MINERAL PHASE MICROSTRUCTURE IN TEETH OF THE SHORT SPINED SEA URCHIN (*Lytechinus variegatus*) STUDIED WITH X-RAY PHASE CONTRAST IMAGING AND WITH ABSORPTION MICROTOMOGRAPHY

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ABSTRACT

X-ray absorption microtomography, a high resolution variant of medical “CT”, and synchrotron x-ray phase contrast imaging reveal the distribution of mineral within teeth of the sea urchin *Lytechinus variegatus*. Data from these imaging modalities on intact teeth are compared with histochemically stained thin sections observed with transmission optical microscopy. Because these teeth grow continuously, the complex variation of attenuation from aboral to incisal end shows how the sea urchin increases mineralization to produce a hard, self-sharpening cutting edge. The phase contrast radiographs appear to show the calcite crystal morphology with greater sensitivity, at least in some portions of the tooth.

INTRODUCTION

The teeth of sea urchins are very complex, self-renewing biocomposites and achieve a high degree of functionality by combining strengthening and toughening strategies often employed in artificial composites. These include varying reinforcement morphology of the calcite mineral phase, high degree of alignment of adjacent crystals, varying composition of the crystalline reinforcement phase, incorporation of toughening inclusions (macromolecules) within individual crystals and noncrystalline CaCO₃ in the composite’s matrix [1-12]. Techniques which have been applied include x-ray diffraction, optical microscopy of thin sections with various types of stains, scanning electron microscopy (SEM) of polished sections and of fractured surfaces.

Very high resolution computed tomography (i.e., microtomography) can image the internal structure of optically opaque samples with spatial resolution approaching that of optical microscopy. Computed tomography can not only employ different imaging “radiation” (x-rays, neutrons, ultrasound), but different phenomena can be used to produce the images. For x-rays, changes in absorptivity, phase or diffraction can be used to produce contrast, and results obtained with the first two modalities are presented below. Absorption-based x-ray microtomography units are now commercially available, and the principles of their operation are similar to those of higher resolution synchrotron radiation systems. In brief, a series of views through the sample (i.e., radiographs taken along different directions) are recombined mathematically into a cross-sectional map of the specimen’s x-ray absorptivity. A review of microtomography instrumentation and earlier materials work can be found elsewhere [13].
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X-ray phase contrast imaging offers considerably greater sensitivity than absorption-based techniques for many samples, particularly those containing different types of soft tissue, and wavefront distortion during propagation through the sample underlies this modality. The origin of this distortion (or phase shift) can either be due to the different sample thickness that the x-ray traverses or to the varying index of refraction within the sample itself. X-ray interferometers directly measure this phase shift, and, when combined with computed tomography, can yield three-dimensional reconstruction of the sample providing information on both sample attenuation and phase [14, 15]. Distorted wavefronts imply local angular variations in the beam direction, and, if sufficient angular collimation exists in the incident beam and crystal analyzers are used to detect small angular changes, phase-enhanced images result [16, 17]. Another technique, used here, is commonly known as the “in-line” or “propagation” method [18, 19]: Fresnel diffraction of the sample as a result of the incoming x-ray beam coherence provides contrast. By placing the detector some distance away from the sample, phase-enhanced contrast can occur due to the interference between various parts of the distorted wavefront.

**MATERIALS AND METHODS**

An entire tooth was removed from an adult sea urchin (*Lytechinus variegatus*); it was about 1.5 mm x 1.5 mm and 19 mm long with a “T”-shaped cross-section over most of its length and was placed in isopropanol in a small sealed vial. The aboral end of the tooth is the youngest portion of the tooth and is expected to contain the least mineral phase.

Microtomography was performed using a Scanco MicroCT-20 system operated at 50 kVp. Two scans of an intact sea urchin tooth were collected, one spanning the ~ 19 mm length of the entire tooth and a second covering 2 mm of the lightly mineralized aboral end of the tooth. The former data set consisted of slices collected every 0.1 mm and the latter of slices spaced 0.011 mm apart. Data were collected in the high resolution mode (500 projections/slice, 1024 samples/projection) with integration times of 0.35 s in the former and 0.3 s in the latter data sets. Reconstruction was with 11 µm in-plane voxel (volume element) size and a 25 µm slice thickness.

Phase contrast radiographs were recorded with the propagation method on station 1-ID of APS (Advance Photon Source) using a 1 mm x 1 mm slit to define the incident beam and a 1K x 1K CCD detector (coupled via an optical lens to a phosphor crystal) to record images. Sample-detector separations were between 15 and 400 mm with photon energies between 12 and 30 keV.

Thin sections of teeth were prepared as follows. After overnight fixation, the teeth were demineralized in a 0.5 M EDTA (Ethylendiaminetetraacetic acid) solution containing 50mM Tris(hydroxymethyl)aminomethane and 2.5% glutaraldehyde (pH 7.4). After dehydration in ethanol, the tissue was placed in embedding medium, sectioned to 4 µm thickness with a microtome and stained with hematoxylin and eosin.

