Formation and fracture mechanics of tough apatite structures in the stomatopod hammer

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2021

Chua, I. J. Q. (2021). Formation and fracture mechanics of tough apatite structures in the stomatopod hammer. Doctoral thesis, Nanyang Technological University, Singapore.
<https://hdl.handle.net/10356/152216>

<https://hdl.handle.net/10356/152216>

<https://doi.org/10.32657/10356/152216>

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**FORMATION AND FRACTURE MECHANICS OF
TOUGH APATITE STRUCTURES IN THE
STOMATOPOD HAMMER**

CHUA JIA QING ISAIAH

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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STOMATOPOD HAMMER**

CHUA JIA QING ISAIAH

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2021

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Assoc Prof. Ali Gilles Tchenguise Miserez

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal in which I am listed as the first author.

Chapter 4 is published as J. Q. I. Chua, D. V. Srinivasan, I. Sridhar, A. Miserez, “Fracture toughness of the stomatopod dactyl club is enhanced by plastic dissipation: A fracture micromechanics study,” *Acta Biomaterialia*, vol. 126, pp. 339–349, Mar. 2021 doi: 10.1016/j.actbio.2021.03.025

The contributions of the co-authors are as follows:

- Assoc Prof. Miserez conceptualized the micro-fracture toughness project.
- Assoc Prof. Miserez and I wrote and edited the manuscript for submission.
- Prof. Sridhar provided ideas and guidance with fracture measurements and FEM analysis.
- All microcantilevers for the micro-fracture study were prepared and tested using by me.
- All FEM models were created and analyzed by me with assistance from Dharun Srinivasan.

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Abstract

The stomatopod dactyl club is one of many materials showing Nature's prowess and ingenuity in creating materials with superior properties using intrinsically weak building blocks. Moreover, they are synthesized under mild environmental conditions compared to the energy-intensive conditions required for many manmade materials. Previous research has shown that the club is a multi-layered structure consisting of a hard and highly mineralized outer region consisting of Fluorapatite (FAP) and a softer inner region made primarily of chitin and CaCO_3 . The hard-outer region can be further divided into the impact surface which starts at the surface and is followed by the impact region. Recent studies focused on understanding and imitating the toughening mechanisms in the club, while little emphasis has been placed on the growth and development of the club. Only a single study has been conducted to date, which found that the club develops from the surface in by a "diecast" method and identified a major protein regulating the biomineralization of apatite. In this thesis, the spatial temporal composition and apatite crystallography are explored in greater detail using primarily synchrotron X-rays. Apatite was found in the club as early as 2 hours after molting. The crystals have different textures in the impact surface and impact region of the club. Results also suggest that the apatite crystals undergo compositional changes throughout its development.

To date, most studies of the mechanical properties of the stomatopod dactyl club were conducted using nanoindentation because the size of the club precludes other commonly used methods. However, properties like fracture toughness are only semi-quantitatively determined using contact mechanics. The reliability of indentation fracture measurements, in particular, is a subject of much debate among the scientific community. Hence, in this project chevron-notched micro-cantilevers were prepared and tested to determine fracture toughness values using linear elastic and elastic plastic fracture mechanics. The fracture behavior in the impact region of the dactyl club was shown to be dominated by plastic dissipation. Using the microcantilevers, fracture toughness in the impact region was also shown to be isotropic and not dependent on the direction of measurement.

Previous studies have shown that the impact region consists of FAP crystals aligned with their strongest *c*-axis toward the surface. SEM images showed that the crystals were misaligned from the surface directly beneath a damaged zone. This is a new toughening mechanism observed in Nature. To verify that this mechanism exists, synchrotron μ XRD mapping was used with monotonically and cyclically loaded samples. The results were inconclusive likely because of the averaging of the X-ray data. The first 3D imaging of the impact region and periodic region was achieved using Ptychographic X-ray Computed Tomography. The 3D reconstruction showed the microchannels present in both layers and a bone osteon-like structure was observed at the impact region, which suggests yet another toughening mechanism employed in the club. Overall, this thesis contributes to understanding the biomineralization of apatite biotools in the marine environment and extends micro-fracture methods to hard biological materials. Future work can focus on expanding the micro-fracture toughness method used in this thesis to other hard mineralized tissues. Many questions remained from the time study of apatite biomineralized in the dactyl club and should be addressed in future studies as well. A physical model capable of predicting the nanocrystal rotation and high-resolution elemental and crystallographic 3D imaging can be another area for further research.

Lay Summary

Mantis shrimps (stomatopods) are shallow water crustaceans, which are more closely related to lobsters than shrimps. The name ‘mantis’ was given because of the strikingly similar pose they make to the praying mantis, when they are about to attack. Instead of two pincers as claws, mantis shrimps are either equipped with a spear with many spines or a bulbous club (hammer); these specialized weapons are launched at bullet speeds to grasp or to pulverize their prey, respectively. Club-wielding mantis shrimps feed on hard shell prey like oysters whose shells are themselves extremely tough and even serve as inspiration for armor used in the military. Using their clubs, these mantis shrimps can break through their tough shells to feed on their flesh. Furthermore, they launch hundreds of high-powered blows before they molt and form a new club completely from scratch. Due to their ultra-tough properties, there is interest to study the mantis shrimp club to create new ultra-tough artificial materials. Previous research showed that the club is made of two main regions, a hard-outer layer containing mineral crystals and a softer inner layer. The hard-outer layer can be further divided into a thin layer from the surface of the club called the impact surface and a thicker layer following it called the impact region. The mineral found in the hard-outer layer and bone share the same mineral group, which is apatite.

Previous research used indentation to measure the mechanical properties of the club. In indentation, a very hard tip, usually diamond, is used to make impressions on the surface of a material. The dimensions of these impressions and the forces measured are used to determine the mechanical properties. While suitable to measure hardness and elastic modulus, other mechanical properties like fracture toughness cannot be reliably determined with indentation methods. However, the mantis shrimp club is too small to be tested with common fracture toughness methods. Therefore, in this thesis, tiny cantilevers cut into the surface were used to measure the fracture toughness of the club. The cantilever lengths were about 10 to 15 times smaller than the diameter of a strand of hair. With this technique the fracture toughness of the impact region of the club was successfully measured. The fracture toughness of the club did not depend on the direction it was measured in, which is contrary to other properties like hardness. And the fracture toughness was attributed mostly to plastic dissipation, similar to how a

metal bar will lengthen when stretched before it separates into two parts compared to glass which will break with no change in length. This is surprising for the club which is made of material similar to bone. The method used in this research can be applied in the future to reliably measure other hard and tough biological materials that cannot be measured using common fracture toughness methods or to measure the fracture toughness of these materials at the microscale.

Although much research was done on the properties of the mantis shrimp club, much less is known about the growth and development of the club. The club, like all natural materials, is formed at ambient pressures and temperatures, instead of at high pressures and temperatures often used in man-made materials. Moreover, it is formed using water-based chemistry; avoiding the need for highly toxic chemicals. Therefore, understanding how the mantis shrimp creates its ultra-tough club may contribute to developing new material synthesis methods that require less energy and are less toxic to the environment. In this thesis, highly focused and intense X-rays were applied to study club samples removed from the mantis shrimp at different time points after molting. Results show that apatite is present in the club as early as 2 hours after molting and suggests that the elements in the apatite mineral changes as the club develops. The element Bromine (Br) was discovered on the surface and at the growth front of new apatite crystals. Proteomics revealed that Br is present as a modification of the main protein controlling the apatite formation. Currently, the reason for Br in the club is not clear; although it is suggested to protect the club from chemical or biological attacks. Future studies can be directed to prove this theory and to understand the elemental changes in the apatite mineral during club growth.

Acknowledgements

The author would like to extend his heartfelt thanks to the following individuals who have been a tremendous help with this thesis:

My supervisor Prof. Ali Miserez. Thank you for accepting me into your team and for obtaining the funding necessary for this work. And also for the many opportunities for overseas research attachments and collaborations. For your advice which helped to steer and keep this project on course; and for your generosity and kind understanding which enabled me to focus on work without much worry.

I am indebted to Dr. Shahrouz Amini for being my mentor. Thank you for freely sharing your knowledge and for guiding me throughout my PhD; even after you accepted a new position overseas. Your willingness to help and share has been a source of inspiration and an invaluable aid to me.

I would also like to thank Prof. Gan Chee Lip and Prof. Martial Duchamp who are my thesis assessment committee members. Thank you for taking time from your busy schedules to listen and provide advice for my PhD. And thank you Prof. Duchamp for teaching me how to cut microcantilevers with focused ion beam (FIB).

Moreover, I am grateful for my colleagues, who have provided prompt assistance whenever requested; among whom I would like to especially mention, Loke Jun Jie, Dr. Hiew Shu Hui, Claire Bui and Soon Wei Long. I would also like to thank the members of the lab aquarium maintenance team, including the four colleagues mentioned above and Lim Zhi Wei, Hur Sun Young, Snehasish Basu, Kenrick Koh, Sun Yue, Abbas Bacha and Dario Heymann. Thank you for giving your time to take care of the animals (including the mantis shrimps) and all the equipment that keep them alive.

I am also very thankful to my overseas collaborators Prof. Henrik Birkedal and his team from iNano including Thorbjørn Christensen, Nina Wittig, Jonas Palle, Mads Jørgensen and Alexander for their tremendous help with the biomineralization project and their friendship. I have learnt so much from working together with you all. Thank you for

your hospitality and for all the wonderful memories together. Additionally, I am thankful for the scientists at the three synchrotrons I visited; Dr. Nobumichi Tamura (ALS), Camelia Stan (ALS), Dr. Esther Tsai (SLS) and Dr. Tilman Gruenwald (ESRF) their help and hospitality are remembered. Special thanks to Dr. Luca Bertinetti and Zhao Yong from Max Planck Institute, Golm for their kind assistance.

There are many more people who have in their own way helped me. I would like to thank Prof. Idapalapati Sridhar and Dharun Srinivasan for providing their resources, guidance and help with the micro-fracture mechanics project. Also Dr. Liu Qing and others at Prof. Gan Chee Lip's lab, Dr. Lek Jun Yan and Alan Lim from FACTS lab who helped tremendously with the FIB equipment. I am thankful to other staff at FACTS lab who have assisted in various ways including Dr. Jason Scott Herrin and Dr. Derrick Lim. I am also grateful for the technical staff at MSE like Gan Zi Li and Patrick Lim who taught me to use the instruments there. I am also thankful for Dr. Loo Chien Wang and Dr. Radoslaw Sobota for their help with the proteomics experiments. Many thanks to Maria Chong and Ong Chee Yung from CEE who performed the ICP measurements; and to Wu Jiang, Ng Fern Lan, Vinod Kumar, Molly Moynihan and Dr. Jeffrey Oalman for helping with EBSD experiments. Special thanks to Lyu Chade who provided the Teflon bombs used in sample digestion for ICP analyses and Rubayn for help also with sample digestion.

Last but far from least; my partner, my friends and family who have in various manners supported me through these past four years. I would not have come so far without their presence in my life. Thank you very much!

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Figure Captions

Figure 1.1. A) *Odontodactylus scyllarus* i.e. Peacock mantis shrimp, arrows indicate the location of the clubs. B) Optical micrograph of mantis shrimp club: D- Dactyl, P- Propulus.

Figure 1.2. Striking mechanism of the hammer stomatopod. a) High-speed camera images of the Stomatopod hitting a target. b) Sketch of the compressed (above) and relaxed (below) positions of the saddle. c) Schematic of the striking mechanism with the saddle modelled as a spring (orange). [9]

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Figure 2.13. Triangular microcantilever schematics. A) Triangular cross-section showing the width (W) and thickness (B), centroid (red dot) and the axis used for second moment of area (I_{xx}) calculations. B) The side view of a section of the cantilever showing the directions of tensile stresses developed in the beam along the length of the

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Figure 2.14. Triangular micro-cantilever prepared using FIB. B) Schematic showing the dimensions used in calculating the fracture toughness, region in red refers to the uncut area. C) Fracture surface of the post-tested micro-cantilever [100].

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Figure 3.1. Type of cantilever notches.

Figure 3.2. A) Zeiss Crossbeam 540X. B) Schematic of dual FIB-SEM setup.

Figure 3.3. Chevron notch cutting optimization. One way to determine if the chevron is fully cut before testing is to measure the distance from the edge of the cantilever to the corner of the ligament (b') and compare it with the thickness of the ligament (b) after the cutting step. The two lengths should be close to each other.

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Figure 3.5. Triboindenter 950 nanoindenter with enclosure.

Figure 3.6. A) 2D bell with loaded from the top. B) FEM mesh of the bell; quadrilateral elements used in creating the mesh, fixed boundary conditions at the bottom nodes (red triangles), load applied at the top most node (red arrow). C) A single element (gray) with four corner nodes.

Figure 3.7. CO₂ vibrational modes. Symmetric stretch (left), anti-symmetric stretch (middle) and in-plane bending (right).

Figure 3.8. The three scattering mechanisms: Rayleigh, Stokes and anti-Stokes. ν_0 – frequency of incident light, ν_s – frequency of scattered light and δE – molecular vibration energy.

Figure 3.9. Results of wet digestion methods. A) White residue remaining after digestion using method 1. B) An almost clear sample solution was obtained with method 2, the control is shown for comparison.

Figure 3.10. Interaction volume of electrons striking a sample surface producing different signals (secondary electrons, backscattered electrons and characteristic X-rays).

Figure 3.11. Shape of light emitted from an electron moving with a velocity of v . A) Dipole distribution of emitted light when the velocity is much smaller than the velocity of light. B) Narrow cone distribution of emitted light in synchrotrons where the electron is moving at relativistic velocities

Figure 3.12. Schematic of powder μ XRD of a polycrystalline sample using using monochromatic X-rays [57].

Figure 3.13. The 2D diffraction patterns of the Stomatopod dactyl club from a single position but integrated along different direction with their corresponding intensity graph below. A) Azimuthal, B) Radial.

Figure 3.14. A) Optical micrograph of sea urchin tooth. B) Diffraction pattern similar to single crystal. [58]

Figure 3.15. Schematic of transmission XRD. A) Experimental setup. B) Example diffraction pattern collected at the detector.

Figure 3.16. A) PIE developed by Rodenburg *et al.* Which is the first use of ptychography for phase retrieval in CDI [48]. B) Ptychographic CDI of a dragonfly wing at different degrees of overlaps showing an optimal ratio of 60% [62].

Figure 3.17. List of the techniques and their use in this work.

Figure 4.1. Overview of the mantis shrimp dactyl club and cantilever preparation. A) Front view of a stomatopod with its dactyl clubs shown (yellow arrows). B) Dactyl club side profile highlighting the impact surface (arrow, top) and a cross-section along the axial plane (yellow dot dash line) (bottom).

Figure 4.2. A) Focused ion beam (FIB) cutting procedure of microcantilevers (see Materials and Methods for details). From left to right; i. trench cuts made using a probe current of 3 nA; ii. shaping of cantilevers with 1.5 nA and 300 pA beams, iii. notch and guide lines cut with 10 pA beam. B) Schematic showing the positions and orientations (0° , 45° , 90° to the surface normal) of representative microcantilevers milled in the impact region of the dactyl club cross-section.

Figure 4.3. Scanning electron microscope (SEM) images of completed microcantilevers. A) Side view. B) Front view; dimensions indicated by letters were measured using SEM. C-D) SEM images (top) and geometrical dimensions (bottom) of chevron (C) and straight notches (D). The fracture surface areas, A , bounded by the green line in the SEM micrographs were measured with ImageJ-based FIJI software [30], and h was determined using trigonometric relationships.

Figure 4.4. Finite element model (FEM) of chevron-notched microcantilevers. A) Side view of the cantilever model (support not shown). The load is applied at the top center node of the free end (right). The gap between the loading position and the actual free end was not modelled as its contribution was deemed insignificant. Magnified images of the mesh near the crack front modelled following the ASTM STP 1074 (yellow and red boxes). i. Full mesh near the crack region; element lengths (l) are at most $0.25d$, where d is the distance from the crack to the element; ii. Mesh at the crack front, a total of 32 wedge elements were used for the singularity; iii. Cross-section view of the notch (at the blue center line in A). The crack front is represented by the yellow line while the areas enclosed by the magenta lines including the crack front is defined as the crack seam. The red arrows indicate the direction of the q vector. B) 3D view of the microcantilever and support. The support is modelled with fixed boundary conditions at the faces that are joined to the surface of the dactyl club (orange line and areas). 15-node wedge elements were used for the singularity elements while 20-node

brick elements were used for the rest of the model. Compliance values are determined using the displacement of the node at the bottom of the free end.

Figure 4.5. Representative FEM model of a straight notch microcantilever. The only difference to the chevron notch model is the crack and seam definition; meshing of the region near the crack is synonymous. (A) Side view. (B) Cross-sectional view at the notch. (C) 3D view of the microcantilever and support.

Figure 4.6. (A) Schematic of the dimensions used in Equation 4.4 and 4.5. (B) Representative LPU curve of a microcantilever tested in hydrated conditions (the same curve as the red curve shown in Figure 4.6A). The numbered points also coincide with those in Figure 4.8.

Figure 4.7. Plots of SL microcantilever testing analysed using LEFM. A, B) Load-displacement curves of the dactyl club (DC) impact region with chevron-notched and straight-notched microcantilevers, respectively. C) Load-displacement curves of chevron-notched microcantilevers of the fused quartz control (QS). Stable crack growth before fracture was observed in all SL microcantilevers. D–F) Compliance calibration curves ($C_v(\tilde{a})$, symbols with solid line) obtained from FEM and geometric functions ($F_v(\tilde{a})$, dashed lines) calculated using Equation 4.2 and 4.3. The minima of $F_v(\tilde{a})$ curves correspond to a_c , which is used to calculate K_{IVb} .

Figure 4.8. Plots of data from LPU microcantilever chevron-notched testing analysed by EPFM. A) Representative LPU curves for microcantilever tested in dry (black) and hydrated (red) conditions, colour coding same throughout. B) Experimental microcantilever compliance change at each unloading segment. C) Non-dimensional compliance calibration curves from FEM correlating compliance with normalized crack length. The numbered points correspond to the same points as in B. The initial stiffness measured from each cantilever was assumed to be the stiffness at the initial crack length a_0 , following which the compliance change of the cantilevers can be determined, and consequently the crack lengths at the other labelled points. D) R -curves plotted from the $\tilde{a}$ values obtained from the compliance calibration curves in C.

Figure 4.9. Statistical and comparative analyses of fracture toughness values obtained using LEFM and EPFM. (A) Statistical inference using two sample t-tests and ANOVA

on the SL microcantilevers (LEFM); the means of samples are not significantly different ($p < 0.05$) when analysed using analytical method or FEM, between chevron and straight notch samples, and with changing orientation. (B) Weibull cumulative function on the SL microcantilevers (LEFM): a median (50% cumulative frequency) fracture toughness of $0.21 \text{ MPa} \cdot \text{m}^{0.5}$ was measured. (C) Comparison of fracture toughness measured using LEFM (K_{IVb}) or EPFM (K_{Jeq}). Fracture toughness of the impact region of the dactyl club is an order of magnitude larger as measured by EPFM, indicating that plastic dissipation is the dominant contribution to the fracture resistance. Control fracture toughness experiments of fused quartz using the same methods (LEFM and EPFM) show values that are close to each other, consistent with the brittle nature of fused quartz. * $p < 0.05$, ** $p < 0.0005$.

Figure 4.10. Additional LPU microcantilever tests. A) Stress-strain curves from measurements of microcantilevers were prepared without notches on fused quartz (black), dactyl club tested dry (red), and dactyl club tested hydrated (green). Little to no deformation was observed in fused quartz before failure, while considerable plastic deformation occurred in the dactyl club microcantilevers. The slight shift for fused quartz at about 0.02 strain is probably due to the tip sliding on the surface of the cantilever. B) Load-displacement curve of fused quartz LPU microcantilever. The material behaves elastic for most of the sample except for the last unloading cycle before the cantilever breaks.

Figure 5.1. Optical images of the stomatopod dactyl club taken from the mantis shrimp at different time points from molting. The two hours (2h), one day (1d) and two days (2d) samples are thinner, softer and more fragile compared to the other three points of development. Hence, they were prepared using cryo-ultramicrotomy while the one week (1w), two weeks (2w) and mature (1 month) samples were prepared by thinning a slice between two abrasive papers until the desired thickness of close to $50 \mu\text{m}$ was achieved. At two weeks, the stomatopod started to use the club as evidenced by the damaged surface on the club.

Figure 5.2. A) The setup used at P06 at PETRA III at DESY, using a MAIA detector for X-Ray Fluorescence (XRF) and an Eiger 4M detector for X-Ray Diffraction (XRD) and B) the setup used at ID13 at the ESRF using a Vortex (V) detector for XRF and an

Eiger 4M detector for XRD. The stage at both beamlines could move in the x, y, and z directions; this was used to raster scan the sample placed on the stage.

Figure 5.3. Diffractogram from a sample; tick marks at the bottom from adapted from [17]. The (002) peak has been marked because that peak was analyzed with the Angular Gaussian Fitter (*AGUF*) software.

Figure 5.4. A) The principle of AGUF. A diffractogram from the detector with hot pixels masked out. B) The diffractogram integrated into 360 azimuthal bins and 900 bins along the diffraction angle 2θ . The red rectangle marks the (002) peak. C) The intensity of the (002) peak as a function of the azimuthal angle χ data as dots and the fit as a line. The red area shows the intensity of the oriented crystals, I_p ; the blue area shows the intensity of the randomly oriented crystals. The data for this figure was taken from the 2w sample.

Figure 5.5. Total XRF spectrum for the sample harvested 2 hours after moulting

Figure 5.6. Identification of precursor ions from stable isotope labeled (SIL) peptides L274-R283, run without native CMP-1. A) Chromatograms with peaks corresponding to the non-brominated peptide precursor L274-R283 from CMNP-1 and their corresponding fragment ions (bottom). *Left*: peaks corresponding to the wild-type (“light”) peptide [L274-R283]_L and *right*: peaks corresponding to the SIL (“heavy”) peptide [L274-R283]_H. B) Chromatograms with peak corresponding to the brominated peptide L274-R283 from CMP-1 (top) and its corresponding fragment ions (bottom). *Left*: peak corresponding to the wild-type (“light”) peptide [L274-R283]_{L,Br} and *right*: peak corresponding to the SIL (“heavy”) peptide [L274-R283]_{H,Br}. Peaks are well-resolved for the SIL peptides, whereas for the wild type peptides they are noisy and close to the baseline, indicating that the heavy SIL peptides are pure and are not contaminated with the light peptides.

Figure 5.7. Overview of samples tested with synchrotron X-rays. A) Schematic showing the different structural layers in the Stomatopod dactyl club; from top to bottom: Initial expanded membrane after molting, impact surface, impact region, periodic region, inner cavity. (B-F) XRF images of clubs: 2h (B), 1d (C), 2d (D), and 1w samples (E); Ca K α (green) and Br K α (red) lines were used to generate these images.

F) Light microscope image of the 2w sample after preparation. The small yellow box marks the region area measured using synchrotron radiation. G) Light microscope image of the mature sample; dash lines showing approximately the thickness of the club one week (cyan) and two weeks (yellow) after molting; the small red box marks the region analysed with synchrotron radiation.

Figure 5.8. 1w sample. A) Large area X-ray map. B) Maps with higher resolution from the white box in A; images from left to right: Polarized light microscope (Pol), Ca K α XRF, Br K α XRF, lattice constant a (a -axis), apparent crystallite size (ACS) ratio, and 002 angle orientation maps. The scale bar can be used to read the a -axis and ACS ratio maps while the colour cylinder below is for the 002 angle orientation maps.

Figure 5.9. Apparent crystallite size of apatite in the [002] direction at different maturation stages of the dactyl club. A) 2h, B) 1d, C) 2d, D) 1w, E) 2w and F) mature samples.

Figure 5.10. Apparent crystallite size of apatite in the [310] direction at different maturation stages of the dactyl club. A) 2h, B) 1d, C) 2d, D) 1w, E) 2w, and F) mature samples.

Figure 5.11. Ratio of the apparent crystallite size of apatite in the [002] and [310] directions at different maturation stages of the dactyl club. A) 2h, B) 1d, C) 2d, D) 1w, E) 2w and F) mature samples.

Figure 5.12. Maps of the lattice constant a . A) 2 hours, B) 1 day, C) 2 days, D) 1 week, E) 2 weeks, and F) mature samples.

Figure 5.13. Maps of the lattice constant c . A) 2 hours, B) 1 day, C) 2 days, D) 1 week, E) 2 weeks, and F) mature samples.

Figure 5.14. EDS on 1w sample. A) Backscatter electron image of a cross-section from the surface to the bulk of the impact region. The yellow box shows the location and dimensions of the EDS map. B) EDS maps of the box in A for elements Ca, P and F. C) The average element wt. % across the short axis of the maps in B plotted against the

depth starting from the sample surface at 0 μm . D) The P and F to Ca ratios against the depth.

Figure 5.15. EDS on mature sample. A) Backscatter electron image of a cross-section from the surface to the bulk of the impact region. The yellow box shows the location and dimensions of the EDS map. B) EDS maps of the box in A for elements Ca, P and F. C) The average element wt. % across the short axis of the maps in B plotted against the depth starting from the sample surface at 0 μm . D) The P and F to Ca ratios against the depth.

Figure 5.16. Raman spectroscopy measurements of dactyl clubs. A) Optical microscope image (left) of the mature club; red dots mark the location for the spectra shown on the intensity vs. wavenumber plots (right). Same for the other samples. B) 2w sample. C) 1w sample.

Figure 5.17. FTIR measurements of dactyl clubs. A) Optical microscope image of the mature club; open circles mark the location for the spectra shown in B. B) FTIR intensity vs. wavenumber plot. Inset: magnified image of the region in the red box showing the assignment of the peaks observed with FTIR.

Figure 5.18. Apatite (002) orientation plots for the A) 1 day and B) mature samples. The characteristic ‘sandwich’ structure of the impact surface layer can be seen in (A) where a sublayer with randomly orientated crystals is sandwiched between two layers with crystals orientated parallel to the surface; in contrast to the impact region (B) where the crystals are generally aligned perpendicular to the surface. 1w orientation plot can be found in Figure 5.8B.

Figure 5.19. Ca $K\alpha$ (8.639 keV) maps. A) Overview of week 1 sample (lower resolution scan), B) 2 hour, C) 1 day, D) 2 day, E) 1 week, F) 2 week G) Mature samples. Intensity is in arbitrary units and can only be compared within a sample and not between samples.

Figure 5.20. Br $K\alpha$ (11.924 keV) maps. A) Overview of week 1 sample (lower resolution scan), B) 2 hour, C) 1 day, D) 2 day, E) 1 week, F) 2 week, G) mature samples.

Intensity is in arbitrary units and can only be compared within a sample and not between samples.

Figure 5.21. Verification of bromination in CMP-1 by the parallel reaction monitoring (PRM) method. SIL peptides were spiked into the wild type CMP-1 sample, followed by identification of the precursor peptides and their fragmentation ions. A) Chromatograms with peaks corresponding to the non-brominated peptide precursor L274-R283 from CMNP-1 and their corresponding fragment ions (bottom). *Left*: peaks corresponding to the wild-type (“light”) peptide [L274-R283]_L and *right*: peaks corresponding to the SIL (“heavy”) peptide [L274-R283]_H. B) Chromatograms with peaks corresponding to the brominated peptide L274-R283 from CMP-1 (top) and their corresponding fragment ions (bottom). *Left*: peaks corresponding to the wild-type (“light”) peptide [L274-R283]_{L,Br} and *right*: peak corresponding to the SIL (“heavy”) peptide [L274-R283]_{H, Br}. For both brominated and non-brominated samples, wild-type precursors and their fragmentation ions could be identified in the wild-type samples, indicating that both brominated and non-brominated peptides are present in the native sample. Based on the precursor peak areas, a higher fraction of peptides was non-brominated.

Figure 5.22. Schematic summary of the development of the dactyl club from the surface to the impact region. Bromine was found in the surface and at the mineralization front and a fraction of CMP-1 proteins were confirmed brominated at tyrosine 275. Apatite crystallites were discovered as early as 2 hours after molting. Apatite crystallites were larger and more isotropic in the impact surface than the impact region. Crystallites formed a unique ‘sandwich’ structure in the impact surface with randomly oriented crystallites sandwiched between two layers of crystallites aligned parallel to the surface. While the crystallites were aligned following the organic matrix in the impact region.

Figure 6.1. A) Optical microscopy image of single loaded sample club. B) Sagittal cross-section club in A, box enclosing the 17.5 N indent. C) Indentation stress strain curve from [2] used to calculate the loads using Equation 2.7 and 2.8, which is printed above A. D) SEM image of the 17.5 N indent – taken in LEI mode at 500× magnification; scale bar is 10 μm.

Figure 6.2. Schematic layout of the ALS beamline 12.3.2 with a superbend dipole magnet source. The beamline acceptance is 0.2 mrad (horizontal and vertical). The M1 toroid mirror focuses the source onto a virtual object. This intermediate image is demagnified by a ratio of 8:1 and 16:1 in horizontal and vertical, respectively, by a pair of KB mirrors. A four-bounce Si(111) monochromator can optionally be brought into the beam path for monochromatic microdiffraction on nanosized grains [3], [4].

Figure 6.3. μ XRD maps of a A) pristine (undamaged) (P1), and a B) native damage (N2) region. The black lines and the coloured squares indicate the mean FAP crystal orientation (ϕ) and the mean maximum intensity ($I_{\max}$) using the 002 diffraction peak measured at the respective (X,Y) coordinates.

Figure 6.4. μ XRD maps from area next to single-loading indents. A) In the elastic regime; 7 N load (SL1). B) In the plastic regime; 65 N load (SL9). The black lines and the coloured squares indicate the mean FAP crystal orientation (ϕ) and the mean maximum intensity ($I_{\max}$) using the 002 diffraction peak measured at the respective (X,Y) coordinates.

Figure 6.5. μ XRD maps from area next to multiple-loading indents. A) In the elastic regime; 600 mN load (ML2) B) In the plastic regime; 4000 mN load (ML3). Coloured boxes enclose the data used for statistical analyses presented in Figure 6.6. One cycle (Red), ten cycles (Yellow), hundred cycles (Blue). The black lines and the coloured squares indicate the mean FAP crystal orientation (ϕ) and the mean maximum intensity ($I_{\max}$) using the 002 diffraction peak measured at the respective (X,Y) coordinates.

Figure 6.6. Comparison between degree of misorientation in A) single-loading samples and B) multiple-loading samples. C) Comparison between signal intensity in single-loading samples and D) multiple-loading samples.

Figure 6.7. A) Schematic of the SLS cSAXS beamline/set up used in PXCT experiment [12]. C) OMNY Pin used with PXCT setup; samples were mounted upright on the tip of the pin [13].

Figure 6.8. X-ray Ptychographic tomograms. A) Cross-sections of the impact surface; inset showing the microchannels within the impact region. B) The impact region; inset showing microchannels which resemble osteons. C) The periodic region. D) Image of the club closer to the surface than A; inset illustrating a lower density of microchannels closer to the surface.

Figure 6.9. 3D stack of tomogram slices reconstructed from PXCT data. The 3D model was generated using FIJI software.

Figure A1. Crack in an infinite plate.

Abbreviations

a	Radius of indenter at arbitrary depth, or crack length
$\tilde{a}$	Arbitrary normalized crack length (a/W)
A	Area
ACS	Apparent Crystallite Size
AFM	Atomic force microscopy
AGUF	Angular Gaussian Fitter
ALS	Advanced Light Source
ANOVA	Analysis of variance
ATR	Attenuated total reflectance
ASTM	American Society for Testing and Materials
B	Width of triangular cantilever
BSE	Backscattered Electron
C	Cantilever compliance
CAP	Carbonated apatite
CDI	Coherent Diffraction Imaging
CEE	School of civil and environmental engineering (NTU)
CMP-1	Club Mineralization Protein-1
CT	Compact tension
CTOD	Crack tip opening displacement
γ_s	Surface energy
DCT	Disk compact tension
DFEA	Dynamic finite element analysis
DI	Deionised or distilled
DoO	Degree of orientation
DSN	Depth-sensing nanoindentation
Δ	Change in-/Displacement
E	Sample elastic modulus
E_t	Tip elastic modulus
E^*	Reduced or Effective Elastic Modulus
EBSD	Electron Backscattered Diffraction
EDS	Energy Dispersive X-ray Spectroscopy

EPFM	Elastic Plastic Fracture mechanics
EPMA	Electron Probe Microanalysis
ESRF	European Synchrotron Radiation Facility
F_V	Geometric function for cantilever
FACTS	Facility for Analysis, Characterization Testing & Simulation
FAP	Fluorapatite
FEI	Field Electron and Ion Company
FEM	Finite element method
FIB	Focused Ion Beam
FTIR	Fourier transform infra-red
FWHM	Full width half maximum
G	Energy release rate
G_c	Critical energy release rate
h	Arbitrary depth of indenter measured from the surface
H	Hardness
HCD	High energy collision dissociation
HR/AM	High Resolution/Accurate Mass
HRR	Hutchinson, Rice and Rosengren
ICP	Inductively coupled plasma
ICP-OES	Inductively coupled plasma optical emission spectroscopy
ICP-MS	Inductively coupled plasma mass spectroscopy
iNano	Interdisciplinary Nanoscience Center at Aarhus University (Denmark)
J	J-integral
JEOL	JEOL Company
K	Stress intensity factor
K_{eq}	Stress intensity equivalent converted from J-integral
K_{Ic}	Plane strain fracture toughness
K_{IVb}	Plane strain fracture toughness (chevron notch)
L	Distance between notch and cantilever fixed end/Fulcrum length
LBNL	Lawrence Berkeley National Laboratory
LC-MS/MS	Liquid chromatography tandem mass spectroscopy
LEFM	Linear Elastic Fracture Mechanics
LEI	Lower secondary electron detector imaging mode
LEM	Lawn, Evans and Marshall

LPU	Loading partial-unloading
λ	Electromagnetic radiation wavelength
MatFRAIA	Matrix-multiplication Facilitated Radial & Azimuthal Integration Algorithm
μ XRD	Micro X-ray Diffraction or Microdiffraction
ν	Poisson's ratio
m/z	Mass-to-charge ratio
micro-CT	micro-computed tomography
MRNP	Multi-range Nanoprobe
MS/MS	Tandem mass spectroscopy
MSE	School of material science and engineering (NTU)
NA	Numerical aperture
NTU	Nanyang Technological University
OCT	Optimal cutting temperature
HAP	Hydroxyapatite
P	Load
PP	Polypropylene
PETRA III	Positron-Elektron-Tandem-Ring-Anlage III
PIE	Ptychographic Iterative Engine
PRM	Parallel reaction monitoring
PSI	Paul Scherrer's Institute
PTFE	Polytetrafluoroethylene (teflon)
PXCT	Ptychographic X-ray Computed Tomography
U	Potential Energy
r	radius of stress tensor from crack tip (polar coordinates)
r_{pl}	Plastic zone size
R	Indenter radius
RF	Radiofrequency
RPM	Revolutions per minute
S	Stiffness from unloading curve, or distance between load and notch
SAXS	Small Angle X-ray Scattering
SIL	Single isotope-labelled
SPM	Surface probe microscopy
SL	Single loading

SLS	Swiss Light Source
SEM	Scanning Electron Microscopy
SENB	Single edge notch beam
SR	Specular reflectance
SRM	Single reaction monitoring
STM	Scanning tunneling microscopy
σ	Stress or indentation stress
σ_f	Fracture strength
σ_{ij}	Stress tensor, where subscript 'ij' is a combination of cartesian coordinates 'x', 'y' or 'z'
σ_∞	External/far-field stresses
σ_{YS}	Yield strength
TEM	Transmission Electron Microscopy
θ	Angle of stress tensor along crack direction (polar coordinates)/Angle of triangular cantilever cross-section
UV	Ultraviolet
UVA	Ultraviolet-A
UVB	Ultraviolet-B
W	Thickness of triangular cantilever
W_s	Work of creating new surfaces
WAXS	Wide Angle X-ray Scattering
XANES	X-ray atomic near edge spectroscopy
XPS	X-ray photoelectron spectroscopy
XRD	X-ray Diffraction
XRF	X-ray Fluorescence Spectroscopy
Y	Fracture toughness geometric factor

Chapter 1

Introduction

This segment of the report provides background information on the project. The problem statement and hypotheses of this thesis is presented. The content in this section includes the problem statement and hypothesis, the project objectives and scope, the findings and outcomes. The subject of this thesis is the stomatopod dactyl club. The club is an example of an ultra-tough and hard biotool found in nature. The impact region of the club consists mainly of bioapatite mineral which is rare among aquatic life. After molting, the stomatopod forms a new club in a span of about a month. The rapid growth of new clubs improves the survivability of the animal and also provides a unique opportunity to study the full growth of the club from cradle to gate over a reasonable timeframe for material research. This work aims to build upon recent work which uncovered that the club is formed by a unique 'diecast mechanism' and sequenced a major protein involved in apatite biomineralization in the club. The limitations of depth-sensing nanoindentation calls for the use of other mechanical test methods to study the club. Focused Ion Beam milling of microscale samples is chosen for its success in studying conventional materials and other biological materials like mammalian teeth

1.1 Hypothesis/Problem Statement

Biological materials are materials made by living organisms [1]. They have many advantages compared to conventional man-made analogues: (i) superior properties from intrinsically weak constituent materials that are made from a limited pool of elements; (ii) multi-functional; (iii) synthesis in aqueous environment under ambient temperature and pressure; (iv) wide range of tunable properties [1]. These advantages provide tremendous impetus to create new materials that incorporate the design principles found in biological materials. This process is known as biomimicry and the material inspired by Nature are termed biomimetic or bio-inspired materials [2].

Biomaterialized tissues are a class of biological materials formed by living creatures for protection (shells, scales, exoskeletons), supporting structures (skeleton) and biotools (teeth, claws, talons). Nacre or mother-of-pearl is one such example. Oysters form shells with nacre to protect its softer body from predators and the harsh underwater environment. Nacre is made primarily of aragonite (CaCO_3 , 95%) and some proteins but it is more than several orders of magnitudes tougher than pure single crystal aragonite [3]. This is because of the multi-scale hierarchy of structures present in Nacre that dramatically increases its fracture toughness [3].

The subject of this study is another ultra-tough biomineral composite, the dactyl club of the hammer-variant mantis shrimp. Mantis shrimps or stomatopods are reef dwelling arthropods with moderately aggressive behavior (Figure 1.1) [4]. Initial interest in this marine creature stemmed for their incredible sight. The eyes of the mantis shrimp are equipped with 16 photoreceptors making them the most complex ocular apparatus in the natural world. In comparison humans have only three photoreceptors [5]. Each eye has three segments which can move independently, affording the stomatopod six views of their surroundings at a point of time. Moreover, stomatopods can see linear and circularly polarized light [6]. Their highly developed visual apparatus was suggested to be used for intra-species communication [7]. They then unleash their deadly pair of clubs at supersonic speeds on their hapless prey. A single blow releases around 1500 N of force which is about 2600 times their body weight [8]. The club reaches speeds greater than 20 m/s^2 upon contact; fast enough to create air cavities around the club [9]. Pistol shrimps, another marine creature, makes use of air cavitation to stun their prey

[10]. Stomatopods can launch their weapons at such high speeds by storing energy in a hyperbolic-paraboloid spring called the saddle, positioned above the dactyl on the merus (Figure 1.2) [11]. Stomatopods also use their clubs to modify their burrows, breaking rock and coral with them. Despite the harsh treatment, the dactyl clubs remain intact for thousands of blows between molting cycles [12]. Moreover, many of their prey (marine bivalves, molluscs and crustaceans) make use of protective armor that are themselves researched for their superior performance (*e.g.* nacre, crab cuticle) [2]. Hence, studying the mantis shrimp dactyl club could provide many lessons in designing tough and impact tolerant composites.

