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14. ABSTRACT Engineered laminate composites have been widely used by public and private sectors due to their high strength-to-weight and high stiffness-to-density ratios. However, engineered composites tend to be brittle, and are consequently vulnerable to delamination when impacted or penetrated when compared to biocomposites. We have completed a careful characterization of the internal structure of one such biolaminate—the scales of the alligator gar fish (<i>Atractosteus spatula</i>). The scales of the alligator gar are similar to teeth and possess remarkable delamination resistance when hydrated. In order to understand this resistance, it was critically important to properly characterize							
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Report Title

Final Report: High Resolution Electron Microbeam Examination and 3D reconstruction of Alligator Gar Scale

ABSTRACT

Engineered laminate composites have been widely used by public and private sectors due to their high strength-to-weight and high stiffness-to-density ratios. However, engineered composites tend to be brittle, and are consequently vulnerable to delamination when impacted or penetrated when compared to biocomposites. We have completed a careful characterization of the internal structure of one such biolaminate—the scales of the alligator gar fish (*Atractosteus spatula*). The scales of the alligator gar are similar to teeth and possess remarkable delamination resistance when hydrated. In order to understand this resistance, it was critically important to properly characterize the various components of the scale and their interfaces. In the course of this study, we have determined: (1) the hierarchical structure of the gar scale from the millimeter down to the nanometer length scale, (2) the first detailed description of chemical variations within the gar scale, (3) the nature of the interface between the hard outside portion of the scale and the boney interior, and (4) provided the detailed structural and chemical information to ERDC for use in the development of the In-House Meso-Scale Scientific and Engineering-Discrete Element Problem Solver (SE-DEPS) model.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
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TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
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TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Spring 2014 Materials Research Society Meeting San Francisco, CA, Abstract and Presentation:

Session U4: Biomolecules, Biomaterials and Medicine I

Abstract Title: Characterization of Alligator Gar Fish Scale as Bioinspired Material

Authors: Kenneth J.T. Livi (presenter), Brandon Lafferty, Jennifer Seiter, Cedric Cedric Bouchet-Marquis, Trevan Landin, Wayne Hodo

2015 TMS Annual Meeting & Exhibition, Orlando, FL, Abstract and Presentation:

Symposium: Biological Materials Science Symposium

Abstract Title: Experimental Characterization of Bone and Exoskeleton Fish

Scale Structures

Authors: Wayne Hodo, Ken Livi (presenter), Jennifer Seiter, Brandon Lafferty, Mark Chappell, Paul Allison, Trevan Landin, Cedric Bouchet-Marquis

2015 Goldschmidt Conference (Biomineralization and Geochemistry meeting) Prague, Czech Republic, Abstract and Presentation:

Symposium: Biomineralization

Abstract Title: Nanoscale Investigation of Hydroxylapatite Formation in Alligator Gar Fish Scale

Authors: Kenneth J.T. Livi (presenter), Quentin Remasse, Cedric Bouchet-Marquis, Phillip McClellan, Brandon Lafferty, Jennifer Seiter, Ling Chen, Trevan Landin, William J. Landis, Nita Sahai, Rik Brydson, Wayne Hodo

Number of Presentations: 3.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received

Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received

Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

<u>NAME</u>
Total Number:

Names of personnel receiving PhDs

NAME

Total Number:

Names of other research staff

NAME

PERCENT SUPPORTED

FTE Equivalent:

Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

Technology Transfer

Transfer of highly detailed description of the internal and external structure of the alligator gar fish scale. This data was to be used for input into mathematical modeling of the physical properties of this scale as an investigation of bio inspirational material to design new delamination resistant materials.

Project Final Report - Grant # 63049-EG

High Resolution Electron Microbeam Examination and 3D reconstruction of Alligator Gar Scale

Kenneth J.T. Livi/ J. Michael McCaffery
Integrated Imaging Center
Johns Hopkins University, Baltimore, Maryland 21228

Objective

Engineered laminate composites have been widely used by public and private sectors due to their high strength-to-weight and high stiffness-to-density ratios. However, engineered composites tend to be brittle, and are consequently vulnerable to delamination when impacted or penetrated when compared to biocomposites. We have completed a careful characterization of the internal structure of one such biolaminate—the scales of the alligator gar fish (*Atractosteus spatula*). The scales of the alligator gar are similar to teeth and possess remarkable delamination resistance when hydrated. In order to understand this resistance, it was critically important to properly characterize the various components of the scale and their interfaces. In the course of this study, we have determined: (1) the hierarchical structure of the gar scale from the millimeter down to the nanometer length scale, (2) the first detailed description of chemical variations within the gar scale, (3) the nature of the interface between the hard outside portion of the scale and the boney interior, and (4) provided the detailed structural and chemical information to ERDC for use in the development of the In-House Meso-Scale Scientific and Engineering-Discrete Element Problem Solver (SE-DEPS) model.

