

## Morphology and Ultrastructure of Prismatic Calcified Cartilage

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The skeleton of sharks, rays and their relatives is composed of a hyaline-like cartilage covered with a thin, highly mineralized calcium phosphate (hydroxyapatite) layer of tiles called tesserae (Fig. 1). Tesserae are believed to form from the fusion of vesicles with high mineral content, secreted by chondrocytes at the mineralizing face [2, 3]. The resultant globular calcospherites merge to form a layer that gradually transforms by further mineralization into prismatic cartilage. This succession occurs as a calcification gradient from the underlying hyaline cartilage to the tesserae. However, the fine-scale morphological and chemical transformations involved in this process have not been studied at the high resolution electron microscopy level due to the difficulty of sample preparation. This research examines the interface between calcified and uncalcified cartilage in order to develop adequate methodology and elucidate the processes involved in cartilage calcification.

Fresh samples of unpreserved material were scanned at the University of Texas high resolution CT scan facility at a z-axis resolution of 60  $\mu\text{m}$ . The 1024x1024 TIFF stack was reconstructed with Amira 3.1 and prototyped with a ZCorp 3-d printer. All remaining samples were fixed in formalin. For light microscopy, samples were decalcified in formic acid and embedded in paraffin. Sections were microtomed at 10 $\mu\text{m}$  and stained with Masson's trichrome, hemotoxylin/eosin, and Verhoeff's elastin. EM samples were prepared by step-wise acetone dehydration. Intact and cross-sectioned SEM samples were sputter-coated with Au/Pd and examined with a SEM. TEM samples were embedded in Spurr resin in BEEM capsules and cured overnight at 70°C. Ultra-thin specimens were cross-sectioned with an Ultramicrotome, and then investigated in a TEM.

The unmineralized and mineralized phases are morphologically and elementally distinct. Ca and P increase dramatically at the mineralization front where globular calcospherites (40-55 nm, shown by arrows) are packed within the collagen fiber network that anchors the tesserae into the underlying hyaline cartilage (Figs. 2A and B). These collagen fibers may guide tesserae formation: individual hydroxyapatite crystals can be seen "walking" out from the mineral border onto the variously mineralized fibers (Fig. 2B). The hydroxyapatite conglomerations are smaller (25-33 nm) and more tightly packed (Fig. 2C) on the superficial surface of the tesserae. Divisions between individual tesserae are sharp, forming a steep crevasse with direct communication to the unmineralized cartilage beneath (Figs. 1C, 2A). As cartilage is avascular, these passageways may communicate nutrients to the interior of the skeletal element. Cross-sectional TEM images reveal that the interface is not linear: isolated mineralized phases are embedded in the unmineralized tissue and vice versa (Fig. 3A and 3B). High magnification TEM and electron diffraction indicate that prismatic cartilage is composed of nanocrystalline hydroxyapatite (Fig. 3C). Further investigation across ontogeny will clarify the mineralization process in developing animals and the transition between phases.

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[4] This work was funded by UCI Biological Sciences and GAANN Fellowships, and a PADI AWARE grant to MND.