Recent studies focused on deriving the composition of the club and relating the material properties to the hierarchical structures observed within the club. Synchrotron X-ray Diffraction (XRD) studies found that the impact region is made of fluorapatite (FAP) crystals arranged radially towards the surface surrounded by an organic matrix mainly composed of chitin fibers and proteins [13]. This discovery makes the stomatopod dactyl club one of the few cases of apatite-based mineral structures found in crustaceans, indicating the apatite structures may be more widespread in invertebrates than previously anticipated. Scanning Electron Microscopy (SEM) images show that FAP crystals are randomly orientated in the regions directly beneath impact by natural use [14]. This suggests that upon impact, the FAP crystals slide and rotate within the pliable chitin matrix, thereby dissipating the impact energy and distributing the load evenly to reduce stress concentration. Apart from their outer shell, stomatopods also shed their hunting appendages when they molt and they must form brand new dactyl clubs quickly in order to resume hunting and for self-defence. Coupled with ease of handling invertebrates in the lab, the stomatopod dactyl club is a unique and convenient model system to study the biomineralization of marine apatite. A previous study by Amini *et al.* identified a key protein named chitin mineralization protein 1 (CMP-1) involved in apatite deposition on the chitin matrix [15]. The study also looked at the chemical composition at various stages of the dactyl club development using electron dispersive X-ray spectroscopy (EDS) and Raman spectroscopy. However, high resolution spatial maps of nanocrystalline apatite distribution and the composition of the apatite was not possible with these methods alone since, both EDS and Raman do not provide crystallographic information but information on the chemical environment.. Moreover, EDS is limited to use in vacuum conditions. Third generation synchrotrons with highly

focused and brilliant X-ray beams to perform a plethora of high spatial resolution mapping experiments overcoming the current limitations of EDS and Raman techniques for crystallography. Hence, providing more information on the biomineralization process of the stomatopod dactyl club.

Using conventional mechanical test methods like uniaxial tensile test or Charpy impact test allow for accurate comparison between the strength and fracture toughness of biological materials and standard materials like steel [16]. However, sample size is a major roadblock to using these techniques on biological materials, which are often too small with non-standard shapes. Furthermore, biological materials are rarely homogenous and have varying properties in different regions [1]. Depth-sensing nanoindentation (DSN) is a popular technique used to characterize materials because: (i) the nano- to micrometer length scale resolution of the technique; (ii) the ease of automation; (iii) and the accuracy of both force and displacement measurements [17]. The hardness, stress-strain and fracture behavior of the Stomatopod dactyl club was determined using nanoindentation. However, other than hardness and elastic modulus, tensile strength and fracture toughness are conventionally determined using different physical models. Conventionally, tensile strength is determined using a uni-axial tensile test. The linear portion of the resulting stress-strain curve can be modelled using Hooke's law while the non-linear curve can be usually modelled with a power law function [18]. On the other hand, Indentation stress-strain is determined using Hertzian contact mechanics [19]. Fracture toughness is conventionally determined by finding the energy release rate or the stress-intensity factor [20] while indentation fracture toughness is calculated from the Lawn-Evans-Marshall equation [21]. The difference in the models used in indentation and conventional methods led to debate in the scientific community regarding the authenticity of for example; tensile strength derived from indentation based models [22]. Focused ion beam (FIB) milling of microscale samples for mechanical testing is a well-established method for studying the mechanical properties of conventional materials like metals, semiconductors at micrometer resolution. Moreover, micro-cantilevers has been successfully employed to study the fracture toughness of biological materials like enamel [23]. Therefore, FIB-milled micro samples may be a suitable alternative to nanoindentation to characterize the tensile strength and fracture toughness of the Stomatopod dactyl hammer. The reader is referred to the literature review section for a more complete discussion on the

advantages of FIB-milled micro samples over nanoindentation for fracture toughness. This thesis has two major thrusts: Firstly, to advance the mechanical test methods of hard biomineralized tissue as well as increase the understanding of the fracture properties of the dactyl club by testing the hypothesis that chevron-notched microcantilevers are a better alternative to nanoindentation for determining fracture toughness in the impact region of the stomatopod dactyl club. Secondly, this thesis aims to contribute to current knowledge of the dactyl club biomineralization by answering the following questions: What is the spatial-temporal organization of the apatite crystals and organic matrix of the developing stomatopod dactyl club? When do the first apatite nanorods appear? And is there an amorphous-to-crystalline transition or are the crystals deposited early on? Nanorods are oriented in the pristine mature club, but how does this preferred orientation occur and when during club formation?

Figure 1.1. A) *Odontodactylus scyllarus* i.e. Peacock mantis shrimp, arrows indicate the location of the clubs. B) Optical micrograph of mantis shrimp club: **D**- Dactyl, **P**- Propus.

Figure 1.2. Striking mechanism of the hammer stomatopod. **a)** High-speed camera images of the Stomatopod hitting a target. **b)** Sketch of the compressed (above) and relaxed (below) positions of the saddle. **c)** Schematic of the striking mechanism with the saddle modelled as a spring (orange). [9]

1.2 Objective and Scope

The objectives and scope of this thesis is presented through the following statements:

- a. Perform fracture toughness measurements of the impact region of the dactyl club using chevron-notched microcantilevers;
- b. Evaluate the fracture toughness with linear-elastic fracture mechanics (LEFM) and elastic-plastic fracture mechanics (EPFM);
- c. Determine the fracture toughness of the impact region in different directions;
- d. Measure the effect of hydration on the fracture toughness of the impact region;
- e. Collection of dactyl club samples at different maturation stages post-molt;
- f. Elucidate the compositional, crystallography, and microstructural changes during the dactyl club biomineralization using synchrotron X-rays and other spectroscopic techniques.

1.3 Dissertation overview

Chapter 1 provides the basis and motivation for this PhD. Two main thrusts of this thesis are presented which will be discussed in Chapters 5 and 6 of this dissertation.

Chapter 2 delves into the literature relevant to this study.

Chapter 3 presents the experiment methods employed to test the hypotheses stated in the first chapter. Working principles for each method as well as the rationale for selecting those methods are discussed

Chapter 4 contains the results and discussion for the first main thrust of this thesis. The chapter compares the use of micro-cantilevers to determine fracture toughness to nanoindentation. The impact region of the stomatopod dactyl club are used in this comparison.

Chapter 5 contains the results and discussion for the second main thrust of this thesis. Details of the study of the Stomatopod dactyl club biomineralization are presented.

Chapter 6 compiles the other research work conducted on the dactyl club that have been investigated in the course of the study. This includes the mechanism of nanocrystal rotation in the impact region and high resolution 3D tomography of the dactyl club.

Chapter 7 concludes the thesis and provides recommendations for further work. This chapter also covers the additional work conducted relating to nanocrystal rotation in the impact region in response to a damage event on the club.

1.4 Findings and Outcomes/Originality

This research led to the novel outcomes listed below:

1. The fracture toughness of hard mineralized tissues like the impact region of the dactyl club can be studied using microcantilever fracture testing.
2. The impact region exhibits elastic-plastic fracture behaviour which could not be determined using indentation methods, showing that notched microcantilever testing is a more suitable method to assess the fracture behaviour of tough biocomposites.
3. Fracture toughness of the impact region at the microscale is isotropic, which is contrary to predictions based on previous elastic modulus and stress-strain measurements.
4. Hydration effects led to little deviation in the fracture toughness of the impact region of the dactyl club.
5. Apatite crystals are present in the dactyl club as early as 2 hours post-molt.
6. More subtle apatite chemistry than previously recognized based on deviation of “ideal” apatite lattices. This is likely because of lattice strain caused by organics between the crystal lattices [24].
7. The outermost surface layer of the dactyl club is composed of apatite crystals with complex texture instead of simply aligned normal to the surface as previously reported [13].
8. The unexpected finding that Br is concentrated in specific locations of the dactyl club. Proteomic studies show that Br is found in Club Mineralization Protein 1 (CMP-1) and concentrated near the mineralization front of the dactyl club.
9. A proposed role of Br is to provide anti-bacterial activity, which would be needed in the early stages of club formation in order to protect the protein matrix regulating club growth/maturation against premature degradation.

References

- [1] M. A. Meyers, P.-Y. Chen, A. Y.-M. Lin, and Y. Seki, “Biological materials: Structure and mechanical properties,” *Progress in Materials Science*, vol. 53, no. 1, pp. 1–206, Jan. 2008, doi: 10.1016/j.pmatsci.2007.05.002.
- [2] P.-Y. Chen, J. McKittrick, and M. A. Meyers, “Biological materials: Functional adaptations and bioinspired designs,” *Progress in Materials Science*, vol. 57, no. 8, pp. 1492–1704, Nov. 2012, doi: 10.1016/j.pmatsci.2012.03.001.
- [3] R. Wang and H. S. Gupta, “Deformation and Fracture Mechanisms of Bone and Nacre,” *Annu. Rev. Mater. Res.*, vol. 41, no. 1, pp. 41–73, Aug. 2011, doi: 10.1146/annurev-matsci-062910-095806.
- [4] R. L. Caldwell and H. Dingle, “Ecology and evolution of agonistic behavior in stomatopods,” *Naturwissenschaften*, vol. 62, no. 5, pp. 214–222, May 1975, doi: 10.1007/BF00603166.
- [5] J. Marshall, T. W. Cronin, and S. Kleinlogel, “Stomatopod eye structure and function: A review,” *Arthropod Structure & Development*, vol. 36, no. 4, pp. 420–448, Dec. 2007, doi: 10.1016/j.asd.2007.01.006.
- [6] T.-H. Chiou *et al.*, “Circular Polarization Vision in a Stomatopod Crustacean,” *Current Biology*, vol. 18, no. 6, pp. 429–434, Mar. 2008, doi: 10.1016/j.cub.2008.02.066.
- [7] T. W. Cronin and N. J. Marshall, “A retina with at least ten spectral types of photoreceptors in a mantis shrimp,” *Nature*, vol. 339, no. 6220, pp. 137–140, May 1989, doi: 10.1038/339137a0.
- [8] S. N. Patek, “Extreme impact and cavitation forces of a biological hammer: strike forces of the peacock mantis shrimp *Odontodactylus scyllarus*,” *Journal of Experimental Biology*, vol. 208, no. 19, pp. 3655–3664, Oct. 2005, doi: 10.1242/jeb.01831.
- [9] S. N. Patek, W. L. Korff, and R. L. Caldwell, “Deadly strike mechanism of a mantis shrimp,” *Nature*, vol. 428, p. 819, Apr. 2004, doi: <http://dx.doi.org/10.1038/428819a>.
- [10] M. Versluis, “How Snapping Shrimp Snap: Through Cavitating Bubbles,” *Science*, vol. 289, no. 5487, pp. 2114–2117, Sep. 2000, doi: 10.1126/science.289.5487.2114.

- [11] M. Tadayon, S. Amini, A. Masic, and A. Miserez, “The Mantis Shrimp Saddle: A Biological Spring Combining Stiffness and Flexibility,” *Adv. Funct. Mater.*, vol. 25, no. 41, pp. 6437–6447, Nov. 2015, doi: 10.1002/adfm.201502987.
- [12] M. L. Reaka, “Molting in stomatopod crustaceans. I. Stages of the molt cycle, setagenesis, and morphology,” *J. Morphol.*, vol. 146, no. 1, pp. 55–80, May 1975, doi: 10.1002/jmor.1051460104.
- [13] J. C. Weaver *et al.*, “The Stomatopod Dactyl Club: A Formidable Damage-Tolerant Biological Hammer,” *Science*, vol. 336, no. 6086, p. 1275, Jun. 2012, doi: 10.1126/science.1218764.
- [14] S. Amini, M. Tadayon, S. Idapalapati, and A. Miserez, “The role of quasi-plasticity in the extreme contact damage tolerance of the stomatopod dactyl club,” *Nat Mater*, vol. 14, no. 9, pp. 943–950, Sep. 2015, doi: <http://dx.doi.org/10.1038/nmat4309>.
- [15] S. Amini *et al.*, “A diecast mineralization process forms the tough mantis shrimp dactyl club,” *Proc Natl Acad Sci USA*, vol. 116, no. 18, pp. 8685–8692, Apr. 2019, doi: 10.1073/pnas.1816835116.
- [16] K. K. Chawla and M. A. Meyers, Eds., “Fracture Testing,” in *Mechanical Behavior of Materials*, 2nd ed., Cambridge: Cambridge University Press, 2008, pp. 525–557.
- [17] A. C. Fischer-Cripps, *Nanoindentation*, 3rd ed. New York, NY: Springer New York, 2011.
- [18] William D. Callister, *Material Science and Engineering: An Introduction*, 7th ed. John Wiley & Sons, Inc., 2007.
- [19] L. H. He and M. V. Swain, “Nanoindentation derived stress–strain properties of dental materials,” *Dental Materials*, vol. 23, no. 7, pp. 814–821, Jul. 2007, doi: 10.1016/j.dental.2006.06.017.
- [20] T. L. Anderson, *Fracture mechanics: fundamentals and applications*, 2nd ed. Boca Raton: CRC Press, 1995.
- [21] B. R. Lawn, A. G. Evans, and D. B. Marshall, “Elastic/Plastic Indentation Damage in Ceramics: The Median/Radial Crack System,” *J American Ceramic Society*, vol. 63, no. 9–10, pp. 574–581, Sep. 1980, doi: 10.1111/j.1151-2916.1980.tb10768.x.

- [22] G. D. Quinn and R. C. Bradt, “On the Vickers Indentation Fracture Toughness Test,” *J American Ceramic Society*, vol. 90, no. 3, pp. 673–680, Mar. 2007, doi: 10.1111/j.1551-2916.2006.01482.x.
- [23] E. D. Yilmaz, S. Bechtle, H. Özcoban, A. Schreyer, and G. A. Schneider, “Fracture behavior of hydroxyapatite nanofibers in dental enamel under micropillar compression,” *Scripta Materialia*, vol. 68, no. 6, pp. 404–407, Mar. 2013, doi: 10.1016/j.scriptamat.2012.11.007.
- [24] W. Huang *et al.*, “A natural impact-resistant bicontinuous composite nanoparticle coating,” *Nature Materials*, Aug. 2020, doi: 10.1038/s41563-020-0768-7.

Chapter 2

Literature Review

This chapter contains a detailed analysis of previous studies of the stomatopod dactyl club. The purpose of this chapter is to provide detailed background information for the reader to understand the significance of the research questions and hypothesis tested within this thesis. The chapter begins with an introduction to stomatopod biology followed by a review on the characterization studies on the dactyl club relating its structure to material properties. A review on fracture toughness theories and conventional methods is presented followed by the microscale fracture toughness testing. Literature regarding the biomineralization of the dactyl club is covered as well. Finally, the research questions are formulated and presented.

2.1 Stomatopod

Stomatopods, commonly known as mantis shrimps, are crustaceans that dwell in tropical shallow waters making their homes within crevices in coral reefs, among rocky rubble or digging burrows in mud or sand [1]. Fossil records date the first stomatopods back to around 300 million years ago during the Permian period [1]. That puts Stomatopods older than the dinosaurs. They are reclusive, territorial and antagonistic in behavior [1]. Like many arthropods, stomatopods molt, forming a new exoskeleton and club. They molt on average every 3 months, though periods as long as 9 months between molting have been observed [2]. When a Stomatopod is about to molt they become increasingly reclusive and docile and they stop feeding, the shell also start to lose its vibrancy [2]. After molting, the newly formed shell is soft and pale, and takes a period of about a month to fully harden [2].

Stomatopods have attracted the interest of the scientific community for many unique features including their visual system, telson, saddle and dactyl club. Two superfamilies of stomatopods possess the most complex eyes known in the animal kingdom. Their eyes have a total of 16 types of photoreceptors; for comparison humans only have three. This complex array of photoreceptors enables them to see 14 colour channels, 8 in the human visible spectrum and 6 in the ultra-violet (UV) spectrum [3], [4]. The eyes of the mantis shrimp are also equipped to see both linearly- and circularly-polarized light (LPL and CPL) [5], the latter is the first and only example present in the animal kingdom yet [4]. The purpose of such complex ocular devices remains elusive. Chiou *et al.*, suggested that the purpose of viewing CPL is for secret mating communication as some of their predators are equipped to view LPL [5]. And another study hypothesizes that CPL may be for inter and intra-species communication [6]. Thoen *et al.* suggested that the purpose of their complex colour vision is not for differentiating colour hues but for quick identification of colour which may prove vital for their survival [7]. A study using UV light showed that stomatopods were able to discern between UVA (longer wavelength) and UVB (shorter wavelength) light, and both UV lights affected feeding, burrowing and behaviour although little is known about the reason for behavioral response to different UV light frequencies [8].

Another distinguishing feature of stomatopods are a pair of raptorial appendages located at the second maxilliped spot in the thorax. These are fashioned into deadly

weapons used in hunting and defense. Stomatopods can be divided into two groups based on their weapons. The spearer stomatopod makes use of an array of spines to grasp passing prey whereas the hammer stomatopod uses a club to crack open the hard protective armor of their prey [9]. The diet of hammer-variant stomatopods are composed of hard-shelled prey like small crabs, marine molluscs and bivalves [1]. Among the protection used by their prey are other ultra-tough biocomposites like nacre, which is also extensively studied and serve as bio-inspiration for tough engineering composites [10]–[14]. Yet the stomatopods are able to break through their formidable defense by striking them with their ultra-tough hammers at staggering speeds. Velocities of around 20 m/s with average strike forces of 700 N were recorded on a flat steel plate [15], [16]. The speeds were so high that cavitation bubbles were produced on the surface of the club generating forces more than half the strike forces on average [16]. Moreover, stomatopods deliver thousands of blows before they molt and form a new club. The hammer, or club, of the mantis shrimp is made up of two parts, the propodus and dactyl (Figure 1.1B). The dactyl is in contact with the target during a strike, and thus it is a model system for the study of ultra-tough and wear resistant materials. Consequently, many new designs and manufacturing processes of engineering composites derived inspiration from the dactyl club [17]–[28]. The focus of this study is the hammer of the hammer variant stomatopod. The species chosen is *Odontodactylus scyllarus*, commonly called peacock mantis shrimp (Figure 1.1A). The following sections will review in greater detail the literature concerning the stomatopod dactyl club and the rest of this section will briefly cover two other features of interest of the stomatopod.

The high-speed motion of the stomatopod hunting appendages cannot be accomplished with muscle contraction alone. Patek *et al.* showed that stomatopod store the energy using an elastic system on their swinging limb consisting of multiple structures including the saddle, a hyperbolic-paraboloid structure sitting atop the merus [15], [29]. Although the saddle does not account for most of the elastic propulsion, removal of the saddle led to 15% lesser forces measured on average [29]. Tadayon *et al.* found with a combination of micro-computed tomography (μ CT), electron dispersive x-ray spectroscopy (EDS) and nanoindentation (NI) that the saddle is made of two layers: a stiffer mineralized outer layer and a softer organic inner layer [30]. They surmised that the outer layer was harder and stiffer because it was loaded in compression compared

with the inner layer that is loaded in tension when the stomatopod readies for a strike. It is interesting that the outer layer is also exposed to the environment and hence requires higher wear resistance and lower permeability. This is another example of natural design incorporating multi-functionality. Often it is a prey that is on the receiving end of the hammer; however during ritualized fighting and territorial disputes, these potent weapons are turned upon their fellow kindred [31]. In these boxing fights, the stomatopods unveil another tool in their arsenal, their telson – a corrugated shield at the tail used to defend against incoming blows and another load-bearing structure of interest. During a stomatopod tussle, the defending stomatopod would curl up around their abdomen to place their telson in front of their attacker [1]. Ball drop tests and μ CT revealed that the telson is highly compliant and dissipates impact energy with close to 70% efficacy [32]. Another recent study compared the telson from an aggressive peacock mantis shrimp and that of a more reclusive spearer species *Lysiosquilla maculata* or zebra mantis shrimp [33]. The telson from the peacock mantis shrimp had the corrugated pattern while the other telson was mostly smooth on the surface. Nanoindentation measurements showed that both telsons have similar moduli and hardness, but bulk compression measurement revealed that the telson of the peacock mantis shrimp is at least three times stiffer than its cousin. Overall, the authors showed that the concave shape of the recesses between the ridges improved the bulk stiffness of the telson while the ridges called carinae imparted higher toughness through the local compliance during a loading event [33]. A short review of the areas of research focus on stomatopods was presented in this section. The next section will cover in greater detail the origin of the high toughness and wear resistance of the Stomatopod dactyl club.

2.2 Structure-property relationship in the Stomatopod dactyl club

2.2.1 Composition

Optical microscopy examinations of the transverse cross-section have revealed three distinct regions (Figure 2.1), the region closest to the club's surface is the "impact region", the region below having a distinct lamellar texture is the "periodic region", and the thick band with fibers aligned toward the surface flanking the sides is the "striated region" [34]. Weaver *et al.* analysed the spatial distribution of elements within the club

with Energy Dispersive X-ray Spectroscopy (EDS) and found that the impact region consisted mainly of calcium and phosphorous in the form of the mineral apatite [34]. Amini *et al.* conducted Electron Probe Microanalysis (EPMA) mapping on the club and identified fluorine and sulfur within the impact region, which was not detected with EDS [9]. Further analysis using Raman spectroscopy identified the apatite to be fluorapatite (FAP), a chemical variant of hydroxyapatite (HAP) where fluoride substitutes the hydroxyl group. Confocal Raman mapping revealed that FAP was found together with calcium sulfate. *Ab initio* calculations suggested that sulfate promotes and stabilizes the formation of the FAP. FAP has two main advantages compared to HAP. Firstly, it does not dissolve in seawater as readily as HAP, which is critical for marine creatures; sharks also use a similar strategy in their teeth [35], [36]. Secondly, it has better mechanical properties, which is attributed to denser packing of the atoms in the FAP crystal and hence a higher modulus and strength compared to HAP [37]. In comparison to the impact region, the periodic region consist of a higher content of carbon and magnesium. Calcium and phosphorous was still present but in lesser quantity and phosphorous content decreased with a gradient inward [9]. X-ray diffraction (XRD) and Raman spectroscopy showed that chitin is present along with amorphous calcium carbonate in the periodic region [9], [34]. Magnesium was found to stabilize the amorphous CaCO_3 phase in biological materials [38].

2.2.2 Toughening mechanisms in the periodic region

One strategy employed by nature in designing materials is a precise control of structures at different length scales referred to as hierarchical organization of structures [10], [39]. The periodic region of the dactyl club provides a good example of hierarchical organization. At the molecular level, the main building blocks of the periodic region is chitin. Chitin, the second most abundant polysaccharides on Earth after cellulose, can exist in three polymorphs, α -chitin which is present in the dactyl club and occurs when the chitin molecules are arranged antiparallel to each other [40]. The antiparallel arrangement allows for the highest number of intermolecular bonds between chitin molecules. Because of this arrangement, α -chitin is the most rigid of the three polymorphs [41]. The chitin molecules assemble into a 3 nm diameter crystalline rod with an orthorhombic structure called microfibrils [42]. The α -chitin crystalline rods have anisotropic mechanical properties with molecular dynamic simulations showing

the highest stiffness along the c -axis, which is an order of magnitude higher than the a -axis and b -axis [39]. Gao *et al.* suggested that the size of mineral crystals within biological materials are optimized for enhanced fracture strength properties [43]. They showed using Griffith criterion that there exists an optimal size where the fracture strength does not decrease despite flaws in the mineral crystals. This could be the case for the size of the chitin microfibril. A cluster of microfibrils to rods with diameters 10-100 nm forms a fibril. Microfibrils are arranged in a hexagonal fashion within a proteinaceous matrix forming an alveolar pattern [42]. Hexagonal arrangement is the best way to pack circular rods into a given volume. This is the likely reason why the chitin microfibrils are arranged into an alveolar pattern. The dense packing also allows for easier load transfer between the chitin microfibrils enhancing its mechanical properties. Amorphous calcium carbonate is deposited over the chitin fibrils increasing the rigidity of the rods [44]. Chitin fibrils arrange into a Bouligand structure (Figure 2.2) [45]. The Bouligand structure can be understood as planes of chitin fibrils stacked on top of each other. Within each plane the long axes of the fibrils are aligned parallel to the plane in a single direction. The fibrils are rotated in fixed steps with each successive plane forming a helicoidal pattern similar to a cholesteric liquid crystal [45]. Bouligand structure improves the fracture toughness of the dactyl by creating an arduous path for cracks. Cracks would have to propagate in a spiral along the weaker interfaces between the chitin fibrils [34]. This was shown to be the case in carbon fiber and epoxy laminate composites [46]; tensile tests were performed on the composite showing that the crack travel along the matrix between the fibers following the Bouligand helicoidal structure. Another study on the dactyl club periodic region [47] showed analytically that a twisted crack path requires more energy to propagate compared to a flat crack path these results were validated with finite-element modelling of the twisting crack. A 180° rotation sees the fibrils return to its initial orientation accomplishing a period. A period relates to a single lamella in the periodic structure of the mantis shrimp [34]. It is obvious that the rotation step of the fibrils determines the length of each lamella, which can be controlled by stomatopods by varying the organic molecules used to direct the organization of the fibrils [42]. Line scan nanoindentation on the periodic region showed an oscillating modulus with maxima in the middle of each lamella and minima at the interfaces between lamellas, corresponding to the orientation of the chitin fibril orientation. Fratzl *et al.* showed that an oscillating modulus provides additional shielding to crack propagation through a material [34], [48]. Microchannels extend from the inner of the

dactyl towards the surface [49]. The microchannels are composed of chitin fibrils orientated orthogonal to the chitin fibrils forming the Bouligand structure [50]. Other than providing nutrients and ions, the microchannels provide additional rigidity to the club and act as channels for load distribution [50]. During loading events, chitin fibrils can rotate and deform which provide energy dissipation and thus toughening the material [39]. Nanoindentation stress-strain studies with Hertzian contact geometry on the periodic region shows that the material strain hardens, this effect can be attributed to microchannel densification. Scanning electron microscopy images of the periodic region after indentation shows the porous periodic region collapsing into a solid mass [49].

Figure 2.1. Surface optical microscopy of characteristic regions of the dactyl club. **A)** Impact region. **B)** Backscatter electron schematic of the dactyl club cross-section. **C)** Periodic region. **D)** Striated region [34].

Figure 2.2. Bouligand structure: **A)** Model of structure. **B)** Fractured surface from periodic region showing the helicoidal pattern. **C)** polished surface from the periodic region showing cracks propagating in a curved manner [34].

2.2.3 Toughening mechanisms in the impact region

The impact region can be separated into two sub-domains based on their structure and mechanical properties [49]. From the surface down 50-70 μm is the impact surface, which has the highest modulus and hardness in the dactyl club [34], [49]. The structure of the impact surface is proposed to be a layer of mineral nanoparticles embedded in an organic matrix, which is a strategy adopted by other dynamic biotools like chiton teeth [50], [51]. A recent study [52] showed with transmission electron microscopy (TEM) that the nanoparticles are polycrystalline consisting of several grains that are slightly misaligned along their (100) planes. Moreover, the crystal lattice is likely interpenetrated with organics creating lattice distortion. From the impact surface down approximately 500 μm is the intermediate impact region. Synchrotron XRD experiments on the impact region reveals that the FAP crystals align with their (002) plane tangent to club surface (Figure 2.4) [34]. The (002) or basal planes of apatite have higher hardness and modulus compared to the prismatic planes [39], [53]. Hence, this arrangement optimizes the club surface's resistance to deformation. Unlike the spears from its 'cousins' species, the stomatopod hammer contains chitin within the impact region [9]. This is likely a functional adaptation; toughness is more important in the hammer than the spear, whereas high modulus and flexural strength matters more in the

spear. The softer chitin phase would improve the toughness of the dactyl club, while compromising modulus. Yaraghi *et al.* showed that chitin is organized differently in the impact than in the periodic region. The regular Bouligand appears to be squashed into a herringbone pattern where the chitin fibrils are curved instead of straight (Figure 2.3) [50]. High resolution modulus and hardness mapping with a nanoindenter shows the mechanical properties varying in the same herringbone pattern. Comparison of the stress distribution during compressive loading between herringbone and Bouligand structures indicated that the herringbone pattern is better at distributing stress, which is important to avoid crack formation within the impact region. Amini *et al.* performed high-load nanoindentation measurements with a cube corner tip on the impact region [49]. The fracture surface of the indents revealed cracks forming a shear-band pattern around the edge of the indent rather than having only cracks extending out from the corners like in brittle materials. Moreover, fibril-bridging and microcracking was observed at the corner cracks, which are well known toughening mechanisms employed in composite materials (Figure 2.5) [11], [49]. This also indicates that *R*-curve behaviour is likely present. High resolution scanning electron microscope images of the region directly next to an indent shows randomly arranged FAP crystals, while an image of an undamaged region showed the crystals aligned toward the surface (Figure 2.5). This suggests that the crystals slide and rotate when the surface is impacted [49], leading to so-called quasi-plastic behavior that delays crack nucleation within the impact region. Studies on nacre, which is composed of aragonite mineral platelets show that the mineral platelets slide in response to loading which dissipate energy and is proposed to contribute to *R*-curve behaviour [39]. Thus, the rotation of FAP crystals is likely to improve the fracture properties of the dactyl club.

Figure 2.3. Scanning electron microscope image of the herringbone pattern. The chitin fibers can be seen to be curved or aligned oblique to the rotation axis [50].

Figure 2.4. A) Debye Scherer ring showing varying intensity. B) A single synchrotron microdiffraction. C) texture mapping of the impact region of a mantis shrimp dactyl club [34].

Figure 2.5. SEM images from the dactyl club cross-section **A)** Aligned FAP crystals from undamaged impact layer. **B)** Misoriented FAP crystals from near the damaged region of the impact layer. **C)** Normal microchannels. **D)** Collapsed microchannels. **E)** Fibril bridging. **F)** Microcracks near the crack front [49].

2.2.4 Synergistic and macroscale toughening mechanisms

Nanoindentation maps of the club have shown that the modulus and hardness is higher in the impact region compared to the periodic region. This is congruent with the composition and structures present in the two regions. The heavily mineralized impact region has a much higher modulus while the softer and compliant chitin fibers with amorphous calcium carbonate exhibits a lower modulus. Mechanical gradients like the stomatopod hammer is observed frequently in Nature [51], [54]. In the stomatopod hammer, high stiffness at the surface is necessary to be used as a biotool. However, the stiffer biominerals are likely to fail catastrophically especially after frequent mechanical impact. Hence, a softer core may help cushion the stresses introduced during loading events through deformation and prevent damage to the more delicate tissue of the animal. Dynamic finite element analysis (DFEA) by Weaver *et al*, estimated that internal cracks are produced within the periodic regions and travel towards the surface [34]. The steep mechanical gradient between the impact and periodic region prevents cracks from traversing up from the periodic region to the

surface through crack deflection [34]. Moreover, DFEA showed that the striated region provides support to the sides of the club during loading [34]. The fibers aligned towards the sides of the club was suggested to provide additional lateral stiffness and rigidity during a loading event. The microstructure of the dactyl club, especially the periodic region, has elements reminiscent of phononic crystals. Phononic crystals are a class of composite materials having a periodic variation of mechanical and stiffness properties that interact with elastic waves in a manner that allows wave dispersion and bandgaps, where wave energies are forbidden [55]. Guarin *et al.* studied the stress wave propagation through the dactyl club using a layered 3D model of Bouligand structure and approximated that the club is able to dampen the travelling stress wave from an impact by up to 30% through this mechanism [56].

2.3 Mechanical properties from nanoindentation

2.3.1 Hardness

Hardness also known as contact stress, is a measure of the resilience of materials to contact loading [57]. Indentation tests were originally created to measure this property. Hardness is measured using the formula below:

$$H = \frac{P_{max}}{A} \quad (2.1)$$

where H is the hardness, P_{max} is the maximum load, and A is the contact area.

2.3.2 Elastic Modulus

In nanoindentation, elastic modulus can be determined using two contact mechanics theories. The first, is Hertzian contact mechanics developed by, Heinrich Hertz in 1881 [58]. He found that for a rigid spherical indenter on a flat surface, the load P is related to the indenter radius R , the deflection from the surface h , and the effective modulus E^* by the following formula:

$$P = \frac{4}{3} E^* R^{0.5} h^{1.5} \quad (2.2)$$

Using the load-displacement curve and knowing the indenter radius, as long as the curve is within the elastic regime the modulus can be calculated using Equation 2.2. E^* combines the moduli and Poisson's ratios of the tip (E_t and ν_t) and sample (E and ν) by this equation:

$$\frac{1}{E^*} = \frac{1 - \nu^2}{E} + \frac{1 - \nu_t^2}{E_t} \quad (2.3)$$

When studying biological materials, because of the difficulty of finding the Poisson's ratio of the test sample, the modulus of the sample is often represented by E^* which is generally accepted because the indenter tip is usually made of diamond which has a modulus several orders larger than the sample modulus. The contact radius a , is related to h and R by $a^2 = h/R$. Substituting this into Equation 2.2 the following formula is obtained [59]:

$$P = \frac{4}{3} \frac{E^* a^3}{R} \quad (2.4)$$

Oliver and Pharr developed another method [60] to determine the elastic modulus of an unknown sample by using the unloading curve of a standard nanoindentation load-displacement graph. They found that a power law relationship $P = \alpha(h - h_f)^m$, fitted the unloading curve, where α , h_f and m are constants that can be found using the least square method. And using the stiffness ($S = dP/dh$) of the upper portion of the unloading curve—found experimentally— E^* can be obtained using Equation 2.6. The area A is calculated from the contact depth h_c using the tip area function, which is detailed in the same paper. h_c is found using Equation 2.5; $h_{\max}$ and $P_{\max}$ are the maximum load and displacements measured from the load displacement curve. With S and A known E^* can be easily calculated from Equation 2.6.

$$h_c = h_{\max} - 0.75 \frac{P_{\max}}{S} \quad (2.5)$$

$$E^* = \left(\frac{\sqrt{\pi}}{2} \right) \frac{S}{\sqrt{A}} \quad (2.6)$$

2.3.3 Indentation stress-strain curve

For a spherical indenter H can be expressed as:

$$H = \frac{P}{\pi a^2} \quad (2.7)$$

substituting Equation 2.7 into 2.4 and rearranging yields the relationship:

This equation is the indentation stress-strain relationship for the elastic portion of the

$$H = \left(\frac{4E}{3\pi} \right) \frac{a}{R} \quad (2.8)$$

curve.

Note that H here is defined as the indentation stress, and indentation strain is a/R . He and Swain derived a method to determine the indentation stress-strain curve using load-displacement curves with a spherical indenter [61], [62].

2.4 Fracture Mechanics

2.4.1 Linear-elastic fracture mechanics (LEFM)¹

2.4.1.1 Energy method

The field of fracture mechanics arose to address the issue of material failure below their theoretical strength due to the presence of cracks within the material body. In his pioneering work in 1920, Griffith applied the first law of thermodynamics and derived the following energy balance at equilibrium for a unit increase in crack area [64]:

$$\frac{d\Pi}{dA} + \frac{dW_s}{dA} = 0 \quad (2.9a)$$

$$-\frac{d\Pi}{dA} = \frac{dW_s}{dA} \quad (2.9b)$$

¹ The majority of this section is summarized from a chapter of the same name in the Fracture Mechanics book by T.L. Anderson [63].

Where Π is the potential energy arising from strain within the material under externally applied forces and W_s is the work of creating new surfaces. Any release in potential energy must be accompanied by a creation of new surfaces and the overall energy of the system should be lower for the process to be spontaneous. Applying his energy approach, Griffith showed that the fracture strength for an infinite plate Figure 2.6A with a center crack of width $2a$ is given by, where γ_s is the surface energy:

$$\sigma_f = \sqrt{\frac{2E\gamma_s}{\pi a_c}} \quad (2.10)$$

In 1956, Irwin defined an energy release rate, G to represent the potential term (LHS) in Equation 2.9b [65]:

$$G = -\frac{d\Pi}{dA} \quad (2.11)$$

Applying Griffith's energy balance, crack extension to failure occurs when G reaches a critical value, G_C , where the energy of creating two surfaces is less than the potential energy. Hence, the fracture toughness of the material body can be characterized by G_C . And considering only the potential term makes it easier to solve fracture related problems since the surface energy needs not be measured and the problem is simplified to an energy balance between the elastic strain and external forces. For both load and displacement control, G can be expressed as:

$$G = \frac{P^2}{2B} \frac{dC}{da} \quad (2.12)$$

Where C is the compliance, a device introduced to simplify the expression and is defined by the instantaneous displacement divided by instantaneous load (i.e. $C = \Delta/P$). For a detailed derivation of Equation 2.12, the reader is referred to Reference [63].

2.4.1.2 Stress method

Irwin and others developed another approach to fracture mechanics by characterizing the stress field produced around the crack tip [66]–[69]. Depending on the complexity of the crack problem, analytical solutions to the stress equation may not always be

possible. However, the first term of the solution in polar coordinates is always in the form (see Appendix A for more details):

$$\sigma_{ij} = \frac{K}{\sqrt{2\pi r}} f_{ij}(\theta) \quad (2.13)$$

Figure 2.6B depicts the polar coordinate system with the origin at the crack tip and the stress tensor (σ_{ij}) notation for the equation above; i and j are the cartesian coordinates (x or y or z), for simplicity first rank stress tensors (i.e. vector) are considered here. The higher order terms of the solutions are geometry dependent and can be disregarded close to the crack tip as the first term is proportional to $r^{-0.5}$ thus, dominating the solutions close to the crack tip. Hence, the first term is sufficient to describe the stress field near the crack trip. K is the stress intensity factor; it is essentially the amplitude of the stress field around a crack. For a given sample configuration and material, there exist a critical K value where fracture would proceed spontaneously. This value is defined as the fracture toughness of the system, K_c is an intrinsic material property. Both K_c and the critical energy release rate, G_c are valid parameters to describe the fracture toughness of a system. In fact, both are related by:

$$K_c = \sqrt{G_c E} \quad (2.14)$$

At this point it is important to consider that fracture can occur from three different modes of loading [70]. Figure 2.7 depicts the different modes of loading. And the K_c for the same material body is different with the different modes. Typically fracture toughness measurements are performed in Mode I as most materials fail by tensile rather than shear stresses (i.e. Mode I K_c is generally smaller than other modes K_c) [63].

Thus far, K has been introduced as another parameter for fracture toughness. However, for K to be practical it must be possible to determine it from external loads and the crack geometry. The can be achieved by solving the Westergaard stress function for a particular crack configuration [68]. Using Westergaard approach for a thick infinite plate with a sharp center crack of length $2a$, Mode I stress intensity factor, K_I can be

calculated from the following equation, assuming $a \gg r$ (see Appendix A for the detailed derivation):

$$K_I = \sigma_\infty \sqrt{\pi a} \quad (2.15)$$

Note that σ_∞ and a here are not the same as σ_{ij} and r ; σ_∞ is the external stress applied on the plate in Mode I and a is the crack length (see Figure 2.6). For some simple configurations, K can be related to σ_∞ and a analytically. However, when the shape of the material body is complicated or when the boundaries are finite and close to the crack tip close-loop solutions are often not possible. And Equation 2.15 is modified by including a geometric function Y .

$$K = Y \sigma_\infty \sqrt{\pi a} \quad (2.16)$$

The geometric function can be solved empirically or numerically for complicated cases. Finite element method (FEM) can also be used.

Figure 2.6. A) Infinite plate with a centre crack loaded in Mode I configuration. B) Schematic of a 2D stress tensor nearby the crack from A. The subscripts used in the stress tensor in Equation 2.13 is ‘ij’ but ‘x’ and ‘y’ were used in the subscripts because the coordinate axes were defined.

Figure 2.7. Fracture testing modes. **I)** Opening, **II)** Sliding and **III)** Tearing.