Approach

The scales of the alligator gar fish are similar to teeth and possess remarkable delamination resistance. Full understanding of the causes for delamination resistance requires careful characterization of the basic components of the scale, how they are spatially arranged, and the nature of their interfaces. These data would then be used as input parameters for computer modeling of the scale's physical properties. In order to accomplish these goals, the exterior and interior of the gar scales were examined by the electron microscopy methods: transmission electron microscopy (TEM), scanning transmission electron microscopy (STEM), analytical electron microscopy (AEM), electron computed tomography (TEM CT), electron energy-loss spectroscopy (EELS), scanning electron microscopy (SEM), electron microprobe analyzer (EMPA), and by X-ray micro computed tomography (μ CT). Each of these methods provided different information pertaining to the structure or composition of the scale.

Background

Atractosteus spatula is a large freshwater fish found in southern rivers of the United States. This gar can reach lengths of up to 3 meters, weigh up to 150 kg and are estimated to live up to 70 years (1)(Fig 1a). The alligator gar is covered by boney scales (Fig 1b) and is thought to be descended from Mesozoic aged ray-finned boney fish palaeoniscoids. The *Atractosteus* scale is composed of two basic components: hydroxyapatite (HAp) and type I collagen. These two materials are combined to form two distinct layers: the thin upper hard highly-mineralized and enamel-like layer called the ganoine, and the bulk of the interior—bone. This composite material resembles, in a gross fashion, mammalian teeth, in that there is a dense outer layer like enamel and a softer boney interior. However, the gar scale lacks the complexity of mammalian teeth. It

appears that the gar scale continues to grow during the lifetime of the fish and preserves growth rings marking the passage of time. The scale bone lacks Haversian structures and osteons found in mammals and is not renewed over time. There is evidence, however, that chemical and structural evolution of the HAp minerals may take place during aging of the fish.

In comparison to avian and mammalian bone, the bone of the gar scale is primitive and simple in design. This more easily lends itself to computer simulation as compared to other bone structures where there are many hierarchical levels making it difficult to properly model.

Mammalian bone is thought to be constructed of seven levels of hierarchical structures (2). The gar scale is a simpler form of this, but contains many of the same elements. At the atomistic level, there are three proteins that combine together in a helical fashion to form the 220 nm long collagen nanofibril. The nanofibrils have different structures at either ends of the molecules. These nanofibrils are themselves twisted into a helical structure that places opposite ends of the nanofibrils adjacent to each other with a space between them. These “holes” are arranged within the collagen fibril in such a way as to create a 66 nm periodicity along the fibril. Within this 66 nm repeat, ~40 nm contains a high concentration of holes creating what is called the “gap” zone. It is within the gap zone that HAp is thought to nucleate. The other ~26 nm constitutes the “overlap” zone. This C/HAp helical structure is the basic building block of bone.

Sample Preparation

The first phase of the project was to determine the proper sample preparation methods that preserve the structural and chemical state of the biomaterial. Several methods were investigated for TEM examination: 1) Preparation of bone material by conventional protocols used for biological materials. This entailed the fixation of bone by Epon plastic and subsequent staining by OsO₄. Although this method produced large areas of relatively thin thicknesses, the protocols inadvertently partially demineralized the sections. Once this was realized, these sections were used to examine the structure of the collagen framework of the bone and to identify some of the relations between HAp and collagen. 2) Preparation of ganoine and bone material by focused ion beam (FIB) methods. This method used a highly focused Ga ion beam to mill portions of the scale and create thin electron transparent membranes. This method proved difficult to produce good quality TEM samples mainly due to the shrinkage of the bone at the final stages of thinning. However, sections from the ganoine were successfully produced since that hard material behaved better under the vacuum of the FIB. 3) Cryo ion milling of mechanically thinned slices of whole scales. This protocol produced the best sections of scale and allowed for thorough examination of both bone and ganoine material. 4) Cryo mechanical crushing and subsequent dissolution of collagen by cold hydrazine. From this method, individual HAp crystals were separated from the bulk (both ganoine and bone) in order to confirm the size and shape of the HAp crystals. 5) Cross sectioning of whole scale and mechanical polishing. This protocol produced flat polished sections of the scales that were examined in the EMPA using backscattered electron imaging and X-ray analysis. 6) Whole scales were also examined in the SEM without any coating or alterations in order to determine the exterior surface structure.

Results

Ganoine Structure

The shape of the ganoine layer was determined by μ CT scans that enabled the 3D rendering of the entire ganoine surface and understructure for the first time (Fig 2a,b,c). The ganoine has a variable thickness that manifests in a thin center and thick ridge that runs around the

circumference of the scale. The lateral extent of the ganoine does not cover the entire scale, leaving a rim of exposed bone around the circumference of the scale. On the underside of the scale there are concentric rasps that serve as structures to anchor the ganoine to the underlying bone. In cross section, the ganoine is comprised of layers that constitute growth rings (Fig 3). Each growth ring terminates in a rasp. The time it takes for the fish to produce one of these growth rings is not known.

