubules is quite different.

This study addresses the following questions: 1) Is diffraction from dentin volumes $(250\ \text{nm})^2 \times 1000\ \text{nm}$ strong enough for identification of crystallographic phases? (Within the irradiated volume, there are $\sim 5 \times 10^3$ variously oriented nanoplatelets of cAp, assuming $\sim 40\%$ vol. % mineral and platelet dimensions of $60\ \text{nm} \times 20\ \text{nm} \times 4\ \text{nm}$). 2) Is cAp the only crystallographic phase detectable? 3) Do the crystallographic orientations in ITD away from the tubules match those adjacent to the tubule lumens? 4) In the near pulp volume of bovine root dentin, is Ca content elevated in the near lumen volumes relative to that far from the tubules, and is the Ca adjacent to the tubule associated only with cAp? 5) Are elevated levels of elements other than Ca, Na, Mg or K associated with the near tubule volumes? Answers to these questions are the first step towards understanding how PTD mineralization proceeds, what processes operate to produce it and what the biological and structural consequences are of having PTD surrounding (and constricting) the tubule lumen and of having it embedded in the ITD.

Materials and Methods

An erupted bovine molar from an animal 12–18 months in age was cast in LR white (Electron Microscopy Sciences, Hatfield, PA). The tooth was sectioned perpendicular to its proximal-distal axis using an Isomet wafering saw (Buehler, Lake Bluff, IL). The cuts were made every 2.0 mm and were numbered from crown to root. The specimens described below were from slab 7, root dentin somewhat below the cervix ($\sim 2\text{--}4\ \text{mm}$, circled area in Fig. 1, left). Slab 7 was then cut into smaller rectangular blocks spanning cementum to pulp and with cross-section $1.7\ \text{mm} \times 1.3\ \text{mm}$ (the former along the crown-root axis). One block was prepared for sectioning by casting it in a mold filled with LR white.

Sections $\sim 1\ \mu\text{m}$ thick were cut (Ultramicrotome UC6 - Leica Microsystems, Bannockburn, IL - and DiAtome Diamond Histoknife - EMS, Hatfield, PA) from one block near the pulp, no closer than $50\ \mu\text{m}$ and no farther than $200\ \mu\text{m}$ from it. The block was oriented so that the tubule axes were perpendicular to the plane of the thin sections. Some thin sections in water droplets were placed on kapton sheets ($12.5\ \mu\text{m}$ thickness, DuPont, Circleville, OH), and the water was allowed to evaporate which deposited the thin section securely onto the kapton. Adjacent sections were placed on glass microscope slides. Optical microscopy was used to identify areas of the thin sections for x-ray mapping and to document the range of structures present.

The specimens were examined in air with the x-ray microprobe at station 2-ID-D of the Advanced Photon Source (APS) which is described elsewhere (Libera et al. 2002). For the data reported below, and the beam size was $250\ \text{nm}$ and energy was $10.1\ \text{keV}$. The sections of dentin were positioned normal to the incident beam, at the focal plane of the x-ray zone plate and at the rotation center of the diffractometer. Fluorescence from the sample was mapped using a Vortex-EX silicon drift detector (SII Nanotechnology USA, Northridge, CA) positioned as close as possible to the specimen and aligned to collect x-rays emerging

nearly parallel to the front surface of the specimen and in the horizontal plane (i.e., the plane of the storage ring) for the best signal-to-noise ratio.

Fluorescence peaks of various strengths were observed from the following elements: Ca, Cu, Mn and Zn. Note that the air path was too long for Na and Mg peaks to be detected. Electronic windows were placed around the four peaks' energies, and the integrated intensity within each window was used to map that element's spatial distribution when the specimen was translated. The Ca signal was by far the largest.

Diffracted intensities were mapped with a QUAD-RO CCD detector (Princeton Instruments, Trenton, NJ) placed behind the specimen, normal to the transmitted beam and centered on it. The specimen-CCD separation was ~60 mm which allowed the 00.2 and unresolved 21.1+11.2+30.0 cAp diffraction rings (20.4 and 25.0–25.9 °2 θ , respectively) to be recorded in their entirety. (Note the use of Miller-Bravais indices for a hexagonal crystal system).