2.4.1.3 Validity of K_{Ic}

Generally, fracture toughness is determined in plane strain conditions as it is smaller than in plane stress conditions. In plane stress conditions, at least one of the principal stresses is equal to zero (e.g. $\sigma_{zz} = 0$). While in plane strain conditions at least one of the principal strains is equal to zero (e.g. $\varepsilon_{zz} = 0$). Plane strain fracture toughness is usually referred to in literature as K_{Ic} . Taking the crack configuration in Figure 2.6A as an example, the sample size in the off-plane direction (B in this case) must be sufficiently large to ensure that the system is mostly in plane strain conditions across the crack front. Apart from plane strain conditions at the crack tip, valid K_{Ic} values require the plastic zone be smaller than the singularity-dominated zone (K zone). For simplicity, we will consider the stresses along the direction parallel to the loading in Mode I (i.e. σ_{yy}). The function f_{yy} is equal to 1 and Equation 2.13 reduces to the following:

$$\sigma_{yy} = \frac{K_I}{\sqrt{2\pi r}} \quad (2.17)$$

It is important to remember that Equation 2.13 only considers the first term of the stress field, which is acceptable when stresses near the crack tip are considered since higher order terms (which depends on the geometry of the object) are negligible near the tip. Figure 2.8A shows a plot of σ_{yy} against r near the crack tip of an isotropic linear elastic material under Mode I loading. The theoretical stresses predicted by Equation 2.17 are plotted as a dashed line. There exists a point where the actual stresses deviate from the theoretical value. The size of r at this point characterizes the K zone where fracture

measurements using the stress model is valid. One issue with the stress model is it predicts infinite stress at the crack tip as the stress tensor is proportional to $r^{-0.5}$ which is not possible. It is more plausible, that the high stresses lead to plastic deformation around the crack tip, a phenomenon called crack tip plasticity [63], which develops a plastic zone at the region surrounding the crack tip even in brittle materials like glass. Crack tip plasticity can be accounted for by the stress model by assuming that the material yields when the stresses reach its yield strength (σ_{YS}). Figure 2.8B shows the plot of σ_{yy} against r but accounting for the crack tip plasticity. The region ahead of the crack tip is assumed to deform plastically when the predicted stresses are equal or above the σ_{YS} of the material. Therefore, the plastic process zone size (r_{pl}) can be estimated by substituting σ_{YS} for σ_{yy} in Equation 2.17. The expression is further divided by 3 to account for the triaxial stress conditions imposed in plane strain conditions.

$$r_{pl} = \frac{1}{6\pi} \left(\frac{K_I}{\sigma_{YS}} \right)^2 \quad (2.18)$$

The criterion for K_{Ic} validity for the ASTM 339 standard [70, p. 399] is where a , B , $W-a$ are the specimen dimensions; crack length, thickness and remaining ligand width respectively (refer to Figure 2.6A for illustration):

$$a, B, (W - a) > 2.5 \left(\frac{K_I}{\sigma_{YS}} \right)^2 \quad (2.19)$$

The off-plane dimensional criterion (i.e. B) accounts for the plane strain conditions, while the in-plane dimensional criterion (i.e. a and $W-a$) is important to ensure that the plastic zone is within the K zone and hence the nominal behavior is linear-elastic. This means that the plastic zone size should be at least around 50 times smaller than the key dimensions of the system for K_{Ic} to be valid. However, studies using finite element method (FEM) have shown that this criterion is overly conservative and plane strain conditions are still obtainable with thinner (smaller B) specimens but not with smaller a and $(W-a)$ [63]. When the size of the plastic zone is larger than the K zone, LEFM cannot be used to describe the material and elastic-plastic fracture mechanics (EPFM) is required. EPFM is discussed in detail in the next section.

Figure 2.8. **A)** Schematic of the singularity-dominated zone (K zone) in the yy direction and Mode I loading. The actual and theoretical stresses in both **A** and **B** are represented by the solid and dashed lines respectively. **B)** Schematic of the plastic zone embedded within the singularity zone which is a critical criterion for the LEFM validity. The modified equation for σ_{yy} can be used to account for crack tip plasticity.

2.4.2 Elastic-plastic fracture mechanics (EPFM)²

2.4.2.1 J -Integral

When the plastic zone is larger than the K zone, fracture toughness determination using LEFM is no longer accurate. Most brittle and semi-brittle materials like ceramics and glasses can be evaluated by LEFM, but many materials do not meet this criterion. EPFM extends fracture toughness measurement to include materials that exhibit larger crack tip plasticity. There are two methods developed for EPFM, namely, crack tip opening displacement (CTOD) and J -integral (J). This section will only cover the latter. Rice extended the energy method introduced by Griffith to include non-linear materials. He defined a path-independent contour integral (J -integral) around the crack tip is equivalent to the energy release rate defined by Griffith [72]. J is a more general expression of the energy release rate, J is equal to G for a linear-elastic material. Therefore, the equations that apply to G are also applicable to J , and J is related to the stress intensity factor, K by Equation 2.14 as follows:

² Majority of this section is paraphrased from a chapter of the same name in the Fracture Mechanics book by T.L. Anderson [63].

$$K = \sqrt{JE} \quad (2.20)$$

However, it is important to note that J is strictly not the energy release rate because energy release rate is the strain energy released through the creation of crack surface, whereas in elastic-plastic materials where J is applied, the strain energy is usually partly absorbed by the material by plastic deformation. The general expression for the J integral on a bend test sample is as follows:

$$J = \frac{\eta A}{Bb} \quad (2.21)$$

Where, A is the area under the load displacement test curve, B is sample thickness, b is the remaining ligament width (note: $b = W-a$) and η is a dimensionless constant that is approximately 2 for pure bending. It can be further separated into elastic and plastic contributions, and using the relationship from Equation 2.20, the elastic component can be expressed in terms of K :

$$J = \frac{\eta_{el} A_{el}}{Bb} + \frac{\eta_{pl} A_{pl}}{Bb} \quad (2.22a)$$

$$J = \frac{K^2}{E} + \frac{\eta_{pl} A_{pl}}{Bb} \quad (2.22b)$$

2.4.2.2 J_{Ic} validity

Hutchinson, Rice and Rosengren (HRR) showed that the stress field around crack tip can be characterized by J and is in the form [73], [74]:

$$\sigma_{hk} = c \left(\frac{J}{r} \right)^{\frac{1}{n+1}} \quad (2.23)$$

c is a constant and n is the strain hardening exponent from the Ramberg-Osgood equation which will not be further discuss for the sake of conciseness. The stress field

characterized by J is known as the HRR singularity or zone after the founding researchers. The HRR zone is generally larger than the K zone and hence extends the usage of EPFM further than the LEFM. However, like for K , the HRR singularity predicts an infinite stress at the crack tip. Equation 2.23 is based on small strain theory and does not account for large strains or crack blunting that occurs at the crack tip due to the large stresses. Hence, there is a region near the crack tip that cannot be characterized by the HRR singularity. Moreover, far from the crack tip the finite geometry effects dominate and J cannot be used to describe the stress state close to the physical boundaries of the material body. According to the ASTM standard for EPFM, for J values tested in Mode I to be considered as plane strain and size-independent fracture toughness values (J_{Ic}) the following criterion must be satisfied [75]:

$$B, b_0 > 10 \frac{J_{Ic}}{\sigma_Y} \quad (2.24)$$

Where b_0 is the initial remain ligament length and σ_Y is the effective yield strength, which is defined as the mean of the material's yield strength and tensile strength. If the criterion is not fulfilled in the test, caution must be taken in interpreting the results of the test and J values obtained can only be termed 'qualified' values (*i.e.* J_Q).

2.4.2.3 *R-curve*

A linear-elastic material with a particular crack size will fail at a critical energy release rate as covered in section 2.4.1. Plotting G_c against the crack extension (Δa) would show a horizontal line at G_c . However, some materials like metals experience strain hardening which increases the amount of stress required to extend the crack further. The G_c vs. Δa graph would show a rising G_c value with crack extension, with usually a decreasing slope as the crack propagates. This curve is called the resistance curve or *R-curve* of the material (Figure 2.9A). *R-curve* behavior can be due to many factors that act on the crack to delay further growth of the crack. The factors can be divided into two broad classes; intrinsic and extrinsic factors. Intrinsic factors typically act ahead of the tip to prevent further crack growth while extrinsic factors act behind the tip to arrest the crack by lowering the driving force for crack growth [76]. Cortical bone is a good example of a material that applies both types of toughening strategies [76]. Intrinsic

factors include unravelling of collagen molecules, fibril sliding and microcracking which increases the force necessary to extend the crack, while extrinsic factors include crack deflection and twisting, crack bridging and collagen fibril bridging. *R*-curve behavior is more often encountered in elastic-plastic materials. For small scale yielding conditions, the *R*-curve of an elastic plastic material can be divided into three segments, namely: crack blunting, fracture initiation and steady state crack propagation (Figure 2.9B) [63]. The *J* rises linearly in the first segment due to blunting of the crack tip, a feature present in elastic-plastic materials. The second segment sees the *J* increasing with decreasing slope. The rising *R*-curve behavior in this segment is due to the plastic wake left behind by the propagating crack. In elastic materials, crack growth is unaffected by the previous state of the system. This is because the elastic strain energy is converted to new surfaces only in purely elastic fracture. However, the strain energy is partially dissipated and absorbed by material that exhibit elastic-plastic fracture behavior. Eventually, the crack propagation reaches the third stage where *J* remains constant. Here the crack is far from the initial crack blunting and crack propagation is in equilibrium with local stresses and strains. For EPFM, *R*-curve behavior can be quantified using:

$$J = \frac{K_i^2}{E} + \frac{\eta_{pl} A_{pl,i}}{B \Delta a_i} \quad (2.25)$$

Where *i* is the index number of the unloading points, η_{pl} is a dimensionless constant for the plastic component, $A_{pl,i}$ is the energy absorbed by the sample during the *i*th crack extension (Δa_i). Unlike calculating *J* in static conditions, where the initiation fracture is determined, the crack growth has to be accounted for. The instantaneous crack growth can be determined by measuring the compliance. This is done by conducting partial unloading at regular intervals during a bend test. The compliance is measured from the unloading segments and used to determine the crack growth. The elastic component of Equation 2.25 can be determined by using the load immediately before unloading and the crack length determined from the compliance. The plastic component is more challenging to determine. Figure 2.10 shows a pictorial representation of the different terms in Equation 2.25. This is usually calculated incrementally since the for EPFM, the fracture toughness is dependent on the loading history. The incremental calculation is complicated and can be found in the ASTM 1820 standard [75]. Anderson documents

the derivation in his book [63]. Instead a simpler calculation is presented here. $A_{pl,i}$ is determined at the current unloading point in similar manner as that for a static J calculation. The denominator Ba_i represents the area of the fractured surface. It is important to note that Equation 2.25 is for a rectangular notch where B does not vary with depth.

Figure 2.9. Typical R-curves for a **A)** linear-elastic and **B)** elastic-plastic material. G and J are the energy release rate or energy absorption rate respectively, while Δa is the crack extension. The R-curve for an elastic-plastic material can be divided into three segments: 1. Crack blunting, 2. Fracture initiation and 3. Steady-state crack propagation.

Figure 2.10. Load-displacement graph for an elastic-plastic material. The blue area is the plastic energy absorbed during crack extension ($A_{pl,i}$). **B)** Schematic of the cross-sectional view of the rectangular notch. The crack area for i^{th} extension can be measured by $B\Delta a_i$.

2.4.3 Indentation fracture

The indentation fracture method was first established by Lawn, Evans and Marshall (LEM) in several works from 1975-1980 [77]–[81]. Fracture toughness (K_c) can be determined by measuring the length of surface cracks (c) that are produced after probing the surface of the material with a sharp indenter tip, usually a Vickers tip [77]. The indentation formula they introduced is as follows:

$$K_c = \alpha \sqrt{\frac{E}{H} \frac{P}{c^2}} \quad (2.26)$$

Where H is the material hardness, P is maximum recorded load and α is a constant measured experimentally. The formula above assumes that two perpendicular cracks are produced by the residual tensile stresses on unloading and that the cracks generated are either median or half-penny cracks Figure 2.11 [78]. The length of the crack produced should be at least 2.5 times the indent size to minimize the plastic zone effect created by the indent [58], [82]. For accurate measurements, the sample should be prepared as flat as possible but more importantly, the surface should be polished well enough so the cracks can be measured using optical methods [83]. Indentation quickly gained popularity and became a common technique used to study fracture behavior because of the small size of indents, its non-destructive nature, the little amount of material required, ease of usage and comparison on different materials and the cracks generated are of the same scale of brittle cracks [84]. Furthermore, with the invention of the nanoindenter, indentation can be used to probe fracture properties at the micrometer scale [59]. However, there are several limitations of fracture toughness from indentation, which are listed below:

- Sample tested must be reasonably homogeneous with structural features much smaller than the indent size [83];
- Cracks are rarely observed to initiate only upon unloading, cracking before unloading could affect the residual tensile stress fields, thus affecting crack growth during unloading [85];
- Data should not be considered if chipping or crack branching occurs [86];

- There is also a large variation in fracture behavior, requiring many different equations to solve for the fracture toughness [83], [86];
- the α constant is determined empirically and not based on a physical model [78], [82];
- The sub-surface fracture pattern and the fracture surface cannot be observed directly in opaque materials unlike for conventional methods [83]. Although, ultrasonication can be used to remove the loose material surrounding an indented site to study the fracture extent below the surface [87];
- Some materials have been found to suffer from post indentation slow crack growth effects [83];
- Not suitable for materials that exhibit *R*-curve behavior, as only a single K_{IC} would be obtained for a given load [82];
- Surface should have no pre-stress, which would introduce errors into the fracture toughness values from the sample [83];
- Equation 2.26 is dependent on hardness which is shown to vary with indentation depth also known as the “indentation size effect”, hence K_{IC} obtained from indentation would also be depth dependent [88].

Hence, there has been much debate in the fracture mechanics community about the accuracy and validity of fracture toughness values measured by indentation fracture techniques [82], [89]–[92]. Studies have shown that indentation produced around a 30–40% agreement in results with other test methods which is statistically a large variance [83], [86]. Errors greater than 50% for K_{IC} were encountered in some studies [90]–[92]. The large spread in values obtained in the literature indicate that caution should be used when applying indentation and when reading studies that apply indentation to study fracture toughness of materials. And indentation fracture methods are suitable only for brittle materials like glasses and ceramics and should be avoided for soft materials. Indentation fracture is also not recommended to be used in hard biomineralized tissues. For example, indentation fracture method produced cracks about the same size as the indents on human cortical bone and thus were well within the plastic zone and fracture toughness from these measurements are questionable [82], [93]. To extend the indentation fracture method to smaller scales on cortical bone, a cube corner tip which produces larger contact pressures than a Vickers tip at the same applied force was

explored [82]. Cube corner indents were made on hydrated human cortical bone with post analysis of indent surface in four conditions: i. on the spot with optical microscopy, ii. alcohol dehydrated with optical microscopy, iii. environmental SEM and high vacuum SEM. Radial cracks from the indent corners were only observed in 2 corners out of 30 and they were only visible with electron microscopy, hence it is unclear if cracks were affected by dehydration in the vacuum conditions. The cracks were also well within the plastic zone.

Another indentation fracture model was introduced by Fett *et al.* which made use of crack opening displacement at the indent corners instead of the crack length to evaluate fracture toughness [94], [95]. A representative solution for fracture toughness using this method is:

$$\delta = \frac{4K_c\sqrt{a}}{\pi E} \left(A \left(1 - \frac{r}{c}\right)^{\frac{1}{2}} + B \left(1 - \frac{r}{c}\right)^{\frac{3}{2}} + C \left(1 - \frac{r}{c}\right)^{\frac{5}{2}} \right) \quad (2.27)$$

Where δ is the crack tip opening displacement (CTOD), and A , B , C are constants calculated from the following expressions [95]:

$$A = \sqrt{\frac{\pi c}{2a}} \quad , \quad B \cong 0.011 + 1.8197 \ln \frac{c}{a} \quad , \quad C \cong -0.6513 + 2.121 \ln \frac{c}{a}$$

Unlike α in the LEM model, none of the parameters in Fett's CTOD solution requires calibration from experiments. Also, the solution is not a function of hardness and thus avoids indentation size effects due to hardness. This method provided fracture toughness values in glass comparable to those obtained using LEFM-based measurements, in comparison to the LEM model which gave a wide spread in fracture toughness values [96]. However, measurements on a silicon carbide ceramic gave values lower than the expected toughness [97]. This was attributed to other cracks forming around the corner crack and the indent. Hence, although Fett's CTOD method is more robust than the LEM method, care must be taken when attempting to apply it and it is unlikely that it is a suitable method to assess the fracture toughness of biomineralized tissues. Although indentation is not ideal it is still possible to adopt an indentation approach to characterize fracture toughness of biomineralized tissues. Miserez *et al.* devised a method to determine the initiation load for cracks using

indentation on fused quartz and the silica skeleton of the sea sponge with has a remarkable fracture toughness for a material made of 99 % silica and 1 % organics [87]. The method involves indenting the material with a sharp tip like the Berkovich or cube corner tips many times at different loads to obtain a large data set. This is possible because the size of each indent is tiny in the nanoindenter. Sharp corners of the residual impression are observed for radial cracks. The load which resulted in half the number of cracks to corners is defined as the initiation load.

Figure 2.11. Lawn-Evan-Marshall (LEM) indentation fracture method. **A)** A Vickers tip pressed into the material creating half-penny crack on the material surface with a crack radius of size c . **B)** View of the indent left on the surface and the cracks extending from the four sharp corners of the Vickers tip.

2.5 Fracture toughness measurements at the microscale

2.5.1 Common K_{Ic} test methods at the macroscale

Figure 2.12 shows a single edge notch beam (SENB), it is a common test sample used to measure the fracture toughness of a material; other standard samples include compact tension (CT) and disk compact tension (DCT) samples [71], [75], [98]. The choice of geometry used depends on the size of the test material and the availability of required equipment to conduct the fracture tests. The compact samples have more complex geometries and generally require more material. However, they can be tested using a tensile tester with an appropriate fixture, but beam samples require the use of three- or

four-point bending equipment. In the ASTM standards the general expression for K is represented in a different form from Equation 2.16 for convenience [71], [75], [98]:

$$K = \frac{P}{B\sqrt{W}} F(a) \quad (2.28)$$

Where $F(a)$ is the geometric function of the beam. For standard samples, this function is determined through rigorous testing and is provided in the standards. If the geometry is non-standard and complex the function has to be determined with numerical methods or FEM. ASTM-399 standard provides information for the testing of metallic materials with LEFM [71]. As mentioned in the section 2.4.1, the material should exhibit small scale yielding with the plastic zone within the K zone. Moreover, the sample dimensions should meet the criterion according to Equation 2.18. For brittle materials like ceramics, ASTM-C1421 standard is suitable and only bend specimens are used in this standard [98]. If the material cannot be analyzed with LEFM, EPFM can be used instead, which is covered in the ASTM-1820 standard. SENB, CT and DCT samples can be used. For tests using EPFM to be valid, Equation 2.24 should be fulfilled for the sample dimensions. The notch geometry and dimensions are critical considerations for successful fracture toughness measurements. The notch is the location of crack growth and eventual failure. For fracture measurements to be accurate, it is imperative to have a sharp starter crack or pre-crack at the notch. For metals or materials that exhibit metallic behaviour, this pre-crack can be created with fatigue testing until the desired crack length is obtained. However, pre-crack generation by fatigue testing is usually not applicable for brittle materials. Instead a sharp pre-crack can be obtained in these materials by indenting with a Vickers or Knoop tip to create median cracks aligned parallel to the thickness of the sample (B), i.e. perpendicular to the long axis of the beam. Another method is to use a notch with a cross-section of an upright triangle; this is called a chevron notch. The high stresses at the tip of the chevron notch is often enough to generate sharp pre-cracks in brittle materials. The chevron notch is advantageous as simpler equipment are required to create the pre-crack necessary for brittle fracture tests. Moreover, the crack width increases with crack depth and hence it is easier to obtain stable crack growth before fracture in brittle ceramics. Furthermore, there is no need to measure the crack lengths in post-test fractography. However, high precision is required for the chevron notch and thus it is difficult to machine with

mechanical cutting tools. Also, substantial crack growth is necessary before failure is required. Finally, the material should not exhibit *R*-curve behavior with chevron notch samples [98]–[100]. The chevron notch is covered in greater detail in section 2.5.3.2. The tests methods covered in this section have been extensively used to characterize materials in the past century and the underlying physics are well understood. However, the standard samples are often in the millimeter scale, which are often not possible to prepare from biological samples because of their size. Moreover, biological materials are often fashioned with hierarchical structures, and to study the properties of the structures at micrometer scale other methods must be used. Nanoindentation can probe materials at the microscale but indentation fracture methods have many limitations as mentioned in section 2.4.3. Hence, other methods should be explored to effectively determine the fracture behaviour of materials at the microscale. Fracture toughness testing using focused ion beam (FIB) milled samples is a recent prospective method and is covered in greater detail in the next section.

Figure 2.12. Single edge notch bend (SENB) specimen. The schematic on the right shows the cross-section at the notch.

2.5.2 Focused ion beam (FIB)

FIB makes use of a stream of charged ions usually Gallium, to remove material from the sample [101]. It is a precision cutting technique, which is frequently used to prepare samples for Transmission Electron Microscopy (TEM) observations [102]. This is both a boon and a bane, as the high resolution of the FIB limits its use to prepare larger samples. Samples several tens of micron large can take several hours to prepare, not to mention that it is a time-consuming process. The cutting time depends on the current

used and the type of material [103]. Monte Carlo analysis is frequently used to estimate the material removal rate using data like the molecular weights of the element cut [103]. The more rudimentary approach of measuring the depth after cutting a sample at a fixed current and time would suffice as well. The accelerating voltage is usually kept at 30 kV for cutting while the current is varied as this is the optimal potential for material removal [104]. There are several considerations when using the FIB on biological materials. Firstly, the sample should be coated with a conductive material [105]. There is a three-fold purpose to this: biological materials are often non-conductive when dry, hence the coating will help to prevent charging, though it should be noted that the ion beam removes the coating at the areas selected for cutting. The second purpose is protection [105]: a Pt coating around 60 nm was shown to be effective in preventing ion damage on a Si substrate [106]. Thirdly, biological materials may be sensitive to radiation damage and sample burning. Therefore, the current used to cut the sample should not be too high to lead to burning [105]. Moreover, the sample should be prepared with progressively lower currents such that the final cut surface has as little radiation damage as possible.

2.5.3 FIB-milled Microcantilever Bending

As mentioned in section 2.4.3, nanoindentation is not an ideal method to evaluate the fracture toughness of hard biological tissues. And standard fracture toughness measurements are too large to be applied to some biological samples including the stomatopod dactyl club. Since the early 2000s, FIB milled samples have been used to study the fracture toughness of artificial materials [107]–[109]. With the use of a FIB it is possible to mill samples at the micrometer scale (the same scale as nanoindentation or smaller). The cantilever geometries prepared are usually rectangular or triangular, triangular cantilevers have the advantage of being easier to prepare compared to rectangular samples [88], [100]. Moreover, they can be prepared anywhere on the sample surface rather than only at the edge [88], [100]. Triangular cantilevers were used by Chan *et al.* to study the properties of teeth a hard biomineral used as a biotool [88]. In their research, the authors prepared different sized micro-cantilevers to study the hierarchical structural properties including fracture strength of human dentine and enamel [88], [110]. Since then, other fracture studies using microcantilevers and microbeams were conducted on structural biomaterials including limpet teeth [111],

bovine enamel [112], rat bone [113], [114], beetle horn cuticle [115], nacre [116]. Another advantage of using microcantilevers and microbeams is they can be milled in any direction enabling the study of anisotropic fracture toughness in different direction [117]. This is advantageous for biological materials as many structural biological materials have anisotropic mechanical properties [10], [13]. Jimenez-Palomar *et al.* used microcantilevers milled in different orientations to study the properties of rat femora samples at the microscale [114]. Therefore, FIB milled microcantilevers are a potential method to study the fracture behaviour of materials at the microscale.

2.5.3.1 Measurements on Unnotched Microcantilevers

Many studies on biological materials covered in the previous section employed unnotched microcantilevers or microbeams and evaluated other properties like elastic modulus and fracture strength. For rigid beams subject to lateral loads and small deformations, the Euler-Bernoulli beam theory or simple beam theory can be used to understand the material properties. For a rigid cantilever with a triangular cross-section loaded with a downward force (P) and corresponding deflection (δ) at the free end, the elastic modulus (E) of the material can be determined by the following expression [118]:

$$E = \frac{L^3}{3I} \left(\frac{P}{\delta} \right) \quad (2.29)$$

Where L is the length from the fixed end to the load and I here is the second moment of area. For a triangular cross-section with width, W and thickness, B and for an axis passing through the centroid and parallel to B (axis xx , see Figure 2.13A), I_{xx} is equal to:

$$I_{xx} = \frac{BW^3}{36} \quad (2.30)$$

However, when the cantilever support is not perfectly rigid but able to deform, the beam theory breaks down, and an additional deflection which scales with lower order terms proportional L^n arises ($n < 3$). At large values of L , this deflection is not significant because the first term scales with L^3 and dominates. At smaller values of L , the

additional terms become significant and should be included in the calculations. Armstrong *et al.* found that cantilever length-to-breadth ratios of at least 6 times are necessary for microcantilevers to retain a linear behavior according to Equation 2.29 [118]. For microcantilevers, the lower order terms can be evaluated by testing at multiple equidistant points along the length of the cantilever [118], [119]. Apart from the elastic modulus, the stress-strain behavior of the material can be determined as well. Figure 2.13B shows a section of the cantilever under bending. The top of the beam is under tension as the load is acting downwards at the free end of the cantilever. At the top surface the stress is maximum and decreases deeper into the cantilever till it reaches a depth where the stress is zero. This point lies upon the neutral axis of the cantilever where the stresses are zero. Below the neutral axis the load stresses become compressive to a maximum compressive stress at the bottom of the cantilever. The flexural stress is measured using the maximum instantaneous tensile stress along the beam (σ_{top}) as materials are more likely to fail in tension. Applying simple beam theory, σ_{top} and the corresponding strain (ϵ_{top}) can be determined for a triangular cross-section using the following formulae:

$$\sigma_{top} = \frac{12PL}{BW^2} \quad (2.31)$$

$$\epsilon_{top} = \frac{W\delta}{L^2} \quad (2.32)$$

Note that by substituting the stress and strain into Hooke's law ($E = \sigma/\epsilon$) for a linear elastic material, Equation 2.29 is obtained. Equations 2.29, 2.31 and 2.32 are only valid when simple beam theory holds, which is for a rigid cantilever and when displacements are small. The flexural strength of the material can be determined using Equation 2.29 by substituting load measured upon fracture. Flexural strength can be used to estimate the toughness of material especially for materials that exhibit high plasticity [120]. However, it is not the ideal method for determining fracture from crack propagation since the crack geometry, location and size is not controlled like in conventional K_{Ic} methods using a well-defined notch. Notwithstanding these limitations, flexural strength measurements are useful in describing interfacial bond strength. Microcantilevers are milled with the interface along the cross-section and the cantilever tested in bending. The interface is usually weaker than the surrounding phase and hence

failure occurs along the interface. There are studies that apply this method to study the interfaces in artificial materials [121], [122], but so far the use of microcantilevers to determine interfacial strength in biomineralized tissues are largely muted and the author was unable to find any study applying this technique.

Figure 2.13. Triangular microcantilever schematics. **A)** Triangular cross-section showing the width (W) and thickness (B), centroid (red dot) and the axis used for second moment of area (I_{xx}) calculations. **B)** The side view of a section of the cantilever showing the directions of tensile stresses developed in the beam along the length of the beam during bending. The downward load is applied perpendicular to the top surface on the far right (not shown on the figure). $\sigma_{TS,max}$ is the maximum tensile stress which is along the top surface of the cantilever during loading.

2.5.3.2 Chevron-notched Microcantilevers

The chevron notch is a method first proposed by Tattersall and Tappin to solve the issue of notch preparation on brittle ceramics [123]. The advantages of the chevron notch are as follows: crack healing is not a problem [58], [86] and there is no need for a separate method to produce a pre-crack [86], [124]. Moreover, the pre-crack length need not be measured. And longer stable crack growth rate which alleviates the undesirable effects of FIB milling on the sample (*i.e.* re-deposition, radiation damage, ion implantation) [99]. However, there are some limitations to using chevron notches:

- Difficulty to produce chevron notch [124]
- Requires the use of shear correction factors [124]

- Cannot be used for materials that toughens with crack growth (i.e. *R*-curve behavior) [99], [124]

Mueller *et al.* explored the use of chevron notches on rectangular micro-cantilevers [99], which was extended to include triangular micro-cantilevers in a following article [100]. Figure 2.14 shows an image of one of the triangular cantilevers prepared for testing with the inset showing the geometry of the chevron notch. The fracture toughness of the material can be determined using the following formula:

$$K_{IC} = \frac{P}{B\sqrt{W}} F_V(\tilde{a}) \quad (2.33)$$

where F_V is the geometrical function for the triangular micro-cantilever determined using the following equation and $\tilde{a}$ is the normalized crack length, a/W :

$$F_V(\tilde{a}) = \sqrt{\frac{1}{2(1-\tilde{a})} \frac{\tilde{a}_1 - \tilde{a}_0}{\tilde{a} - \tilde{a}_0} \frac{dC_V}{d\tilde{a}}} \quad (2.34)$$

where C_V is the compliance of the triangular micro-cantilever which is found by finite element analysis using the distance from the notch to the location of indentation S , as well as the distance from the notch to the fixed end of the cantilever L (Figure 2.14). S is determined by SEM inspection of the broken cantilever [99]. Stable crack growth through the ligament is necessary [86]. This can be determined by checking if a proper succession of events occurred during the load displacement curves, namely: 1) crack nucleation; 2) stable crack growth; and 3) unstable crack growth [99].

The method by Mueller *et al.* was applied to materials that follow LEFM. For EPFM, methods largely make use of notches with a straight (at least theoretically) initial crack front created by FIB. Testing is performed by partially unloading the microcantilevers at different time points to determine the change in compliance of the beam [125]–[127]. Wurster *et al.* studied the fracture toughness of tungsten (W) with microcantilever. They determined the fracture toughness using in-situ measurement of the CTOD during testing and showed that crack propagation in W for FIB-milled notches and notches from natural cracks behave similarly [127]. Hence, pre-cracking the test sample may not be necessary for notches produced using FIB. Ast *et al.* applied this method to study

the fracture toughness of single crystal NiAl showing good agreement between microcantilever measured fracture toughness and fracture toughness evaluated using conventional methods [125]. They also introduced a method to continuously monitor the stiffness of the microcantilever during testing by applying a harmonic oscillation to the nanoindenter tip during the bend tests, and they showed good agreement between their method and the partial unloading method [126]. Moreover, the fracture behavior of NiAl is characterized by LEFM at the macroscale but at the microscale NiAl is more accurately represented by EPFM [126], [128]. Bohnert *et al.* performed similar measurements on tungsten as Wurster *et al.* but other than straight-notched they used chevron-notched microcantilevers [129]. The fracture toughness values they measured experimentally using EPFM were similar to results from their FEM simulations as well as those from macroscopic measurements [130]. In their experiments, Bohnert *et al.* measured the CTOD by in-situ testing with a nanoindenter and imaged it with SEM [129]. Li *et al.* performed loading and partial unloading measurements on W chevron-notched microcantilevers, but instead of measuring the CTOD they determined the crack propagation using FEM modelling and compliance measured during the unloading segments of the bend tests [131].

Figure 2.14. Triangular micro-cantilever prepared using FIB. **B)** Schematic showing the dimensions used in calculating the fracture toughness, region in red refers to the uncut area. **C)** Fracture surface of the post-tested micro-cantilever [100].

2.6 Biomineralization of the dactyl club

Biological materials offer many benefits to their man-made counterparts, including synthesis occurring exclusively in aqueous environment without the use of harsh chemicals and extreme conditions (high temperature and pressure). Additionally, the signature synthesis method employed by nature is self-assembly; a bottom-up approach instead of top-down [10]. Moreover, biological materials often incorporate multifunctionality in structures. For example, the ridges found on some of the scales of some sharks not only helps to propel the shark through the water with less effort by reducing underwater drag, but prevents the attachment and growth of cellular organisms on them hence keeping the body of the shark clean [132], [133]. A better understanding of how natural materials are formed will provide inspiration for new material synthesis

methods. The study of nature forming hard mineralized tissues is called biomineralization. This section will review the biomineralization of the Stomatopod dactyl club.

The dactyl club is composed of three major minerals: calcium carbonate mainly in the amorphous state, amorphous calcium phosphate and crystalline apatite. Calcium carbonate is commonly observed in the cuticle of crustaceans [134]. Apatite however, is much more rare. Since stomatopods are crustaceans, they molt every 1-3 months and develop brand new fully functional dactyl clubs quickly as these biotools are critical for their survival. A time study of the dactyl club formation showed that a new club matures over a period of approximately one month [135]. This is much faster than the time needed for the development of bone and other bioapatite structures [136], [137]. Hence, the entire biomineralization process of this predominantly apatite structure can be documented in the laboratory with ease. Moreover, since they are invertebrate lesser restrictions are placed on obtaining and handling stomatopods. Therefore, the dactyl club is a convenient, quick, and unique system to understand bioapatite formation. Amini *et al.* discovered that contrary to previously thought [56], the club develops from the outside in [135]. The pre-molt membrane hidden in the inner cavity of the club is shown to expand to form the outer boundary of the new club within the first hour after molting. After this, the club is formed by layer-by-layer deposition of new material on the expanded membrane till the club is complete (**Figure 2.15**). The impact layer is completed within the first two weeks, at which point the mantis shrimp begin to use the clubs. The club fully matures within a month. Amini *et al.* also performed EDS and Raman measurements on the club at the different stages of the club's development [135]. With Raman, they found that the symmetric P-O stretch (strongest Raman band in apatite) was not present before the second day post-molt. EDS measurements showed that Ca and P was only observed on the membrane 1 day after molting. The authors further identified a major protein contributing to apatite formation on the club; termed Club Mineralization Protein 1 (CMP-1). CMP-1 has the following features that suggest that it plays an important role in apatite mineralization: i) chitin-binding domain; ii) many acidic residues; iii) phosphorylation and iv) and the presence of aspartic acid-rich regions alike to those found in Osteopontin – a major player in the biomineralization of bone [138], [139]. It is hypothesized that the protein should be able to bind to chitin in order to deposit the minerals on the protein-chitin matrix of the expanded membrane.

Veis *et al.* showed that the proteins coordinating apatite mineralization are rich in acidic residues [140]. Additionally, phosphorylated proteins are shown to modulate the growth of apatite [141]–[143]. *In vitro* analysis with recombinant CMP-1 and a buffer saturated with Ca and PO₄ ions proved CMP-1's ability to nucleate rod-shaped apatite crystals [135]. Another intriguing feature observed was spherical globules about 50–100 nm in size in the hemocyanin within the cavity of the expanded membrane. TEM and EDS measurements showed that these spherical grains are amorphous and rich in Ca and P [135]. Other studies on crustacean hemocyanin support the idea that they may play a role in Ca storage and delivery to accelerate the biomineralization of the club [144]–[147].

Figure 2.15. Structural changes of the dactyl club during its develop post molting. **A)** Optical microscope images, **B)** SEM images; **C)** Carbon elemental map showing the pre-molt membrane within the inner cavity of the fully developed dactyl club. **D)** Schematic of the growth process of the new dactyl club [135].

2.7 Questions to answer based on literature

2.7.1 Micro-fracture toughness of the stomatopod dactyl club

The stomatopod dactyl club is a robust biological composite. It is able to shatter the tough armor employed by other crustaceans, marine mollusks and bivalves, which includes nacre, another tough biological composite that serves as inspiration for many tough artificial composites. Even after thousands of high powered blows (a single blow could be fatal to it's own kind [1]) the club is still intact, a testament to its remarkable toughness. Hence, the mechanical properties of the stomatopod dactyl club have been heavily assessed in the past decade by electron, X-ray and vibrational imaging techniques to understand the origins of its superior toughness. In these studies, nanoindentation is by far the most common method used to evaluate the mechanical properties including fracture toughness. However, the validity of fracture toughness measured by indentation methods have been met with much controversy, with errors up to 50% of the measured values [90]–[92]. Moreover, indentation fracture methods are not suitable to assess materials with anisotropic properties [88]. However, the size of the dactyl club precludes the use of standard fracture toughness methods which require samples at least several millimeters in size. With the advent of FIB, it is possible to fabricate microscale samples for mechanical characterization of materials. Particularly of interest is the method presented by Mueller and Zagar *et al.*, who used chevron-notched microcantilevers to evaluate the fracture toughness of fused quartz and alumina [99], [100]. The chevron-notch confer certain advantages for fracture toughness determination including: i) not needing a separate technique to introduce a sharp precrack; ii) steady-state fracture in brittle materials; iii) fracture occurring after considerable crack propagation and hence, measurements are less affected by FIB damage at the original crack front. Apart from fused quartz and alumina, FIB milled notched microcantilevers, have been used to study other artificial materials like ceramic and semi-brittle metals [125]–[127], [129]. However, only a few studies have applied notched microcantilevers to study the fracture mechanics of biological materials [112], [116]. Based on the literature reviewed in this chapter, the present work endeavors to answer the thesis: Chevron-notched microcantilevers are a better alternative to measure the fracture toughness of hard mineralized tissues than nanoindentation. The subject of this study is the impact region of the stomatopod dactyl club of the species

Odontodactylus scyllarus or peacock mantis shrimp. Included in this thesis are other questions: can hard mineralized tissues be evaluated with LEFM or EPFM or are both not suitable? How does hydration affect the mechanical properties of the impact region? Is the fracture toughness of the impact region homogenous and is it isotropic or anisotropic?

2.7.2 Biomineralization of the dactyl club

As a crustacean with a hard exoskeleton, stomatopods have to molt in order to grow. When molting the stomatopod sheds its entire cuticle including their hunting appendages. These tools are critical for the animal's survival hence they have to quickly develop new hunting tools. Amini *et al.* showed that the peacock mantis shrimp is able to develop a fully mature dactyl club within a month from molting [135]. Moreover, the impact region of the stomatopod dactyl club is composed of apatite nanofibrils within a chitin-protein matrix [9], [34]. Hence, the stomatopod dactyl club is a unique model system to study the biomineralization of apatite. To date, only a single time study of the development of the dactyl club was conducted [135]. Amini *et al.* showed that the club is formed from the outer boundary in by a “diecasting” mechanism instead of from inside out as previously thought [56]. In their work, the mineral phases were determined by Raman spectroscopy which can provide qualitative information on the degree of crystallinity of the material and the average crystal orientation. Moreover, the size of the laser probe is several micrometers hence crystal structure details in submicron scale is not discernable with Raman. EDS was used to evaluate the composition of the club at different developmental stages [135]. While EDS is semi-quantitative and possibly quantitative with an applicable standard used, the limitation is measurements are performed in high vacuum conditions which may affect the structure of biological materials, especially in softer materials. While the impact region is mostly stable under electron beam owing to its high mineral content, the dactyl club is non-conductive which would lead to charging issues even with a conductive coating applied. X-ray techniques like X-ray fluorescence (XRF) X-ray diffraction (XRD) are advantages in this respect as they can be used in ambient temperature and pressure and does not cause sample charging. Moreover, highly intense and collimated X-ray beams are achievable with 3rd generation synchrotrons, leading to greater spatial resolution and faster scan times. Synchrotron X-rays have been used successfully in previous

studies to characterize many biological materials including, bone [148]–[150], nacre [151], enamel [152], sponge glass spicules [153], parrotfish teeth [154], pistol shrimp claw [155] and the stomatopod dactyl club [34], [156]. A variety of X-ray techniques were used like ptychographic X-ray computed tomography (PXCT), combined micro-small angle X-ray scattering and wide-angle X-ray scattering (μ SAXS-WAXS) and μ XRF which allowed for high resolution 2D and 3D mapping of the microstructure, composition gradient and crystallography at the microscale and in some cases even nanoscale. With the use of synchrotron X-ray techniques to study the development of the stomatopod dactyl club this work endeavors to answer the following questions: What is the spatial-temporal organization of the apatite crystals and organic matrix of the developing stomatopod dactyl club? When do the first apatite nanorods appear? And is there an amorphous-to-crystalline transition or are the crystals deposited early on? Nanorods are oriented in the pristine mature club, but how does this preferred orientation occur and when during club formation?