From TEM examination, it was determined that the ganoine was comprised of tablets of HAp crystals that were highly oriented with the HAp *c*-axis oriented perpendicular to the growth layer. The HAp crystals in the ganoine were the largest found in the scale and are approximately 200 nm by 100 nm by 30 nm in size. The HAp crystals are tightly bound to each other forming a dense ceramic material (Fig 4a,b). Little collagen is found in the ganoine. The composition of HAp in the ganoine is distinct from HAp found in most of the bone material. Ganoine HAp is Na rich, Mg poor as compared to bone HAp and has an average Ca/P ratio (atomic %) of 1.55. Well-crystallized geologic apatite has little Na or Mg and a Ca/P of 1.66. Ganoine HAp contains some amount of CO₃, which has yet to be determined.

Bone Structure

The gar scale is mainly composed of bone material that at a first approximation resembles most other vertebrate bone in that it is composed of HAp and collagen. The local structure of the collagen/HAp (C/HAp) composite varies from top to bottom of the scale with the top most layer being a transitional region that is harder and with lower porosity than lower regions. Backscattered images of the bone show that it contains ~20 μm layers of alternating bright and dark material (Fig 5). This contrast is produced by regions of higher and lower density bone. These are presumed to be growth layers representing some unknown time period (possibly seasonal). Under the transition layer, the porosity of the bone increases away from the ganoine.

Transition Bone Structure

The bone that underlies the ganoine is composed of well oriented type I collagen and HAp crystals (Fig 5). The Transition Bone is demarcated by loci of rings that were exposed during earlier growth stages. Thus, the Transition layer represents bone that required physical properties conducive to exposure to external elements. Presumably, this would favor harder and stiffer material. The direction of elongation of the C/HAp is roughly parallel to the ganoine-bone interface (Fig 6) and wraps around the ganoine protuberances. The Transition Bone is the densest bone in the scale and also contains HAp compositions that can be similar to both the overlying ganoine and the underlying bone. In the TEM, the interface between the Transition Bone and the ganoine is gradual on the nanometer scale, but completes the transition over a few micrometers.

Interior Bone Structure

Further away from the ganoine, the C/HAp becomes more of a woven texture with an increase in porosity and an increase in the sizes of the pores. This creates a more compressible material. HAp crystals wrap around pores and will follow the collagen orientation in most regions with the *c*-axis oriented along the fiber axis (Fig 7). In between C/HAp fibers, more randomly oriented HAp can crystallize. In some areas, the overall texture of the bone can be described as consisting

of ropes of C/HAp fibers running in one general direction, with more randomly organized C/HAp connecting the ropes (Fig 8a,b).

The composition of bone HAp was determined from crystal separates in the TEM. Bone HAp is Mg-rich and Na-poor relative to ganoine HAp with an average Ca/P of 1.45. The bone HAp crystals average around 40 nm in diameter (Fig 9). STEM HAADF/EELS images of the nanometer scale compositional variations clearly delineate the presence of HAp crystals within the collagen fibrils (Fig 10). This figure exhibits two important features: 1) The compositional maps generated by EELS analyses are consistent with presence of two zones within the collagen fibrils. The high calcium and oxygen bands that run horizontally in this image correspond to the gap zone of the collagen, while the high carbon bands correspond to the overlap zone. However, these bands do not correspond to collections of HAp crystals, but to regions of high Ca and O within the collagen fibrils themselves. 2) The bright thin lines in the HAADF image represent the HAp platelets imbedded in the collagen fibrils. These platelets are found at an angle to the collagen elongation direction (vertical). This has not been previously identified. This implies that the collagen fibril molecules may pass through the HAp crystals. If this is correct, then such an intimate intertwining of organic molecules and inorganic mineral sheets would have profound effects on the strength of bone materials and represents a previously unknown structural component of bone.

Hierarchical Structure of Gar Scale

The arrangement of C/HAp fibrils varies in different places in the scale, but follows two basic schemes. Bundles (fibers) of C/HAp fibrils can be arranged in a linear fashion with little space between them, as in the Transition Bone region, or in a more woven fashion with higher porosity and cross-linked C/HAp between the fibers, as in the lower bone region. The numbers of pores and their sizes increase towards the bottom of the scale bone.

The ganoine lacks collagen, making it harder, but more brittle. The layered and textured nanoparticle nature of the ganoine, however, presumably adds strength to the ganoine as compared to a single crystal of HAp. The curved top surface of the ganoine may play a part in not only keeping adjacent scales in place, but also to distribute load during compression. The attachment of the ganoine to the bone is accomplished by the presence of rasps at the bottom of the ganoine. The rasps point towards the outer rims of the scale creating strong anchoring points. The dense well-oriented bone in the Transition Bone wraps around the rasps at the ganoine-bone interface. In this interface, the HAp crystals reflect a gradational change from the larger, Na-rich ganoine crystals to the more Mg-rich smaller bone crystals. There is a gradual change in the degree of preferred orientation of C/HAp in the Transition Bone towards a more woven texture in the bone. These gradational changes in texture and composition likely serve to distribute loads applied from the scale exterior (as in a predator bite) and the most likely contribute to the high resistance to delamination demonstrated by the gar scale.