Sample positioning in the beam was accomplished by moving the order sorting aperture out of the beam and observing radiographs of the specimen produced by the unfocused beam. Few features could be observed in the radiographs except those which were reasonably large (much larger than individual tubules) and which produced strong phase contrast at their borders: tears on one side of the thin section, a fold or two where the section rucked up and the edges of the block of mineralized tissue. After all four of its edges were located, the specimen was translated to an area that prior optical microscopy suggested would be of interest, that is, where there were no folds or tears, where the tubules were nearly perpendicular to the section plane and where the tubules were not too tightly clustered.

Rapid scans over areas of (25 μm)² identified individual tubules for mapping (400 nm translation steps, acquisition of only Ca fluorescent signal, 1 s/position) and could be completed in ~1.5 hr. High resolution scans (250 nm steps; for the data in Fig. 3, 80 s integration for fluorescence; for Fig. 4 and 5, 60 s/position integration for the diffraction pattern simultaneous with 40 s integration for fluorescence signals) covering one or two tubules typically spanned 6–9 μm across the section and required 12–16 hr to complete. This report describes x-ray fluorescence and diffraction maps for three fields of view (FOV) of one thin section; these maps are typical of those obtained from other cows.

Results

Figure 2 presents optical micrographs of a typical 1 μm thick section. The entire 1.7 mm $\times$ 1.3 mm cross-section of the block covers most of the field of view in Fig. 2a. Part of the mineralized tissue is missing (* in the lower part of Fig. 2a), and the partial infill of plastic indicates that the void was present when the block was cast in plastic. Figure 2b shows an enlarged view of the boxed area in Fig. 2a; Fig. 2c shows the boxed area of Fig. 2b at twice the magnification and Fig. 2d enlarges the boxed area of Fig. 2c. In Fig. 2c there are 4.3×10^4 tubules/mm², and, if the tubules were regularly distributed in a rectangular array as suggested by (Kinney et al. 2003), this corresponds to a mean center-to-center tubule spacing of 4.8 μm . The tubules are irregularly distributed in all portions of this and adjacent sections, ranging from tightly clustered to isolated and from regularly spaced to seemingly randomly distributed. The five nearest neighbors of the tubule identified with an arrow in Fig. 2d are separated (center-to-center) from it by between 5.8 and 8.1 μm , mean 6.8 μm . This central tubule, therefore, is more isolated than average. The pairs of tubules at F and G are more closely spaced (4.2 and 3.2 μm center-to-center, respectively) and are not atypical of closely clustered tubules.

In Fig. 2 and other sections, some tubule lumens appear to be unfilled and some to be filled. Figure 2d shows the margins of the tubule are the darkest features in the FOV. The tubule

identified with the black arrow in Fig. 2d intersects the section obliquely (more so than almost all of the other tubules in this section) and is perhaps a bit larger than is typical of this section. Measuring from the center of the dark ring, the major and minor diameters are 2.2 and 1.8 μm , respectively. The corresponding diameters measured from the inner edge of dark ring are 1.5 and 1.2 μm . If the tubule were a circular cylinder, these dimensions correspond to $\sim 35^\circ$ tilt of its axis from perpendicular to the plane of the section and represent an extreme misorientation. The filled tubule A is slightly larger and C is smaller than the tubule labeled with the arrow.

X-ray fluorescence maps are shown in Fig. 3 for two FOV of a single thin section. Panels a and b are from FOV 1 (Ca and Zn, respectively), and c-f are from FOV 2 (Ca, Zn, Mn and Cu, respectively). Except where there were surface contaminants on the thin section or on the underlying kapton film, Mn and Cu maps reflected no concentration of these elements and an absence where the Ca maps indicated positions of tubule lumens. Given that the other Mn and Cu maps add nothing beyond what is seen in Fig. 3e,f, no additional maps of these elements are shown.

The Ca maps of Fig. 3 show 50–60 times greater peak signal than the corresponding Zn maps. The tubule lumens appear as dark blue to black in both maps, and Zn appears to extend away from the lumen's edges. In the ITD, i.e., away from the near-tubule concentrations, Zn appears uniform and is significantly higher than in the tubule lumens, see description of Fig. 5. In some places Zn appears concentrated where the Ca signal is lower, elsewhere the Zn concentration appears independent of Ca. Zn is not uniformly distributed around any of the tubules in Fig. 3 and drops to background for at least part of the perimeter of each of the tubules.