References

- [1] R. L. Caldwell and H. Dingle, “Ecology and evolution of agonistic behavior in stomatopods,” *Naturwissenschaften*, vol. 62, no. 5, pp. 214–222, May 1975, doi: 10.1007/BF00603166.
- [2] M. L. Reaka, “Molting in stomatopod crustaceans. I. Stages of the molt cycle, setagenesis, and morphology,” *J. Morphol.*, vol. 146, no. 1, pp. 55–80, May 1975, doi: 10.1002/jmor.1051460104.
- [3] J. Marshall, T. W. Cronin, and S. Kleinlogel, “Stomatopod eye structure and function: A review,” *Arthropod Structure & Development*, vol. 36, no. 4, pp. 420–448, Dec. 2007, doi: 10.1016/j.asd.2007.01.006.
- [4] T. W. Cronin, M. Garcia, and V. Gruev, “Multichannel spectrometers in animals,” *Bioinspir. Biomim.*, vol. 13, no. 2, p. 021001, Feb. 2018, doi: 10.1088/1748-3190/aaa61b.
- [5] T.-H. Chiou *et al.*, “Circular Polarization Vision in a Stomatopod Crustacean,” *Current Biology*, vol. 18, no. 6, pp. 429–434, Mar. 2008, doi: 10.1016/j.cub.2008.02.066.
- [6] R. M. Templin, M. J. How, N. W. Roberts, T.-H. Chiou, and J. Marshall, “Circularly polarized light detection in stomatopod crustaceans: a comparison of

- photoreceptors and possible function in six species,” *J Exp Biol*, vol. 220, no. 18, pp. 3222–3230, Sep. 2017, doi: 10.1242/jeb.162941.
- [7] H. H. Thoen, M. J. How, T.-H. Chiou, and J. Marshall, “A Different Form of Color Vision in Mantis Shrimp,” *Science*, vol. 343, no. 6169, pp. 411–413, Jan. 2014, doi: 10.1126/science.1245824.
- [8] M. J. Bok, N. W. Roberts, and T. W. Cronin, “Behavioural evidence for polychromatic ultraviolet sensitivity in mantis shrimp,” *Proc. R. Soc. B.*, vol. 285, no. 1884, p. 20181384, Aug. 2018, doi: 10.1098/rspb.2018.1384.
- [9] S. Amini *et al.*, “Textured fluorapatite bonded to calcium sulphate strengthen stomatopod raptorial appendages,” *Nature Communications*, vol. 5, p. 3187, Jan. 2014, doi: 10.1038/ncomms4187.
- [10] M. A. Meyers, P.-Y. Chen, A. Y.-M. Lin, and Y. Seki, “Biological materials: Structure and mechanical properties,” *Progress in Materials Science*, vol. 53, no. 1, pp. 1–206, Jan. 2008, doi: 10.1016/j.pmatsci.2007.05.002.
- [11] J. W. C. Dunlop and P. Fratzl, “Biological Composites,” *Annu. Rev. Mater. Res.*, vol. 40, no. 1, pp. 1–24, Jun. 2010, doi: 10.1146/annurev-matsci-070909-104421.
- [12] U. G. K. Wegst, H. Bai, E. Saiz, A. P. Tomsia, and R. O. Ritchie, “Bioinspired structural materials,” *Nature Mater*, vol. 14, no. 1, pp. 23–36, Jan. 2015, doi: 10.1038/nmat4089.
- [13] P.-Y. Chen, J. McKittrick, and M. A. Meyers, “Biological materials: Functional adaptations and bioinspired designs,” *Progress in Materials Science*, vol. 57, no. 8, pp. 1492–1704, Nov. 2012, doi: 10.1016/j.pmatsci.2012.03.001.
- [14] A. Wat *et al.*, “Bioinspired nacre-like alumina with a bulk-metallic glass-forming alloy as a compliant phase,” *Nat Commun*, vol. 10, no. 1, p. 961, Dec. 2019, doi: 10.1038/s41467-019-08753-6.
- [15] S. N. Patek, W. L. Korff, and R. L. Caldwell, “Deadly strike mechanism of a mantis shrimp,” *Nature*, vol. 428, p. 819, Apr. 2004, doi: <http://dx.doi.org/10.1038/428819a>.
- [16] S. N. Patek, “Extreme impact and cavitation forces of a biological hammer: strike forces of the peacock mantis shrimp *Odontodactylus scyllarus*,” *Journal of Experimental Biology*, vol. 208, no. 19, pp. 3655–3664, Oct. 2005, doi: 10.1242/jeb.01831.
- [17] M. R. Abir, T. E. Tay, and H. P. Lee, “On the improved ballistic performance of bio-inspired composites,” *Composites Part A: Applied Science and*

- Manufacturing*, vol. 123, pp. 59–70, Aug. 2019, doi: 10.1016/j.compositesa.2019.04.021.
- [18] D. Ginzburg, F. Pinto, O. Iervolino, and M. Meo, “Damage tolerance of bio-inspired helicoidal composites under low velocity impact,” *Composite Structures*, vol. 161, pp. 187–203, Feb. 2017, doi: 10.1016/j.compstruct.2016.10.097.
- [19] L. K. Grunenfelder *et al.*, “Bio-inspired impact-resistant composites,” *Acta Biomaterialia*, vol. 10, no. 9, pp. 3997–4008, Sep. 2014, doi: 10.1016/j.actbio.2014.03.022.
- [20] Q. Han *et al.*, “Study on mechanical properties of multi-structure dactyl-inspired sandwich honeycomb with basalt fiber,” *Composite Structures*, vol. 247, p. 112467, Sep. 2020, doi: 10.1016/j.compstruct.2020.112467.
- [21] L. Mencattelli and S. T. Pinho, “Realising bio-inspired impact damage-tolerant thin-ply CFRP Bouligand structures via promoting diffused sub-critical helicoidal damage,” *Composites Science and Technology*, vol. 182, p. 107684, Sep. 2019, doi: 10.1016/j.compscitech.2019.107684.
- [22] L. Mencattelli and S. T. Pinho, “Herringbone-Bouligand CFRP structures: A new tailorable damage-tolerant solution for damage containment and reduced delaminations,” *Composites Science and Technology*, vol. 190, p. 108047, Apr. 2020, doi: 10.1016/j.compscitech.2020.108047.
- [23] M. Mroz, J. L. Rosenberg, C. Acevedo, J. J. Kruzic, B. Raeymaekers, and S. E. Naleway, “Ultrasound freeze-casting of a biomimetic layered microstructure in epoxy-ceramic composite materials to increase strength and hardness,” *Materialia*, vol. 12, p. 100754, Aug. 2020, doi: 10.1016/j.mtla.2020.100754.
- [24] B. Natarajan and J. W. Gilman, “Bioinspired Bouligand cellulose nanocrystal composites: a review of mechanical properties,” *Phil. Trans. R. Soc. A.*, vol. 376, no. 2112, p. 20170050, Feb. 2018, doi: 10.1098/rsta.2017.0050.
- [25] L. Ren *et al.*, “3D magnetic printing of bio-inspired composites with tunable mechanical properties,” *J Mater Sci*, vol. 53, no. 20, pp. 14274–14286, Oct. 2018, doi: 10.1007/s10853-018-2447-5.
- [26] D. Wang, A. Zaheri, B. Russell, H. Espinosa, and P. Zavattieri, “Fiber reorientation in hybrid helicoidal composites,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 110, p. 103914, Oct. 2020, doi: 10.1016/j.jmbbm.2020.103914.

- [27] K. Wu *et al.*, “Discontinuous fibrous Bouligand architecture enabling formidable fracture resistance with crack orientation insensitivity,” *Proc Natl Acad Sci USA*, vol. 117, no. 27, pp. 15465–15472, Jul. 2020, doi: 10.1073/pnas.2000639117.
- [28] X. Yang, J. Ma, Y. Shi, Y. Sun, and J. Yang, “Crashworthiness investigation of the bio-inspired bi-directionally corrugated core sandwich panel under quasi-static crushing load,” *Materials & Design*, vol. 135, pp. 275–290, Dec. 2017, doi: 10.1016/j.matdes.2017.09.040.
- [29] S. N. Patek, M. V. Rosario, and J. R. A. Taylor, “Comparative spring mechanics in mantis shrimp,” *Journal of Experimental Biology*, vol. 216, no. 7, pp. 1317–1329, Apr. 2013, doi: 10.1242/jeb.078998.
- [30] M. Tadayon, S. Amini, A. Masic, and A. Miserez, “The Mantis Shrimp Saddle: A Biological Spring Combining Stiffness and Flexibility,” *Adv. Funct. Mater.*, vol. 25, no. 41, pp. 6437–6447, Nov. 2015, doi: 10.1002/adfm.201502987.
- [31] P. A. Green and S. N. Patek, “Mutual assessment during ritualized fighting in mantis shrimp (Stomatopoda),” *Proc. R. Soc. B.*, vol. 285, no. 1871, p. 20172542, Jan. 2018, doi: 10.1098/rspb.2017.2542.
- [32] J. R. A. Taylor and S. N. Patek, “Ritualized fighting and biological armor: the impact mechanics of the mantis shrimp’s telson,” *Journal of Experimental Biology*, vol. 213, no. 20, pp. 3496–3504, Oct. 2010, doi: 10.1242/jeb.047233.
- [33] N. A. Yaraghi *et al.*, “The Stomatopod Telson: Convergent Evolution in the Development of a Biological Shield,” *Adv. Funct. Mater.*, vol. 29, no. 34, p. 1902238, Aug. 2019, doi: 10.1002/adfm.201902238.
- [34] J. C. Weaver *et al.*, “The Stomatopod Dactyl Club: A Formidable Damage-Tolerant Biological Hammer,” *Science*, vol. 336, no. 6086, p. 1275, Jun. 2012, doi: 10.1126/science.1218764.
- [35] J. Enax, O. Prymak, D. Raabe, and M. Epple, “Structure, composition, and mechanical properties of shark teeth,” *Journal of Structural Biology*, vol. 178, no. 3, pp. 290–299, Jun. 2012, doi: 10.1016/j.jsb.2012.03.012.
- [36] E. C. Moreno, M. Kresak, and R. T. Zahradnik, “Fluoridated Hydroxyapatite Solubility and Caries Formation,” *Nature*, vol. 247, no. 5435, pp. 64–65, Jan. 1974, doi: 10.1038/247064a0.
- [37] T. Aoba, “The Effect of Fluoride On Apatite Structure and Growth,” *Critical Reviews in Oral Biology & Medicine*, vol. 8, no. 2, pp. 136–153, Apr. 1997, doi: 10.1177/10454411970080020301.

- [38] E. Loste, R. M. Wilson, R. Seshadri, and F. C. Meldrum, “The role of magnesium in stabilising amorphous calcium carbonate and controlling calcite morphologies,” *Journal of Crystal Growth*, vol. 254, no. 1–2, pp. 206–218, Jun. 2003, doi: 10.1016/S0022-0248(03)01153-9.
- [39] W. Huang *et al.*, “Multiscale Toughening Mechanisms in Biological Materials and Bioinspired Designs,” *Adv. Mater.*, vol. 31, no. 43, p. 1901561, Oct. 2019, doi: 10.1002/adma.201901561.
- [40] N. H. Daraghmeh, B. Z. Chowdhry, S. A. Leharne, M. M. Al Omari, and A. A. Badwan, “Chitin,” in *Profiles of Drug Substances, Excipients and Related Methodology*, vol. 36, Elsevier, 2011, pp. 35–102.
- [41] R. Minke and J. Blackwell, “The structure of α -chitin,” *Journal of Molecular Biology*, vol. 120, no. 2, pp. 167–181, Apr. 1978, doi: 10.1016/0022-2836(78)90063-3.
- [42] M.-M. Giraud-Guille, “Fine structure of the chitin-protein system in the crab cuticle,” *Tissue and Cell*, vol. 16, no. 1, pp. 75–92, Jan. 1984, doi: 10.1016/0040-8166(84)90020-X.
- [43] Huajian Gao, Baohua Ji, Ingomar L. Jäger, Eduard Arzt, and Peter Fratzl, “Materials become insensitive to flaws at nanoscale: Lessons from nature,” *Biomaterials*, vol. 23, pp. 1873–1882, Mar. 2003.
- [44] M.-M. Giraud-Guille, “Plywood structures in nature,” *Current Opinion in Solid State and Materials Science*, vol. 3, no. 3, pp. 221–227, Jun. 1998, doi: 10.1016/S1359-0286(98)80094-6.
- [45] Y. Bouligand, “Twisted fibrous arrangements in biological materials and cholesteric mesophases,” *Tissue and Cell*, vol. 4, no. 2, pp. 189–217, Jan. 1972, doi: 10.1016/S0040-8166(72)80042-9.
- [46] T. Apichattrabrut and K. Ravi-Chandar, “Helicoidal Composites,” *Mechanics of Advanced Materials and Structures*, vol. 13, no. 1, pp. 61–76, Jan. 2006, doi: 10.1080/15376490500343808.
- [47] N. Suksangpanya, N. A. Yaraghi, D. Kisailus, and P. Zavattieri, “Twisting cracks in Bouligand structures,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 76, pp. 38–57, Dec. 2017, doi: 10.1016/j.jmbbm.2017.06.010.
- [48] P. Fratzl, H. S. Gupta, F. D. Fischer, and O. Kolednik, “Hindered Crack Propagation in Materials with Periodically Varying Young’s Modulus—Lessons

- from Biological Materials,” *Adv. Mater.*, vol. 19, no. 18, pp. 2657–2661, Sep. 2007, doi: 10.1002/adma.200602394.
- [49] S. Amini, M. Tadayon, S. Idapalapati, and A. Miserez, “The role of quasi-plasticity in the extreme contact damage tolerance of the stomatopod dactyl club,” *Nat Mater*, vol. 14, no. 9, pp. 943–950, Sep. 2015, doi: <http://dx.doi.org/10.1038/nmat4309>.
- [50] N. A. Yaraghi *et al.*, “A Sinusoidally Architected Helicoidal Biocomposite,” *Adv. Mater.*, vol. 28, no. 32, pp. 6835–6844, Aug. 2016, doi: 10.1002/adma.201600786.
- [51] L. K. Grunenfelder *et al.*, “Stress and Damage Mitigation from Oriented Nanostructures within the Radular Teeth of *Cryptochiton stelleri*,” *Adv. Funct. Mater.*, vol. 24, no. 39, pp. 6093–6104, Oct. 2014, doi: 10.1002/adfm.201401091.
- [52] W. Huang *et al.*, “A natural impact-resistant bicontinuous composite nanoparticle coating,” *Nature Materials*, Aug. 2020, doi: 10.1038/s41563-020-0768-7.
- [53] B. Viswanath, R. Raghavan, U. Ramamurty, and N. Ravishankar, “Mechanical properties and anisotropy in hydroxyapatite single crystals,” *Scripta Materialia*, vol. 57, no. 4, pp. 361–364, Aug. 2007, doi: 10.1016/j.scriptamat.2007.04.027.
- [54] A. Miserez, T. Schneberk, C. Sun, F. W. Zok, and J. H. Waite, “The Transition from Stiff to Compliant Materials in Squid Beaks,” *Science*, vol. 319, no. 5871, pp. 1816–1819, Mar. 2008, doi: 10.1126/science.1154117.
- [55] M. I. Hussein, M. J. Leamy, and M. Ruzzene, “Dynamics of Phononic Materials and Structures: Historical Origins, Recent Progress, and Future Outlook,” *Applied Mechanics Reviews*, vol. 66, no. 4, p. 040802, Jul. 2014, doi: 10.1115/1.4026911.
- [56] N. Guarán-Zapata, J. Gomez, N. Yaraghi, D. Kisailus, and P. D. Zavattieri, “Shear wave filtering in naturally-occurring Bouligand structures,” *Acta Biomaterialia*, vol. 23, pp. 11–20, Sep. 2015, doi: 10.1016/j.actbio.2015.04.039.
- [57] William D. Callister, *Material Science and Engineering: An Introduction*, 7th ed. John Wiley & Sons, Inc., 2007.
- [58] P. Ostojic and R. McPherson, “A review of indentation fracture theory: its development, principles and limitations,” *Int J Fract*, vol. 33, no. 4, pp. 297–312, Apr. 1987, doi: 10.1007/BF00044418.

- [59] A. C. Fischer-Cripps, *Nanoindentation*, 3rd ed. New York, NY: Springer New York, 2011.
- [60] W. C. Oliver and G. M. Pharr, “An improved technique for determining hardness and elastic modulus using load and displacement sensing indentation experiments,” *J. Mater. Res.*, vol. 7, no. 6, pp. 1564–1583, Jun. 1992, doi: 10.1557/JMR.1992.1564.
- [61] L. H. He and M. V. Swain, “Nanoindentation derived stress–strain properties of dental materials,” *Dental Materials*, vol. 23, no. 7, pp. 814–821, Jul. 2007, doi: 10.1016/j.dental.2006.06.017.
- [62] L. H. He, N. Fujisawa, and M. V. Swain, “Elastic modulus and stress–strain response of human enamel by nano-indentation,” *Biomaterials*, vol. 27, no. 24, pp. 4388–4398, Aug. 2006, doi: 10.1016/j.biomaterials.2006.03.045.
- [63] T. L. Anderson, *Fracture mechanics: fundamentals and applications*, 2nd ed. Boca Raton: CRC Press, 1995.
- [64] A. A. Griffith, “The phenomena of rupture and flow in solids,” *Phil. Trans. R. Soc. Lond. A*, vol. 221, no. 582–593, pp. 163–198, 1920, doi: 10.1098/rsta.1921.0006.
- [65] G. R. Irwin, “Onset of Fast Crack Propagation in High Strength Steel and Aluminum Alloys,” *Sagamore Research Conference Proceedings*, vol. 2, pp. 289–305, 1956.
- [66] G. R. Irwin, “Analysis of Stresses and Strains near the End of a Crack Traversing a Plate,” *Journal of Applied Mechanics*, vol. 24, pp. 361–364, 1957.
- [67] I. N. Sneddon, “The Distribution of Stress in the Neighbourhood of a Crack in an Elastic Solid,” *Proceedings Royal Society of London*, vol. A-187, pp. 229–260, 1946.
- [68] H. M. Westergaard, “Bearing Pressures and Cracks,” *Journal of Applied Mechanics*, vol. 6, pp. 49–53, 1939.
- [69] M. L. Williams, “On the Stress Distribution at the Base of a Stationary Crack,” *Journal of Applied Mechanics*, vol. 24, pp. 109–114, 1957.
- [70] B. R. Lawn, *Fracture of brittle solids*, 2nd ed. Cambridge; New York: Cambridge University Press, 1993.
- [71] “Standard Test Method for Linear-Elastic Plane-Strain Fracture Toughness of Metallic Materials,” *ASTM E399-17*, Feb. 2018.

- [72] J. R. Rice, “A Path Independent Integral and the Approximate Analysis of Strain Concentration by Notches and Cracks,” *Journal of Applied Mechanics*, vol. 35, no. 2, pp. 379–386, Jun. 1968, doi: 10.1115/1.3601206.
- [73] J. W. Hutchinson, “Singular behaviour at the end of a tensile crack in a hardening material,” *Journal of the Mechanics and Physics of Solids*, vol. 16, no. 1, pp. 13–31, Jan. 1968, doi: 10.1016/0022-5096(68)90014-8.
- [74] J. R. Rice and G. F. Rosengren, “Plane strain deformation near a crack tip in a power-law hardening material,” *Journal of the Mechanics and Physics of Solids*, vol. 16, no. 1, pp. 1–12, Jan. 1968, doi: 10.1016/0022-5096(68)90013-6.
- [75] “Standard Test Method for Measurement of Fracture Toughness,” *ASTM E1820-20*, Jan. 2020.
- [76] M. E. Launey, M. J. Buehler, and R. O. Ritchie, “On the Mechanistic Origins of Toughness in Bone,” *Annu. Rev. Mater. Res.*, vol. 40, no. 1, pp. 25–53, Jun. 2010, doi: 10.1146/annurev-matsci-070909-104427.
- [77] A. G. Evans and E. A. Charles, “Fracture Toughness Determinations by Indentation,” *J American Ceramic Society*, vol. 59, no. 7–8, pp. 371–372, Jul. 1976, doi: 10.1111/j.1151-2916.1976.tb10991.x.
- [78] B. R. Lawn, A. G. Evans, and D. B. Marshall, “Elastic/Plastic Indentation Damage in Ceramics: The Median/Radial Crack System,” *J American Ceramic Society*, vol. 63, no. 9–10, pp. 574–581, Sep. 1980, doi: 10.1111/j.1151-2916.1980.tb10768.x.
- [79] B. R. Lawn and A. G. Evans, “A model for crack initiation in elastic/plastic indentation fields,” *J Mater Sci*, vol. 12, no. 11, pp. 2195–2199, Nov. 1977, doi: 10.1007/BF00552240.
- [80] B. Lawn and R. Wilshaw, “Indentation fracture: principles and applications,” *J Mater Sci*, vol. 10, no. 6, pp. 1049–1081, Jun. 1975, doi: 10.1007/BF00823224.
- [81] D. B. Marshall, B. R. Lawn, and P. Chantikul, “Residual stress effects in sharp contact cracking: Part 2 Strength degradation,” *J Mater Sci*, vol. 14, no. 9, pp. 2225–2235, Sep. 1979, doi: 10.1007/BF00688429.
- [82] J. J. Kruzic, D. K. Kim, K. J. Koester, and R. O. Ritchie, “Indentation techniques for evaluating the fracture toughness of biomaterials and hard tissues,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 2, no. 4, pp. 384–395, Aug. 2009, doi: 10.1016/j.jmbbm.2008.10.008.

- [83] G. R. Anstis, P. Chantikul, B. R. Lawn, and D. B. Marshall, “A Critical Evaluation of Indentation Techniques for Measuring Fracture Toughness: I, Direct Crack Measurements,” *J American Ceramic Society*, vol. 64, no. 9, pp. 533–538, Sep. 1981, doi: 10.1111/j.1151-2916.1981.tb10320.x.
- [84] K. K. Chawla and M. A. Meyers, Eds., “Fracture Testing,” in *Mechanical Behavior of Materials*, 2nd ed., Cambridge: Cambridge University Press, 2008, pp. 525–557.
- [85] R. F. Cook and G. M. Pharr, “Direct Observation and Analysis of Indentation Cracking in Glasses and Ceramics,” *J American Ceramic Society*, vol. 73, no. 4, pp. 787–817, Apr. 1990, doi: 10.1111/j.1151-2916.1990.tb05119.x.
- [86] P. F. Cesar *et al.*, “ADM guidance—Ceramics: Fracture toughness testing and method selection,” *Dental Materials*, vol. 33, no. 6, pp. 575–584, Jun. 2017, doi: 10.1016/j.dental.2017.03.006.
- [87] A. Miserez *et al.*, “Effects of Laminate Architecture on Fracture Resistance of Sponge Biosilica: Lessons from Nature,” *Advanced Functional Materials*, vol. 18, no. 8, pp. 1241–1248, Apr. 2008, doi: 10.1002/adfm.200701135.
- [88] Y. L. Chan, A. H. W. Ngan, and N. M. King, “Use of focused ion beam milling for investigating the mechanical properties of biological tissues: A study of human primary molars,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 2, no. 4, pp. 375–383, Aug. 2009, doi: 10.1016/j.jmbbm.2009.01.006.
- [89] A. Ghosh, Z. Li, C. Henager, A. Kobayashi, and R. C. Bradt, “Vickers microtoughness of SiC in the median-crack regime,” in *Fracture Mechanics of Ceramics*, vol. 11, Karlsruhe, Germany: Plenum Press, New York, 1996, pp. 219–231.
- [90] Z. Li, A. Ghosh, A. S. Kobayashi, and R. C. Bradt, “Indentation Fracture Toughness of Sintered Silicon Carbide in the Palmqvist Crack Regime,” *J American Ceramic Society*, vol. 72, no. 6, pp. 904–911, Jun. 1989, doi: 10.1111/j.1151-2916.1989.tb06242.x.
- [91] C. B. Ponton and R. D. Rawlings, “Vickers indentation fracture toughness test Part 2 Application and critical evaluation of standardised indentation toughness equations,” *Materials Science and Technology*, vol. 5, no. 10, pp. 961–976, Oct. 1989, doi: 10.1179/mst.1989.5.10.961.

- [92] G. D. Quinn and R. C. Bradt, “On the Vickers Indentation Fracture Toughness Test,” *J American Ceramic Society*, vol. 90, no. 3, pp. 673–680, Mar. 2007, doi: 10.1111/j.1551-2916.2006.01482.x.
- [93] L. P. Mullins, M. S. Bruzzi, and P. E. McHugh, “Measurement of the microstructural fracture toughness of cortical bone using indentation fracture,” *Journal of Biomechanics*, vol. 40, no. 14, pp. 3285–3288, Jan. 2007, doi: 10.1016/j.jbiomech.2007.04.020.
- [94] T. Fett, “Computation of the crack opening displacements for Vickers indentation cracks,” Institute for materials research, Karlsruhe, Germany, FZKA-6757, 2002. [Online]. Available: http://inis.iaea.org/search/search.aspx?orig_q=RN:34001846.
- [95] T. Fett, A. B. Kouna Njiwa, and J. Rödel, “Crack opening displacements of Vickers indentation cracks,” *Engineering Fracture Mechanics*, vol. 72, no. 5, pp. 647–659, Mar. 2005, doi: 10.1016/j.engfracmech.2004.07.003.
- [96] Z. Burghard, A. Zimmermann, J. Rödel, F. Aldinger, and B. R. Lawn, “Crack opening profiles of indentation cracks in normal and anomalous glasses,” *Acta Materialia*, vol. 52, no. 2, pp. 293–297, Jan. 2004, doi: 10.1016/j.actamat.2003.09.014.
- [97] J. J. Kruzic and R. O. Ritchie, “Determining the Toughness of Ceramics from Vickers Indentations Using the Crack-Opening Displacements: An Experimental Study,” *Journal of the American Ceramic Society*, vol. 86, no. 8, pp. 1433–1436, Aug. 2003, doi: 10.1111/j.1151-2916.2003.tb03490.x.
- [98] “Standard Test Methods for Determination of Fracture Toughness of Advanced Ceramics at Ambient Temperature,” *ASTM C1421-18*, Jan. 2018.
- [99] M. G. Mueller, V. Pejchal, G. Žagar, A. Singh, M. Cantoni, and A. Mortensen, “Fracture toughness testing of nanocrystalline alumina and fused quartz using chevron-notched microbeams,” *Acta Materialia*, vol. 86, pp. 385–395, Jan. 2015, doi: 10.1016/j.actamat.2014.12.016.
- [100] G. Žagar, V. Pejchal, M. G. Mueller, L. Michelet, and A. Mortensen, “Fracture toughness measurement in fused quartz using triangular chevron-notched micro-cantilevers,” *Scripta Materialia*, vol. 112, pp. 132–135, Sep. 2015, doi: 10.1016/j.scriptamat.2015.09.032.

- [101] Z. Cui, “Nanofabrication by Ion Beam,” in *Nanofabrication: Principles, Capabilities and Limits*, 2nd ed. 2017., Cham: Springer International Publishing : Imprint: Springer, 2017, pp. 149–175.
- [102] M. H. F. Overwijk, “Novel scheme for the preparation of transmission electron microscopy specimens with a focused ion beam,” *J. Vac. Sci. Technol. B*, vol. 11, no. 6, p. 2021, Nov. 1993, doi: 10.1116/1.586537.
- [103] L. A. Giannuzzi and F. A. Stevie, Eds., “Ion - Solid Interactions,” in *Introduction to focused ion beams: instrumentation, theory, techniques, and practice*, New York: Springer, 2005, pp. 13–52.
- [104] L. Frey, C. Lehrer, and H. Ryssel, “Nanoscale effects in focused ion beam processing,” *Applied Physics A: Materials Science & Processing*, vol. 76, no. 7, pp. 1017–1023, May 2003, doi: 10.1007/s00339-002-1943-1.
- [105] D. Drobne, M. Milani, V. Lešer, and F. Tatti, “Surface damage induced by FIB milling and imaging of biological samples is controllable,” *Microsc. Res. Tech.*, vol. 70, no. 10, pp. 895–903, Oct. 2007, doi: 10.1002/jemt.20494.
- [106] B. W. Kempshall, L. A. Giannuzzi, B. I. Prenitzer, F. A. Stevie, and S. X. Da, “Comparative evaluation of protective coatings and focused ion beam chemical vapor deposition processes,” *J. Vac. Sci. Technol. B*, vol. 20, no. 1, p. 286, 2002, doi: 10.1116/1.1445165.
- [107] D. Di Maio and S. G. Roberts, “Measuring fracture toughness of coatings using focused-ion-beam-machined microbeams,” *J. Mater. Res.*, vol. 20, no. 2, pp. 299–302, Feb. 2005, doi: 10.1557/JMR.2005.0048.
- [108] J. McCarthy, Z. Pei, M. Becker, and D. Atteridge, “FIB micromachined submicron thickness cantilevers for the study of thin film properties,” *Thin Solid Films*, vol. 358, no. 1, pp. 146–151, Jan. 2000, doi: 10.1016/S0040-6090(99)00680-X.
- [109] K. Minoshima, T. Terada, and K. Komai, “Influence of nanometre-sized notch and water on the fracture behaviour of single crystal silicon microelements,” *Fatigue & Fracture of Engineering Materials & Structures*, vol. 23, no. 12, pp. 33–104, 2000, doi: <https://doi.org/10.1111/j.1460-2695.2000.00354.x>.
- [110] Y. L. Chan, A. H. W. Ngan, and N. M. King, “Nano-scale structure and mechanical properties of the human dentine–enamel junction,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 4, no. 5, pp. 785–795, Jul. 2011, doi: 10.1016/j.jmbbm.2010.09.003.

- [111] D. Lu and A. H. Barber, “Optimized nanoscale composite behaviour in limpet teeth,” *Journal of The Royal Society Interface*, vol. 9, no. 71, pp. 1318–1324, Jun. 2012, doi: 10.1098/rsif.2011.0688.
- [112] S. Bechtle *et al.*, “A method to determine site-specific, anisotropic fracture toughness in biological materials,” *Scripta Materialia*, vol. 66, no. 8, pp. 515–518, Apr. 2012, doi: 10.1016/j.scriptamat.2011.12.027.
- [113] I. Jimenez-Palomar, A. Shipov, R. Shahar, and A. H. Barber, “Mechanical Behavior of Osteoporotic Bone at Sub-Lamellar Length Scales,” *Frontiers in Materials*, vol. 2, p. 9, 2015, doi: 10.3389/fmats.2015.00009.
- [114] I. Jimenez-Palomar, A. Shipov, R. Shahar, and A. H. Barber, “Structural orientation dependent sub-lamellar bone mechanics,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 52, pp. 63–71, Dec. 2015, doi: 10.1016/j.jmbbm.2015.02.031.
- [115] H.-T. Lee *et al.*, “Site-specific characterization of beetle horn shell with micromechanical bending test in focused ion beam system,” *Acta Biomaterialia*, vol. 57, pp. 395–403, Jul. 2017, doi: 10.1016/j.actbio.2017.04.026.
- [116] J. Liu, Y. Xu, H. Yang, Y. Liu, P. KDV. Yarlagaadda, and C. Yan, “Investigation of failure mechanisms of nacre at macro and nano scales,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 112, p. 104018, Dec. 2020, doi: 10.1016/j.jmbbm.2020.104018.
- [117] S. Brinckmann, C. Kirchlechner, and G. Dehm, “Stress intensity factor dependence on anisotropy and geometry during micro-fracture experiments,” *Scripta Materialia*, vol. 127, pp. 76–78, Jan. 2017, doi: 10.1016/j.scriptamat.2016.08.027.
- [118] D. E. J. Armstrong, A. J. Wilkinson, and S. G. Roberts, “Measuring anisotropy in Young’s modulus of copper using microcantilever testing,” *J. Mater. Res.*, vol. 24, no. 11, pp. 3268–3276, Nov. 2009, doi: 10.1557/jmr.2009.0396.
- [119] W. A. Sasangka, C. L. Gan, D. Lai, C. S. Tan, and C. V. Thompson, “Characterization of the Young’s modulus, residual stress and fracture strength of Cu–Sn–In thin films using combinatorial deposition and micro-cantilevers,” *J. Micromech. Microeng.*, vol. 25, no. 3, p. 035023, Mar. 2015, doi: 10.1088/0960-1317/25/3/035023.

- [120] D. Taylor, “Measuring fracture toughness in biological materials,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 77, pp. 776–782, Jan. 2018, doi: 10.1016/j.jmbbm.2017.07.007.
- [121] K. Matoy, T. Detzel, M. Müller, C. Motz, and G. Dehm, “Interface fracture properties of thin films studied by using the micro-cantilever deflection technique,” *Surface and Coatings Technology*, vol. 204, no. 6–7, pp. 878–881, Dec. 2009, doi: 10.1016/j.surfcoat.2009.09.013.
- [122] J. Schaufler, C. Schmid, K. Durst, and M. Göken, “Determination of the interfacial strength and fracture toughness of a-C:H coatings by in-situ microcantilever bending,” *Thin Solid Films*, vol. 522, pp. 480–484, Nov. 2012, doi: 10.1016/j.tsf.2012.08.031.
- [123] H. G. Tattersall and G. Tappin, “The work of fracture and its measurement in metals, ceramics and other materials,” *J Mater Sci*, vol. 1, no. 3, pp. 296–301, Aug. 1966, doi: 10.1007/BF00550177.
- [124] P. A. Withey, R. L. Brett, and P. Bowen, “Use of chevron notches for fracture toughness determination in brittle solids,” *Materials Science and Technology*, vol. 8, no. 9, pp. 805–810, Sep. 1992, doi: 10.1179/mst.1992.8.9.805.
- [125] J. Ast, T. Przybilla, V. Maier, K. Durst, and M. Göken, “Microcantilever bending experiments in NiAl – Evaluation, size effects, and crack tip plasticity,” *J. Mater. Res.*, vol. 29, no. 18, pp. 2129–2140, Sep. 2014, doi: 10.1557/jmr.2014.240.
- [126] J. Ast, B. Merle, K. Durst, and M. Göken, “Fracture toughness evaluation of NiAl single crystals by microcantilevers—a new continuous J-integral method,” *J. Mater. Res.*, vol. 31, no. 23, pp. 3786–3794, Dec. 2016, doi: 10.1557/jmr.2016.393.
- [127] S. Wurster, C. Motz, and R. Pippan, “Characterization of the fracture toughness of micro-sized tungsten single crystal notched specimens,” *Philosophical Magazine*, vol. 92, no. 14, pp. 1803–1825, May 2012, doi: 10.1080/14786435.2012.658449.
- [128] G. Bergmann and H. Vehoff, “Effect of environment on the brittle-to-ductile transition of pre-cracked NiAl single and polycrystals,” *Materials Science and Engineering: A*, vol. 192–193, pp. 309–315, Feb. 1995, doi: 10.1016/0921-5093(95)80018-2.
- [129] C. Bohnert, N. J. Schmitt, S. M. Weygand, O. Kraft, and R. Schwaiger, “Fracture toughness characterization of single-crystalline tungsten using notched micro-

- cantilever specimens,” *International Journal of Plasticity*, vol. 81, pp. 1–17, Jun. 2016, doi: 10.1016/j.ijplas.2016.01.014.
- [130] J. Riedle, P. Gumbsch, and H. F. Fischmeister, “Cleavage Anisotropy in Tungsten Single Crystals,” *Phys. Rev. Lett.*, vol. 76, no. 19, pp. 3594–3597, May 1996, doi: 10.1103/PhysRevLett.76.3594.
- [131] B.-S. Li, T. J. Marrow, S. G. Roberts, and D. E. J. Armstrong, “Evaluation of Fracture Toughness Measurements Using Chevron-Notched Silicon and Tungsten Microcantilevers,” *JOM*, vol. 71, no. 10, pp. 3378–3389, 2019, doi: 10.1007/s11837-019-03696-1.
- [132] B. Dean and B. Bhushan, “Shark-skin surfaces for fluid-drag reduction in turbulent flow: a review,” *Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences*, vol. 368, no. 1929, pp. 4775–4806, 2010, doi: 10.1098/rsta.2010.0201.
- [133] A. Kesel and R. Liedert, “Learning from nature: Non-toxic biofouling control by shark skin effect,” *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, vol. 146, no. 4, p. S130, Apr. 2007, doi: 10.1016/j.cbpa.2007.01.246.
- [134] P.-Y. Chen, A. Y.-M. Lin, J. McKittrick, and M. A. Meyers, “Structure and mechanical properties of crab exoskeletons,” *Acta Biomaterialia*, vol. 4, no. 3, pp. 587–596, May 2008, doi: 10.1016/j.actbio.2007.12.010.
- [135] S. Amini *et al.*, “A diecast mineralization process forms the tough mantis shrimp dactyl club,” *Proc Natl Acad Sci USA*, vol. 116, no. 18, pp. 8685–8692, Apr. 2019, doi: 10.1073/pnas.1816835116.
- [136] A. G. Fincham, W. Luo, J. Moradian-Oldak, M. L. Paine, M. L. Snead, and M. Zeichner-David, “Enamel biomineralization: the assembly and disassembly of the protein extracellular organic matrix,” in *Development, Function and Evolution of Teeth*, M. F. Teaford, M. W. J. Ferguson, and M. Meredith Smith, Eds. Cambridge: Cambridge University Press, 2000, pp. 37–62.
- [137] P. Fratzl, N. Fratzl-Zelman, K. Klaushofer, G. Vogl, and K. Koller, “Nucleation and growth of mineral crystals in bone studied by small-angle X-ray scattering,” *Calcif Tissue Int*, vol. 48, no. 6, pp. 407–413, Jun. 1991, doi: 10.1007/BF02556454.

- [138] A. George and A. Veis, "Phosphorylated Proteins and Control over Apatite Nucleation, Crystal Growth, and Inhibition," *Chem. Rev.*, vol. 108, no. 11, pp. 4670–4693, Nov. 2008, doi: 10.1021/cr0782729.
- [139] J. Sodek, B. Ganss, and M. D. McKee, "Osteopontin," *Critical Reviews in Oral Biology & Medicine*, vol. 11, no. 3, pp. 279–303, Jul. 2000, doi: 10.1177/10454411000110030101.
- [140] A. Veis, "A Window on Biomineralization," *Science*, vol. 307, no. 5714, pp. 1419–1420, Mar. 2005, doi: 10.1126/science.1109440.
- [141] L. Glazer *et al.*, "A Protein Involved in the Assembly of an Extracellular Calcium Storage Matrix," *J Biol Chem*, vol. 285, no. 17, pp. 12831–12839, Apr. 2010, doi: 10.1074/jbc.M109.071068.
- [142] G. He *et al.*, "Phosphorylation of phosphophoryn is crucial for its function as a mediator of biomineralization," *J Biol Chem*, vol. 280, no. 39, pp. 33109–33114, Sep. 2005, doi: 10.1074/jbc.M500159200.
- [143] C. Holt, N. M. Wahlgren, and T. Drakenberg, "Ability of a beta-casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters," *Biochem J*, vol. 314 (Pt 3), pp. 1035–1039, Mar. 1996, doi: 10.1042/bj3141035.
- [144] K. Adachi, H. Endo, T. Watanabe, T. Nishioka, and T. Hirata, "Hemocyanin in the exoskeleton of crustaceans: enzymatic properties and immunolocalization," *Pigment Cell Res*, vol. 18, no. 2, pp. 136–143, Apr. 2005, doi: 10.1111/j.1600-0749.2005.00217.x.
- [145] L. Glazer *et al.*, "Hemocyanin with phenoloxidase activity in the chitin matrix of the crayfish gastrolith," *J Exp Biol*, vol. 216, no. Pt 10, pp. 1898–1904, May 2013, doi: 10.1242/jeb.080945.
- [146] S. Omelon and W. Habraken, "Influence of Condensed Phosphates on the Physical Chemistry of Calcium Phosphate Solids," in *Inorganic Polyphosphates in Eukaryotic Cells*, T. Kulakovskaya, E. Pavlov, and E. N. Dedkova, Eds. Cham: Springer International Publishing, 2016, pp. 177–205.
- [147] A. Ziegler, H. Fabritius, and M. Hagedorn, "Microscopical and functional aspects of calcium-transport and deposition in terrestrial isopods," *Micron*, vol. 36, no. 2, pp. 137–153, Feb. 2005, doi: 10.1016/j.micron.2004.09.006.