Aspects of this Study that Require Further Investigation

We have elucidated the basic structural components of *Atractosteus spatula* and identified the main feature that are likely to contribute to the superior delamination resistance of its scale. These features must now be used in computer modeling of the scale to determine which aspects are most important. The questions raised in this report are: 1) How do gradual transitions between layers that contain the same components, but with different concentrations and textures

of these components, distribute load and increase desired physical properties? 2) Is the concentric rasp structure the best design to anchor a hard top layer to a more compliant underpinning? 3) What are the advantages to having cross-linked ropes? 4) Is there a role of porosity in the durability of bone, besides being channels for transport of extra cellular fluids? 5) How does the layered and textured HAp nanocrystals in the ganoin change the physical properties of this protective layer? 6) What advantage is there to having thick crests in the ganoin layer? 7) Are bone HAp crystals porous, thus allowing collagen molecules to pass through them? 8) What is the difference in strength gained by having HAp crystals grow at an angle to the collagen fibril axis? 9) What is the nature of the Ca/O-rich bands within the collagen fibrils? 10) What aspects of the gar scale can be directly incorporated into new designs to create stronger, more impact-resistant materials?

Relevance to Army

This effort is directly relevant to the Army goals of conducting basic research to better understand material behavior, performance, and durability under extreme loading conditions. A fundamental understanding of how nature creates these bio-laminates can potentially lead to the design of engineered layered systems that can better mitigate damage caused by penetration and impact. Engineered laminate composites have been widely used by public and private sectors for marine, automotive, transportation, and aerospace industries. Laminate materials are very attractive due to their high strength-to-weight and high stiffness-to-density ratios. However, engineered composites tend to be brittle, and are consequently vulnerable to delamination when impacted or penetrated. Delamination at the interface is the primary mechanism that leads to the failure of composite materials. The interface is an important factor controlling the stiffness, strength, and fracture properties for composite materials at the macro scale.

The data gathered here on the structure of the gar scale has been provided as input for mathematical models utilized by ERDC to predict the physical properties of gar scales and bioinspired materials. It is apparent that Transition Bone is important to mitigating cracking and delamination through the solid. ERDC is using computational tools to ascertain what are the rate independent properties that govern fracture resistance in biomineralized solids. The new discovery of mechanisms provided by this research has allowed ERDC to precisely model the physical state of the system. As part of ERDC's military engineering mission, they are using the fish scale as means to understand and define the internal state variables that are appropriate for designing multiphase high-performance composites that can effectively operate in extreme dynamic environments. The first step in modeling is to understand how energy is deflected and damping through the fish scales layers. Thus, this fundamental research has provided specific details of the gar scale so that elastic-plastic-viscous contributions from the isolated structural components can be better identified at each length scale.

Collaborations and Technology Transfer

- The internal structure of gar scale has been determined, and this data has been supplied to Wayne Hodo at the Army Corps of Engineers, ERDC as input for computer simulations of gar scale and bioinspired designs. Mr. Hodo, and a group of researchers from EDRC, have joined Dr. Ken Livi and Trevan Landin at the headquarters of the FEI company where the 3-D reconstruction was performed. The ERDC group has also attended an Alligator Gar Scale Symposium held at Johns Hopkins University in May of 2015 to present and discuss the results of this project.

Resulting Journal Publications During Reporting Period

- Spring 2014 Materials Research Society Meeting San Francisco, CA, Abstract and Presentation:
Session U4: Biomolecules, Biomaterials and Medicine I
Abstract Title: Characterization of Alligator Gar Fish Scale as Bioinspired Material
Authors: Kenneth J.T. Livi (presenter), Brandon Lafferty, Jennifer Seiter, Cedric Cedric Bouchet-Marquis, Trevan Landin, Wayne Hodo

- 2015 TMS Annual Meeting & Exhibition, Orlando, FL, Abstract and Presentation:
Symposium: Biological Materials Science Symposium
Abstract Title: Experimental Characterization of Bone and Exoskeleton Fish Scale Structures
Authors: Wayne Hodo, Ken Livi (presenter), Jennifer Seiter, Brandon Lafferty, Mark Chappell, Paul Allison, Trevan Landin, Cedric Bouchet-Marquis

- 2015 Goldschmidt Conference (Biomineralization and Geochemistry meeting) Prague, Czech Republic, Abstract and Presentation:
Symposium: Biomineralization
Abstract Title: Nanoscale Investigation of Hydroxylapatite Formation in Alligator Gar Fish Scale
Authors: Kenneth J.T. Livi (presenter), Quentin Remasse, Cedric Bouchet-Marquis, Phillip McClellan, Brandon Lafferty, Jennifer Seiter, Ling Chen, Trevan Landin, William J. Landis, Nita Sahai, Rik Brydson, Wayne Hodo

Graduate Students Involved During Reporting Period

- None

Awards, Honors and Appointments

- None

References

- (1) Garcia de Leon, F. J., L. Gonzalez-Garcia, J. M. Herrera-Castillo, K. O. Winemiller, and A. Banda-Valdes (2001) Ecology of the alligator gar, *Atractosteus spatula*, in the Vicente Guerrero Reservoir, Tamaulipas, Mexico. *The Southwestern Naturalist* **46**:151-157.
- (2) Buehler, M., and Keten S. (2010) Colloquium: Failure of Molecules, Bones, and the Earth Itself. *Reviews of Modern Physics*. Vol. 82, pp. 1459—1487.