The tubule in Fig. 3a,b is nearly perpendicular to the sectioning plane (round lumen) and, based on the Ca signal, has a diameter of 1.5–1.75 μm . The tubule in the middle of Fig. 3c–f appears to be cut obliquely and that in the upper left corner is cut quite obliquely. In the Ca maps, the former tubule has major and minor diameters of 1.75 μm and 1.0 μm , and these diameters cannot be accurately determined for the latter because it is partly out of the FOV.

Figure 4 shows Zn, Ca and diffracted intensity maps for FOV 3 of the same thin section as Fig. 3. The FOV is centered on one tubule, and a second barely intersects the upper right of the FOV. The Ca intensity is very high on the left side central tubule and at the lower right of the out-of-view tubule. The Zn signal around the tubule perimeters is not uniform, does not drop to background and is about one-seventh that of the Ca.

Continuous diffraction rings are present for all positions except where there was no diffracted signal at all, i.e., the same positions where absence of Ca fluorescence indicated tubule lumens (data not shown). These rings index as cAp 00.2 and the cAp unresolved 2.11+11.2+30.0 triplet, and the absence of other rings demonstrates that no other crystallographic phases are present in detectable amounts. Integrating the intensity within the detector regions of interest (ROI) shown in Fig. 4c produces the maps of diffraction intensity in Fig. 4d–f. Figure 4d maps intensity for the unresolved 21.1+11.2+30.0 diffraction ring, and Fig. 4e,f map the two orthogonally-oriented 00.2 ROI. In Fig. 4d, the intensity is relatively uniform although it is somewhat lower at the 12 o'clock position than elsewhere. The maximum intensities in Fig. 4e are on either side of the tubule at the 1 o'clock – 7 o'clock positions but are not quite identical. For the orthogonally-oriented ROI (Fig. 4f), the intensity maxima are also on approximately opposite sides of the tubule at the 4 o'clock – 9 o'clock positions and are also not quite identical in magnitude. Thus, the orthogonally-oriented ROI produce maxima in diffracted intensity that are also orthogonally oriented relative to the tubule.

Figure 5 shows line plots of Ca and Zn fluorescence and 211+11.2+30.0 diffracted intensity along the lines indicated by arrows in Fig. 4a, b, d, i.e., through the center of the tubule. The minima in Ca, Zn and diffracted intensity are exactly aligned. In the horizontal direction of Fig. 4 (Fig. 5a), the Ca peak is only slightly more pronounced on the left side of the tubule as compared to the right side, while the Zn signal is quite a bit larger on the left side as compared to the right side. In the vertical direction, the Zn peak intensity is symmetric on either side (but not the background) and Ca is not peaked. The peak Zn and the Ca bulk intensities are about five times larger than values in the tubule lumen. The peak Zn intensity (adjacent to the tubule) ranges from 20% to 90% greater than that at the ends of the 1D plots. Along both directions, the 21.1+11.2+30.0 diffracted intensity is uniform outside the tubule's lumen. For all three signals, the full-width at half maximum (FWHM) of the tubule lumen is just over 7 pixels ($\sim 1.8 \mu\text{m}$) along the horizontal direction and about 12.5 pixels ($\sim 3.1\text{--}3.2 \mu\text{m}$) along the vertical direction. The FWHM of the Zn peaks range between 2 and 4 pixels ($0.5\text{--}1.0 \mu\text{m}$).

Discussion

Five important results appear above: 1) Zn surrounds around all of the tubules examined and completely encircles some of the tubule lumens. 2) Elevated Ca fluorescence (relative to that from the ITD far removed from the tubules) is observed around portions of some but not all of the tubules. 3) The only crystallographic phase producing detectable diffracted intensity is cAp. 4) Diffraction from cAp 00.2 shows pronounced preferred orientation and from 21.1+11.2+30.0 does not. 5) Ca, Zn and 21.1+11.2+30.0 profiles show identical transitions from bulk dentin (ITD) through volumes adjacent to the tubule lumens. Whether the specimens are representative of root dentin near the pulp cavity and sample preparation introduces artefacts are discussed after the main results. Lateral resolution and through-thickness averaging interact in these transmission measurements and are discussed next. The effect of beam and specimen instabilities, if any, are discussed in the penultimate subsection, and the discussion concludes with suggestions for future work.