- [148] C. Giannini *et al.*, “Scanning SAXS–WAXS microscopy on osteoarthritis-affected bone – an age-related study,” *J Appl Crystallogr*, vol. 47, no. 1, pp. 110–117, Feb. 2014, doi: 10.1107/S1600576713030215.
- [149] M. Liebi *et al.*, “Nanostructure surveys of macroscopic specimens by small-angle scattering tensor tomography,” *Nature*, vol. 527, no. 7578, pp. 349–352, Nov. 2015, doi: 10.1038/nature16056.
- [150] C. A. Stiffler *et al.*, “X-ray Linear Dichroism in Apatite,” *J. Am. Chem. Soc.*, vol. 140, no. 37, pp. 11698–11704, Sep. 2018, doi: 10.1021/jacs.8b05547.
- [151] F. Mastropietro *et al.*, “Revealing crystalline domains in a mollusc shell single-crystalline prism,” *Nature Mater*, vol. 16, no. 9, pp. 946–952, Sep. 2017, doi: 10.1038/nmat4937.
- [152] T. Sui *et al.*, “In situ monitoring and analysis of enamel demineralisation using synchrotron X-ray scattering,” *Acta Biomaterialia*, vol. 77, pp. 333–341, Sep. 2018, doi: 10.1016/j.actbio.2018.07.027.
- [153] M. E. Birkbak, M. Guizar-Sicairos, M. Holler, and H. Birkedal, “Internal structure of sponge glass fiber revealed by ptychographic nanotomography,” *Journal of Structural Biology*, vol. 194, no. 1, pp. 124–128, Apr. 2016, doi: 10.1016/j.jsb.2016.02.006.
- [154] M. A. Marcus *et al.*, “Parrotfish Teeth: Stiff Biominerals Whose Microstructure Makes Them Tough and Abrasion-Resistant To Bite Stony Corals,” *ACS Nano*, vol. 11, no. 12, pp. 11856–11865, Dec. 2017, doi: 10.1021/acsnano.7b05044.
- [155] S. Amini, M. Tadayan, J. Q. I. Chua, and A. Miserez, “Multi-scale structural design and biomechanics of the pistol shrimp snapper claw,” *Acta Biomaterialia*, vol. 73, pp. 449–457, Jun. 2018, doi: 10.1016/j.actbio.2018.04.038.
- [156] Y. Zhang *et al.*, “Towards in situ determination of 3D strain and reorientation in the interpenetrating nanofibre networks of cuticle,” *Nanoscale*, vol. 9, no. 31, pp. 11249–11260, 2017, doi: 10.1039/C7NR02139A.

Chapter 3

Experimental Methods

This chapter describe the design of experiments to study the stomatopod dactyl club. The purpose of this chapter is to provide the reader with the working principles of the experimental methods used in this thesis. The rationale for selecting these methods over others are given as well. The chapter begins with the methods used for the micro fracture toughness experiments like focused ion beam milling, followed by material characterization methods like scanning electron microscopy, Raman and FTIR spectroscopy and inductively coupled plasma techniques. Finally, methods used in the time study of the dactyl club growth and development are covered including for the X-ray measurements at synchrotrons: X-ray microdiffraction, X-ray fluorescence and ptychographic X-ray computed tomography; as well as the method used for proteomics study, parallel reaction monitoring.

3.1 Microscale fracture toughness testing

3.1.1 Chevron-notched microcantilever bending

Nanoindentation and more recently picondentation has been widely used to measure the mechanical properties of biological materials including the Stomatopod dactyl club [1]–[5]. However, the reliability of fracture toughness values by indentation methods have been in question [6], [7]. Conventional methods used in fracture toughness measurements require samples that are in the several millimeters and larger. This is not feasible for the dactyl club and many other biological materials which are around the same size or smaller. Furthermore, biological materials are known to have complex structures with different material properties at different length scales [8], [9]. Therefore, other methods are explored to measure the fracture toughness of biological materials at the microscale, which was covered in greater detail in the literature review. Presented here is a summary of the key reasons for the selection of chevron-notched microcantilevers for evaluating the fracture toughness of the impact region of the stomatopod dactyl club. To evaluate fracture toughness from a notched microcantilever, a load is applied on one end of the microcantilever. The stress concentration at the notch leads to the crack propagating through the notch until the cantilever is separated from the support. Load and displacement data are usually measured throughout the testing. With this configuration and considering mode I fracture, the fracture toughness of a material can be determined using linear-elastic fracture mechanics (LEFM) by evaluating the critical stress intensity factor (K_{Ic}), and with elastic-plastic fracture mechanics (EPFM) by evaluating the J -integral (J_{Ic}). The two parameters are related by the relationship $K = \sqrt{JE}$, where E is the elastic modulus of the material. When the material is linear-elastic J is the energy release rate of a propagating crack, G . And J is an extension of the energy method in evaluating fracture toughness originally introduced by Griffith [10]. Brittle and semi-brittle materials can be assessed with LEFM. On the other hand, materials that exhibit plastic behavior must be evaluated using EPFM. Figure 3.1 shows the two types of notch geometries that can be used for testing, namely straight and chevron notches. The advantage of using a chevron notch over a straight notch is that a sharp pre-crack does not need to be created, which is difficult for microcantilevers. Moreover, because the crack width extends with crack

depth, it is possible to achieve stable crack growth even in brittle materials, which leads to more accurate measurement of material fracture toughness. Another benefit of using chevron notches is that fracture occurs within the bulk of the material, a region unaffected by FIB damage that remains localized on the surface of the notch. The following sections cover the methods used to prepare, test, and analyze the data from chevron-notched microcantilevers.

Figure 3.1. Type of cantilever notches.

3.1.2 Sample preparation with focused ion beam (FIB)

3.1.2.1 Working principle and rationale

In FIB, a beam of charged ions is used to remove material from a sample surface [11]. The ions are accelerated onto the sample surface with electromagnets. When they collide with the surface several reactions can ensue. The ions can knock off the atoms of the surface in a process called ion sputtering, which is the desired effect. Other effects of ion collision include ion implantation and damage of surface structure like loss of crystallinity (amorphization). These are often undesirable effects because they modify the material properties at the surface of the milled sample. The depth of the modified surface or the damage zone is dependent on the accelerating voltage and probe current used [12]. At a higher accelerating voltage the colliding ion is more likely to be implanted than to sputter the sample, while at a lower accelerating voltage structure damage by heat is more likely to happen than sputtering. The material removal rate is proportional to the probe current used. However, higher probe currents lead to larger spot sizes and damage zones. In a typical FIB experiment, the accelerating voltage is

usually kept at 30 keV for cutting and trimming while the current is varied from high to low [13]. After the cutting and trimming a cleaning step with a low accelerating voltage and current (*e.g.* 2kV, 10 pA) is used to reduce the damage zone size. The damage zone created by FIB is on the order of 10 nm after finishing steps. This is much smaller compared to the approximately 2-6 μm damage zones created using femtosecond laser cutting [14], another technique that can be used to prepare small samples. Another method used to prepare tiny samples is photolithography. This involves the use of chemicals and light to pattern materials – usually semiconductor materials. Photolithography can produce extremely fine (around 5 nm) structures [15]. However, such techniques cannot be easily applied on biological materials, which have very complex hierarchical structures. Physical removal methods like FIB is more versatile and less sensitive to material type. Because of these advantages FIB has been extensively for the preparation of samples from a variety of materials for mechanical testing including biological materials [16]–[22]. Due to the reasons mentioned above, FIB was used to prepare microcantilevers for testing.

3.1.2.2 Limitations of FIB

However, there are several drawbacks to FIB. Firstly, the use of ions can lead to issues like sample charging and beam shifts. This happens due to the accumulation or loss of charge on the sample surface. Secondly, a high vacuum environment is required for FIB to minimize collisions between the ions and other molecules in the FIB chamber. These issues are especially important for biological materials because they are usually non-conductive, and they are almost exclusively found with water within. Hence, as mentioned in section 2.5.2, the sample should be coated with a conductive material to avoid charging, and for protection of the regions of interest. Fortunately, the impact region of the dactyl club studied mostly consists of mineral which is less sensitive to radiation damage and dehydration compared to organic material like cells. A third drawback of FIB is that it is time and labour intensive and by extension expensive. To address this issue the cutting process is optimized for the dactyl club and is covered in greater detail in the next section.

3.1.2.3 Instrumentation

The FIB used was the Zeiss Crossbeam 540X, a dual FIB-SEM system equipped with a Ga^+ source (Figure 3.2). The SEM module is useful for selecting the area to be cut and measuring the dimensions of the finished samples since electrons do not damage the sample surface as much as Ga^+ ions. The control software provided by Zeiss has a function to correct for the angle between the SEM and the surface of the sample so that images taken are upright and the dimensions measured from the images are accurate.

Figure 3.2. A) Zeiss Crossbeam 540X. B) Schematic of dual FIB-SEM setup.

3.1.2.4 Optimization of FIB milling microcantilevers

Use of the FIB can be very costly both in time and money. Hence, it is important to streamline the process for its use on the dactyl club. The objective was to reduce the cutting time to a maximum of 6 hours to increase the throughput of sample. Monte Carlo analysis is frequently used to estimate the material removal rate using data like the molecular weights of the element cut [12]. However, Monte Carlo analysis was not performed on the dactyl club. This is because the dactyl club has an extremely complex structure with new insights reported on a regular basis since this work has begun and finished [5]. Generating the model for the Monte Carlo analysis would not be an

efficient use of time. Instead a more rudimentary approach of measuring the depth after cutting a sample at a fixed current and time was adopted. An accelerating voltage of 30 kV was used for optimal cutting while the current was varied [13]. The maximum current allowable was determined by trial-and-error. A cut was performed using preset currents on the FIB from high current to low current. Beam shifts were observed with probe currents above 3 nA. The FIB beam is tapered [11]. To adjust for this, cutting angles were varied and it was found that an angle of 1.5 degrees compensated well for the beam taper. No formal inquiry was done to determine if the taper angle was accurate, but judgement was based on the operator. Cutting the chevron-notch was the most difficult step of the microcantilever preparation. Some of the cantilevers presented in Chapter 4 did not have fully developed chevron notches but had notches that were more similar to straight notches. There was no simple way to determine if the notches were chevron before breaking the cantilever and observing the cross-section at the notch. Figure 3.3 shows how trigonometry was used to estimate if the chevron notch was cut properly, the following sentences explains this in words. The thickness of the notch (b) from the top can be measured with the SEM and compared to the calculated thickness using trigonometry. If the notch was fully cut through, the lengths should be comparable. Another issue encountered when cutting the notch was beam drift. These led to a wider notch which is undesirable. Long cutting processes were shown to result in more beam drift. Hence, the notch cutting was split into many multiple lines of shallower cuts instead of a single deep cut. The optimal procedure used for cutting the microcantilever are shown in Table 3.1. The schematics in Figure 3.4 are provided to help readers understand the scanning modes and raster types in Table 3.1. However, because the material properties may vary at different locations, the exact procedure for cutting may change slightly. And the operator involvement is still necessary to fabricate the samples. With this procedure a single cantilever can be prepared in about 4.5 – 6 hours depending on whether many adjustments were necessary during the cutting process.

Figure 3.3. Chevron notch cutting optimization. One way to determine if the chevron is fully cut before testing is to measure the distance from the edge of the cantilever to the corner of the ligament (b') and compare it with the thickness of the ligament (b) after the cutting step. The two lengths should be close to each other.

Table 3.1. Details of optimal FIB milling procedure for chevron notched microcantilevers.

Step	Acc V (kV)	Current (pA)	Pattern Shape	Cycle Depth (um)	Cycles	Scanning Mode	Rastering Type	Est. Time
Trench cuts	30	3000	3 Trapezium	2	4	Bi-directional	Cross-section	1.2 hr
45° rough cut (left)	30	3000	Rectangle	8	1	Uni-directional	Cross-section	10 min
45° rough cut (right)	30	3000	Rectangle	5	1	Uni-directional	Cross-section	6 min
Fine cut (front)	30	1500	Rectangle	8	1	Uni-directional	Cross-section	2 min
Fine cut (right)	30	1500	Rectangle	5	1	Uni-directional	Cross-section	5 min
Fine cut (left)	30	1500	Rectangle	5	1	Uni-directional	Cross-section	5 min
Finishing cut (left)	30	300	Rectangle	5	1	Uni-directional	Cross-section	7 min
Finishing cut (right)	30	300	Rectangle	5	1	Uni-directional	Cross-section	15 min
Finishing cut (front)	30	300	Rectangle	3.5	1	Uni-directional	Cross-section	2 min
III Guidelines	30	10	3 Lines	0.2	1	Uni-directional	-	16 s
O Guidelines	30	10	Circles	0.2	1	Uni-directional	-	5 s
R Notch	30	10	Line	1	20	Uni-directional	-	8 min
L Notch	30	10	Line	1	15	Uni-directional	-	6 min
Setup and focusing	-	-	-	-	-	-	-	1-1.5 hr
Positioning	-	-	-	-	-	-	-	1 hr
Adjustment cuts	-	-	-	-	-	-	-	0.8-1.2 hr

Figure 3.4. Schematic illustrating the different scanning and rastering modes used in FIB Milling. Bottom figures are the side views of the top figures.

3.1.3 Testing using nanoindentation

3.1.3.1 Working Principle and rationale

Nanoindentation stems from conventional hardness tests, where a stiff probe (usually diamond) is pressed into a surface to measure hardness and elastic modulus. But in nanoindentation, contact depth is measured with nanometer rather than micro- or millimeter precision [23]. At this length scale, accurately measuring the dimensions of the indent by light microscopy is difficult [23]. Hence, depth sensing technology is incorporated in nanoindenters to measure the load and displacement into the sample, by using the geometry of the probe to calculate the contact area or the area of the residual impression [24]. Another challenge at this length scale is the effect of environment. Errors introduced by thermal drift and external vibrations are significant and hence should be accounted for. Vibration dampening modules and enclosures are used along with corrections by software to minimize and manage these undesirable factors. One added benefit of these countermeasures is surface probe microscopy (SPM), which can be used with the same equipment setup. In this work, nanoindentation was used to test the FIB-milled microcantilevers and measure the elastic modulus of the sample. The following lists the advantages and rationale of using nanoindentation technique: The nanoscale control of load and displacement achievable in nanoindenters makes it invaluable in testing microscale samples prepared using FIB which are too small for conventional indentation to be used. Moreover, load-displacement curves obtained from a nanoindentation test provide a wealth of information which cannot be obtained easily using conventional hardness tests [23]. Additionally, nanoindenter tip can be accurately placed on the desired location for testing on the FIB milled microscale samples using surface probe microscopy (SPM), which is covered in greater detail in the next section.

3.1.3.2 Surface Probe Microscopy (SPM) Imaging

SPM is a broad class of techniques that makes use of a probe to observe a surface. The two main classes are atomic force microscopy (AFM) and scanning tunneling microscopy (STM) [25]. In AFM images are formed using the variation in electrostatic forces between the atoms at a sharp tip and a surface while in STM electric current

variation between the tip and the surface is used to generate an image. Due to the nature of STM, only semi-conductive or conductive materials can be imaged; thus biological samples which are mostly not conductive cannot be studied easily with STM. In contrast, AFM can be used on all solid surfaces albeit the surface must be flat. Another requirement for AFM is good environmental control. The signal measured by AFM are in the nanonewton range and very easily influenced by heat and vibrations, hence a controlled environment is necessary. A sharp probe is also preferred as the spatial resolution of AFM is directly influenced by the sharpness of the tip. Since nanoindenters are equipped with a vibration dampener, an outer shell or enclosure and a sharp diamond tip, they have been adapted to include AFM functionality. SPM images have nanometer resolutions, which are on the length scale of the indents made with nanoindentation. Hence, SPM imaging can be used for more accurate positioning of the nanoindenter probe and for defining preferred indent locations.

3.1.3.3 Instrumentation

Hysitron TI950 nanoindenter was used in this work. Figure 3.5 shows the nanoindenter with the enclosure and the instruments within the enclosure. Samples are usually mounted on a ferromagnetic AFM disc which can then be placed on one of the magnetic spots on the stage. The tip is attached on a transducer which measures the force and displacement during a test. The transducer is attached on a piezoscanner which allows for the smaller but more accurate movement through piezoelectric actuation. And the piezoscanner is attached on the load head together with the microscope optics for initial optical positioning of the sample.

Figure 3.5. Triboindenter 950 nanoindenter with enclosure.

3.1.4 Compliance from Finite Element Method (FEM)

Although FIB can prepare much smaller samples compared to conventional machining techniques, it is not as accurate. With FIB, it is not possible to prepare the microscale samples to specified dimensions with low tolerance for error. Since fracture toughness is a function of the sample geometry, using a single geometric function for all the microscale samples would introduce errors. Therefore, geometric functions provided in standards cannot be used. Moreover, an analytical solution is not possible because of the complex geometry of the microcantilever; instead approximate solutions for the geometric function should be determined for each microcantilever and FEM can be used to do so without performing any further experiments. Figure 3.6A shows a two-

dimensional (2D) bell under a load from the top, obtaining an analytical solution for the displacement of the bell would be difficult because of its complex geometry. FEM can be applied to solve this problem. The bell here can be considered the domain with cartesian coordinates x and y . The deformation of the bell is governed by a mathematical equation; in this case Hooke's Law ($F = ku$), assuming that the bell only deforms elastically. The deformation or displacement is the dependent variable. The domain is divided into many simpler elements, quadrilaterals in this example Figure 3.6B. The elements are joined at their corners by nodes. The nodes are points where the math equation is applied to determine the dependent variable. Applying Hooke's Law, the displacements at every node can be calculated by solving the matrix equation:

$$\mathbf{u} = \mathbf{K}^{-1}\mathbf{F} \quad (3.1)$$

where $\mathbf{u}$ and $\mathbf{F}$ are vectors of all the nodal displacements and internal forces. While $\mathbf{K}$ is the stiffness matrix which contains all the geometry and material properties of the domain. The displacement (u) of any point within a single element like shown in Figure 3.6C can be described by the following expression:

$$u(x, y) = N_1u_1 + N_2u_2 + N_3u_3 + N_4u_4 \quad (3.2)$$

where N and u are the shape functions and displacements for the corresponding nodes in Figure 3.6C. The shape functions interpolate the nodal displacements to determine the displacement at any non-nodal point within the element. With the displacements of every point known the deformation of the complex 2D bell can be drawn. Here a brief description of the theory of FEM was presented. For this study, the FEM is used to determine the compliance of the microcantilevers at different crack lengths. Using the compliance values the geometric function can be determined and used to calculate the fracture toughness of the material. In ASTM standards for fracture toughness measurements, samples cut to precise dimensions are tested and a single geometric function can be determined and used. However, identical microcantilevers cannot be milled with FIB. Hence, a separate geometric function needs to be determined for each cantilever. FEM solves this problem. Abaqus CAE software (Dassault Systèmes) was used for modeling and finite element analyses.

Figure 3.6. A) 2D bell with loaded from the top. B) FEM mesh of the bell; quadrilateral elements used in creating the mesh, fixed boundary conditions at the bottom nodes (red triangles), load applied at the top most node (red arrow). C) A single element (gray) with four corner nodes.

3.2 Composition and Structural Analysis

3.2.1 FTIR Microscopy

Fourier Transform Infra-Red (FTIR) spectroscopy uses electromagnetic radiation (light) from the infra-red (IR) spectrum to probe the vibrations of interatomic bonds. Consider a permanent dipole like a hydrogen chloride molecule; at temperatures above absolute zero the atoms will vibrate (bond stretching and bond angle bending) creating an oscillating electric field with a particular frequency. Light energy with the same frequency and phase as the electric field would be absorbed, amplifying the vibrations [26]. The resonant frequencies of atomic vibrations are in the IR spectrum and thus IR radiation is associated with atomic vibrations. The frequency or energy of the infra-red light absorbed or scattered changes depending on the ‘stiffness’ of the bond and the chemical environment. Analyzing these IR frequencies provides information on the atomic bonds present which is a kind of fingerprint that can be used to identify the molecule. However, since IR light interaction with atomic vibrations is condition upon the atomic vibration generating an electric field; homonuclear diatoms which generate no electric field when they vibrate are not IR-active. For polyatomic molecules, if the bond movement generates a dipole the vibrational mode is IR-active. For example, CO_2 is a linear molecule with three vibrational modes as shown in Figure 3.7: symmetric

stretch, antisymmetric stretch and symmetric bending [26]. In symmetric stretching, the bond extends and retracts in sync leading to no temporary dipole created. While in antisymmetric stretching, one bond extends while the other retracts creating a temporary dipole. Therefore, the CO₂ symmetric stretch is not IR-active while the antisymmetric stretch is IR-active. Similar to the antisymmetric stretch, the symmetric bending mode is IR-active. A typical IR experiment measures the IR signal with respect to time. To obtain the frequencies absorbed, a Fourier transform is performed on the raw signal. Fourier transformation is a mathematical operation that converts a temporal signal into a frequency graph. Hence, the method is called Fourier transform Infra-red Spectroscopy. FTIR measurements can be performed in transmission on liquid samples or on powder sample compressed into a pellet [27]. Drawbacks of transmission measurement is that the sample must allow some IR light through. Therefore, good sample preparation is required for transmission FTIR. Solid samples must be sufficiently thin and powder samples should have an appropriate ratio of sample and an IR neutral filler is necessary to ensure that good intensities are measured. Solid and liquid samples can be probed without further preparation by using attenuated total reflectance (ATR) FTIR [27]. However, the trade-off of this technique is the ATR crystal must be in contact with the sample. A final method is using specular reflectance (SR) FTIR. In this method the IR light scattered back is recorded. The IR frequencies related to the bond vibrations are absorbed by the sample and hence missing from the recorded signal [28]. This method, like ATR-FTIR, does not need much sample preparation and can be performed on bulk samples but contrary to ATR-FTIR, a probe in contact with the sample is not required. However, the limitation is the data have lower accuracy and more noise. Apart from point spectroscopy, FTIR can be used in conjunction with optical microscopy to image the sample providing spatially resolved chemical information on the sample. In this study, SR-FTIR microscopy was used because of its ease of application on thick samples without the risk of damage due to ATR crystal surface contact. And for a non-homogenous sample like the stomatopod dactyl club this method can be used to differentiate the molecular bonding of the constituents at different specified sites.

Figure 3.7. CO₂ vibrational modes. Symmetric stretch (left), anti-symmetric stretch (middle) and in-plane bending (right).

3.2.2 Raman Spectroscopy

Raman spectroscopy, like FTIR, spectroscopy is used to analyze the atomic and molecular vibrations in a sample. However, instead of measuring the frequency or energy of the molecular vibrations directly, Raman spectroscopy detects the frequency shifts in the scattered light. When a strong external electric field is applied to a molecule the charge distribution will shift creating a small dipole within the molecule. The induced dipoles can generate oscillating electric fields similar to permanent dipoles when they vibrate [26]. Light can interact with these molecular vibrations and be scattered by three mechanisms: Rayleigh, Stokes and anti-Stokes scattering [29]. Figure 3.8 is a schematic of the three scattering mechanisms using an energy level diagram. δE is the characteristic molecular vibration frequency. Rayleigh scattering is considered an elastic process since the frequency of the light emitted (ν_s) is equal to the frequency of the incident beam (ν_0). Stokes scattering and anti-Stokes scattering occur when the light emitted has a frequency lower and higher (lower and higher energy) than the incident light frequency by δE . Therefore, the frequency shifts ($\nu_0 - \nu_s$) of the Stokes and anti-Stokes scattering can be used to differentiate the molecular vibrations and hence identify the molecular species. Since light is an electromagnetic wave, a strong enough Raman light source can both polarize and interact with the polarized molecule. Therefore, Raman spectroscopy is usually performed using a high-power incident laser to probe the sample surface. An advantage of Raman is that the frequency of the incident laser light is not critically important because it does not affect the Raman measurements since frequency shifts are measured. This is unlike FTIR, where δE is determined directly by probing with a range of IR radiation frequencies [26]. However, Rayleigh scattering occurs most commonly out of the three processes and thus, Raman spectroscopy has generally lesser sensitivity compared to FTIR [29]. Nevertheless, Raman can be used to detect vibration modes that do not generate a net dipole moment

and thus are not IR-active. On the other hand, according to the rule of mutual exclusion, vibration modes in molecules or molecular groups that possess a centre of symmetry cannot be both IR and Raman-active [29]. Hence, Raman and FTIR are complimentary methods and it was deemed beneficial to use both techniques to obtain a complete picture of the chemical environment of the stomatopod dactyl club using vibrational spectroscopy.

Figure 3.8. The three scattering mechanisms: Rayleigh, Stokes and anti-Stokes. ν_0 – frequency of incident light, ν_s – frequency of scattered light and δE – molecular vibration energy.

3.2.3 Inductively Coupled Plasma (ICP)

ICP optical emission spectroscopy (ICP-OES) and mass spectroscopy (ICP-MS) are techniques used in determining elemental composition of a sample. The techniques can be further divided into a two-step process. First, plasma is used to ionize a sample. Plasma is cloud of ionized gas at very high temperatures of 7000 K and hotter [30]. The plasma is generated by applying a strong alternating electric field using a radiofrequency (RF) generator. A few high energy electrons are introduced to begin the ionization process. The electrons collide with the inert gas ionizing it while ejecting more electrons that ionize more of the gas. This process is called “inductive coupling” and it is self-sustaining as long as the RF is applied. The high temperatures achieved in the plasma coupled with the minimal reactivity of Ar makes it an excellent tool to ionize samples including refractory materials [31]. Therefore, since its inception, ICP-based techniques have steadily replaced older technology as the industry standard tool for

composition analyses [32]. After ionization, the elemental composition can be determined using optical emission spectroscopy (OES) or mass spectroscopy (MS). Apart from ionization, the plasma interacts with the sample atoms raising their electronic state by promoting outer shell electrons to higher orbitals or by knocking electrons from lower orbitals. The electron clouds of these atoms are highly unstable and will return to a more stable state by the electrons falling from the higher orbitals to the lower orbitals releasing photons in the process. The photons are emitted at characteristic energies as according to atomic theory, electrons within an atom can only occupy discrete energy levels [26]. Therefore, by determining the characteristic photon energies emitted from the sample atoms, the electron transition energies are known, and the elements can be identified. Moreover, the intensity of the characteristic photon energies can be used to determine the amount of the elements present making this method a quantitative one [33]. This is the working principle of ICP-OES. In ICP-MS, instead of the electronic transitions, the mass-to-charge ratio (m/z) of the sample ions are determined. Ions are usually separated with a quadrupole, which separates the ions based on their m/z . The m/z is obviously characteristic to the ion and the intensity is the number of ions counted making ICP-MS a quantitative technique as well [31]. The advantage of ICP-MS is greater sensitivity compared to ICP-OES. However, ICP-MS is more expensive to use and to maintain than ICP-OES. ICP-OES is a well-established and robust method as well and thus it is widely used in routine analysis [34]. One limitation of both methods is the difficulty in measuring halogen quantities in a sample due to the presence of spectra interference in ICP-OES and interference from many overlapping m/z bands from polyatomic ions [34], [35]. Fortunately, the ICP-MS equipment (ELAN DRC-e, Perkin-Elmer-SCIEX) available for this work is equipped with another component (dynamic reaction cell) right before the quadrupole and mass analyzer that removes the polyatomic ions and hence reducing the interference [36]. Therefore, in this work ICP-MS was applied to measure Br which is in trace amounts within the stomatopod dactyl club and ICP-OES was used to measure the other elements present in the stomatopod dactyl club.

3.2.3.1 Optimization of digestion of dactyl club for ICP

The entire ICP analysis consist of two main parts: sample digestion, and then measurement with ICP-OES and ICP-MS. The ICP experiment is the easiest of the two

since the protocol is well-established. Digestion of the dactyl club posed a challenge in this work. This is because the dactyl club is a biological material composed of organic compounds like chitin and proteins, as well as inorganic compounds like apatite mineral, and each component has different optimal conditions for digestion. Before optimizing the method for digestion, the club sample should be broken into smaller pieces so that chemical digestion is easier because of the increased surface area. The important parameters for consideration in grinding the sample are the degree of contamination, the speed of the method and the size of the final ground product. The first method proposed was to use a bead beater. Bead beaters are often used for lysing biological samples for RNA analyses. It was successfully used to pound the dactyl club for RNA analysis in a previous study [37]. In this work, 0.5 mm ZrO_2 beads were used to pound the fully developed dactyl club. After 30 mins in the bead beater, the club was still intact and only a whitish slush was observed along with the ZrO_2 beads. The sample could be too large to be ground effectively using the bead beater method and another method should be used instead. The next method tested was crushing with a mortar and pestle. The mortar and pestle were thoroughly rinsed with Milli-Q water and isopropanol, followed by cleaning in 5 M HNO_3 for at least 24 hours. The crushing was performed at cryogenic temperatures so that it was easier to obtain a fine powder from the sample. The sample and the pestle were cooled using liquid N_2 (LN_2) till vigorous boiling of the LN_2 was not observed. Immediately after all the LN_2 vaporizes, the sample was ground using the pestle. The sample was first pounded several times with the pestle, followed by grinding by moving the pestle in circles while it was in contact with the fragmented sample. After grinding, the powder was transferred from the mortar using a sterilized spatula. Some of the powder could not be removed using the spatula, thus Milli-Q water was added, and a micropipette was used to transfer as much of the remaining sample from the mortar. Centrifugation with subsequent freeze drying was used to dry the sample. The yield from this method is lower than half but the method is quick and applicable to most labs as specialized equipment is not required. Wet chemical digestion is the next step in this digestion method.

HNO_3 is often used in digestion because it can be readily obtained with high purity and it can oxidize most of the organic compounds [38]. Moreover, a 1-5% w/w HNO_3 solution can keep most metals in solution and prevent attachment onto the container surface [39]. Often, nonoxidizing acids like HCl and HF are added to the digestion

mixture to chelate the inorganic compounds. However, HCl and HF are known to produce polyatomic molecules during ICP measurement which may contribute to interferences [40], [41]. Additionally, it is unlikely that they are necessary as the dactyl club is not known to contain refractory materials like silicates, and apatite and calcium carbonate can be digested in hot concentrated HNO₃ [42]. Most methods applied to digest hard mineralized tissues like bone (another apatite-based biological material) used concentrated HNO₃ with other acids like concentrated HCl and 30-50% H₂O₂ solution in a closed container heated with microwave digesters [38], [42]–[48]. Monitoring and control of the pressure and temperature in the digestion chamber is possible using these specialized equipments and hence, these experiments are relatively safe. Unfortunately, the author did not have access to a microwave digester and hence, the closed container digestion can be very risky. Furthermore, the H₂O₂ will oxidize organic compounds rapidly producing a lot of vapor in the process. However, closed container digestion is desirable because there is lesser loss of analytes from volatile compounds and lesser contamination from air and dust. Therefore, two methods were explored to determine a possible closed container digestion method for the dactyl stomatopod dactyl club.

The first method involves using only concentrated HNO₃ (69%) in a capped polypropylene (PP) tube. The polypropylene tubes used in this method were cleaned with HNO₃ in similar fashion to the mortar and pestle. 87.35 mg of the powder sample was measured into PP tube. The PP tube was filled almost to the brim with 5 M HNO₃. They were left in 70°C oven for 5 days. Figure 3.9A shows the sample digested with this method. White residue was found in the PP tube indicating the club was not completely dissolved and thus this method was not suitable to digest the club. The temperature and acid concentration were likely too low to fully digest the sample. For the second method, Teflon bombs from Parr Instruments were used. Since Teflon bombs were used, a much harsher environment was possible and a combination of concentrated HNO₃ and H₂O₂ was used to digest the club sample. 55.1 mg of the ground powder sample was measured into the Teflon bombs and 2 ml of 30% H₂O₂ was added followed by 3 ml concentrated HNO₃ (70%). The boiling point of HNO₃ is 121°C, when mixed in equal ratio with 50% H₂O₂ the boiling point drops considerably to 85°C [38], [49], this greatly limits the temperature that can be used to digest the dactyl club in open systems. Therefore, the Teflon bomb was placed into a 95°C oven for at least 4 hours.

The temperature was kept close to the boiling temperature of the HNO_3 and H_2O_2 mixture to reduce the experimental risk because there was no way to determine the pressure within the Teflon bombs without the appropriate equipment. The final product using this method showed that no white residue; however, the colour of the solution was just slightly murky compared to a control with the same solution without the sample tested alongside Figure 3.9B. Thus, the sample was fully digested using the second method. This sample was diluted to 1% w/w HNO_3 for analysis with ICP-OES and ICP-MS.

Figure 3.9. Results of wet digestion methods. **A)** White residue remaining after digestion using method 1. **B)** An almost clear sample solution was obtained with method 2, the control is shown for comparison.

3.2.4 Scanning Electron Microscopy (SEM)

SEM is a ubiquitous technique in science used primarily for imaging of samples in the microscale and nanoscale. Optical microscopy makes use of visible light scattering off the sample surface to directly produce a magnified image of the sample surface. The limitations of optical microscopy include the resolution limit and poor depth-of-field of optical images. Resolution is the minimum distance between two objects for them to be distinguishable. According to Abbe's formula the resolution (d) of a perfect optical

system is dependent on the wavelength of the incident light (λ) and the numerical aperture (NA) [50]:

$$d = 0.612 \frac{\lambda}{NA} \quad (3.3)$$

Numerical aperture is a constant dependent on the objective lens used and the medium where the light is travelling through. The visible spectrum has wavelengths around 380 – 700 nm, this translate to a resolution limit of around 200 nm [50]. In electron microscopy, electrons are focused on to a sample surface using powerful electromagnets as objectives. The electron beam is scanned on the sample generating a raster image [50]. The wavelength of an electron is proportional to its potential energy or accelerating voltage (V) and given by [25]:

$$\lambda = \frac{h}{\sqrt{2meV}} \quad (3.4)$$

Where, h is the planck's constant, m is the electron mass and e is the electron's charge. For a V of 5 keV, λ is 0.0173 nm. Thus, according to Abbe's formula the resolution achievable with electron microscopy is about at least 10,000 times smaller. Electrons from the electron beam interact with matter to produce many different signals including secondary electrons, backscattered electrons, transmitted electrons, auger electrons and characteristic x-rays [50]. Figure 3.10 shows a typical interaction zone of an electron striking the sample surface and the different possible signals produced. The origin and application of secondary electrons, backscattered electrons and characteristic x-rays will be discussed in the following sections.

3.2.4.1 Secondary Electron and Backscatter Electron Imaging

Secondary electrons are generated when the incident electron knocks off the loosely bound outer shell electrons of the atoms near the sample surface [50]. These electrons are differentiated from other electron signals by their low energies. The low energies of secondary electrons also prevent them from escaping from deeper within the sample and hence secondary electrons are useful to probe the surface topology and are usually

first used to select the region of interest for further probing. Backscattered electrons are electrons that undergo elastic scattering events that divert the electrons back from deeper within the sample at wide angles [25]. The electrons trajectory is changed by interacting with positive nuclei of the atoms and hence, heavier atoms will produce larger backscattered electron signals [25]. Therefore, backscatter electron images do not have topographic contrast and the image contrast is indicative of atomic mass variation across the imaged region.

3.2.4.2 Electron Dispersive X-ray Spectroscopy (EDS)

When electrons are dislodged from the inner shells of the sample atoms, the atoms become unstable and will seek to return to a stable electronic structure by electrons in outer shells falling to fill the vacancies left by dislodged electrons. The energy loss by the electron falling from a higher to a lower energy level is given of as characteristic X-rays that can be used to identify the atom. This is similar to how elemental composition is determined with OES.

Figure 3.10. Interaction volume of electrons striking a sample surface producing different signals (secondary electrons, backscattered electrons and characteristic X-rays).

3.2.4.3 Electron Backscattered Diffraction (EBSD)

EBSD arises from backscattered electrons from a flat polished sample surface placed at an angle usually at 20° from the incident electron beam [51]. EBSD produces the

“Kikuchi Patterns”, named after the scientist who first reported this phenomenon [52]. These patterns are the result of low angle diffraction of the incident electron beam; the backscattered electrons undergo minimal scattering and loss of energy within the structure and exits the sample surface with different harmonic phases [52]. Destructive interferences eliminate electrons that are out-of-phase, only electrons diffracted in-phase by crystalline structures in the sample create the diffraction or Kikuchi pattern on a phosphor screen. Therefore, Kikuchi patterns can be used to image the grain size and orientation as well as internal strains in the crystalline phases. As an SEM technique, EBSD benefits from the ease of operation and can be conducted simultaneously with EDS and secondary electron imaging. However, EBSD is highly sensitive to the flatness and roughness of the surface. The surface should have no more than 0.5° angle variation for measurable results from EBSD [52]. Moreover, the surface should not be highly charging. From experience, charging may pose an issue with the dactyl club sample. Thus, sample preparation would most likely be the main challenge faced in using EBSD.

3.2.4.4 Rationale for SEM

SEM is a ubiquitous technique, widely available in many laboratories including the Facility for Analysis Characterization Testing and Simulation (FACTS), which can be accessed by the author. SEM techniques are used in this study because the stomatopod dactyl club contain structural features from the micrometer even to the nanometer range and thus optical light microscopy is not able to provide the desired resolution. Moreover, it is easy and quick to use. However, the stomatopod dactyl club is a biological material and hence the main challenge encountered will be sample preparation as the dactyl club is non-conducting and maybe effected by dehydration. Nonetheless, the benefits of high resolution and collocal and temporal multi-signal measurement outweighs the disadvantages.

3.4 Synchrotron X-ray Techniques

Synchrotrons are large ring facilities that accelerate electrons to near relativistic speeds in a closed loop. Powerful electromagnets bend the electrons around the synchrotron storage ring. The Lorentz force from the magnetic field pulls the electrons towards the center of rotation this change in electron acceleration generate photons [53]. For

electrons travelling at non-relativistic speeds, the photons emitted in a dipolar distribution as shown in Figure 3.11. At relativistic speeds, the photons are emitted in a cone in the direction tangent to the orbit of the electron with a half angle, $\theta \approx 1/\gamma$. γ is the dimensionless Lorentz factor given by [53]:

$$\gamma = \frac{E}{mc^2} \quad (3.5)$$

where E is the energy of the particles travelling round the storage ring, m is the mass of the particles and c is the speed of light. From Equation 3.5, it is obvious that the higher the E the narrower the photon beam produced. At a typical storage ring energy of 4 GeV, the angle of the photon emission cone is around 0.26 mrad or 4×10^{-6} degrees [53]. Hence a tightly focused X-ray beam is produced from synchrotron sources. The photon flux generated by electrons circulating within the storage ring scales with γ^4 [53]. The combination of a tightly focused beam and the high flux generated in synchrotron lead to extremely brilliant X-rays. The brilliance of a typical third generation synchrotron is around 10^{20} photons $\text{s}^{-1}\text{mrad}^{-2}\text{mm}^{-2}(0.1\% \text{ bandwidth})^{-1}$ which is around 10 orders of magnitude larger than those from conventional anode Cu K α lab sources [53]. The bright and coherent X-ray beams generated by the synchrotron make it possible to conduct experiments usually not achievable by conventional lab X-ray sources like microscale large area mapping of samples, nanoscale tomography using ptychography and *in-situ* strain measurements.