Figures



Figure 1a. Alligator Gar fish caught in Brazos River, Texas. Six feet long and 129 lbs. Credit: Clinton and Charles Robertson

https://www.flickr.com/photos/dad_and_clint/12716099/in/album-72057594067873642/



Figure 1b. Gar fish scales. Credit: <http://shadyufo.tumblr.com/post/35927882044/gar-fish-scales-these-things-are-wicked-sharp>

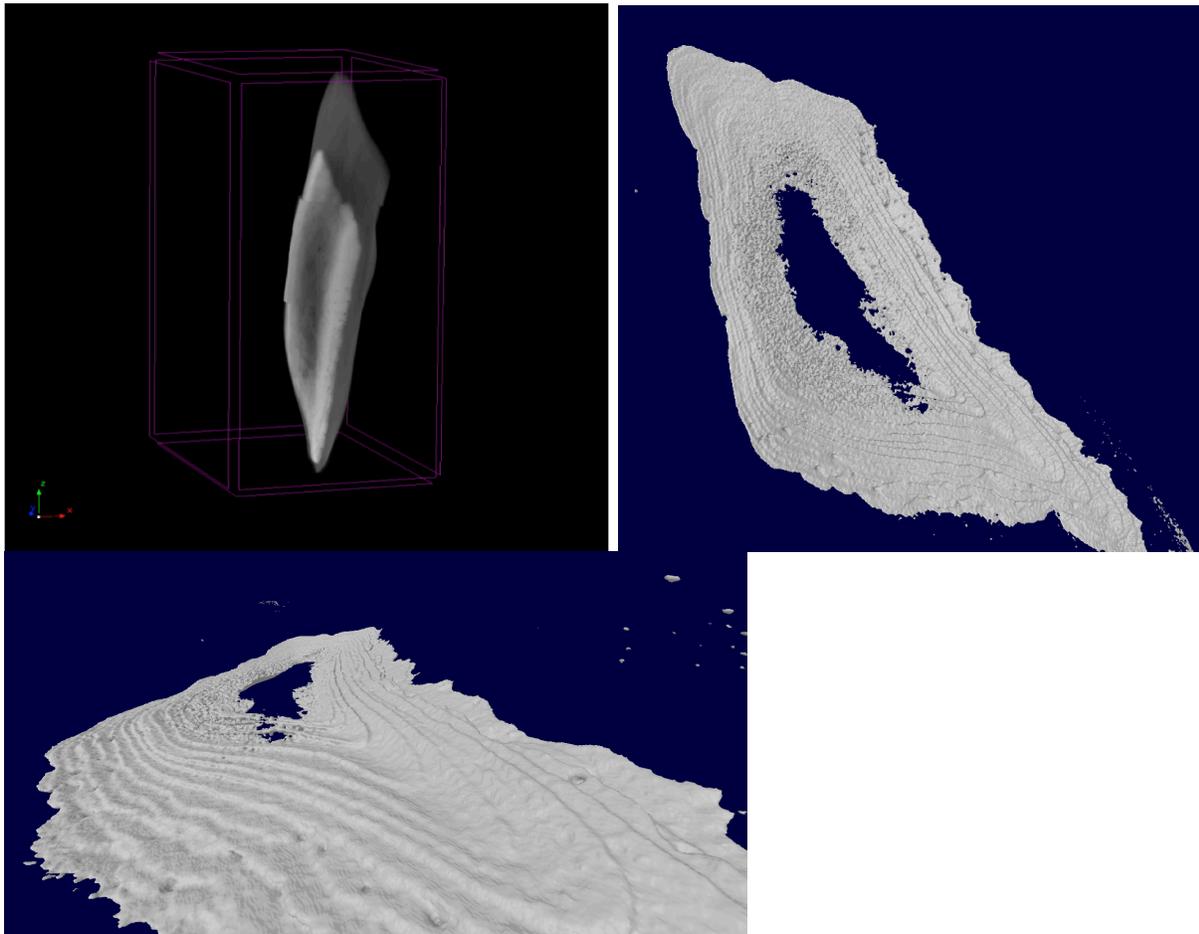


Figure 2a (top left). μ CT scan of gar scale showing the dense ganoine layer (bright). The gar scale is 2 mm long. Figure 2b (top right). 3D reconstruction showing the rasps that run concentrically around the underside of the ganoine. Length of ganoine is 1.5 mm. Figure 2c (bottom). Inclined view of ganoine underside.