Presence and distribution of Zn

Zinc is strongly concentrated in the volume of material immediately outside the tubule lumen, and concentrations drop to ITD levels outside of the collar with thickness typical of PTD. The authors have found no prior reports of Zn localized to the near tubule volumes although Zn has been mapped near carious lesions where Hg was associated with the tubules and Zn was reported to be more evenly distributed (Harris et al. 2008).

In human dentin, Zn is the most abundant trace element and observations of mean concentration range from 100 to 700 $\mu\text{g g}^{-1}$ dry weight (Driessens and Verbeeck 1990). Zinc is evenly distributed in dentin ($\sim 225 \mu\text{g g}^{-1}$) except near the pulp where its concentration rises rapidly to 560–1400 $\mu\text{g g}^{-1}$ (Brudevold et al. 1963). In bone, Zn is concentrated only within areas of active calcification, and diffusive uptake is not seen elsewhere in the bone (Vincent 1963). Zinc is also concentrated at the boundary between unmineralized and mineralized articular cartilage (Zoeger et al. 2006; Zoeger et al. 2008). Zinc in the near tubule volumes may, therefore, be the hallmark of ongoing mineralization or it may help mineralization or hinder it (preventing mineral from occluding the tubules).

Zinc near the tubule lumens may be incorporated within the cAp crystal lattice or associated with the surrounding macromolecules.

X-ray diffraction demonstrates that Zn can substitute into the bulk of synthetic hydroxyapatite crystals at 15–20 mol. % (Ren et al. 2009). The signal-to-noise ratio is too

low in the present diffraction patterns to reveal changes in lattice parameters and peak widths and intensities that would reflect positional variation of Zn substitution in cAp.

Zinc can influence apatite mineralization kinetics, even if it were not incorporated into the crystal lattice, and its role is often contrasted with that of Mg. Bovine PTD is rich in Mg^{2+} and K^+ associated primarily with the organic matrix (Gotliv et al. 2006). In synthetic cartilage lymph, both Mg^{2+} and Zn^{2+} caused similar reductions in the rate and duration of rapid hydroxyapatite formation, but Zn^{2+} was a more important inhibitor on a molar basis (Wu et al. 2009). In phosphatidylserine- Ca^{2+} -Pi complexes, Mg^{2+} significantly affected mineralization but Zn^{2+} did not (Wu et al. 2009); the mineral fraction of embryonic bovine dentin contains phosphatidylserine and significant amounts of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol and phosphatidic acid (Goldberg and Septier 2002; Gotliv and Veis 2007). Zinc and calcium are intrinsic to the function of the endogenous matrix metalloproteinases (MMP) and are associated with resin-dentin bond weakening via collagen solubilization (Tezvergil-Mutluay et al. 2010).

Calcium near the tubules

The high Ca signals observed around part of the tubules in Fig. 4 suggests these tubules have partial hypercalcified collars whereas the tubules in Fig. 3 have not developed even partial collars. In root dentin, hypercalcified collars can be quite irregular (Weber 1968) or even absent (Takuma and Eda 1966). When other positions within the tooth are examined (i.e., near the DCJ or in the crown), we expect the PTD to be much more calcified relative to the ITD than is the case in the present data. In these other locations, it also will be interesting to observe whether Zn localization remains at the levels of the present study.

Diffraction and mineral organization

The only crystallographic phase detected is cAp, and the expected 00.2 and 21.1+11.2+30.0 peaks are observed (Almer and Stock 2010; Deymier et al. 2010). The patterns have very high background, and the authors expect to improve the signal-to-noise ratio in the future. Much like in bone, dentin patterns recorded with high energy photons ($E > 65$ keV) are much cleaner today than there were at the start of those experiments several years ago (Almer and Stock 2005; Almer and Stock 2007). At present, it is difficult to set a precise detection limit for crystalline phases other than cAp, but the authors expect they would detect Ca-containing crystals amounting to one-quarter of the cAp.

The diffraction data clearly show that the cAp content and orientations (Fig. 4, 5) do not differ appreciably from the remote ITD to the edge of the tubule lumen. This continuum is consistent with that inferred for human ITD and PTD from electron diffraction (Weiner et al. 1999).