Figure 3.11. Shape of light emitted from an electron moving with a velocity of v . **A)** Dipole distribution of emitted light when the velocity is much smaller than the velocity of light. **B)** Narrow cone distribution of emitted light in synchrotrons where the electron is moving at relativistic velocities

3.4.1 X-ray Fluorescence Spectroscopy (XRF)

X-rays interact with matter in several ways. The high energies present in the X-rays can dislodge inner orbital electrons. As previously explained, higher orbital electrons will fill the vacancies left by the dislodged electrons and emit characteristic X-rays in the process. The characteristic X-rays provide information on the elemental composition of the sample. This technique is called X-ray fluorescence (XRF). XRF can be easily applied to measure sample composition. Compared to EDS, it does not require a high vacuum environment to operate. Additionally, the flux generated by the synchrotron X-rays are much larger than those from a typical scanning electron microscope. Consequently, the desired characteristic X-rays lines are visible with lower overvoltages applied than in EDS, where as a rule of thumb for EDS, the overvoltage should be around 2.5 times the energy of the characteristic X-ray line for it to be observed [25]. However, electron microscopy has better spatial resolution compared to conventional XRF. Moreover, a secondary electron image of the region of interest can be easily taken and compared with EDS data to obtain a spatial map of elemental composition when using a scanning electron microscope. Synchrotrons enable large area micrometer spatially resolved XRF maps to be taken within realistic times, hence bridging the gap between EDS and conventional XRF. Hence, synchrotron μ -XRF is a viable technique to study biological materials.

3.4.2 X-ray Microdiffraction (μ XRD)

3.4.2.1 Working Principle and Rationale

The wavelength of X-rays is comparable to the size of interatomic spacings. Hence, X-rays will diffract as they propagate through the sample, like how visible light is diffracted by millimeter-sized slits [54]. In an amorphous sample (where there is no periodic arrangement of atoms), the X-rays will be randomly diffracted or scattered and interfere with each other leading to a broad signal captured at a detector. However, when X-rays travel through a periodic arrangement of atoms (*i.e.* a crystal lattice), according to Bragg's law, the diffracted X-rays that travel path lengths that integer (n) multiples of the spacings between crystallographic planes (d) will undergo constructive

interference and defined X-ray intensity peaks can be observed with a detector [55]. Bragg's law is given by:

$$n\lambda = 2d \sin \theta \quad (3.6)$$

where λ is the X-ray wavelength and θ is the angle of incidence. X-ray diffraction (XRD) is used to determine the position of atoms with respect to each other in a crystalline material. XRD provides information on the crystal structure, lattice strain, crystal orientation and the positions of all atoms in a crystal [56]. Figure 3.12 shows the experimental setup of transmission μ -XRD in a synchrotron. The detector is placed at an angle to the incident beams to collect diffracted beams. Monochromatic X-rays are used for Bragg diffraction experiments. When the crystallites are randomly oriented and smaller than the X-ray beam spot size, continuous circular disks concentric around the incident beam form on the detector, known as Debye-Scherrer Rings [56]. The incident ray diffracting off a crystallographic plane creates each ring. Because the crystals are oriented randomly, the beam is diffracted in all directions in a conical manner. A conventional 1D diffraction graph can be produced by integrating the data in the radial direction of the rings (Figure 3.12). The sharp peaks can be correlated to the lattice parameters of the crystal, which can be used to determine an unknown crystal structure. The size of crystals affects the width of the peaks. Broadening of peaks in the radial directions occurs as a function of grain size and can be calculated using the Scherrer equation [56]:

$$W = \frac{k\lambda}{B \cos \theta} \quad (3.7)$$

where W is the full width half maximum of the peak, k a geometric constant, and B is the crystal size. This equation is quantitative for crystallites size approximately below 100 nm [44].

Figure 3.12. Schematic of powder μ XRD of a polycrystalline sample using using monochromatic X-rays [57].

Figure 3.13. The 2D diffraction patterns of the Stomatopod dactyl club from a single position but integrated along different direction with their corresponding intensity graph below. **A)** Azimuthal, **B)** Radial.

3.4.2.2 Texture analysis

Preferred orientation or texture is one of the stratagems often employed in structural biological materials to obtain desired mechanical properties. Figure 3.13 presents two 2D diffraction patterns from a single point scan of a Stomatopod dactyl club. Texture is clearly present because the diffraction rings contain varying intensity. By resolving the maximum intensity along the rings (known as integration along χ), it is possible to determine the preferred orientation of the crystals within the material (Figure 3.13A). If the diffraction pattern is resolved radially (Figure 3.13B), a diffractogram is obtained which can be used to determine crystal structure. Weaver *et al.* used μ XRD to generate an angular map of apatite crystals within the impact region of a Stomatopod dactyl club [1]. A texture map of the impact region was created by stitching multiple adjacent scans together (Figure 2.4C). Texture mapping is possible by using a synchrotron because of the fast scan rates and high spatial resolution. In cases where the crystals are highly textured, a spot pattern similar to a single crystal can be produced (Figure 3.14B). Sea urchin teeth are an example of such a material in Nature (Figure 3.14A) [58]. The sample had to be rotated so that the detector can collect diffracted beams from all atomic planes since the material is nearly single crystalline (Figure 3.14B).

Figure 3.14. A) Optical micrograph of sea urchin tooth. B) Diffraction pattern similar to single crystal. [58]

3.4.3 Ptychographic X-ray Computed Tomography

3.4.3.1 The Phase Problem

In X-ray imaging, the primary goal is to obtain the position and type of atoms within a sample. One issue encountered with imaging techniques that use diffracted X-rays is the so called “phase problem” [53]. For simplicity, consider a single crystal sample measured in transmission and the diffraction pattern is collected using a 2D detector like in Figure 3.15. The diffraction pattern recorded at 2D detector is the Fourier transform of the crystal structure. Each diffraction spot represents an atomic plane within the single crystal. The intensity (I_{hkl}) of each spot is equal to the square of the structure factor of the atomic plane (F_{hkl}) and is dependent on the type, position, and quantity (n) of atoms on that plane [55]:

$$F_{hkl} = f_1 + f_2 + f_3 \dots + f_n \quad (3.8)$$

$$I_{hkl} = |F_{hkl}|^2 \quad (3.9)$$

F_{hkl} is the vector sum of all the X-rays scattered by the atoms within the atomic plane. Therefore, the phase/angle of the scattered X-rays with respect to the incident X-rays is lost and the atomic positions cannot be simply determined from the diffraction pattern. Ptychographic X-ray Computed Tomography (PXCT) makes use of ptychography for phase retrieval and hence, the 3D sample structure can be imaged with high resolution.

3.4.3.2 Coherent Diffraction Imaging

PXCT is a Coherent Diffraction Imaging (CDI) technique [59]. In CDI, a spatially coherent beam of particles is used to image an object without using any lens to focus the beam. Instead computation is used to recover the real space image of the object [53]. The major advantage of CDI is that it removes the need for aberration correctors, which are expensive and difficult to manufacture, as in the case of X-rays [53]. Without the use of lenses, the far-field (Fraunhofer) diffraction 2D image is collected at the detector.

Using an iterative algorithm the real space image of the object can be calculated from the diffracted patterns [40].

Figure 3.15. Schematic of transmission XRD. **A)** Experimental setup. **B)** Example diffraction pattern collected at the detector.

3.4.3.3 Ptychography Iterative Engine (PIE)

Ptychography is a technique first introduced by Walter Hoppe [60]. The term “Ptycho” comes from the greek word meaning “to fold”. In ptychography, scans are overlapped over each other and the constraints from a previous scan is applied to the overlapping scans [61]. Since the overlapped regions should have the same phase, the iterative algorithm can use the regions as constraints to help achieve convergence. Figure 3.16A, shows the first iterative algorithm introduced for CDI. A study on the effect of the degree of overlapping found that 60% overlap is the sweet spot for the PIE algorithm (Figure 3.16B) [62]. Another added advantage of ptychography is the scan region can be extended to large scan areas depending only bounded by the time allowable for the scan. This makes Ptychographic CDI a suitable candidate for imaging of large samples like the stomatopod dactyl club. Ptychographic CDI provides two types of X-ray images: one using the attenuation of the amplitude, which corresponds to imaging using absorption contrast, and the other one using attenuation of the phase which is phase imaging. Phase imaging was found to be better for imaging objects of biological origin because of the poor absorption of X-rays in lighter elements that are dominant in biological tissues and the high sensitivity of light elements to phase attenuation, which is about four order of magnitude larger than the amplitude contrast [53]. Ptychographic

X-ray CDI can be easily extended in the three dimension by computed tomography. Holler *et al.* to obtained 3D structure of sea sponge skeleton to up to 16 nm resolution [63] as well as an integrated circuit with resolution up to 14.6 nm using this technique [64]. Hence, PXCT is believed to also be a suitable technique for 3D imaging of the stomatopod dactyl club

Figure 3.16. A) PIE developed by Rodenburg *et al.* Which is the first use of ptychography for phase retrieval in CDI [48]. B) Ptychographic CDI of a dragonfly wing at different degrees of overlaps showing an optimal ratio of 60% [62].

3.5 Proteomics

3.5.1 Parallel Reaction Monitoring (PRM)

Identification of proteins (qualitative analysis) within a mixture of proteins can be achieved by liquid chromatography tandem mass spectrometry (LC-MS/MS). Tandem mass spectroscopy (MS/MS) is a class of methods that combine two or more mass analyzers in sequence. In LC-MS/MS, Liquid chromatography (LC) is first used to separate the proteins by their mass or other properties like ionic charge or hydrophobicity. The proteins are ionized and the ions enter into the first mass analyzer which filter the precursor ions by mass-to-charge (m/z). The selected precursor ion is fragmented into smaller ions and these fragmented ions pass through a second mass analyzer. The filtered fragmented ion is measured at the detector. Performing LC-

MS/MS by selecting a precursor ion with the first mass analyzer followed by selecting a fragmented ion with the second mass analyzer for detection in a single cycle is called single reaction monitoring (SRM) [65]. Systematic quantification of peptides within complex biological samples is difficult using LC-MS/MS because of the abundance of proteins creating a cloud of signals that is hard to deconvolute [66]. Thus, intelligent selection of peptides for analysis coupled with methods with higher sensitivity were developed. Currently, triple quadrupole SRM is used for routine protein quantification and as the reference method [65]. However, this technique has limited mass sensitivity and interferences cannot be avoided [67], [68]. High resolution/accurate mass (HR/AM) techniques arose as a promising alternative. One such method is PRM which uses a quadrupole to filter desired precursor ions into a high energy collision dissociation (HCD) cell for ion fragmentation and accumulation before high resolution/accurate mass (HR/AM) analysis in an orbitrap-based mass spectrometer [66]. The mass filtering at the front allows the selection of desired precursors for HR/AM measurement at the end. Additionally, multiple peptides and fragmented ions can be monitored simultaneously with PRM, greatly improving the throughput without diminishing the sensitivity of the technique with respect to triple quadrupole SRM. Due to its advantages, the PRM method was used to quantify the peptide of interest from the club mineralization protein-1 (CMP-1).

3.6 Methods Summary

Figure 3.17 summarizes the experimental techniques introduced in this section as well as their purposes for this thesis. Readers are referred to the respective sections above for the working principles and rationale for using these techniques.

Technique	Purpose
Focused Ion Beam (FIB) Milling	Fracture toughness determination using microcantilevers
Nanoindentation	
Finite Element Method (FEM)	
Fourier Transform Infra Red (FTIR) Spectroscopy	Molecular Bonding Environment
Raman Spectroscopy	
Inductively Coupled Plasma (ICP)	Elemental Composition
Energy Dispersive X-ray Spectroscopy (EDS)	
X-ray Fluorescence (XRF)	
X-ray Diffraction (XRD)	Crystal structure determination, apparent crystallite size, texture
Ptychographic X-ray Computed Tomography (PXCT)	High Resolution 3D Imaging
Parallel Reaction Monitoring	Protein sequencing, detection and quantification of Protein residues and peptide sequences

Figure 3.17. List of the techniques and their use in this work.

References

- [1] J. C. Weaver *et al.*, “The Stomatopod Dactyl Club: A Formidable Damage-Tolerant Biological Hammer,” *Science*, vol. 336, no. 6086, p. 1275, Jun. 2012, doi: 10.1126/science.1218764.
- [2] S. Amini *et al.*, “Textured fluorapatite bonded to calcium sulphate strengthen stomatopod raptorial appendages,” *Nature Communications*, vol. 5, p. 3187, Jan. 2014, doi: 10.1038/ncomms4187.
- [3] S. Amini, M. Tadayon, S. Idapalapati, and A. Miserez, “The role of quasi-plasticity in the extreme contact damage tolerance of the stomatopod dactyl club,” *Nat Mater*, vol. 14, no. 9, pp. 943–950, Sep. 2015, doi: <http://dx.doi.org/10.1038/nmat4309>.
- [4] N. A. Yaraghi *et al.*, “A Sinusoidally Architected Helicoidal Biocomposite,” *Adv. Mater.*, vol. 28, no. 32, pp. 6835–6844, Aug. 2016, doi: 10.1002/adma.201600786.
- [5] W. Huang *et al.*, “A natural impact-resistant bicontinuous composite nanoparticle coating,” *Nature Materials*, Aug. 2020, doi: 10.1038/s41563-020-0768-7.
- [6] J. J. Kruzic, D. K. Kim, K. J. Koester, and R. O. Ritchie, “Indentation techniques for evaluating the fracture toughness of biomaterials and hard tissues,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 2, no. 4, pp. 384–395, Aug. 2009, doi: 10.1016/j.jmbbm.2008.10.008.
- [7] G. D. Quinn and R. C. Bradt, “On the Vickers Indentation Fracture Toughness Test,” *J American Ceramic Society*, vol. 90, no. 3, pp. 673–680, Mar. 2007, doi: 10.1111/j.1551-2916.2006.01482.x.
- [8] M. A. Meyers, P.-Y. Chen, A. Y.-M. Lin, and Y. Seki, “Biological materials: Structure and mechanical properties,” *Progress in Materials Science*, vol. 53, no. 1, pp. 1–206, Jan. 2008, doi: 10.1016/j.pmatsci.2007.05.002.
- [9] P.-Y. Chen, J. McKittrick, and M. A. Meyers, “Biological materials: Functional adaptations and bioinspired designs,” *Progress in Materials Science*, vol. 57, no. 8, pp. 1492–1704, Nov. 2012, doi: 10.1016/j.pmatsci.2012.03.001.
- [10] A. A. Griffith, “The phenomena of rupture and flow in solids,” *Phil. Trans. R. Soc. Lond. A*, vol. 221, no. 582–593, pp. 163–198, 1920, doi: 10.1098/rsta.1921.0006.

- [11] Z. Cui, “Nanofabrication by Ion Beam,” in *Nanofabrication: Principles, Capabilities and Limits*, 2nd ed. 2017., Cham: Springer International Publishing : Imprint: Springer, 2017, pp. 149–175.
- [12] L. A. Giannuzzi and F. A. Stevie, Eds., “Ion - Solid Interactions,” in *Introduction to focused ion beams: instrumentation, theory, techniques, and practice*, New York: Springer, 2005, pp. 13–52.
- [13] L. Frey, C. Lehrer, and H. Ryssel, “Nanoscale effects in focused ion beam processing,” *Applied Physics A: Materials Science & Processing*, vol. 76, no. 7, pp. 1017–1023, May 2003, doi: 10.1007/s00339-002-1943-1.
- [14] B. Girard, D. Yu, M. R. Armstrong, B. C. Wilson, C. M. L. Clokie, and R. J. D. Miller, “Effects of femtosecond laser irradiation on osseous tissues,” *Lasers Surg. Med.*, vol. 39, no. 3, pp. 273–285, Mar. 2007, doi: 10.1002/lsm.20466.
- [15] Q. Wu, Y. Li, Y. Yang, and Y. Zhao, “A Photolithography Process Design for 5 nm Logic Process Flow,” *Journal of Microelectronic Manufacturing*, vol. 2, no. 4, pp. 1–8, 2019, doi: 10.33079/jomm.19020408.
- [16] Y. L. Chan, A. H. W. Ngan, and N. M. King, “Use of focused ion beam milling for investigating the mechanical properties of biological tissues: A study of human primary molars,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 2, no. 4, pp. 375–383, Aug. 2009, doi: 10.1016/j.jmbbm.2009.01.006.
- [17] Y. L. Chan, A. H. W. Ngan, and N. M. King, “Nano-scale structure and mechanical properties of the human dentine–enamel junction,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 4, no. 5, pp. 785–795, Jul. 2011, doi: 10.1016/j.jmbbm.2010.09.003.
- [18] D. Lu and A. H. Barber, “Optimized nanoscale composite behaviour in limpet teeth,” *Journal of The Royal Society Interface*, vol. 9, no. 71, pp. 1318–1324, Jun. 2012, doi: 10.1098/rsif.2011.0688.
- [19] I. Jimenez-Palomar, A. Shipov, R. Shahar, and A. H. Barber, “Mechanical Behavior of Osteoporotic Bone at Sub-Lamellar Length Scales,” *Frontiers in Materials*, vol. 2, p. 9, 2015, doi: 10.3389/fmats.2015.00009.
- [20] I. Jimenez-Palomar, A. Shipov, R. Shahar, and A. H. Barber, “Structural orientation dependent sub-lamellar bone mechanics,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 52, pp. 63–71, Dec. 2015, doi: 10.1016/j.jmbbm.2015.02.031.

- [21] H.-T. Lee *et al.*, “Site-specific characterization of beetle horn shell with micromechanical bending test in focused ion beam system,” *Acta Biomaterialia*, vol. 57, pp. 395–403, Jul. 2017, doi: 10.1016/j.actbio.2017.04.026.
- [22] B. Bayerlein, L. Bertinetti, B. Bar-On, H. Blumtritt, P. Fratzl, and I. Zlotnikov, “Inherent Role of Water in Damage Tolerance of the Prismatic Mineral-Organic Biocomposite in the Shell of *Pinna Nobilis*,” *Adv. Funct. Mater.*, vol. 26, no. 21, pp. 3663–3669, Jun. 2016, doi: 10.1002/adfm.201600104.
- [23] A. C. Fischer-Cripps, *Nanoindentation*, 3rd ed. New York, NY: Springer New York, 2011.
- [24] W. C. Oliver and G. M. Pharr, “An improved technique for determining hardness and elastic modulus using load and displacement sensing indentation experiments,” *J. Mater. Res.*, vol. 7, no. 6, pp. 1564–1583, Jun. 1992, doi: 10.1557/JMR.1992.1564.
- [25] William D. Callister, *Material Science and Engineering: An Introduction*, 7th ed. John Wiley & Sons, Inc., 2007.
- [26] R. J. Le Roy, *A Spectroscopy Primer: An Introduction to Atomic, Rotational, Vibrational, Raman, Electronic, Photoelectron and NMR Spectroscopy*. Waterloo, Ontario, Canada: University of Waterloo, 2011.
- [27] E. A. Taylor and E. Donnelly, “Raman and Fourier transform infrared imaging for characterization of bone material properties,” *Bone*, vol. 139, p. 115490, Oct. 2020, doi: 10.1016/j.bone.2020.115490.
- [28] A. McClelland, E. Bulat, B. Bernier, and E. L. Murphy, “Specular Reflection FTIR: A Non-Contact Method for Analyzing Coatings on Photographs and Other Cultural Materials,” *Journal of the American Institute for Conservation*, vol. 59, no. 2, pp. 123–136, Apr. 2020, doi: 10.1080/01971360.2019.1660546.
- [29] J. R. Ferraro, K. Nakamoto, and C. W. Brown, *Introductory Raman spectroscopy*, 2nd ed. Amsterdam ; Boston: Academic Press, 2003.
- [30] G. L. Moore, “Inductively Coupled Plasmas,” in *Introduction to Inductively Coupled Plasma Atomic Emission Spectrometry*, Elsevier, 1989, pp. 119–133.
- [31] H. E. Taylor, *Inductively Coupled Plasma-Mass Spectrometry: Practices and Techniques*. Elsevier, 2001.
- [32] O. Butler *et al.*, “Atomic Spectrometry Updates: A 25-year retrospective,” *J. Anal. At. Spectrom.*, vol. 25, no. 10, p. 1546, 2010, doi: 10.1039/c005518m.

- [33] R. E. Wainerdi and E. A. Uken, Eds., “Optical Emission Spectroscopy,” in *Modern Methods of Geochemical Analysis*, Boston, MA: Springer US, 1971, pp. 169–204.
- [34] G. L. Donati, R. S. Amais, and C. B. Williams, “Recent advances in inductively coupled plasma optical emission spectrometry,” *J. Anal. At. Spectrom.*, vol. 32, no. 7, pp. 1283–1296, 2017, doi: 10.1039/C7JA00103G.
- [35] X. Bu, T. Wang, and G. Hall, “Determination of halogens in organic compounds by high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS),” *J. Anal. At. Spectrom.*, vol. 18, no. 12, p. 1443, 2003, doi: 10.1039/b306570g.
- [36] “Expanding the capabilities of ICP-MS: ELAN-DRC-e.” PerkinElmer SCIEX, Accessed: Dec. 15, 2020. [Online]. Available: https://www.perkinelmer.com/CMSResources/Images/44-74438BRO_ExpandingtheCapabilitiesofICP-MS.pdf.
- [37] S. Amini *et al.*, “A diecast mineralization process forms the tough mantis shrimp dactyl club,” *Proc Natl Acad Sci USA*, vol. 116, no. 18, pp. 8685–8692, Apr. 2019, doi: 10.1073/pnas.1816835116.
- [38] E. I. Muller, M. F. Mesko, D. P. Moraes, M. das G. A. Korn, and É. M. M. Flores, “Wet Digestion Using Microwave Heating,” in *Microwave-Assisted Sample Preparation for Trace Element Analysis*, Elsevier, 2014, pp. 99–142.
- [39] R. S. Pappas, “Sample Preparation Problem Solving for Inductively Coupled Plasma-Mass Spectrometry with Liquid Introduction Systems I. Solubility, Chelation, and Memory Effects,” *Spectroscopy (Springf)*, vol. 27, no. 5, pp. 20–31, May 2012.
- [40] S. Fitz-James, “Use of Hydrochloric Acid (HCl) in digests for ICP-MS analysis,” Jul. 26, 2013.
- [41] T. W. May and R. H. Wiedmeyer, “A table of polyatomic interferences in ICP-MS,” *Atomic Spectroscopy*, vol. 19, no. 5, p. 6, 1998.
- [42] S. Ashoka, B. M. Peake, G. Bremner, K. J. Hageman, and M. R. Reid, “Comparison of digestion methods for ICP-MS determination of trace elements in fish tissues,” *Analytica Chimica Acta*, vol. 653, no. 2, pp. 191–199, Oct. 2009, doi: 10.1016/j.aca.2009.09.025.
- [43] C. Ishii *et al.*, “Characterization and imaging of lead distribution in bones of lead-exposed birds by ICP-MS and LA-ICP-MS,” *Chemosphere*, vol. 212, pp. 994–1001, Dec. 2018, doi: 10.1016/j.chemosphere.2018.08.149.

- [44] S. Rattanachongkiat, G. E. Millward, and M. E. Foulkes, "Determination of arsenic species in fish, crustacean and sediment samples from Thailand using high performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICP-MS)," *J. Environ. Monit.*, vol. 6, no. 4, pp. 254–261, 2004, doi: 10.1039/B312956J.
- [45] A. C. Raffalt, J. E. T. Andersen, and S. Christgau, "Application of inductively coupled plasma–mass spectrometry (ICP–MS) and quality assurance to study the incorporation of strontium into bone, bone marrow, and teeth of dogs after one month of treatment with strontium malonate," *Anal Bioanal Chem*, vol. 391, no. 6, pp. 2199–2207, Jul. 2008, doi: 10.1007/s00216-008-2171-0.
- [46] N. B. Roberts, H. P. J. Walsh, L. Klenerman, S. A. Kelly, and T. R. Helliwell, "Determination of elements in human femoral bone using inductively coupled plasma atomic emission spectrometry and inductively coupled plasma mass spectrometry," *J. Anal. At. Spectrom.*, vol. 11, no. 2, p. 133, 1996, doi: 10.1039/ja9961100133.
- [47] S. Dillane, M. Thompson, J. Meyer, M. Norquay, and R. C. O'Brien, "Inductively coupled plasma – atomic emission spectroscopy (ICP-AES) as a method of species differentiation of bone fragments," *Australian Journal of Forensic Sciences*, vol. 43, no. 4, pp. 297–312, Dec. 2011, doi: 10.1080/00450618.2011.595429.
- [48] H. S. Mahanti and R. M. Barnes, "Determination of major, minor and trace elements in bone by inductively-coupled plasma emission spectrometry," *Analytica Chimica Acta*, vol. 151, pp. 409–417, 1983, doi: 10.1016/S0003-2670(00)80103-8.
- [49] E. Mohammed, T. Mohammed, and A. Mohammed, "Optimization of an acid digestion procedure for the determination of Hg, As, Sb, Pb and Cd in fish muscle tissue," *MethodsX*, vol. 4, pp. 513–523, 2017, doi: 10.1016/j.mex.2017.11.006.
- [50] W. Zhou, Z. L. Wang, R. Apkarian, and D. Joy, "Fundamentals of Scanning Electron Microscopy (SEM)," in *Scanning microscopy for nanotechnology: techniques and applications*, New York, NY: Springer, 2007, pp. 1–40.
- [51] V. Randle, "Recent Developments in Electron Backscatter Diffraction," in *Advances in Imaging and Electron Physics*, vol. 151, Elsevier, 2009, pp. 363–416.

- [52] T. Maitland and S. Sitzman, “Backscattering Detector and EBSD in Nanomaterials Characterization,” in *Scanning Microscopy for Nanotechnology*, W. Zhou and Z. L. Wang, Eds. New York, NY: Springer New York, 2006, pp. 41–75.
- [53] P. Willmott, *An Introduction to Synchrotron Radiation: Techniques and Applications*, 1st ed. Wiley, 2011.
- [54] J. M. Cowley, *Diffraction Physics*. Burlington: Elsevier, 1995.
- [55] J. Als-Nielsen and D. McMorrow, *Elements of modern X-ray physics*, 2. ed. Chichester: Wiley, 2011.
- [56] E. J. Mittemeijer and U. Welzel, Eds., *Modern diffraction methods*. Weinheim: Wiley-VCH, 2013.
- [57] N. Tamura and P. U. P. A. Gilbert, “X-Ray Microdiffraction of Biominerals,” in *Methods in Enzymology*, vol. 532, Elsevier, 2013, pp. 501–531.
- [58] Y. Ma *et al.*, “The grinding tip of the sea urchin tooth exhibits exquisite control over calcite crystal orientation and Mg distribution,” *Proceedings of the National Academy of Sciences*, vol. 106, no. 15, pp. 6048–6053, Apr. 2009, doi: 10.1073/pnas.0810300106.
- [59] M. E. Birkbak, M. Guizar-Sicairos, M. Holler, and H. Birkedal, “Internal structure of sponge glass fiber revealed by ptychographic nanotomography,” *Journal of Structural Biology*, vol. 194, no. 1, pp. 124–128, Apr. 2016, doi: 10.1016/j.jsb.2016.02.006.
- [60] W. Hoppe, “Beugung im inhomogenen Primärstrahlwellenfeld. I. Prinzip einer Phasenmessung von Elektronenbeugungsinterferenzen,” *Acta Cryst A*, vol. 25, no. 4, pp. 495–501, Jul. 1969, doi: 10.1107/S0567739469001045.
- [61] J. M. Rodenburg *et al.*, “Hard-X-Ray Lensless Imaging of Extended Objects,” *Phys. Rev. Lett.*, vol. 98, no. 3, p. 034801, Jan. 2007, doi: 10.1103/PhysRevLett.98.034801.
- [62] O. Bunk, M. Dierolf, S. Kynde, I. Johnson, O. Marti, and F. Pfeiffer, “Influence of the overlap parameter on the convergence of the ptychographical iterative engine,” *Ultramicroscopy*, vol. 108, no. 5, pp. 481–487, Apr. 2008, doi: 10.1016/j.ultramic.2007.08.003.
- [63] M. Holler *et al.*, “X-ray ptychographic computed tomography at 16 nm isotropic 3D resolution,” *Sci Rep*, vol. 4, no. 1, p. 3857, May 2015, doi: 10.1038/srep03857.

- [64] M. Holler *et al.*, “High-resolution non-destructive three-dimensional imaging of integrated circuits,” *Nature*, vol. 543, no. 7645, pp. 402–406, Mar. 2017, doi: 10.1038/nature21698.
- [65] R. A. Yost and C. G. Enke, “Triple quadrupole mass spectrometry for direct mixture analysis and structure elucidation,” *Anal. Chem.*, vol. 51, no. 12, pp. 1251–1264, Oct. 1979, doi: 10.1021/ac50048a002.
- [66] S. Gallien, E. Duriez, C. Crone, M. Kellmann, T. Moehring, and B. Domon, “Targeted Proteomic Quantification on Quadrupole-Orbitrap Mass Spectrometer,” *Mol Cell Proteomics*, vol. 11, no. 12, pp. 1709–1723, Dec. 2012, doi: 10.1074/mcp.O112.019802.
- [67] J. Sherman, M. J. McKay, K. Ashman, and M. P. Molloy, “How specific is my SRM?: The issue of precursor and product ion redundancy,” *Proteomics*, vol. 9, no. 5, pp. 1120–1123, Mar. 2009, doi: 10.1002/pmic.200800577.
- [68] S. E. Abbatiello, D. R. Mani, H. Keshishian, and S. A. Carr, “Automated Detection of Inaccurate and Imprecise Transitions in Peptide Quantification by Multiple Reaction Monitoring Mass Spectrometry,” *Clinical Chemistry*, vol. 56, no. 2, pp. 291–305, Feb. 2010, doi: 10.1373/clinchem.2009.138420.

Chapter 4*

Micro Fracture Toughness Study

This chapter presents the details of the fracture toughness study of the impact region of the dactyl club using chevron-notched microcantilevers. A brief introduction providing the motivation of this study is presented. The experimental methods were covered followed by the results of this study. Fracture toughness values were one order of magnitude larger with EPFM than with LEFM, suggesting that plastic dissipation is the primary mode of fracture resistance in the impact region. Orientation did not have a significant effect on fracture toughness which is unlike other mechanical properties like the hardness and elastic modulus.

*This section published substantially as J. Q. I. Chua, D. V. Srinivasan, I. Sridhar, A. Miserez, "Fracture toughness of the stomatopod dactyl club is enhanced by plastic dissipation: A fracture micromechanics study," *Acta Biomaterialia*, vol. 126, pp. 339–349, Mar. 2021 doi: 10.1016/j.actbio.2021.03.025

4.1 Introduction

Mantis shrimps (stomatopods) (Figure 4.1A) are aggressive marine crustaceans that employ a distinctive hunting strategy. They are equipped with a pair of raptorial hammer-like dactyl clubs (Figure 4.1A and B) that they deploy at high velocity in order to shatter the hard protective shells of their prey [1], [2]. Because a stomatopod club typically sustains hundreds of high-energy impacts without exhibiting macroscopic fracture before being replaced by new ones during molting [3], it has emerged in recent years as a stimulating model biological material of impact-resistant bioceramics [4]–[7]. In order to fulfill their demanding biomechanical function, the clubs must in particular be imparted with superior impact and fracture resistance. In a previous study [6], we demonstrated that the outer layer of the club –which is made of preferentially oriented fluorapatite (FAP) nanorods surrounded by a thin protein/chitin organic matrix– displays a quasi-plastic contact mechanical response endowing the club with the ability to internally dissipate repetitive mechanical impacts. This characteristic was linked to FAP nanorods that undergo to interfacial sliding and rotation upon contact loading facilitated by the organic matrix.

Fracture resistance is another critical property required for the club to achieve its mechanically-active predatory role. However, accurate fracture toughness measurements of the dactyl club material with conventional fracture mechanics methods is very challenging due to the clubs' small size, an issue that is common to all biological structures with millimeter length scales [8]. Reliable fracture toughness measurements have been carried out on biological materials that are sufficiently large for standardized “macro” fracture mechanics specimens to be extracted, such as large seashells [9], nacre [10] or bone [11], [12]. Careful fracture toughness measurements of teeth using miniature compact tension specimens have also recently been carried out [13], [14]. However, these protocols are not applicable for the dactyl club because they still require samples of at least a few mm long, whereas the impact region of dactyl clubs are just a few hundred microns thick, making it exceedingly challenging to obtain suitable samples by conventional machining methods.

Owing to these inherent difficulties associated with sample size and geometry, many investigators have used the simpler-to-implement nanoindentation fracture techniques

[15]–[17] to evaluate the fracture toughness of hard biological tissues, including for stomatopod dactyl clubs [5]. While indentation methods are versatile tools to characterize the contact mechanics and fracture properties of hard biological tissues [18], they remain semi-quantitative for fracture toughness evaluation and cannot differentiate between elastic and plastic dissipation contributions to the fracture resistance. Recent advances in focused ion beam (FIB) machining and nanomechanical instrumentation have resulted in the development of micromechanical fracture methods that more closely follow standardized fracture mechanics protocols [19], including for biological samples [20]–[25]. Among micro-fracture sample geometries, microcantilevers specimens with a chevron-notched crack geometry established by Mortensen and co-workers [26]–[27] are particularly appealing in fracture studies for several reasons. First, the chevron geometry alleviates the need for a pre-crack –a pre-requisite for valid fracture toughness measurements– because the crack initiates at low loads from the sharp apex but remains stable since the crack width increases as it propagates through the triangular ligament, until it reaches a critical length a_c at which point catastrophic fracture occurs. For linear elastic materials, this occurs at a peak load P_c which is then used to compute the plain strain fracture toughness from chevron notched samples, K_{IVb} [28]. A corollary is that stable crack propagation is achieved even for very brittle materials. An additional benefit for FIB-milled specimens is that the critical fracture criteria – K_{Ic} for linear elastic materials or J_{Ic} for elastic-plastic materials– is obtained after the crack has propagated well beyond the FIB-damaged zone, thereby circumventing any effect due to potential damage introduced during the milling irradiation process.

Enticed by these benefits, here we conducted a fracture mechanics study of the impact region material of the dactyl club using chevron-notched microcantilevers with triangular cross-sections. The microcantilevers were tested with a depth-sensing nanoindenter and experimental load-displacement curves were calibrated by Finite Element Modelling (FEM) to account for variation in individual microbeam geometries following the method by Žagar *et al.* [27] Chevron-notched microcantilevers were tested with a linear elastic fracture mechanics (LEFM) protocol as well as with an elastic-plastic fracture mechanics (EPFM) [29] method using a J -integral approach. Using these methods, we find that –similar to the contact damage resistance– plastic dissipation plays a major role in enhancing the fracture resistance of the dactyl club,

leading to an *R*-curve behavior. Our study also suggests that the microcantilever chevron-notched geometry, which to the best of our knowledge has previously not been employed for biological samples, is well-suited for fracture studies of hard biological materials.

Figure 4.1. Overview of the mantis shrimp dactyl club and cantilever preparation. **A)** Front view of a stomatopod with its dactyl clubs shown (yellow arrows). **B)** Dactyl club side profile highlighting the impact surface (arrow, top) and a cross-section along the axial plane (yellow dot dash line) (bottom).

Figure 4.2. A) Focused ion beam (FIB) cutting procedure of microcantilevers (see Materials and Methods for details). From left to right; i. trench cuts made using a probe current of 3 nA; ii. shaping of cantilevers with 1.5 nA and 300 pA beams, iii. notch and guide lines cut with 10 pA beam. **B)** Schematic showing the positions and orientations (0°, 45°, 90° to the surface normal) of representative microcantilevers milled in the impact region of the dactyl club cross-section.

Figure 4.3. Scanning electron microscope (SEM) images of completed microcantilevers. **A)** Side view. **B)** Front view; dimensions indicated by letters were measured using SEM. **C-D)** SEM images (top) and geometrical dimensions (bottom) of chevron (**C**) and straight notches (**D**). The fracture surface areas, A , bounded by the green line in the SEM micrographs were measured with ImageJ-based FIJI software [30], and h was determined using trigonometric relationships.

4.2 Materials and methods

4.2.1 Materials

Live *Odontodactylus Scyllarus* were acquired from a local aquarium supplier and kept in the custom aquarium in the lab. Three dactyl clubs were obtained from freshly molted mantis shrimps. The clubs were rinsed in DI water to remove salt water and stored immersed in DI water.

4.2.2 Sample preparation

The dactyl clubs were mounted in cold curing epoxy resin (EpoFix™ Resin, Struers). 15 parts resin was added to 2 parts hardener and stirred vigorously for 5 mins before the mixture was poured into a mould containing the sample, which was pre-positioned with common cyanoacrylate glue (super glue). The mould containing the sample and mixture was placed in vacuum for 30 mins and left to cure for at least 20 hours. Slices around 1-1.5 mm thick were cut along the axial plane of the dactyl clubs with an Isomet 1000 Precision Saw equipped with a Struers 50A15 alumina cut-off wheel. Cutting speeds of 200 RPM were used; the feed rate could not be precisely controlled but were managed by hand with assistance of a counterbalance weight. The slices were temporarily attached to a block for easier handling during grinding and polishing. Plane grinding was carried out on Metken Forcipol 1V at 250 RPM under running water using silica paper of 1000 and 1200 Grit paper. Fine grinding was carried out at 150 RPM using 2500 and 4000 Grit paper. A damp polishing cloth impregnated with 40 nm Silica colloidal solution was used for final polishing on Struers RotoPol-35. Samples were rotated by 180° within each step and 90° between steps. Each step is completed when marks from the previous step has been removed. Samples were washed in running water between every grinding and polishing step, this is critical to prevent abrasives and debris from the previous step affecting the current step. Samples were sonicated in DI water for 2 mins to remove any silica nanoparticles remaining on the samples and left to air dry. The dry samples were attached using carbon glue to a standard pin stub used with FEI systems.

4.2.3 Secondary electron microscopy (SEM)

SEM images were obtained using two equipments; JEOL 7600F Field Emission (FE) SEM and FEI Nova Nanolab 600I. In both equipments, an accelerating voltage of 5 kV was used. Lower accelerating voltages were used when samples were found to over-charge. Probe currents of around 680 pA and 98 pA were used respectively. In the 7600F FESEM, the lower secondary electron detector imaging mode (LEI) was employed. This helped to reduce the effects of charging present in the non-conductive biological samples. For general viewing, samples were sputter-coated with platinum for 40 seconds.

4.2.4 FIB milling micro-cantilevers

Microcantilevers were prepared from the axial cross-section of dactyl clubs (Figure 4.1C) as well as from fused quartz for control experiments. The cantilevers were fabricated with a notch-to-indent distance (L) at least twice the width of the cantilever (B), see Figure 4.1D for details. A Zeiss Crossbeam 540X FIB-SEM with a Ga ion source was used for cutting the microcantilevers. This equipment has a dual beam set up which enables convenient SEM imaging along with ion beam cutting (Figure 3.2B). This allows the user to view and select the location for milling as well as observe the results of milling directly after the cutting. Figure 4.1E shows the machining procedure of the microcantilevers. Triangular cantilevers were cut because of the relative ease of preparation compared to rectangular counterparts. The micro-cantilevers were prepared perpendicular and parallel to the surface to measure anisotropy in the fracture toughness of the club. A carbon coat of about 20 nm was applied to protect the sample surface and to provide better electric conductivity during the milling process. An ion accelerating voltage of 30 kV and probe current of 3 nA were used for rough shaping of the cantilever. First, two sloped trenches were milled with their deep end facing each other leaving a bar with a length, width and depth of $20\text{ }\mu\text{m} \times 8\text{ }\mu\text{m} \times 12\text{ }\mu\text{m}$ between them. The third sloped trench is milled with its deep end facing the short cross-section of the gap. After a 1.5 nA beam was used to make two rectangular cuts at 45° angles from the surface normal following the first two sloped trenches; creating a cantilever with a triangular cross section. The ion beam current was reduced to 0.3 nA and the cantilever was trimmed to its final dimensions. After which three guiding lines for cutting the

chevron notch and for positioning the nanoindenter tip were etched on the surface of the cantilever using a 10 pA beam. A ring was also milled to mark the testing spot. Finally, the notch was cut using the same probe current of 10 pA to produce a thin “crack” which is critical for successfully initiating crack propagation during a loading event. Microcantilevers were milled around 100 μm from the surface of the club to stay within the impact region and to avoid the effects of mechanical gradient as much as possible. They were also fabricated in three orientations: normal, tangent and at 45° to the club surface (Figure 4.1C). For more details on the FIB milling of microcantilevers the reader is directed to section 3.1.2.4 covering the optimization of this experiment.