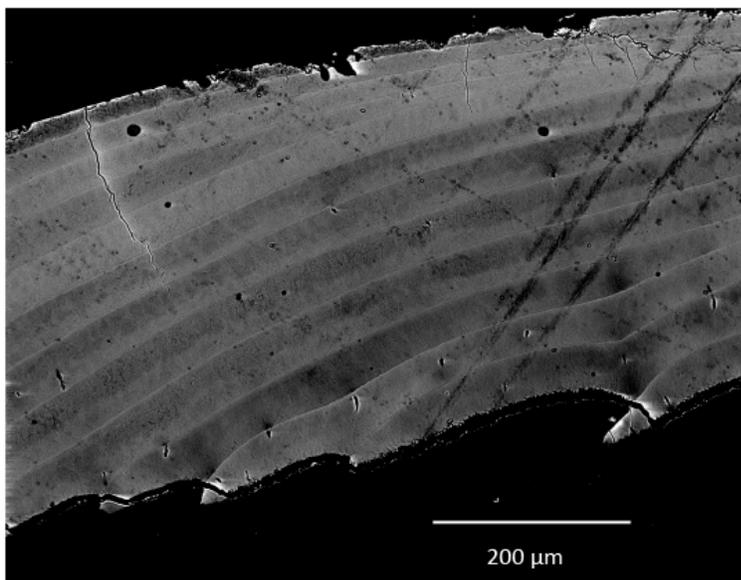


Figure 3. Backscattered SEM image of ganoine in cross section showing growth rings.

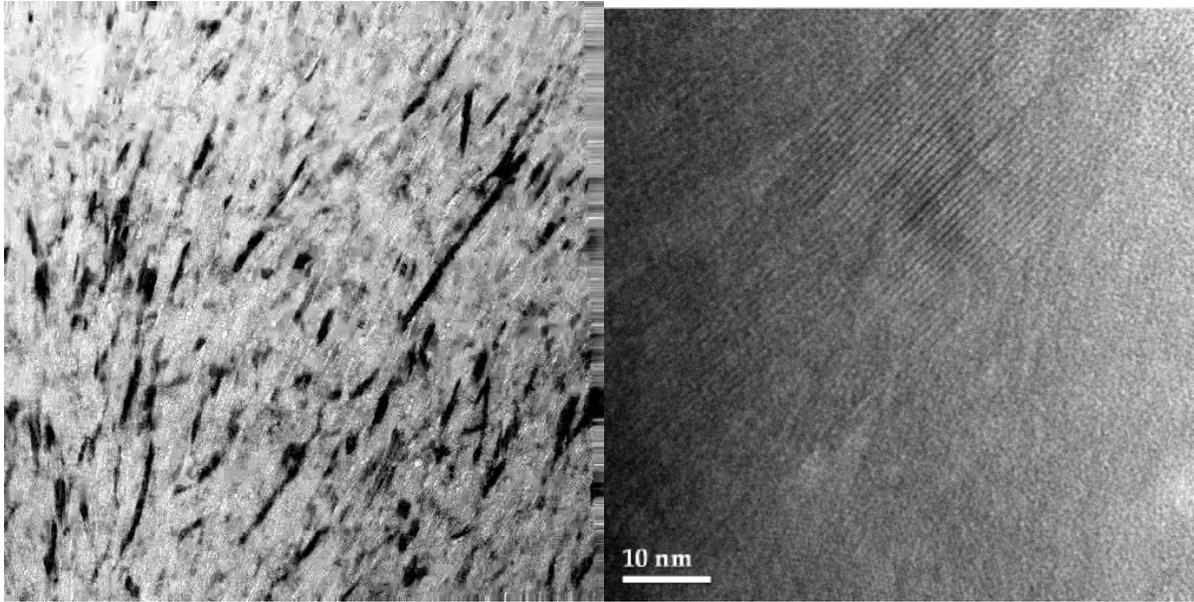


Figure 4a (left). Bright-field TEM image showing the orientation of HAp crystals in the ganoine. Figure 4b. (right) High-resolution TEM image of ganoine HAp. Adjacent crystals are packed with low angle rotations of the c -axis.