Diffraction from polycrystalline specimens produces Debye rings whose azimuthal intensity (i.e., around the rings' circumferences) varies little if the diffracting particles are randomly oriented and substantially if some orientations are over-represented, i.e., if there is preferred orientation (Cullity and Stock 2001). Such crystallographic texture is reflected in the 00.2 maps' positional variation diffracted intensity (Fig. 4e,f). With the collagen fibril axes tangential to the tubule axis and the cAp *c*-axes parallel to the fibrils, biaxial texture is expected and is observed in the cAp 00.2 diffraction maps. In other words, one expects a) a given 00.2 ROI map to have roughly equal intensities on diametrically opposite sides of the tubule and b) the two orthogonally-oriented 00.2 ROI to have regions of maximum integrated intensity rotated 90° from each other. Both a) and b) are observed, but the crudely defined and aligned ROI (rectangles rather than sectors of a torus, no background subtraction) have produced some deviations from exact symmetry. Scatter between

diffraction rings varies considerably for different azimuths, and the quality of the present diffraction patterns does not allow meaningful quantitative measures of texture, e.g., like in (Al-Jawad et al. 2007).

The rate limiting step in mapping the region around tubules is the time required to collect diffraction patterns. Because diffracted intensity scales with volume of material diffracting and absorption is quite low, doubling specimen thickness from 1 to 2 μm would double the number of tubules that could be mapped. Attempts to produce 2 μm thick dentin sections have been unsuccessful to date. The sections roll into “scrolls” (Fig. 6a), and, where small regions of a section adhered to a glass slide, features within the section are largely obscured in optical microscopy and strong striations are observed in rapid Ca maps (data not shown). Use of thicker specimens would also affect the lateral resolution (see below).

Transitions: ITD to tubule lumen

The Ca, Zn and 21.1+11.2+30.0 maps show the same gradual changes from positions far from the tubule to adjacent to the lumen (Fig. 4a,b,d). Within the near-tubule volumes, the FWHM of the peaks in these three signals also match (Fig. 5a,b). Similarly, the crystallographic texture (Fig. 5e,f) does not change from adjacent to the tubule to the remote ITD. These maps of an intact section of dentin confirm the inferences drawn in (Weiner et al. 1999) on the edges of fragments of dentin.

The intensities (Ca, Zn, 21.1+11.2+30.0) in both the vertical and horizontal plots of Fig. 5 rise from the lumen to bulk levels over about 4 translation steps (1 μm) and remain at the lumen levels over 5–7 steps (1.25–1.75 μm). The matching Ca and diffraction profiles (Fig. 5) indicate that all of the Ca is within cAp or that any non-cAp calcium (i.e., as might be present in amorphous Ca-P or complexed with macromolecules) is uniformly distributed throughout the dentin.

Specimens and preparation

The present observation of 4.3×10^4 tubules/ mm^2 is consistent with the range cited by Schilke et al. (Schilke et al. 2000) for bovine dentin: 2.1 to 4.7×10^4 tubules/ mm^2 . Schilke et al. also list bovine tubule diameters of 1.3 to 3.5 μm , a range into which the present optical and x-ray measurements fall.

The void observed in Fig. 2a shows that the section is very close to the pulp cavity. The slab from which this section was cut, possessed a pulp cavity with three diverticula, one of which was intersected by this section shown in Fig. 2a.

There is no evidence that suggests that microtome sectioning of the dentin specimens produces enough damage to affect the x-ray mapping. If the surface were rough, transmission optical micrographs would not show the uniform background observed: high spatial frequency interference fringes would have been prominent. If significant knife marks were present, linear traces should have been observed in the different fluorescent and diffraction maps. There is no obvious evidence in the diffraction patterns of damage to the cAp nanoplatelets or their collagen matrix, but more detailed analysis of diffraction ring width or ellipticity is required, e.g. (Almer and Stock 2005; Almer and Stock 2010), before this can be regarded as firmly established.

Atomic force microscopy (AFM) of metallographically polished surfaces of human dentin shows tubule structures similar to those seen in the present study (Kinney et al. 2003). An AFM comparison of metallographically polished lamellar bone surface and one prepared by microtome revealed that the latter surface was significantly smoother with surface roughness less than 14 nm (Xu et al. 2003). Differences in surface height of 20 nm on the front surface

would not be expected to affect fluorescence intensity, and variation of 40 nm thickness (4% of $\sim 1\ \mu\text{m}$) would be difficult to detect in diffracted intensity especially with canaliculi ($> 100\ \text{nm}$ diameter) running from tubules.