**RESULTS AND DISCUSSION**

Figure 1 shows 3-D renderings of the tooth produced from the microtomography data sets. Differences in mineralization are clearly visible in the slices of Fig. 2. It is interesting to note that the pattern of high and low attenuation varies with distance from the soft end of the tooth,
i.e., with age in the continuously growing sea urchin tooth. There is less variation when the keel ("K" in Fig. 2d) is fully formed, but two zones of lower attenuation coefficient can be discerned. The first is rather subtle and lies on either side of keel (above and below “K” in Fig. 2d) and may correspond to the transition between different cellular structures (Fig. 2f). The second is much more prominent and is discussed in the following paragraph.

A low attenuation zone parallel to the top of the “T” of the tooth (extending from the upper left to lower right in Fig. 2) appears throughout the length of the tooth. This low attenuation zone may correspond to a local decrease in mineral content (and increase in organic matrix), to a change in mineral phase present or to mineral with a different composition. X-ray diffraction, however, revealed only calcite in sea urchin teeth [1-10], and no other crystalline phase appears to be present. Considerable variation in the amount of Mg incorporated in the calcite crystals in different sections of the sea urchin teeth have been reported, and the maximum Mg content of sea urchin tooth calcite was 43.5 mol. % in *L. variegatus* at and around the positions where the low attenuation zone was observed [20]. For Mo Kβ radiation (19.6 keV), the mass attenuation coefficient of Mg is about 2.8 cm$^2$/g and of Ca is about 13.6 cm$^2$/g [21], and it is not surprising, therefore, that contrast is seen. Staining of thin sections reveals that organic matrix and cellular activity are not insignificant within the tooth, especially within the center of the top of the “T”.

Also of interest is the way the low attenuation band changes along the length of the tooth: its prominence decreases from the tooth’s softer portion to its cutting edge (i.e., from Fig. 2a to 2e).

![Fig. 1. Renderings from 3-D microtomography data set from a sea urchin tooth showing the soft, aboral end (left, 1.7 mm tooth length shown) which contains little mineral and the heavily mineralized main section of the tooth (right, 18 mm of the 19 mm long tooth shown) with the cutting edge indicated. There is slightly less than 1mm overlap of the images, and the scale of the renderings differ. The number plus letter combinations indicate the approximate position where data shown in the figure of the same number were obtained. The discontinuities in the curved aboral end of the tooth reflect both the threshold level selected for the rendering and the relatively low levels of mineralization in this portion of the tooth.](image-url)
Fig. 2. (a-e) Five microtomographic slices at the approximate positions indicated in Fig. 1 and (f) a 4 µm thick section imaged with transmission optical microscopy. The positions of a-e are approximately 0.9, 1.7, 3.4, 13.1 and 18.3 mm from the end of the tooth, respectively, and the lighter the pixel, the higher the attenuation. The optical section is of decalcified tissue.
Fig. 3. Phase contrast radiographs at the positions indicated in Fig. 1. The field of view in each is 1 mm, and the lighter the pixel, the greater the intensity.

Perhaps the organic content of the tooth is shrinking (and being replaced by mineral) as the tooth ages and a particular section approaches the end of the tooth. It could be that the cells within the low attenuation zone serve to transport nutrients to the growing tooth, or that the localized high-organic content zone serves to increase toughness of the tooth as a structural element, or both.

Figure 3 shows phase contrast radiographs recorded from the same tooth seen in Fig. 1 and 2, and the positions at which the images were recorded are indicated by “3a” and “3b” in Fig. 1. The striations within the keel region (“K” in Fig. 3a) appear to be related to the long, single-crystal calcite fibers comprising the keel, but more work is required to determine whether each striation in the image corresponds to a single fiber or whether the striations result from interference effects. It is unclear whether the ridge (“R” in Fig. 3b) is an intrinsic part of the tooth’s soft tissue or is an artefact of fixation or handling. The striations “S” near “R” appear to be early stages of calcite crystal formation, but more work is required to establish this.

CONCLUSIONS

X-ray absorption microtomography of an intact sea urchin tooth (Lytechinus variegatus) reveal several zones of low attenuation. The more prominent is within the top of the “T” of the tooth, i.e., within that part of the tooth which becomes the cutting edge, and may result from calcite with a much higher fraction of Ca replaced with Mg, consistent with reports of the composition of this portion of the tooth. Staining reveals a non-mineral organic component of the tooth at about the same position within the tooth, and the data are not inconsistent with gradual change of an organic section to higher mineral content as the tooth ages and a particular section approaches the cutting edge. A pair of less prominent low attenuation zones are parallel to the sides of the keel and correlate with a transition in soft tissue morphology seen in decalcified thin sections. Phase contrast radiographs appear to reveal calcite crystal morphology with greater sensitivity
than absorption-based x-ray imaging, but further work is required to determine the origin of the observed contrast.

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REFERENCES