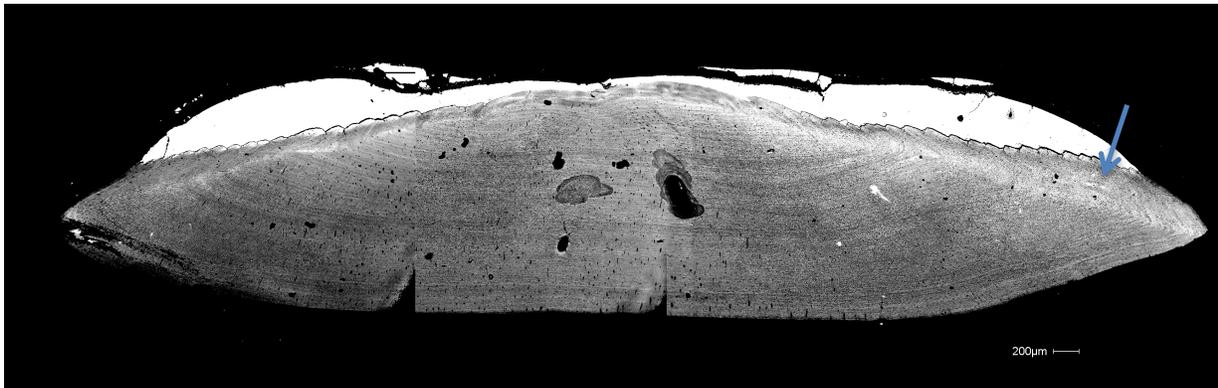


Figure 5. Backscattered SEM image of scale cross section. The bright top layer is the ganoine with its saw-tooth underpinning. Below the ganoine is the bone. The bone is marked by growth rings identified by alternating bright and dark lines that follow the circumference of the scale. The blue arrow points to the transition zone containing dense bone.

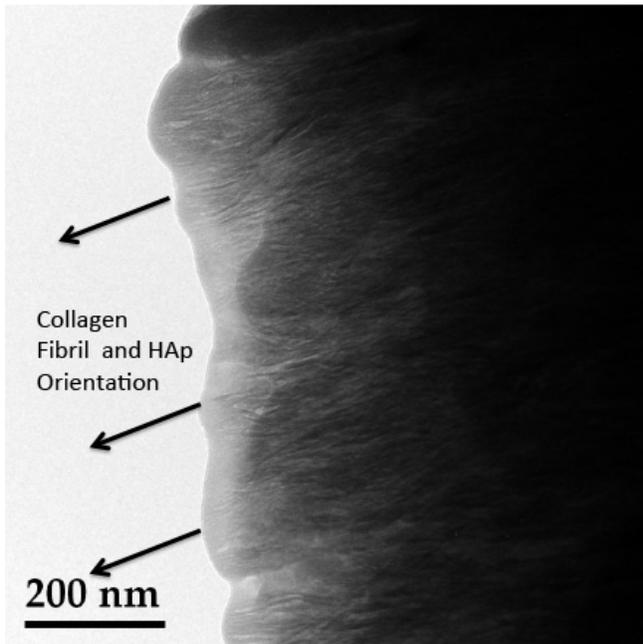


Figure 6. TEM image of the transition zone bone structure. This bone contains highly oriented HAp and collagen fibrils.

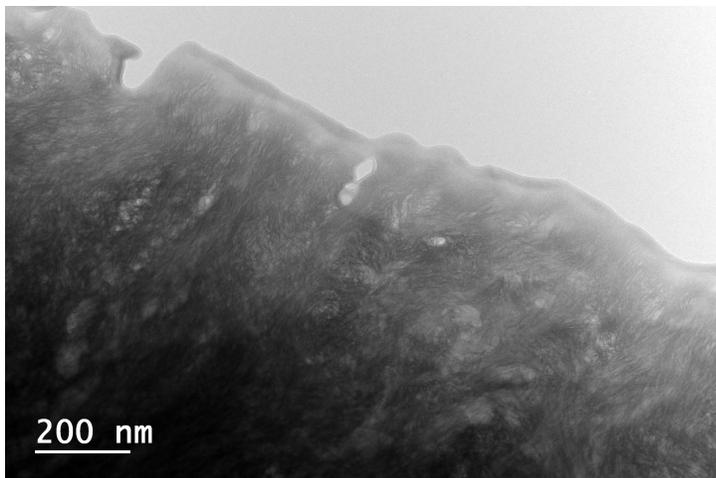


Figure 7. TEM image of the bone interior showing more chaotic texture of C/HAp and pores (light features).

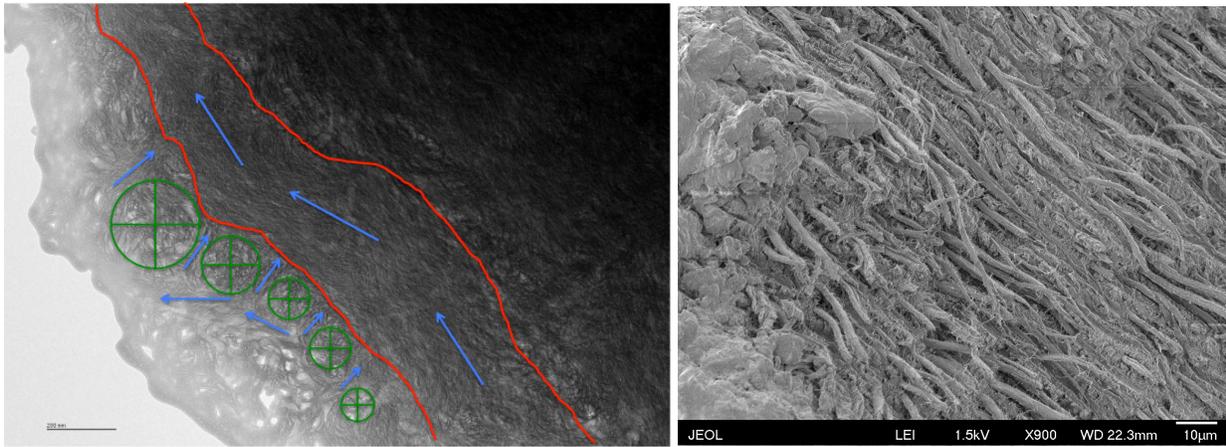


Figure 8a (left). TEM image of the bone interior area containing a C/HAp fiber (outlined in red) next to a region where fibrils are chaotically arranged. Blue arrows indicate the elongation of the collagen fibrils. Green circles and ovals contain fibrils running perpendicular to the image plane. Figure 8b (right) SEM image of fractured gar scale showing the presence of fiber “ropes” with disorganized C/HAp material between them. Compare the SEM image with the TEM image.