In any physical sectioning preparation, one must consider whether any of the components can be removed preferentially from the section, particularly at interfaces such as the PTD-tubule lumen. It is possible that small portions of the PTD could be missing from the sections, but this is hard to confirm or refute based on the present data and the resolution of the x-ray mapping and the optical microscopy. Comparing the present data with nanoCT (nanoComputed Tomography with volume element, voxel, sizes $< 100\ \text{nm}$) of subsurface tubules is one direct way of confirming (or refuting) the absence of chipping in the sections, but such data ($\sim 60\ \text{nm}$ voxels in tube-based and $\sim 30\ \text{nm}$ voxels in synchrotron nanoCT) are not yet ready for comparison with the results of this study.

Lateral and through-thickness microstructural averaging

In the transmission maps, beam dimensions, tubule inclination and sample thickness can combine so that depth averaging can obscure lateral variation in microstructure. Figure 2, typical of the sections used for x-ray mapping, shows most of the tubules were nearly perpendicular to the sectioning plane. Some tubules are significantly inclined to the plane of the thin section (such as that identified by the black arrow in Fig. 2d at 35° and apparently those in Fig. 3c–f and perhaps that in Fig. 4); interpretation of maps of these structures must carefully compare variation along the major and minor axes of the tubules.

The 60 s/pt required integration of diffraction patterns seriously limits the number of tubules which can be mapped, and use of thicker specimens would increase the diffracted intensity at the cost of lateral averaging. The 250 nm wide beam and $1\ \mu\text{m}$ sample thickness represent a 4:1 ratio of thickness to beam diameter. A ratio up to 10:1 ($2.5\ \mu\text{m}$ thickness) could probably be tolerated in these mapping experiments, at least for tubules nearly normal to the section, and a 2.5 times increase in tubules mapped would be very valuable but Fig. 6a shows the difficulty in making such sections.

Instability: Beam or sample drift

The tubules in maps of Fig. 3c–f appear far from equiaxed, but the rapid scan including this area (Fig. 6b) demonstrates that the tubules are, in fact, not too elliptical. Over the $> 12\ \text{hrs}$ irradiation of the small area, the kapton film can be damaged (orange in Fig. 6c), and the thin section can *sometimes* pull away from the kapton film (elliptical ring from changing optical interference, Fig. 6c). The accompanying lateral shifts can distort the maps, at least if the sections are placed on kapton. Shifts of a few hundred nm over many hours would be all that would be required to produce the observed effect. A more stable substrate such as amorphous silicon nitride should eliminate the effect seen in Fig. 3c–f.

All of the fluorescence and diffraction data are recorded simultaneously from the same irradiated volume, and in many data sets no drift was noted when rapid scans were compared to the long scans. If precise geometry were essential (e.g. dimensions of tubule lumens, tubule-tubule separations), the rapid Ca scans would allow correction for the drift in the longer scans.

Future studies

More precise analysis of the diffraction data will potentially be very valuable and will require detailed peak fitting and background subtraction similar to that used previously at APS (Almer and Stock 2005). A better point-by-point representation would be in terms of texture indices (e.g., the ratio of the maximum to minimum intensity within each ring,

azimuthal FWHM for each maximum) and of direction of the maximum (as in (Al-Jawad et al. 2007)). It would be interesting to see if residual stresses in dentin's phases (cAp and collagen) are preserved in the thin sections, and diffraction analysis of cAp and collagen could be employed, e.g. (Almer and Stock 2005; Almer and Stock 2007; Almer and Stock 2010). The observation of similar cAp (and also Ca) levels in the ITD and near the tubule (early stage of PTD?) suggests traversing the tubules from pulp to DEJ would add invaluable information about PTD maturation, and this will be a priority in the authors' future investigations.

Improved mounting of the thin sections will also be valuable. Placing the freshly cut thin section and its water droplet onto supported amorphous silicon nitride layers or onto TEM microscope grids should be investigated.

Synchrotron microCT with 1–2 μm voxels has revealed tubule paths in the interior of millimeter-sized dentin samples, e.g. (Stock et al. 2008). NanoCT with 60 nm voxels in tube-based systems and 30 nm voxels with synchrotron x-radiation would add much more information and complement the present study. For example, differences in mineralization may be quantifiable around tubules 10–20 μm away from prepared surfaces, and the resolving power and x-ray phase-contrast edge enhancement should allow 3D mapping of the canaliculi network.