4.2.5 Microcantilevers fracture testing

Microcantilevers were tested with a Hysitron (Minneapolis, MN, USA) TI 950 Triboindenter equipped with a two axis 10 mN transducer and a cube corner tip. The in-built surface probe microscopy (SPM) imaging feature, which uses the nanoindenter tip to probe the specimen surface, allows for accurate placement of the tip on the indentation spot. The microcantilevers were tested in two different ways, namely: single loading (SL) and loading partial-unloading (LPU). In SL, testing was done in quasi-static indentation mode with a three segment load function: i) loading to a peak load, ii) holding for 2 s, iii) followed by quick unloading. A loading rate $1\text{--}3\ \mu\text{Ns}^{-1}$ was selected in accordance to the method by Žagar *et al.* [27] For LPU testing, instead of a single loading cycle to failure multiple partial unloading cycles were done at regular intervals. The unloading cycles were set at 50% of the current maximum load and both loading and unloading were performed at a rate of $3\ \mu\text{Ns}^{-1}$. SL measurements were analysed with LEFM methods, while LPU measurements were analysed with an EPFM procedure. Microcantilevers were tested in both dry and hydrated conditions. Because of the micron size of the cantilevers, simply immersing the entire sample surface in water could generate forces large enough to break or deform the microcantilevers before testing [31]. Therefore, an alternative method was established to hydrate the sample. The sample was placed in a small container above water heated with a hotplate set to 40°C for an hour. Water vapour condensed on the surface of the sample, hydrating it. Single microcantilevers were then tested immediately after the hydration step and the sample rehydrated before the next test.

4.2.6 Finite element method (FEM)

With current FIB technology, it is not possible to produce two exactly identical microcantilevers. Therefore, a single geometric function cannot be used for different cantilevers. One method to circumvent this is to use FEM in order to compute the compliance curve for every cantilever tested, and in turn the geometric function of the chevron-notched geometry [26]. Abaqus CAE® software (Dassault Systèmes) was used to create at least 30 FEM models for each microcantilever, varying the crack length between a_0 and a_1 for the chevron notch, while arbitrary values for crack length were used for the straight notch. Figure 4.2 shows a typical FEM model of a chevron-notched microcantilever; an example FEM model for straight-notched microcantilever can be found in Figure 4.3. The cantilever was modelled following the dimensions measured from SEM images. The cantilever support was also included in each model to account for support deformation during testing. The dimensions of the support were arbitrary but equal for all the cantilevers. For the fused quartz samples, the elastic modulus and Poisson's ratio provided by the supplier were used, whereas for the dactyl club material the elastic modulus (61.1 GPa) and Poisson's ratio (0.25) were taken from our previous study [5]. The cross-sectional view of the model at the notch is shown in Figure 4.2A. The crack and seam are also highlighted in the figure. Partitioning was used to create the seam for the crack and the crack front. The seam was assumed to be infinitesimally small, meaning the adjacent nodes on the seam are overlapping with each other. The geometry was discretised using 3D 20-node quadratic brick elements (C3D20R) for most of the mesh, except for the crack singularity where 15-node quadratic wedge elements (C3D15) were used. For up to 12 elements away from the crack front, the mesh was modelled according to ASTM STP 1074 [32]. The size of the crack singularity zone was kept to at least 20 times smaller than the least dimension of the model; the least dimension is either the crack width (a_0) or the ligament width ($W-a_0$). Moreover, for a 1% error margin the element sizes (l) were kept to at least one quarter its distance (d) from the crack (*i.e.* $l/d \leq 0.25$) (Figure 4.2A). The number of wedge elements (N) required in the singularity was estimated from the equation: $N = 2\pi/(l/d)$. [32] 32 wedge elements were created within the singularity. To achieve the LEFM singularity, the midpoint nodes of the singularity elements are shifted to $0.25d$ from the crack front as shown on the wedge element in Figure 4.2A. A completed model had approximately 100,000 – 130,000 elements. Clamped boundary conditions were

applied to the support faces that are attached to the sample surface (Figure 4.2B), and a 1 μN load was applied at the mid-point of cantilever free end corresponding to the indentation site of the experiment. The cantilever deflection was determined from the node at the bottom of the cantilever free end, as shown in Figure 4.2B. The displacements from the 30+ simulations were recorded and used to find the compliance at different crack lengths for a single microcantilever. The crack lengths and compliance values were converted to dimensionless values for dimensional analysis: $\tilde{a} = a/W$, $C_V = CBE^*$, $E^* = E/(1-\nu^2)$ [26]. Variation of C_V with $\tilde{a}$ was fitted with a fifth-order polynomial (using OriginPro® software) to obtain the compliance calibration curve for each micro-cantilever.

Figure 4.4. Finite element model (FEM) of chevron-notched microcantilevers. **A)** Side view of the cantilever model (support not shown). The load is applied at the top center node of the free end (right). The gap between the loading position and the actual free end was not modelled as its contribution was deemed insignificant. Magnified images of the mesh near the crack front

modelled following the ASTM STP 1074 (yellow and red boxes). i. Full mesh near the crack region; element lengths (l) are at most $0.25d$, where d is the distance from the crack to the element; ii. Mesh at the crack front, a total of 32 wedge elements were used for the singularity; iii. Cross-section view of the notch (at the blue center line in **A**). The crack front is represented by the yellow line while the areas enclosed by the magenta lines including the crack front is defined as the crack seam. The red arrows indicate the direction of the $\mathbf{q}$ vector. **B**) 3D view of the microcantilever and support. The support is modelled with fixed boundary conditions at the faces that are joined to the surface of the dactyl club (orange line and areas). 15-node wedge elements were used for the singularity elements while 20-node brick elements were used for the rest of the model. Compliance values are determined using the displacement of the node at the bottom of the free end.

Figure 4.5. Representative FEM model of a straight notch microcantilever. The only difference to the chevron notch model is the crack and seam definition; meshing of the region near the crack is synonymous. **(A)** Side view. **(B)** Cross-sectional view at the notch. **(C)** 3D view of the microcantilever and support.

4.2.7 K_{IVb} determination with LEFM

For LEFM materials, the fracture toughness for chevron-notched specimens (K_{IVb}) can be obtained from Equation 2.33 repeated here for ease of reading:

$$K_{IC} = \frac{P_C}{B\sqrt{W}} F_V(\tilde{a}_c) \quad (4.1)$$

The geometric function, $F_V(\tilde{a})$, for a chevron notch can be determined using Equation 2.34 (also repeated here):

$$F_V(\tilde{a}) = \sqrt{\frac{1}{2(1-\tilde{a})} \frac{\tilde{a}_1 - \tilde{a}_0}{\tilde{a} - \tilde{a}_0} \frac{dC_V}{d\tilde{a}}} \quad (4.2)$$

If instead the notch is straight $F_V(\tilde{a})$ is given by the following equation:

$$F_V(\tilde{a}) = \sqrt{\frac{1}{2(1-\tilde{a})} \frac{dC_V}{d\tilde{a}}} \quad (4.3)$$

Theoretically, the critical load is achieved when $F_V(\tilde{a})$ reaches its minima, which has been shown to be the case with fused quartz [27] and other brittle materials [26], [33]–[37]. The crack length at this minima is the critical crack length a_c , which is then used to compute K_{IVb} using Equation 2.33 and P_c . Importantly, Equation 2.33 assumes that there is no R -curve behaviour in the material, which is a limitation since R -curve behaviour is hallmark of elastic-plastic materials, and to some extent semi-brittle materials. To overcome this drawback, Li *et al.* [29] have expanded the microcantilever chevron-notch method to EPFM, which we adopted in this study as explained below.

4.2.8 R -curve behaviour and EPFM

EPFM was used to analyze data from LPU microcantilever tests and obtain R -curves. The advantage of LPU testing compared to SL testing is that the stiffness of the cantilever can be monitored at regular load intervals from the unloading segments of LPU load-displacement curves. With the compliance curves obtained from FEM, the crack length at the unloading points can be determined, and thus the incremental crack area can be estimated assuming the crack front remains straight as the crack advances. In EPFM, the J -integral encompasses the energy released during crack advancement (linear elastic contribution), and the energy absorbed by the material by deformation (plastic contribution) [38]. J characterizes the crack tip conditions in elastic-plastic materials just as the stress intensity factor K does in linear-elastic materials. Both

parameters are uniquely related by the intrinsic relationship, $K = \sqrt{JE^*}$ (Equation 2.20). In the case of a linear elastic material J becomes the energy release rate G [38]. The R -curve for each microcantilever can be determined by computing J at the unloading segments using the following equations [29]:

$$K_i = \frac{P_i}{B\sqrt{W}} F(\tilde{a}_i) \quad (4.4)$$

$$J_i = J_{i,el} + J_{i,pl}$$

$$J_i = \frac{K_i^2}{E^*} + \frac{2\eta A_{i,pl}}{\hat{b}_i \hat{a}_i} \quad (4.5)$$

J_i consists of the elastic component $J_{i,el}$, which can be calculated by substituting the normalized crack length at the i^{th} unloading segment into Equation 4.4, as well as the plastic component $J_{i,pl}$ obtained from the area ($A_{i,pl}$) under the load displacement curve up to the i^{th} unloading segment multiplied by a constant η divided by the crack area ($\hat{a}\hat{b}$). The dimensionless constant η is equals to 2 for this test configuration [29], and $\hat{a}$ and $\hat{b}$ are the incremental crack length and width from a_0 . Figure 4.4 shows a visual representation of J_i calculation for a typical curve in hydrated conditions used to obtain an R -curve.

Figure 4.6. (A) Schematic of the dimensions used in Equation 4.4 and 4.5. **(B)** Representative LPU curve of a microcantilever tested in hydrated conditions (the same curve as the red curve shown in Figure 4.6A). The numbered points also coincide with those in Figure 4.8.

4.2.9 Data treatment and statistics

Since the material surrounding the location of indent experiences a stress field and can deform plastically, the indenter tip might travel into the cantilever during testing. Hence, the total displacement measured by the nanoindenter is a combination of the deflection of the cantilever and the indentation depth of the tip on the cantilever. To account for the indentation depth contribution, several indents were made in the region next to the support of the cantilever named peripheral indents. The region next to the support was chosen because it resembles the cantilever surface the most, being exposed to similar damage from FIB milling. The same load function used in testing the cantilever was used for the peripheral indents and the average of the load displacement curves were taken and fitted with a polynomial function. The displacement contribution from indentation was then found by substituting the peak load into the function. Statistical inference were performed using the statistics module in OriginPro software. A two samples t-test was employed to compare the fracture toughness of straight-notched cantilevers found using the two different methods (FEM and analytical solution), as well as to analyse the difference between chevron and straight-notched microcantilevers. ANOVA was used to study the effect of cantilever orientation on the fracture toughness. OriginPro was also used to fit a line to the unloading segments in the LPU tests to extract the stiffness. Artefacts due to hysteresis were observed at the start of each unloading segment during the LPU measurements; these were ignored in line fitting. A custom Python code was used to determine $A_{i,pl}$, and calculate K_i and J_i using Equations 4.4 and 4.5, respectively. OriginPro was used to generate all plots in the figures unless otherwise stated.

4.3 Results and discussion

A total of 27 notched microcantilevers were successfully prepared and tested in this study, namely 23 from the impact region of the stomatopod dactyl club in both dry and wet conditions as well as 4 fused quartz samples for control experiments. Furthermore, dactyl club samples in SL were tested under three orientations (Figure 4.1C). Table 4.1 and 4.3 summarizes the notch type, orientation, measured dimensions, load and fracture toughness results of the SL and LPU microcantilevers that were successfully milled and tested, respectively. Figure 4.5 shows typical load-displacement curves from tests

carried out on both dactyl club samples as well as the control fused quartz. Three representative curves are displayed for the chevron-notched (Figure 4.5A and D), and another three for chevron-notched microcantilevers (Figure 4.5B and E). A typical successful test curve [28] begins with a linear extension representing the recoverable extension of the cantilever, followed by a non-linear extension with a decreasing slope. At this point stable, crack growth through the ligament occurs until the load reaches a maximum. The system then becomes unstable and the crack begins to rapidly propagate without increase of load until the cantilever is separated from the support.

Table 4.1. Results from single loading cycle microcantilever chevron-notch measurements on the impact region of the dactyl club and control fused quartz. Physical dimensions for chevron and straight-notch microcantilevers are as defined in Figure 4.1D, F and G. $\dot{P}$: loading rate for cantilever testing; P_c : critical load from the load-displacement curves corrected for indenter tip embedding; $F_V(\tilde{a}_c)$: geometric functions at the critical crack length $\tilde{a}_c$ determined using FEM; $B, a_0, W-a_0 > r_k = 2.5 \left(\frac{K_{Ivb}}{\sigma_{ys}} \right)^2$: minimum dimensions for LEFM to be valid according to ASTM 399 [39] where σ_{ys} is the indentation yield strength taken from Reference [6].

Sample	Material	Notch	W	B	a_0	a_1	L	S	$\dot{P}$	P_c	$\tilde{a}_c$	$F_V(\tilde{a}_c)$	K_{Ivb}	r_k
			μm	μm	μm	μm	μm	μm	μNs^{-1}	μN			$\frac{\text{MPa}}{\text{m}^{0.5}}$	μm
QS1	Quartz	Chevron	3.0	5.2	0.9	1.9	10.7	0.5	2	24.69	0.43	187.0	0.51	0.93
QS2	Quartz	Chevron	2.8	5.0	1.0	1.8	9.5	1.0	3	26.72	0.46	209.7	0.66	1.55
QS3	Quartz	Chevron	2.9	5.1	1.1	1.9	11.3	0.8	2	17.79	0.47	245.9	0.50	0.87
DC1	MSDC	Straight	2.9	4.6	0.9	-	15.0	0.9	1	14.40	0.31	94.1	0.17	0.06
DC2	MSDC	Straight	3.2	4.8	0.8	-	15.1	1.2	1	37.70	0.24	63.00	0.28	0.14
DC3	MSDC	Straight	3.4	5.4	1.3	-	15.1	1.1	1	20.90	0.39	110.8	0.24	0.10
DC4	MSDC	Chevron	3.6	4.5	0.8	1.9	14.3	2.9	1	17.50	0.35	128.9	0.26	0.13
DC5	MSDC	Chevron	3.9	6.6	1.3	2.6	15.3	2.9	1	20.40	0.45	254.3	0.40	0.29
DC6	MSDC	Chevron	3.2	4.6	0.5	1.4	14.8	2.2	1	14.30	0.22	131.9	0.23	0.10
DC7	MSDC	Chevron	2.7	4.5	0.3	1.3	14.4	1.9	1	12.10	0.22	121.0	0.19	0.07
DC8	MSDC	Chevron	3.0	4.8	0.4	1.5	14.5	1.9	1	10.70	0.28	137.1	0.18	0.06
DC9	MSDC	Chevron	3.3	5.6	1.0	1.9	15.2	1.6	1	11.92	0.41	206.5	0.24	0.11
DC10	MSDC	Straight	2.8	4.8	1.2	0.0	14.5	1.0	1	5.88	0.43	157.8	0.12	0.03
DC11	MSDC	Straight	2.2	4.2	0.9	0.0	9.1	1.3	1	11.50	0.42	123.5	0.23	0.10
DC12	MSDC	Chevron	2.8	4.8	0.8	1.7	9.7	1.4	1	5.09	0.40	150.4	0.10	0.02
DC13	MSDC	Chevron	2.7	4.6	0.8	1.5	11.0	1.2	1	1.28	0.40	174.3	0.03	0.02
DC14	MSDC	Straight	2.8	5.4	1.0	0.0	11.1	1.2	1	11.55	0.36	89.5	0.11	0.02
DC15	MSDC	Straight	3.3	5.7	1.4	0.0	11.6	0.9	1	12.83	0.44	111.5	0.14	0.04

Table 4.2. Fracture toughness determined analytically using Equation 4.6 and 4.7 on the straight-notched SL microcantilevers.

Sample	Material	Notch	W	B	a_0	a_1	L	S	P_c	$\tilde{a}_c$	$F_V(\tilde{a}_c)$	K_{IVb}	r_K
			μm	μm	μm	μm	μm	μm	μN			$\text{MPa} \cdot \text{m}^{0.5}$	μm
DC1	MSDC	Straight	2.9	4.6	0.9	0.0	15.0	0.9	14.40	0.31	1.45	0.17	0.05
DC2	MSDC	Straight	3.2	4.8	0.8	0.0	15.1	1.2	37.70	0.24	1.30	0.28	0.15
DC3	MSDC	Straight	3.4	5.4	1.3	0.0	15.1	1.1	20.90	0.39	1.66	0.21	0.08
DC10	MSDC	Straight	2.8	4.8	1.2	0.0	14.5	1.0	5.88	0.43	1.81	0.10	0.02
DC11	MSDC	Straight	2.2	4.2	0.9	0.0	9.1	1.3	11.50	0.42	1.78	0.19	0.07
DC14	MSDC	Straight	2.8	5.4	1.0	0.0	11.1	1.2	11.55	0.36	1.57	0.10	0.02
DC15	MSDC	Straight	3.3	5.7	1.4	0.0	11.6	0.9	12.83	0.44	1.86	0.12	0.03

4.3.1 Microcantilever chevron-notched LEFM experiments

Of the 15 SL microcantilevers on the dactyl club, 7 were straight-notched cantilevers. According to Ast *et al.* [19], geometry does not affect the fracture toughness values measured from straight-notched microcantilevers significantly. Therefore, a single geometric function can in principle be applied to determine the fracture toughness from all the straight-notched cantilevers. Bechtle *et al.* [20] applied this concept to obtain the fracture toughness of enamel from straight-notched cantilevers using:

$$F_{I,V}(\tilde{a}) = \frac{13.458\sqrt{\pi\tilde{a}}}{(1-\tilde{a})^{3/2}} (1 + 0.87\tilde{a}) \left[\frac{5}{8} - \frac{5}{12}\tilde{a} + \frac{1}{8}\tilde{a}^2 + 5\tilde{a}^2(1-\tilde{a})^6 + \frac{3}{8}e^{-\frac{6.1342\tilde{a}}{1-\tilde{a}}} \right] \quad (4.6)$$

By substituting the normalized initial crack length ($\tilde{a}_0$) into Equation 4.6, the geometric constant can be determined for a straight notch triangular cantilever. Consequently, substituting the fulcrum length (L), cantilever width (B), thickness (W), and breaking load (P_C), the fracture toughness of the cantilever can be determined as:

$$K_{IC} = \frac{PL}{BW^{3/2}} F_{I,V}(\tilde{a}) \quad (4.7)$$

Table 4.2 shows the fracture toughness values from the same straight SL microcantilevers but using the aforementioned analytical solution. The average fracture toughness values from both methods were compared (Figure 4.7A, left) and found to

be statistically similar ($p_{2S} = 0.62$). Therefore, the mean fracture toughness from FEM and analytical solutions are both viable to analyse the straight notched cantilevers. For further analyses, only FEM solutions were considered. Fracture toughness measurements ranged from a minimum of $0.03 \text{ MPa m}^{0.5}$ to a maximum of $0.340 \text{ MPa m}^{0.5}$. For the K_{IVb} values to be qualified under ASTM 399 [39], the following criterion for the minimum dimension size should be met:

$$B, W-a_0 \text{ and } a_0 > r_K = 2.5 \left(\frac{K_{IVb}}{\sigma_{ys}} \right)^2 \quad (4.8)$$

where σ_{ys} is the yield strength, which was estimated from our previous indentation stress-strain measurements [6] following the relationship derived by Tabor [40], $\sigma_{ys} = P_m/3$, where P_m is the mean contact pressure at yielding ($\sigma_{ys} = 1167 \text{ MPa}$ in dry conditions and $\sigma_{ys} = 867 \text{ MPa}$ in hydrated conditions). The criterion was met for all SL cantilevers. Comparison between straight and chevron notched samples (Figure 4.7A, center) revealed that on average, fracture toughness values were not significantly different from each other ($p_{2S} = 0.11$), indicating that the straight notch approximation could be used for cantilevers where the chevron notch had not been completely cut out during FIB milling. The fracture toughness values were further analysed using the method described in Reference [17] by plotting the data points as a cumulative distribution plot and fitted with a Weibull Function (Figure 4.7B). The fracture toughness at 50% of micro-cantilever failure rate was found to be $0.21 \pm 0.08 \text{ MPa m}^{0.5}$. In previous nanoindentation fracture experiments [5], fracture toughness values of $2.02 \text{ MPa m}^{0.5}$ were obtained (dry conditions), which is about 10 times larger. In a separate study, the fracture toughness of pure (non-biogenic) FAP determined by microindentation fracture was found to be $0.30 \text{ MPa m}^{0.5}$ [41]. In order to verify the validity of our experimental procedure and FEM model, control experiments were carried out on fused quartz SL microcantilevers and compared to the work of Zagar *et al.* [27] Their load-displacement and compliance curves are shown in Figure 4.5C and F. An average value of $0.56 \pm 0.09 \text{ MPa m}^{0.5}$ was obtained, which is in the lower end of the range of values obtained in other studies [42]–[48]. Tests on fused quartz were conducted in an enclosure at 63% humidity, making the samples susceptible to environmentally assisted subcritical crack growth [26], which could give rise to some embrittlement. Nevertheless, the fracture toughness values are close to those reported

in the literature for fused quartz, giving us confidence in the accuracy of our experimental and FEM protocols.

A median value of fracture toughness for the impact region below that of geological FAP is clearly not consistent with the high impact resistance of the club, and must be attributed to other factors. One possibility is that the measurements were performed on cracks subjected to uniaxial loading so as to obtain mode I fracture toughness (K_{IVb}). The club is designed to withstand loading in compression and to impact objects that are much larger than the scale at which the experiments were conducted. In this respect, indentation methods are more akin to the club's natural use. While the loading mode may play a role, it cannot explain fracture toughness values that are 10-fold lower than those obtained by indentation fracture, especially since low fracture toughness values (*ca.* 0.30 MPa m^{0.5}) have also been reported for geological FAP measured by an indentation fracture method. Instead, we surmised that these results are due to the data analysis treatment of the chevron-notched geometry, which ignores non-elastic dissipation at the crack tip. Indeed, the shape of the load-displacement curves, with stable crack propagation after the peak load, did suggest significant plastic dissipation (see Figure 4.7A and B), prompting us to conduct EPFM testing with partial loading/unloading cycles.

Figure 4.7. Plots of SL microcantilever testing analysed using LEFM. **A, B)** Load-displacement curves of the dactyl club (DC) impact region with chevron-notched and straight-notched microcantilevers, respectively. **C)** Load-displacement curves of chevron-notched microcantilevers of the fused quartz control (QS). Stable crack growth before fracture was observed in all SL microcantilevers. **D–F)** Compliance calibration curves ($C_v(\tilde{a})$, symbols with solid line) obtained from FEM and geometric functions ($F_v(\tilde{a})$, dashed lines) calculated using Equation 4.2 and 4.3. The minima of $F_v(\tilde{a})$ curves correspond to a_c , which is used to calculate K_{IVb} .

4.3.2 Microcantilever chevron-notched EPFM experiments

To test the above hypothesis that non-elastic dissipation mechanisms occur at the crack tip, microcantilevers specimens were micromachined from the impact region of the club. As a control, one specimen was also prepared from the fused quartz material, and all specimens were tested by LPU. The fused quartz cantilever was tested dry while club samples were tested in both dry and hydrated conditions. Figure 4.6A compares two load-displacement curves of the impact region of dry and hydrated microcantilevers. Significant non-linear behaviour was clearly detected in both conditions, suggesting that plastic dissipation strongly contributes to the fracture resistance of the dactyl club material. While dissipative behaviour was detected in both dry and hydrated conditions, the latter exhibited more stable crack propagation behaviour after the maximum load. The compliance of the cantilevers was found to increase with the load (Figure 4.6B), confirming (stable) crack growth through the microcantilevers (Figure 4.6B). Crack growth resistance curves were obtained using the compliance calibration curves for each cantilever, and representative curves are shown in Figure 4.6D (the procedure to compute the R -curves from LPU data at each unloading point, including the crack length and area under the curve A_{pl} , is depicted in Figure 4.4; see also section 4.2.8 for fuller details). Under both conditions, a marked R -curve behaviour was identified for all tests and the J -value at fracture instability, J_q , was taken as the critical criterion. The cantilever dimensions, test conditions, and results of the LPU microcantilever tests are tabulated in Table 4.3.

For the J -integral values to be qualified according to ASTM 1820 [49], the following criteria should be met: B and $W-a_0 > r_J = \frac{\lambda J_q}{\sigma_Y}$, where λ is a constant equal to 10 and σ_Y is the effective yield stress, calculated from $\sigma_Y = \frac{\sigma_{YS} + \sigma_{max}}{2}$. Here σ_{max} can be taken as the maximum flow stress [49] obtained from the indentation stress-strain curves as described above for σ_{YS} [5], [40] ($\sigma_Y = 1317$ MPa in dry conditions and $\sigma_Y = 1000$ MPa in hydrated conditions). According to this criterion, J_q can be qualified as the plane strain fracture energy, which can be also converted into an equivalent fracture toughness K_{Jeq} . Comparison of fracture toughness values measured using LEFM and EPFM is presented in Figure 4.7C. The average fracture toughness ($K_{Jeq} = 1.89 \pm 0.41$ MPa m^{0.5}) is about 8.5 times that obtained using LEFM ($K_{IVb} = 0.22 \pm 0.08$ MPa m^{0.5}), confirming

that plastic dissipation is the dominant contribution to the fracture resistance of the dactyl club material. As a control, we tested a fused quartz microcantilever by LPU, and the load-displacement curve is shown in Figure 4.8. Figure 4.8A shows that for fused quartz there is hardly any plastic deformation before sudden fracture. The loading, holding, and unloading segments coincided with each other except for the last cycle, which indicated minor non-linear behaviour. Using the EPFM method, a J -integral at the final unloading point before instability J_q of 6.20 J/m^2 was obtained for fused quartz, corresponding to an equivalent fracture toughness K_{Jeq} of $0.68 \text{ MPa m}^{0.5}$. As expected for a brittle material such as fused quartz, the fracture toughness values measured using LEFM and EPFM are close to each other, since for a linear elastic material J is equal to the energy release rate, G . Hence, nearly all the stored elastic energy within the fused quartz is released by crack growth at the microcantilever notch with little plastic deformation. This control experiment gives us further confidence that the plastic dissipation measured for the dactyl club material is not an experimental artefact of our EPFM protocol and that the measured J -integral values can be used to quantify the fracture characteristics of the dactyl club material.

Amini *et al.* [6] found that FAP nanocrystallites of the impact region rotate and slide under contact stress field, thereby dissipating impact energy by a so-called quasi-plastic behaviour. Such a dissipative deformation mechanism of nanocrystalline sliding and rotation fracture energy may also be activated by the tensile crack tip field, similar to tablet sliding identified in nacre [10]. Other inelastic deformation processes documented in tough biomineralized structures [50], [51] are likely to contribute to crack tip plasticity of the impact region. Plausible mechanisms include deformation and strain hardening of the thin organic layer located between FAP nanocrystals and micro-fracture in the crack-tip process zone. While the relative contributions of these phenomena to the total fracture resistance remain unknown, the key conclusion from EPFM analysis is that plastic dissipation plays a dominant role in imparting fracture resistance to the impact region of the dactyl club.

It is noteworthy that the K_{Jeq} values inferred from our EPFM testing yields values are comparable to those obtained by indentation fracture methods [5]. However, the results are not directly comparable because indentation fracture testing does not explicitly separate elastic from plastic contributions and thus cannot be used to obtain the R -curve

behaviour [52]. Nevertheless, during indentation fracture testing, plastic dissipation is indirectly captured because it increases the contact load to initiate crack initiation, which is itself used to obtain the fracture toughness [5], [53]. Thus, measuring fracture toughness values that are close to each with both methods is not surprising. We also note that the average K_{eq} from dry and hydrated measurements on the dactyl club are similar ($p = 0.98$). While dried biological materials are usually more brittle than when hydrated [54], the differences between dry and hydrated samples become much less apparent for hypermineralized structures containing a low amount of organic phase (such as the impact region of the dactyl club), because it is mostly the latter phase that is susceptible to hydration effects. This is also consistent with the small variation in elastic modulus of the impact region between dry and hydrated conditions [4]. The notion that hydration affects the mechanical properties of apatite structures is not fully agreed upon in the literature. Hua *et al.* showed that dehydration affects the mechanical properties of porcine enamel using nanoindentation and nanowear experiments; hardness increased by around 30% after 48 h drying [55]. Giant panda teeth have also been shown to exhibit over 70% increase in fracture toughness with indentation fracture measurements and marginal recovery from wear damage when hydrated [56]. However, the elastic modulus of rat femur did not vary significantly (about 2%) when dried in an SEM or hydrated [31]. From a biological perspective, it is important for the stomatopod that its dactyl clubs do not vary considerably with environmental conditions, especially in the impact region of the club.

Table 4.3. Results from LPU chevron-notched microcantilevers measurements on the impact region of the dactyl club and control fused quartz (EPFM measurements). Microcantilever and notch physical dimensions are defined as in Figure 4.1D, F and G. $\dot{P}$: loading rate during testing; $P_{\max}$: maximum load before the final unloading cycle; Δa : crack length increments from a_0 at the final unloading cycle; J_q : J -integral at the maximum load; K_{Jeq} : corresponding equivalent fracture toughness. $B, W-a_0 > \frac{\lambda J_c}{\sigma_Y}$: minimum dimensions for validity under EPFM from ASTM 1820.

Sample	Material	W	B	a_0	a_I	L	S	$\dot{P}$	$P_{\max}$	Δa	J_q	K_{Jeq}	$\frac{\lambda J_q}{\sigma_Y}$
		μm	μm	μm	μm	μm	μm	$\mu\text{N/s}$	μN	μm	J/m^2	$\text{MPa m}^{0.5}$	μm
QS4	Quartz	3.0	5.3	1.0	1.9	14.2	0.9	3	16.90	0.36	6.20	0.68	0.74
DC16	MSDC	3.2	5.2	1.2	2.1	12.0	1.2	3	7.21	0.56	53.0	1.75	0.40
DC17	MSDC	2.9	5.1	0.9	1.9	11.8	1.0	3	7.84	0.58	35.5	1.55	0.27
DC18	MSDC	2.2	4.0	0.6	1.4	9.9	0.8	3	7.38	0.38	79.8	2.35	0.61
DC19	MSDC	2.6	4.8	0.9	1.6	11.0	1.1	3	5.69	0.33	59.4	1.61	0.59
DC20	MSDC	2.7	4.9	0.9	1.7	11.5	1.0	3	5.64	0.24	70.0	1.80	0.70
DC21	MSDC	2.6	4.9	0.8	1.6	11.4	1.2	3	5.71	0.51	46.7	1.64	0.47
DC22	MSDC	2.7	5.3	1.0	1.7	11.5	1.0	3	6.68	0.39	96.2	2.13	0.96
DC23	MSDC	2.8	5.1	0.9	1.8	11.5	1.0	3	5.62	0.57	112.8	2.27	1.13

Figure 4.8. Plots of data from LPU microcantilever chevron-notched testing analysed by EPFM. **A)** Representative LPU curves for microcantilever tested in dry (black) and hydrated (red) conditions, colour coding same throughout. **B)** Experimental microcantilever compliance change at each unloading segment. **C)** Non-dimensional compliance calibration curves from FEM correlating compliance with normalized crack length. The numbered points correspond to the same points as in B. The initial stiffness measured from each cantilever was assumed to be the stiffness at the initial crack length a_0 , following which the compliance change of the cantilevers can be determined, and consequently the crack lengths at the other labelled points. **D)** R-curves plotted from the $\tilde{a}$ values obtained from the compliance calibration curves in C.

Figure 4.9. Statistical and comparative analyses of fracture toughness values obtained using LEFM and EPFM. **(A)** Statistical inference using two sample t-tests and ANOVA on the SL microcantilevers (LEFM); the means of samples are not significantly different ($p < 0.05$) when analysed using analytical method or FEM, between chevron and straight notch samples, and with changing orientation. **(B)** Weibull cumulative function on the SL microcantilevers (LEFM): a median (50% cumulative frequency) fracture toughness of $0.21 \text{ MPa} \cdot \text{m}^{0.5}$ was measured. **(C)** Comparison of fracture toughness measured using LEFM (K_{IVb}) or EPFM (K_{Jeq}). Fracture toughness of the impact region of the dactyl club is an order of magnitude larger as measured by EPFM, indicating that plastic dissipation is the dominant contribution to the fracture resistance. Control fracture toughness experiments of fused quartz using the same methods (LEFM and EPFM) show values that are close to each other, consistent with the brittle nature of fused quartz. * $p < 0.05$, ** $p < 0.0005$.

Figure 4.10. Additional LPU microcantilever tests. **A)** Stress-strain curves from measurements of microcantilevers were prepared without notches on fused quartz (black), dactyl club tested dry (red), and dactyl club tested hydrated (green). Little to no deformation was observed in fused quartz before failure, while considerable plastic deformation occurred in the dactyl club microcantilevers. The slight shift for fused quartz at about 0.02 strain is probably due to the tip sliding on the surface of the cantilever. **B)** Load-displacement curve of fused quartz LPU microcantilever. The material behaves elastic for most of the sample except for the last unloading cycle before the cantilever breaks.

4.3.3 Orientational effect on fracture toughness

It was previously found [4], [5] that the apatite crystals in the impact region are preferentially oriented with their *c*-axis aligned normal to the surface. In this present study, five cantilevers were prepared, normal, tangent and at 45° to the surface, to quantify possible fracture toughness anisotropy in the impact region (Figure 4.1C). The data analysed using ANOVA (Figure 4.7A, right) indicated no significant difference ($p_{anova} = 0.39$) between fracture properties within any of the three orientations, despite the elastic anisotropic properties of this region [5], [6]. A recent study [7] showed that the impact region is composed of mineralized microchannels aligned towards the surface with interwoven chitin fibers. The chitin fibers form wavy sheets that are stacked with a small angle between each layer, forming a herringbone pattern when observed in cross-sectional orientation. This interweave pattern of chitin fibers may result in the isotropic fracture properties measured here, in contrast to the anisotropic elastic properties governed by the aligned mineralized microchannels. These results also indicate that micromechanical measurements are useful to understand the local

mechanical properties of biological materials but may not be extrapolated to assess the orientational effects at the meso- and macro-scale. Jimenez-Palomar *et al.* reached the same conclusion for rat femoral bone [57]. Using microbeams, they found that the work-of-fracture was independent of orientation although fracture toughness of bone at the macroscale was very dependent on the direction of measurements. The work of fractures values varied from 24.8 to 40.6 Jm⁻² from 6 measurements which when plotted against elastic modulus showed a rough scatter, although elastic modulus shows a downward trend with angle. The angle here is the angle from the *c*-axis of the collagen fibrils. The fracture toughness measurements in this study provides an understanding of the fracture toughness of the impact region at the submicron level.

4.4 Conclusion

The impact region of mantis shrimp dactyl clubs is highly mineralized and hard, yet as many previous studies have shown it is capable of fracturing other tough biocomposites like nacre. This study set out to study the fracture mechanics of this region using microcantilever bending, with the goal of avoiding the pitfalls of indentation fracture measurements. Chevron-notched triangular notched cantilevers were successfully used to characterize the fracture toughness of the impact region of dactyl clubs using both LEFM and EPFM procedures. With LEFM, a median K_{IVb} value of 0.21 ± 0.08 MPa m^{0.5} was measured, whereas with EPFM the equivalent fracture toughness at fracture instability K_{Jeq} was 1.89 ± 0.41 MPa m^{0.5}. The large difference in fracture toughness values obtained between LEFM and EPFM procedures show that plastic dissipation at the crack tip is the main contribution to the fracture resistance, which parallels the quasi-plastic behaviour occurring during contact loading [6s]. Fracture toughness measured at different angles to the surface showed no statistically significant differences. FEM models assume an isotropic and homogenous material which is true for a material like fused but not fully accurate for a complex biomineral structure like the dactyl club. More accurate measurements of fracture toughness of biomineral structures may be achieved by refining the FEM model to be as close to the native structure. Nonetheless, this work presents a more robust method to quantify the fracture mechanics response of biomineral structures at the microscale using chevron-notched microcantilevers and could be expanded to other hard biotools such as protective shells or dental materials.

References

- [1] S. N. Patek, W. L. Korff, and R. L. Caldwell, “Deadly strike mechanism of a mantis shrimp,” *Nature*, vol. 428, p. 819, Apr. 2004, doi: <http://dx.doi.org/10.1038/428819a>.
- [2] S. J. Longo *et al.*, “Beyond power amplification: latch-mediated spring actuation is an emerging framework for the study of diverse elastic systems,” *J Exp Biol*, vol. 222, no. 15, Aug. 2019, doi: 10.1242/jeb.197889.
- [3] S. Amini *et al.*, “A diecast mineralization process forms the tough mantis shrimp dactyl club,” *Proc Natl Acad Sci USA*, vol. 116, no. 18, pp. 8685–8692, Apr. 2019, doi: 10.1073/pnas.1816835116.
- [4] J. C. Weaver *et al.*, “The Stomatopod Dactyl Club: A Formidable Damage-Tolerant Biological Hammer,” *Science*, vol. 336, no. 6086, p. 1275, Jun. 2012, doi: 10.1126/science.1218764.
- [5] S. Amini *et al.*, “Textured fluorapatite bonded to calcium sulphate strengthen stomatopod raptorial appendages,” *Nature Communications*, vol. 5, p. 3187, Jan. 2014, doi: 10.1038/ncomms4187.
- [6] S. Amini, M. Tadayon, S. Idapalapati, and A. Miserez, “The role of quasi-plasticity in the extreme contact damage tolerance of the stomatopod dactyl club,” *Nat Mater*, vol. 14, no. 9, pp. 943–950, Sep. 2015, doi: <http://dx.doi.org/10.1038/nmat4309>.
- [7] N. A. Yaraghi *et al.*, “A Sinusoidally Architected Helicoidal Biocomposite,” *Adv. Mater.*, vol. 28, no. 32, pp. 6835–6844, Aug. 2016, doi: 10.1002/adma.201600786.
- [8] D. Taylor, “Measuring fracture toughness in biological materials,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 77, pp. 776–782, Jan. 2018, doi: 10.1016/j.jmbbm.2017.07.007.
- [9] S. Kamat, X. Su, R. Ballarini, and A. H. Heuer, “Structural basis for the fracture toughness of the shell of the conch *Strombus gigas*,” *Nature*, vol. 405, no. 6790, pp. 1036–1040, Jun. 2000, doi: 10.1038/35016535.
- [10] F. Barthelat and H. D. Espinosa, “An Experimental Investigation of Deformation and Fracture of Nacre—Mother of Pearl,” *Experimental Mechanics*, vol. 47, no. 3, pp. 311–324, Jun. 2007, doi: 10.1007/s11340-007-9040-1.