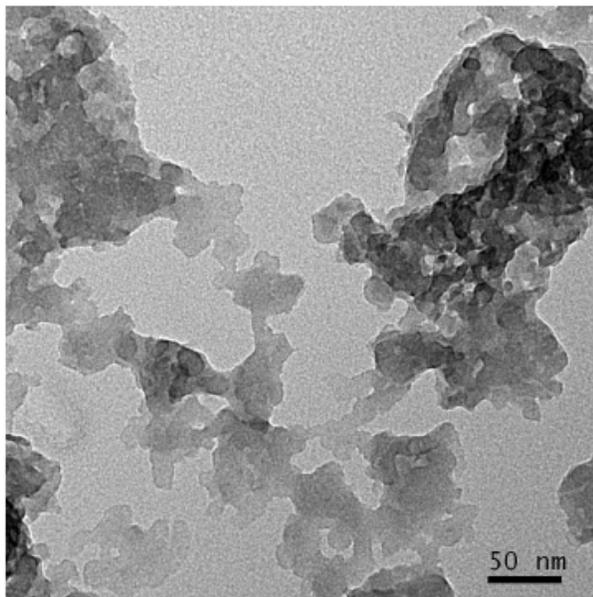


Figure 9. TEM image of bone HAp crystals separated from the collagen.

Electron Energy-Loss Maps

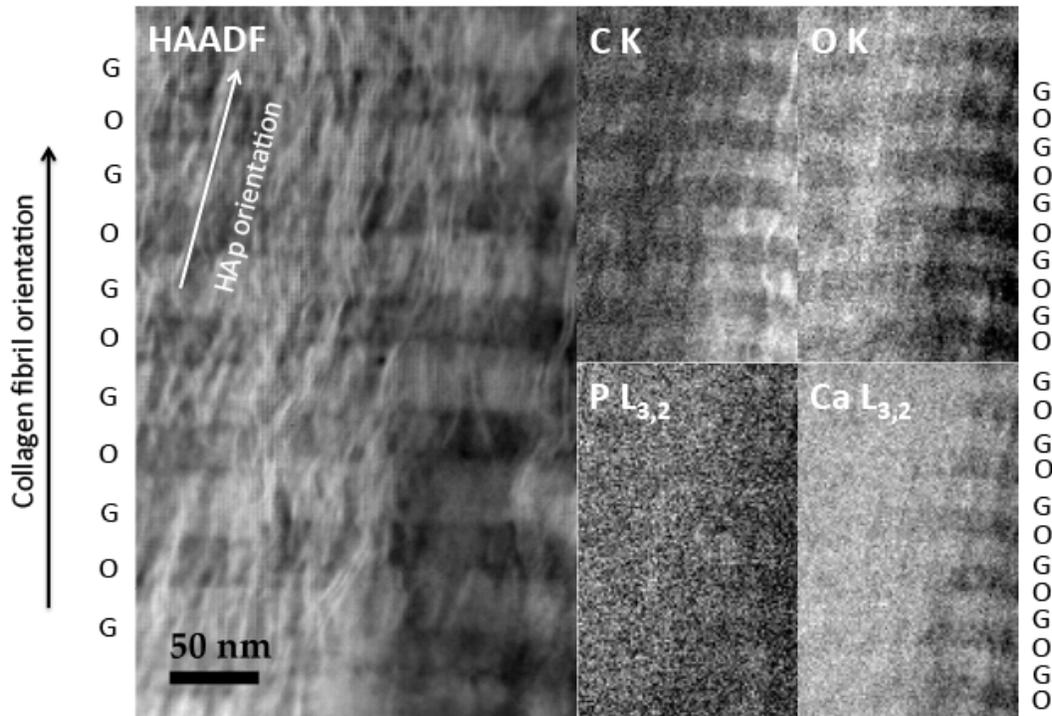


Figure 10. STEM High-Angle Annular Dark-Field (HAADF) image of C/HAp ultrastructure (left) and EELS compositional maps of C, O, P and Ca. The bright nearly vertical lines in the HAADF image are thin HAp crystals. The bright and dark horizontal bands represent the gap (G) and overlap (O) zones within the collagen fibrils. The collagen fibrils are oriented vertically, but at an angle to the HAp crystals. The compositional maps on the right are consistent with higher concentrations of Ca, P, and O in the gap zones, while C is higher in the overlap zones.