It would be of great import to determine whether the Zn concentration around tubules was associated with the mineral phase, with the non-collagenous proteins or with phospholipids in the PTD. It may be possible to ascertain this by immersing the thin sections briefly in methanol-chloroform solutions tailored for extracting lipids (Dirksen and Marinetti 1970), but the presence of the embedding plastic would be a complication.

The near lumen concentration of Zn may vary with anatomical position within a given tooth and with tooth age. Hypercalcification in PTD has been observed to be greater in coronal dentin than root dentin and near the DEJ or DCJ than near the pulp root dentin (Takuma and Eda 1966; Weber 1968); a peak in Zn concentration has been seen very near the pulp but not elsewhere in the dentin (Brudevold et al. 1963). In addition to investigating Zn levels, future x-ray submicrometer mapping of different anatomical positions would also simultaneously probe any changes in ITD-PTD crystallographic texture and in partition of Ca between crystalline and noncrystalline forms. Another important question for future investigation is whether bovine molar and incisor PTD differ in ways measurable by the approach used in the present study. Finally, human and rat tooth structures differ from bovine structure in important ways, and extension of this mapping approach to other species' dentin should be very valuable.

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Figure 1. Hierarchy of structures in tooth. (left) Entire tooth. (center) Intertubular dentin (ITD), peritubular dentin (PTD) and tubules. (upper right) Mineralized collagen fibrils around a tubule. (lower right) Collagen triple helices and carbonated apatite (cAp) platelets within each fibril; for simplicity, extrafibrillar mineral and mineral within the collagen gaps regions are not shown. The cAp c-axes lies along the long direction of black rectangles.

Figure 2.

Optical micrographs of a 1 μm thick section from the vicinity of the sections mapped with x-ray fluorescence and with diffraction. a. Low magnification view of the entire block. The block of dentin is 1.7 mm wide horizontally, i.e., along crown-root axis. b. Magnified view of the boxed area in a. c. Magnification of the boxed area in b. d. Enlargement of the boxed area in c. The symbols are explained in the text, and the horizontal FOV in b–d equal 440 μm , 220 μm and 55 μm , respectively.

Figure 3.

Fluorescence maps of FOV 1 (a, b) and FOV 2 (c–f) with the element mapped labeled in white. a. Ca map and b. corresponding Zn map for a $6.5\ \mu\text{m}$ horizontal $\times$ $5.5\ \mu\text{m}$ vertical area. c–f. Ca, Zn, Mn, Cu maps, respectively of a $6.5\ \mu\text{m}$ horizontal $\times$ $5.25\ \mu\text{m}$ vertical area. Intensities for each map are represented by a linear color table (bar with units in 10^3 cts).

Figure 4.

Fluorescence and diffracted intensity maps for FOV 3. a. Ca, b. Zn, c. schematic of identifying the diffraction ROI, d. 21.1+ 11.2+30.0, e. 00.2, f. 00.2. The horizontal width of map is 5 μm , the vertical height is 8.5 μm , and the pixels in this image are longer horizontally than vertically. The linear color table is the same as in Fig. 3; the fluorescence maps are in units of 10^3 cts and the diffracted intensity maps are in units of 10^7 cts. The arrows identify the positions of the linear plots of intensity in Fig. 5.

Figure 5.

a. Horizontal and b. Vertical scans through the center of the tubule at the positions of the arrows in Fig. 4. For each, Ca, Zn and 21.1+ 11.2+30.0 intensities are plotted with intensities rescaled for clarity. The horizontal axis is in units of 250 nm pixels (below the plots) and in μm (above the plots).

Figure 6.

a. Optical micrograph of a scroll of dentin. The horizontal FOV is 315 μm . b. Rapid Ca map (300 nm pixels, 1 s/pt) recorded prior to the detailed mapping of Fig. 3c–f. The color bar shows the intensity range in cts, and the white dashed box indicates the approximate position of the Fig. 3c–f maps. c. Optical micrograph of the mapped area of a section on kapton. Orange/dark brown is damaged kapton; the elliptical interference rings show where the beam damage pulled the kapton away from the dentin. The horizontal FOV equals 79 μm .