Abbreviations: T = tesserae; UC = uncalcified cartilage

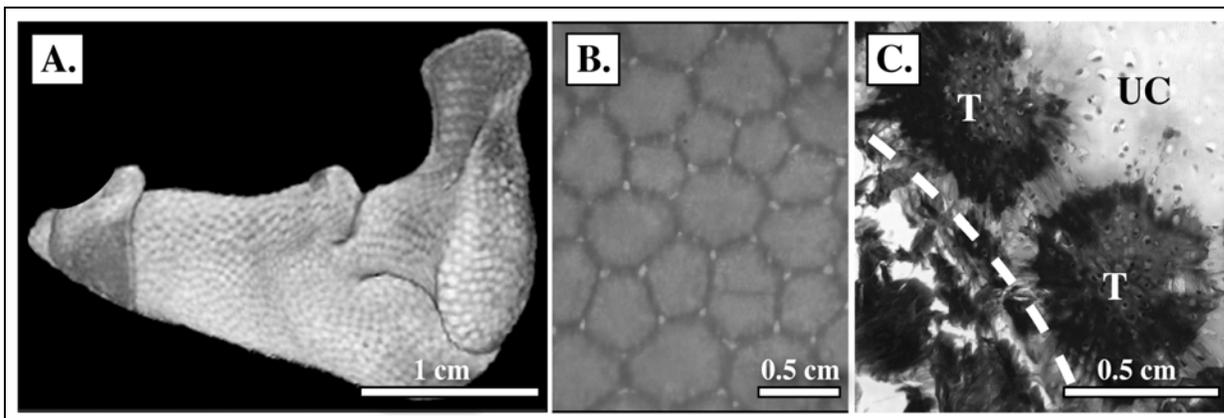


Fig. 1. Mineralized blocks (tesserae) covering the surface of skeletal elements of sharks and rays. A. Micro-CT scan, lower jaw, left lateral view; B. cleared and stained dorsal chondrocranial cartilage; C. Cross-section, hemotoxylin and eosin stain of lower jaw cartilage. White dashed line = perichondrium.

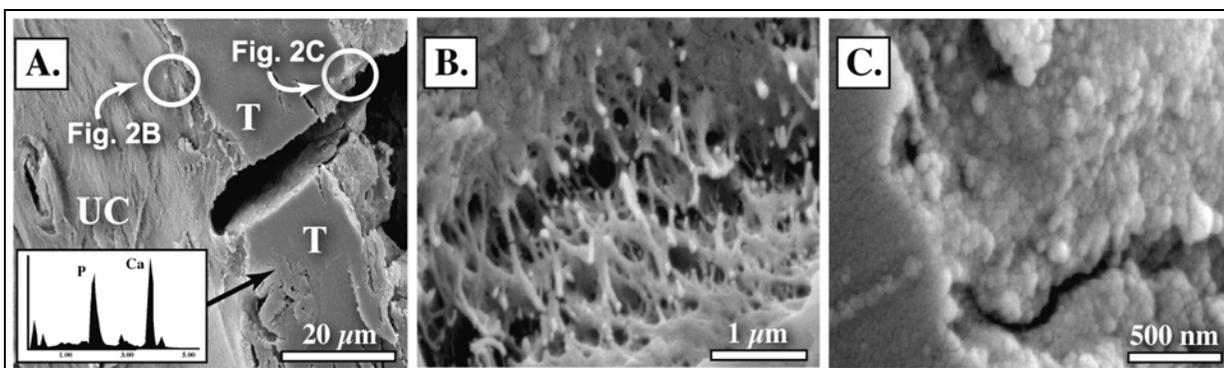


Fig. 2. SEM micrographs of calcified cartilage (cross-section, pelvic girdle). The crevasse separating mineralized blocks in A corresponds to the region between the tesserae in Fig. 1C.

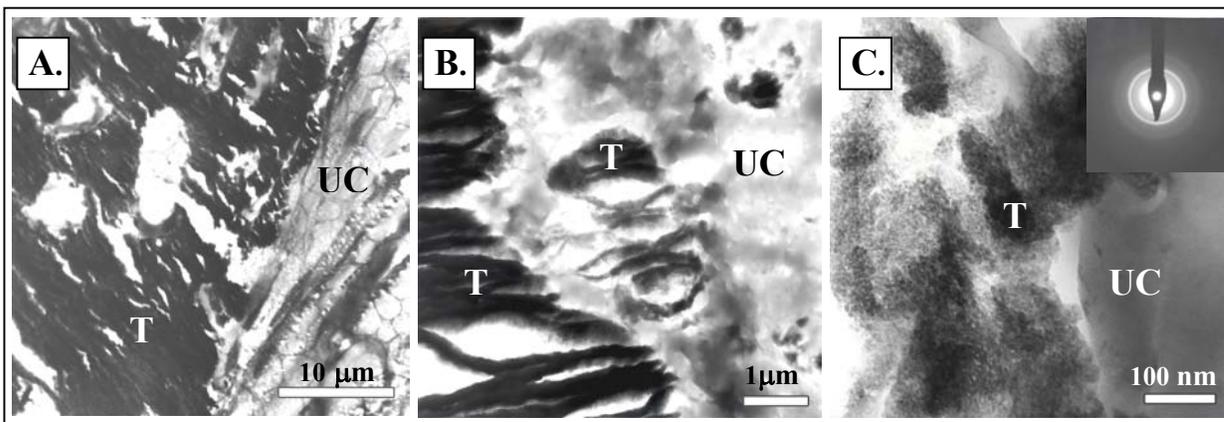


Fig. 3. TEM micrographs of calcified cartilage (cross-section, pelvic girdle) depicting interfaces between mineralized/unmineralized tissues; ED pattern in 3C indicates hydroxyapatite.