- [11] R. K. Nalla, J. J. Kruzic, J. H. Kinney, and R. O. Ritchie, “Mechanistic aspects of fracture and R-curve behavior in human cortical bone,” *Biomaterials*, vol. 26, no. 2, pp. 217–231, Jan. 2005, doi: 10.1016/j.biomaterials.2004.02.017.
- [12] R. K. Nalla, J. J. Kruzic, J. H. Kinney, M. Balooch, J. W. Ager, and R. O. Ritchie, “Role of microstructure in the aging-related deterioration of the toughness of human cortical bone,” *Materials Science and Engineering: C*, vol. 26, no. 8, pp. 1251–1260, Sep. 2006, doi: 10.1016/j.msec.2005.08.021.
- [13] B. An, Y. Liu, D. Arola, and D. Zhang, “Fracture toughening mechanism of cortical bone: An experimental and numerical approach,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 4, no. 7, pp. 983–992, Oct. 2011, doi: 10.1016/j.jmbbm.2011.02.012.
- [14] J. Ivancik, N. K. Neerchal, E. Romberg, and D. Arola, “The Reduction in Fatigue Crack Growth Resistance of Dentin with Depth,” *J Dent Res*, vol. 90, no. 8, pp. 1031–1036, May 2011, doi: 10.1177/0022034511408429.
- [15] V. Imbeni, J. J. Kruzic, G. W. Marshall, S. J. Marshall, and R. O. Ritchie, “The dentin–enamel junction and the fracture of human teeth,” *Nature Materials*, vol. 4, no. 3, pp. 229–232, Mar. 2005, doi: 10.1038/nmat1323.
- [16] J. J. Kruzic, D. K. Kim, K. J. Koester, and R. O. Ritchie, “Indentation techniques for evaluating the fracture toughness of biomaterials and hard tissues,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 2, no. 4, pp. 384–395, Aug. 2009, doi: 10.1016/j.jmbbm.2008.10.008.
- [17] A. Miserez *et al.*, “Effects of Laminate Architecture on Fracture Resistance of Sponge Biosilica: Lessons from Nature,” *Advanced Functional Materials*, vol. 18, no. 8, pp. 1241–1248, Apr. 2008, doi: 10.1002/adfm.200701135.
- [18] D. B. Marshall *et al.*, “The Compelling Case for Indentation as a Functional Exploratory and Characterization Tool,” *J. Am. Ceram. Soc.*, vol. 98, no. 9, pp. 2671–2680, Sep. 2015, doi: 10.1111/jace.13729.
- [19] J. Ast, M. Ghidelli, K. Durst, M. Göken, M. Sebastiani, and A. M. Korsunsky, “A review of experimental approaches to fracture toughness evaluation at the micro-scale,” *Materials & Design*, vol. 173, p. 107762, Jul. 2019, doi: 10.1016/j.matdes.2019.107762.
- [20] S. Bechtle *et al.*, “A method to determine site-specific, anisotropic fracture toughness in biological materials,” *Scripta Materialia*, vol. 66, no. 8, pp. 515–518, Apr. 2012, doi: 10.1016/j.scriptamat.2011.12.027.

- [21] D. Lu and A. H. Barber, “Optimized nanoscale composite behaviour in limpet teeth,” *Journal of The Royal Society Interface*, vol. 9, no. 71, pp. 1318–1324, Jun. 2012, doi: 10.1098/rsif.2011.0688.
- [22] A. H. Barber, D. Lu, and N. M. Pugno, “Extreme strength observed in limpet teeth,” *Journal of The Royal Society Interface*, vol. 12, no. 105, p. 1326, Apr. 2015, doi: 10.1098/rsif.2014.1326.
- [23] A. C. Deymier *et al.*, “Micro-mechanical properties of the tendon-to-bone attachment,” *Acta Biomaterialia*, vol. 56, pp. 25–35, Jul. 2017, doi: 10.1016/j.actbio.2017.01.037.
- [24] H.-T. Lee *et al.*, “Site-specific characterization of beetle horn shell with micromechanical bending test in focused ion beam system,” *Acta Biomaterialia*, vol. 57, pp. 395–403, Jul. 2017, doi: 10.1016/j.actbio.2017.04.026.
- [25] J. Liu, Y. Xu, H. Yang, Y. Liu, P. KDV. Yarlagadda, and C. Yan, “Investigation of failure mechanisms of nacre at macro and nano scales,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 112, p. 104018, Dec. 2020, doi: 10.1016/j.jmbbm.2020.104018.
- [26] M. G. Mueller, V. Pejchal, G. Žagar, A. Singh, M. Cantoni, and A. Mortensen, “Fracture toughness testing of nanocrystalline alumina and fused quartz using chevron-notched microbeams,” *Acta Materialia*, vol. 86, pp. 385–395, Jan. 2015, doi: 10.1016/j.actamat.2014.12.016.
- [27] G. Žagar, V. Pejchal, M. G. Mueller, L. Michelet, and A. Mortensen, “Fracture toughness measurement in fused quartz using triangular chevron-notched microcantilevers,” *Scripta Materialia*, vol. 112, pp. 132–135, Sep. 2015, doi: 10.1016/j.scriptamat.2015.09.032.
- [28] “Standard Test Methods for Determination of Fracture Toughness of Advanced Ceramics at Ambient Temperature,” *ASTM C1421-18*, Jan. 2018.
- [29] B.-S. Li, T. J. Marrow, S. G. Roberts, and D. E. J. Armstrong, “Evaluation of Fracture Toughness Measurements Using Chevron-Notched Silicon and Tungsten Microcantilevers,” *JOM*, vol. 71, no. 10, pp. 3378–3389, 2019, doi: 10.1007/s11837-019-03696-1.
- [30] J. Schindelin, I. Arganda-Carreras, and E. Frise, “Fiji: an open-source platform for biological-image analysis,” *Nature methods*, vol. 9, no. 7, pp. 676–682, 2012, doi: 10.1038/nmeth.2019.

- [31] I. Jimenez-Palomar, A. Shipov, R. Shahar, and A. H. Barber, “Influence of SEM vacuum on bone micromechanics using in situ AFM,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 5, no. 1, pp. 149–155, Jan. 2012, doi: 10.1016/j.jmbbm.2011.08.018.
- [32] W. H. Gerstle and J. E. Jr. Abdalla, “Finite Element Meshing Criteria for Crack Problems,” *ASTM STP 1074*, 1990.
- [33] H. G. Tattersall and G. Tappin, “The work of fracture and its measurement in metals, ceramics and other materials,” *J Mater Sci*, vol. 1, no. 3, pp. 296–301, Aug. 1966, doi: 10.1007/BF00550177.
- [34] M. G. Mueller, G. Žagar, and A. Mortensen, “Stable room-temperature micron-scale crack growth in single-crystalline silicon,” *Journal of Materials Research*, vol. 32, no. 19, pp. 3617–3626, 2017, doi: 10.1557/jmr.2017.238.
- [35] N. L. Okamoto, S. Michishita, Y. Hashizume, and H. Inui, “Fracture Toughness of the Fe–Zn Intermetallic Compounds Measured by Bend Testing of Chevron-Notched Single-Crystal Microbeams,” *ISIJ International*, vol. 58, no. 9, pp. 1569–1577, 2018, doi: 10.2355/isijinternational.ISIJINT-2018-068.
- [36] R. Henry *et al.*, “Local fracture toughness measurements in polycrystalline cubic zirconia using micro-cantilever bending tests,” *Mechanics of Materials*, vol. 136, p. 103086, Sep. 2019, doi: 10.1016/j.mechmat.2019.103086.
- [37] M. O. Eremin, E. E. Deryugin, and S. Schmauder, “Evaluation of fracture toughness of $\text{ZrO}_2 - 3.0 \text{ mol}\% \text{Y}_2\text{O}_3$ ceramics utilizing wedge splitting loading of double cantilever specimen with a chevron notch,” *Engineering Failure Analysis*, vol. 110, p. 104409, Mar. 2020, doi: 10.1016/j.engfailanal.2020.104409.
- [38] T. L. Anderson, “Elastic-Plastic Fracture Mechanics,” in *Fracture mechanics: fundamentals and applications*, 2nd ed., Boca Raton: CRC Press, 1995, pp. 125–126.
- [39] “Standard Test Method for Linear-Elastic Plane-Strain Fracture Toughness of Metallic Materials,” *ASTM E399-17*, Feb. 2018.
- [40] D. Tabor, *The hardness of metals*. Oxford : New York: Clarendon Press; Oxford University Press, 2000.
- [41] K. A. Gross and L. M. Rodríguez-Lorenzo, “Sintered hydroxyfluorapatites. Part II: Mechanical properties of solid solutions determined by microindentation,” *Biomaterials*, vol. 25, no. 7, pp. 1385–1394, Mar. 2004, doi: 10.1016/S0142-9612(03)00636-7.

- [42] D. S. Harding, W. C. Oliver, and G. M. Pharr, “Cracking During Nanoindentation and its Use in the Measurement of Fracture Toughness,” *MRS Proceedings*, vol. 356, p. 663, 1994, doi: 10.1557/PROC-356-663.
- [43] T. Scholz, G. A. Schneider, J. Muñoz-Saldaña, and M. V. Swain, “Fracture toughness from submicron derived indentation cracks,” *Appl. Phys. Lett.*, vol. 84, no. 16, pp. 3055–3057, Apr. 2004, doi: 10.1063/1.1711164.
- [44] L. Barker, “Short bar specimens for KIC measurements,” in *Fracture mechanics applied to brittle materials*, vol. ASTM STP 678, Conshohocken, PA: ASTM International, 1979, pp. 73–82.
- [45] S. M. Wiederhorn, H. Johnson, A. M. Diness, and A. H. Heuer, “Fracture of Glass in Vacuum,” *J American Ceramic Society*, vol. 57, no. 8, pp. 336–341, Aug. 1974, doi: 10.1111/j.1151-2916.1974.tb10917.x.
- [46] J. J. Mecholsky, R. W. Rice, and S. W. Freiman, “Prediction of Fracture Energy and Flaw Size in Glasses from Measurements of Mirror Size,” *J American Ceramic Society*, vol. 57, no. 10, pp. 440–443, Oct. 1974, doi: 10.1111/j.1151-2916.1974.tb11377.x.
- [47] J. A. Salem, “Transparent Armor Ceramics as Spacecraft Windows,” *J. Am. Ceram. Soc.*, vol. 96, no. 1, pp. 281–289, Jan. 2013, doi: 10.1111/jace.12089.
- [48] J. P. Lucas, N. R. Moody, S. L. Robinson, J. Hanrock, and R. Q. Hwang, “Determining fracture toughness of vitreous silica glass,” *Scripta Metallurgica et Materialia*, vol. 32, no. 5, pp. 743–748, Mar. 1995, doi: 10.1016/0956-716X(95)91596-H.
- [49] “Standard Test Method for Measurement of Fracture Toughness,” *ASTM E1820-20*, Jan. 2020.
- [50] R. Wang and H. S. Gupta, “Deformation and Fracture Mechanisms of Bone and Nacre,” *Annu. Rev. Mater. Res.*, vol. 41, no. 1, pp. 41–73, Aug. 2011, doi: 10.1146/annurev-matsci-062910-095806.
- [51] Z. Liu, Z. Zhang, and R. O. Ritchie, “On the Materials Science of Nature’s Arms Race,” *Adv. Mater.*, vol. 30, no. 32, p. 1705220, Aug. 2018, doi: 10.1002/adma.201705220.
- [52] B. R. Lawn, *Fracture of brittle solids*, 2nd ed. Cambridge ; New York: Cambridge University Press, 1993.

- [53] S. Amini, M. Tadayon, J. Q. I. Chua, and A. Miserez, “Multi-scale structural design and biomechanics of the pistol shrimp snapper claw,” *Acta Biomaterialia*, vol. 73, pp. 449–457, Jun. 2018, doi: 10.1016/j.actbio.2018.04.038.
- [54] S. Amini and A. Miserez, “Wear and abrasion resistance selection maps of biological materials,” *Acta Biomaterialia*, vol. 9, no. 8, pp. 7895–7907, Aug. 2013, doi: 10.1016/j.actbio.2013.04.042.
- [55] L. C. Hua *et al.*, “The dehydration effect on mechanical properties of tooth enamel,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 95, pp. 210–214, Jul. 2019, doi: 10.1016/j.jmbbm.2019.04.013.
- [56] Z. Liu *et al.*, “Hydration-induced nano- to micro-scale self-recovery of the tooth enamel of the giant panda,” *Acta Biomaterialia*, vol. 81, pp. 267–277, Nov. 2018, doi: 10.1016/j.actbio.2018.09.053.
- [57] I. Jimenez-Palomar, A. Shipov, R. Shahar, and A. H. Barber, “Structural orientation dependent sub-lamellar bone mechanics,” *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 52, pp. 63–71, Dec. 2015, doi: 10.1016/j.jmbbm.2015.02.031.

Chapter 5

Time Evolution Study of the Stomatopod Dactyl Club

This chapter covers the time study on the stomatopod dactyl club mineralization. A brief background is presented together with the motivation and significance of this research. The materials and methods used in this study and their corresponding results are covered and discussed. Apatite was shown to be present in the club as early as 2 hours post-molt. Apatite crystals were present with unique differing textures in the surface impact and impact regions. Results also suggest that the crystal structure of apatite changes as the club grows. The reasons are not immediately obvious and could not be ascertained in this study. Br was discovered for the first time in the dactyl club and found to be incorporated as a post-translational modification of the main protein regulating biomineralization of the club and may play a role in protecting the dactyl club against chemical and microbial attacks during its development.

5.1 Introduction

Mineralized hard tissues continue to represent a valuable source of inspiration for tough composite materials manufactured using “green chemistry” principles. Over the past decade, the dactyl club of stomatopods has been the subject of many studies for its impressive impact tolerance and wear resistance. The club remains largely intact even after thousands of high energy blows delivered at targets, which include other natural materials known for their formidable toughness like nacre [1]. The club has three distinct regions at the mesoscale; a hard mineralized layer from the surface followed by a softer lamellar layer and two thick bands of aligned chitin fibers on the flanks of the club [2]. Previous works showed that the hard mineralized layer consist of FAP nanorods embedded in a chitin-protein matrix [3], [4]. As a crustacean, the stomatopod molts its entire shell and fashions a completely new exoskeleton including the dactyl clubs regularly [5]. The formation of the new shell occurs over a period of about a month, which is much faster compared to other apatite tissues like vertebrates’ bones that develops over a longer time [6]. Moreover, stomatopods are invertebrates and hence there are lesser restrictions to perform experiments on them. This allows this study to be conducted within the timeframe of this research project. With our research setting being strategically located close to their natural habitat, it is easy to obtain *Odontodactylus scyllarus* and keep them in a seawater aquarium in the lab. With their close proximity, the stomatopods can be monitored hourly, and clubs obtained at different time points during its development. Herein lies a unique opportunity to study apatite-based biomineralization. The entire process of tissue growth, mineral deposition, and apatite crystallization can be studied. As of this writing, only a single study looked into the biomineralization of the dactyl club [6]. The dactyl club is formed by an intriguing growth mechanism akin to a “die-casting” process. This previous study also included obtaining the full-length sequence of an important protein that regulates the apatite biomineralization in the dactyl club, namely CMP-1 which is short for club mineralization protein-1 [6]. After molting the new membrane unfolds from the inner cavity and expands to form the outer envelope of the new club; the outer mineralized region first (within a few days), then the inner lamellar or “periodic” region is deposited. In this previous study, mineral phases and composition at various stages detected using mostly confocal Raman spectroscopy (Raman) and electron dispersive spectroscopy (EDS) [6]. Raman can provide rapid information on the crystallinity and crystal

orientation of the material, but the data is generally qualitative while EDS requires the samples to be placed in high vacuum and charging is also an issue for non-conductive samples. Synchrotron X-rays have been successfully used to perform crystallographic and elemental analyses on biological samples including sponge glass spicules [7], parrotfish teeth [8], pistol shrimp claw [9], and the stomatopod dactyl club [2]. In this work synchrotron X-rays were applied to provide greater detail into the development of the FAP crystals and the spatial-temporal organization of the organic phase within the dactyl club. Position resolved combined XRF and WAXS at micrometer resolution were used to study the samples. XRF provides information on distribution of elements within the scanned area. WAXS provided crystallographic information on the mineral phase during the club development. Using Rietveld refinement, texture information, variation in lattice constants, the size of the apatite crystals in the club can also be obtained.

5.2 Materials and Methods

5.2.1 Materials

Live *Odontodactylus Scyllarus* were acquired from a local aquarium supplier and kept in a custom-built marine aquarium in the laboratory. Dactyl clubs were removed from the Stomatopods at different maturation periods after molting (Figure 5.1): 2 hours (2h), 1 day (1d), 2 days (2d), 1 week (1w), 2 weeks (2w). The mature/molting (> 1 month) dactyl clubs were also collected upon molting. The clubs were rinsed thoroughly in DI water to remove salt water and stored immersed in DI water at -80°C. Furthermore, the 1d sample was separated into two halves for comparison, one half was stored in DI water while the other half was soaked in 5 M Guanidine Thiocyanide for 20 hours, rinsed and stored at -80°C. Fluorapatite (FAP) standard was obtained from Electron Microscopy Sciences. Reagents used: Conc. HNO₃ (70%), H₂O₂ (30%) and absolute ethanol (Emsure®) from Sigma-Aldrich, and isopropanol (J.T.Baker®, Avantor). Royal Nature Pro Advance Formula Salt used in our aquarium system was obtained from a local supplier. Sterile polypropylene (PP) centrifuge tubes were purchased from Sigma-Aldrich while 0.40 µm syringe filters were obtained from Norm-Ject. All PP tubes and other apparatus (e.g. mortar and pestle) used in sample digestion and ICP-MS analysis were cleaned with 5 M HNO₃ acid for at least 24 hrs. Two 45 ml PTFE

bombs (Parr Instruments) were cleaned with 30 ml conc. HNO_3 by heating in a 140°C furnace for 2 hr.

Figure 5.1. Optical images of the stomatopod dactyl club taken from the mantis shrimp at different time points from molting. The two hours (2h), one day (1d) and two days (2d) samples are thinner, softer and more fragile compared to the other three points of development. Hence, they were prepared using cryo-ultramicrotomy while the one week (1w), two weeks (2w) and mature (1 month) samples were prepared by thinning a slice between two abrasive papers until the desired thickness of close to $50\ \mu\text{m}$ was achieved. At two weeks, the stomatopod started to use the club as evidenced by the damaged surface on the club.

5.2.2 Sample Preparation

The hemolymph and tissue were removed from the cavity of the dactyl clubs. The remaining outer membrane remained soft and pliable when harvested two hours after molting (2h). The samples harvested a day (1d) and two days (2d) post-molt were brittle and could be easily cut using a sharp scalpel. Therefore, 2h, 1d and 2d club samples were mounted in Optimal Cutting Temperature (OCT) compound (Tissue-Tek) and axial cross sections about $10\ \mu\text{m}$ thick were obtained from the 1d sample using a cryo-microtome and from the 2h and 2d samples with a rotary cryo-microtome (Leica RM2265) equipped with a liquid N_2 cooling unit (Leica LN22). The 1w, 2w and mature clubs were much harder and tougher and had to be prepared differently. The mature and

2w clubs were prepared as follow: The whole club was attached from its base with cyanoacrylate glue to a hard substrate and cut with a lower speed precision saw using a diamond cut-off wheel. Sections about 150 μm were made on the axial plane of the clubs. The sections were further thinned with SiC paper to around 50-80 μm . Having hardness toughness between the softer membrane samples and the harder ready clubs, the 1w club was mounted in Struers EpoFixTM resin and cured overnight before the section was prepared equivalently to the 2w and mature samples. The 2h, 2d and 2w samples where mounted on SiN₄ Windows for synchrotron X-ray measurements whereas the 1d, 1w and the mature samples where mounted between two sheets of ultralene foil.

For EDS, FTIR and Raman spectroscopy measurements, the samples were prepared as follow: The same 1w used for synchrotron measurements were used without further preparation as the sample was fragile. A new two-week and mature club samples were prepared similarly as for synchrotron measurements, except the grinding and polishing steps were accomplished differently. The protocol used for polishing all samples was as follows: Plane grinding was carried out on Metken Forcipol 1V at 250 RPM under running water using silica paper of 1000 and 1200 Grit paper. Fine grinding was carried out at 150 RPM using 2500 and 4000 Grit paper. A damp polishing cloth impregnated with 40 nm Silica colloidal solution was used for final polishing on Struers RotoPol-35. Samples were rotated by 180° within each step and 90° between steps. Each step is completed when marks from the previous step has been removed. Samples were washed in running water between every grinding and polishing step, this is critical to prevent abrasives and debris from the previous step affecting the current step. Samples were sonicated in DI water for 2 mins to remove any silica nanoparticles remaining on the samples. After the samples were dry, they were coated with approximately 15 nm carbon (JEOL JEE-600) for EDS.

For inductive coupled plasma analysis, a molted (mature) stomatopod club was rinsed then sonicated in DI water for 5 mins followed by 5 mins in absolute ethanol. The club was then cryo-grounded to powder with liquid N₂ in a mortar and pestle. To digest the sample, 55.1 mg of the powder was measured into a 45 ml PTFE Bomb (Parr Instruments), to which a solution of 3 ml concentrated nitric acid (69%) and 2 ml hydrogen peroxide (30%) was added in succession. A control was prepared using the

same reagents in the other PTFE Bomb. Both PTFE bombs were sealed and placed in a furnace at 95 degrees Celsius for 4 hrs. The sample solution after digestion was slightly translucent while the control was completely colourless. Both solutions were diluted to approximately 1% w/w nitric acid. The marine salt control was prepared by dissolving the marine salt (Royal Nature) in Milli-Q water to a solution specific gravity of 1.024-1.025 and filtered with a 0.45 μm syringe filter. Artificial seawater from our aquarium system was heated at 70°C overnight and filtered with a 0.45 μm syringe filter.

5.2.3 Synchrotron X-ray Measurements

The 1d, 1w, and the mature samples were measured at P06 at PETRA III at DESY. The scanning was done at 16.5 KeV at a beam size of $500 \times 380 \text{ nm}^2$ ($H \times V$) and a motor step size of 500 nm in both directions. The diffraction data was collected with an EIGER x 4M detector and the XRF data with a MAIA XRF detector the setup is shown on Figure 5.2A. The 2h, 2d, and 2w samples were measured at the ID13 at the ESRF with a $250 \times 250 \text{ nm}^2$ ($H \times V$) beam and a motor step size of 250 nm in both directions. The diffraction data was collected with an EIGER X 4M detector and the XRF data was collected using a vortex XRF detector. The scanning was done with an energy of 15.2 KeV. A schematic of the experimental setups at both beamlines are shown in Figure 5.2.

Figure 5.2. **A)** The setup used at P06 at PETRA III at DESY, using a MAIA detector for X-Ray Fluorescence (XRF) and an Eiger 4M detector for X-Ray Diffraction (XRD) and **B)** the setup used at ID13 at the ESRF using a Vortex (V) detector for XRF and an Eiger 4M detector for XRD. The stage at both beamlines could move in the x, y, and z directions; this was used to raster scan the sample placed on the stage.

5.2.4 X-ray Data Analysis

The Diffraction data was integrated using the novel in house integration software package Matrix-multiplication Facilitated Radial and Azimuthal Integration Algorithm (*MatFRALA*) written by Jensen *et al.* [10]. The data was Integrated into both a single azimuthal bin, for Rietveld refinement, and into 360 azimuthal bins for the angular analysis. A sample diffractogram is shown in Figure 5.3. The Rietveld refinement was done using the *MultiRef* software package [11]. Rietveld refinement is an iterative method that finds the atomic positions and quantity of unit cells that will generate a diffraction pattern closest to those from the experiment given an initial crystal structure and composition. The lattice parameters and full width half maximum (FWHM) of the peaks can be found from Rietveld refinement. The apparent crystallite size (ACS) of the apatite crystals can be calculated using the FWHM and Scherrer's formula:

$$\tau = \frac{K\lambda}{\beta \cos \theta} \quad (6.1)$$

Where τ is the ACS, K is a dimensionless crystallite shape factor with typical values close to 1, λ is the X-ray wavelength, β the FWHM and θ is half the angle of diffraction with both units in radians. ACS can be understood as the number of the planes multiplied by the interplane spacing in a small crystal thus giving the size of the crystal with respect to the plane selected. ACS was determined for two orthogonal planes, (002) and (310), providing the dimensions of the crystals in the directions normal to the selected planes.

The angular analysis was done using the in-house software package Angular Gaussian Fitter (*AGUF*) as described by J. Palle *et al.* [12] based on the technique described by Gourrier *et al.* [13], the (002) peak was fitted angularly as shown in Figure 5.4. First the local background in the 2θ direction was removed, then the data was fitted with the angular gaussian:

$$I = A_1 e^{-\frac{\text{dist}(\chi, \chi_0)^2}{2\sigma^2}} + A_2 e^{-\frac{\text{dist}(\chi, \chi_0 + \pi)^2}{2\sigma^2}} + A_{\text{bkg}} \quad (6.2)$$

where χ_0 is the angle of the peaks along the χ axis (representative peaks shown in Figure 5.4C), A_1 and A_2 are the amplitudes of the peaks and A_{bkg} is the amplitude of the background. For the angular gaussian function the distance function used is:

$$\text{dist}(\chi, \chi_0) = \cos^{-1} \left((\cos \chi \quad -i \sin \chi) \begin{pmatrix} \cos \chi_0 \\ i \sin \chi_0 \end{pmatrix} \right) \quad (6.3)$$

Which allows for circle wrapping, unlike the normal distance function:

$$\text{dist}(\chi, \chi_0) = \chi - \chi_0 \quad (6.4)$$

From integrating the angular gaussian function over 2π the areas of oriented crystal peaks (I_p) and the area of the randomly oriented crystals (I_{bkg}) were obtained. Thus, we can find the total summed intensity (Figure 5.4C): $I_T = I_p + I_{\text{bkg}}$. This allows us to find the Degree of Orientation (DoO) in any given point:

$$DoO = \frac{I_p}{I_T} \quad (6.5)$$

The X-Ray-Fluorescence (XRF) data from P06 at PETRA was analyzed with the *GEO-PIXE software* (CSIRO, c/o CMSE, Clayton VIC, Australia) [14], [15] and the XRF data from ID13 at the ESRF was analyzed using *pyMCA* [16]. Figure 5.5 shows the summed XRF spectrum for a sample

Figure 5.3. Diffractogram from a sample; tick marks at the bottom from adapted from [17]. The (002) peak has been marked because that peak was analyzed with the Angular Gaussian Fitter (*AGUF*) software.

Figure 5.4. **A)** The principle of AGUF. A diffractogram from the detector with hot pixels masked out. **B)** The diffractogram integrated into 360 azimuthal bins and 900 bins along the diffraction angle 2θ . The red rectangle marks the (002) peak. **C)** The intensity of the (002) peak as a function of the azimuthal angle χ data as dots and the fit as a line. The red area shows the intensity of the oriented crystals, I_p ; the blue area shows the intensity of the randomly oriented crystals. The data for this figure was taken from the 2w sample.

Figure 5.5. Total XRF spectrum for the sample harvested 2 hours after moulting

5.2.5 Electron Dispersive Spectroscopy (EDS)

EDS hyperspectroscopy maps on the 1w and mature samples were taken with a field-emission SEM (JEOL 7800F) equipped with a 170 mm² X-ray area detector (Oxford Instruments). An accelerating voltage of 10 kV and an emission current of 900 pA was used. Beam measurement was first conducted on a Silicon sample attached to the sample stub next to the sample. Spectra of at least a million total counts was first measured on the FAP standard (Electron Microscopy Sciences) at three different spots and used for composition determination in post-processing. Strip maps were taken on the stomatopod samples covering the surface of the sample to the periodic region for mature sample and up to the inner cavity for the 1w samples. The maps were converted to linescans by binning along the horizontal axis.

5.2.6 Vibrational Spectroscopy

Raman spectroscopy was conducted on the 1w and mature samples with a confocal raman microscope (alpha300, WiTec, Ulm, Germany) equipped with a 633 nm laser source and 600 g/mm grating. A 20x magnification objective lens and an integration time of 3.6 s was used. Raman mapping of the 2w sample was performed using an

Nd:YAG laser source with an excitation wavelength of 532 nm and a piezo scanner. An integration time of 3 s were used to map the selected zones, each with a 10 mm x 200 mm area. The Spectroscopy Plus software (*WITec Control*) was used for measurements and *WITec Project Plus* for spectrum processing. FTIR measurements were done on the 1w and mature samples only. A FTIR microscope (Nicolet Continuum) with a mid-IR source (Polaris) and KBr beamsplitter was used; range of 4000 – 650 cm⁻¹ could be achieved. Mapping was done on the samples from the surface up to the periodic region. *OMNIC* software was used for data collection and pre-processing. Representative points along the map from the surface in were background subtracted and smoothed with a 5-point symmetric window, Fast Fourier Transform filter using OriginPro.

5.2.7 Inductively Coupled Plasma (ICP)

ICP Optical Emission Spectroscopy (ICP-OES) was applied to measure the Ca, P, Mg, Sr and S concentrations of the digested sample along with a HNO₃ + H₂O₂ control and other samples for comparison: i. Milli-Q water, ii. Marine salt solution, iii. Artificial seawater. Sample preparation is detailed in Section 5.2.2 and 3.2.3.1. Elemental concentrations from previous EDS and EPMA data [3] were used along with calibration solutions (PerkinElmer/SciexTM) to calibrate the range for ICP-OES. The ICP-OES instrument (PerkinElmer/SciexTM model Optima 8300) fitted with a standard quartz torch, double-pass Scott-type spray chamber and GemTip cross-flow nebulizer. The ICP-OES setup was not able to measure Br⁻ ions. Hence, ICP Mass Spectrometry (ICP-MS) was done to measure the Br concentration. The ICP-MS instrument (PerkinElmer/SciexTM model ELANDRC-e) was equipped with a standard quartz torch, borosilicate glass nebulizer (Meinhard® model TR-30-C3) and nickel sampler and skimmer cones. Calibration for Br range from 1 ppb to 100 pb was performed using diluted Br solution (High Purity Standards). The Ca concentration from ICP-OES was used in conjunction with Br/Ca concentration from synchrotron XRF measurements done in this work to determine the calibration range for ICP-MS. Triplicate measurements were done on each sample and the average used to determine the Br concentration. Table 5.1 shows the parameters used for the ICP-OES and ICP-MS measurements.

Table 5.1. Parameters used for ICP-OES and ICP-MS measurements on digested mature dactyl club sample and control samples.

Parameter	ICP-OES	ICP-MS
RF power wavelength (W)	1300	1400
Plasma gas flow rate (L/min)	10	17
Auxiliary gas flow rate (L/min)	0.2	1
Nebulizer gas flow rate (L/min)	0.6	0.87
Element wavelength (nm)	S - 181.975 P - 213.617 Ca - 317.933 Mg - 279.077 Sr - 460.733	
Isotope (m/z)		Br79

5.2.8 Parallel Reaction Monitoring (PRM)

To further confirm bromination of Y₂₇₅ in CMP-1, we used the mass spectrometry method parallel reaction monitoring (PRM) [18]. A set of stable isotope-labelled (SIL) peptides corresponding to L274-R283 peptide from CMP-1 was synthesized and used for identification of bromination. The method aims to identify two target precursors of L274-R283 from wild type CMP-1 and their corresponding synthetic SIL analogues simultaneously. The masses for the peptides were as follow. Wild type (“light”) peptides [L274-R283]_L: L $\underline{\mathbf{M}}^{+16}$ YVENVLTD $\underline{\mathbf{R}}$ (non-brominated; $m/z = 684.8$, $z = +2$), and [L274-R283]_{L,Br}: L $\underline{\mathbf{M}}^{+16}$ $\underline{\mathbf{Y}}^{+78}$ VENVLTDR (brominated Tyr, $m/z = 723.8$, $z = +2$). The corresponding synthetic SIL (“heavy”) peptides containing heavy-labeled Arg at the C-terminus were the following: [L274-R283]_H: LMYVENVLTD $\underline{\mathbf{R}}^{+10}$ ($m/z = 689.8$; $z = +2$), and [L274-R283]_{H,Br}: LMY $^{+78}$ VENVLTDR $^{+10}$ ($m/z = 728.8$; $z = +2$). SIL peptides were first identified by the PRM method to detect all four precursors without background proteome from the wild type CMP-1 (Figure 5.6). The peaks corresponding to the non-brominated peptides are shown in Figure 5.6A (left panel: wild type and right panel: SIL) whereas these peaks from the brominated peptides are shown in Figure 5.6B (left panel: wild type and right panel: SIL). On these figures, the top panels are the target precursor peptides and the bottom panels are their corresponding fragment ions. These chromatograms show well-resolved peaks for the SIL (heavy) peptides, while the wild-type (light) peptides displayed noisy signals close to the baseline, indicating that the latter were not present or below the detection limit in the SIL peptides. This

purity allowed us to use SIL peptides according to PRM without concern that the wild type peptides were present as contaminants in the SIL peptides.

Figure 5.6. Identification of precursor ions from stable isotope labeled (SIL) peptides L274-R283, run without native CMP-1. **A)** Chromatograms with peaks corresponding to the non-brominated peptide precursor L274-R283 from CMNP-1 and their corresponding fragment ions (bottom). *Left:* peaks corresponding to the wild-type (“light”) peptide [L274-R283]_L, and *right:* peaks corresponding to the SIL (“heavy”) peptide [L274-R283]_H. **B)** Chromatograms with peak corresponding to the brominated peptide L274-R283 from CMP-1 (top) and its corresponding fragment ions (bottom). *Left:* peak corresponding to the wild-type (“light”) peptide [L274-R283]_{L,Br} and *right:* peak corresponding to the SIL (“heavy”) peptide peptide [L274-R283]_{H,Br}. Peaks are well-resolved for the SIL peptides, whereas for the wild type peptides they are noisy and close to the baseline, indicating that the heavy SIL peptides are pure and are not contaminated with the light peptides.

5.3 Results and Discussion

5.3.1 General observations

Optical examination shows that the 2w club was slightly damaged suggesting that at two weeks after molting the club has developed enough to be functional (Figure 5.1), albeit the club was worn with a large portion of the club chipped off. Figure 5.7 provides an overview of the samples measured at the synchrotrons (ESRF and PETRA). Optical microscope and XRF images show that at about two hours from molting the club was about 26 μm thick. The club then grows threefold to about 77 μm thick in a day, and to 107 μm two days after molting, respectively. In a full week after molting, the club grew about sixfold to 680 μm and to 1290 μm after another week. The mature or fully developed dactyl club is about 1960 μm thick excluding the inner cavity. Figure 5.7A is a schematic showing the progression in the dactyl club development at the different time points measured. The 2h and 1d sample are within the impact surface where the crystallites are speculated to be nanospheres [19], [20]. The 2d sample is just beginning to construct the impact region with larger crystals interweaved in chitin in a herringbone pattern [4], [19]. At one week the club should be forming the periodic region, and by two weeks half the periodic region should be developed. As the 2w and mature samples are large, high resolution mapping across the full depth is not practical with synchrotron X-rays, instead selected regions from these dactyl club were mapped; these are indicated by the yellow and red boxes in Figure 5.7F and G. These measurements were confined to mostly the surface and impact regions where apatite is abundant. Nevertheless, a full depth high resolution map of the 1w sample was conducted. Figure 5.8A shows a low resolution combined XRF image of the 1w dactyl club, where the white box indicates where high resolution mapping was performed. XRF maps and the different maps from processing the X-ray diffractograms (WAXS data) are shown in Figure 5.8B; these maps were generated for all developmental stages studied in this research work.

Figure 5.7. Overview of samples tested with synchrotron X-rays. **A)** Schematic showing the different structural layers in the Stomatopod dactyl club; from top to bottom: Initial expanded membrane after molting, impact surface, impact region, periodic region, inner cavity. **(B-F)** XRF images of clubs: 2h (B), 1d (C), 2d (D), and 1w samples (E); Ca $K\alpha$ (green) and Br $K\alpha$ (red) lines were used to generate these images. **F)** Light microscope image of the 2w sample after preparation. The small yellow box marks the region area measured using synchrotron radiation. **G)** Light microscope image of the mature sample; dash lines showing approximately the thickness of the club one week (cyan) and two weeks (yellow) after molting; the small red box marks the region analysed with synchrotron radiation.

Figure 5.8. 1w sample. **A)** Large area X-ray map. **B)** Maps with higher resolution from the white box in A; images from left to right: Polarized light microscope (Pol), Ca $K\alpha$ XRF, Br $K\alpha$ XRF, lattice constant a (a -axis), apparent crystallite size (ACS) ratio, and 002 angle orientation maps. The scale bar can be used to read the a -axis and ACS ratio maps while the colour cylinder below is for the 002 angle orientation maps.

5.3.2 Spatial Distribution of Apatite and Crystallite Size

Synchrotron WAXS measurements show that apatite was present even at the earliest stage of club growth, which is approximately 2 hours after molting. This was deduced from the presence of signals (coloured regions) from any of the 2h XRD maps like the 2h ACS map (Figure 5.9A). Figure 5.8 shows the location of the high resolution synchrotron X-ray scan on the 1w sample and the corresponding XRF and XRD maps. Since Ca is present in most regions of the club the 1w Ca XRF map shows the extent of the club development 1 week after molting. Comparing the Ca XRF with any of diffraction maps show that around 2/3 of the 1w sample is mineralized (Figure 5.8B). This suggests that the organic matrix may be created first before the apatite crystals were deposited over the matrix and the transition between the mineralized region and the unmineralized region is the mineralization front. Additionally, similar diffraction maps obtain between the 1d sample treated and untreated with a denaturing agent (5 M guanidine thiocyanide) showed that negligible mineralization occurs after the sample was removed and rinsed with Milli-Q water. Therefore, the sample remained unchanged during the transportation to the synchrotron facility. Figure 5.9 and 6.10 shows the ACS of apatite crystallites in the dactyl club at the different maturation stages in two orthogonal directions (i.e. [002] and [310]) (refer to Section 5.2.4 for more information on X-ray data treatment). The crystallite size was found to vary from 3 nm to 50 nm. It is important to note that the ACS presented here do not account for instrument broadening and thus provide the lower bound of the crystallite size. This explains why the ACS was smaller than previously reported [3]. One intriguing feature observed was the presence of a band or dual-bands with higher ACS within the sample. This feature is clearly seen in ACS_[310] for the 2h, 1d and 2d samples (Figure 5.10). They seem to coincide with the shallow region next to the surface with higher elastic modulus than the impact region observed in previous studies [2], [3]. Another observation is the ACS is smaller in the [310] direction compared with the [002] direction. Therefore, the crystallites are likely needle-like rather than spherical in shape. Figure 5.11 shows the ratio between the ACS in the [002] and [310] directions: the larger the ratio the greater the anisotropy along the [002] direction. The crystals have an aspect ratio of around 2.5 – 3.5 near the surface of the club and decreases exponentially after the first 10 μm into the within the 2h and 2d samples. However, the opposite trend was observed in the 1d sample. In the 1w sample, there is a surface layer about 80 μm thick that is has a smaller

average aspect ratio. Interestingly, this layer is within the size of the 2h and 1d samples. Therefore, this surface layer seems to be a defining feature in the dactyl club. In fact, recent publications indicate that there are spherical nanocrystals in the top 70 μm layer of the dactyl club and is termed the impact surface region [19], [20]. However, contrary to these articles, observations in this study and previous work on molted dactyl clubs did not possess this impact surface and the nanocrystals were likely worn off and not present in SEM images of the molted dactyl club which appeared used by stomatopods. Hence the impact surface may not possess the wear-resistant characteristics mentioned in the articles [2], [4]. Nonetheless, the results of ACS mapping support the presence of this spherical nanocrystal region near the surface. Deeper within the 1w and 2w and mature samples the crystallites were more elongated, in agreement with SEM images of the impact region by Amini *et al.* that showed columnar crystals within the impact region [4]. Additionally, the ACS in the impact region were generally smaller than those in the impact surface as well.

Figure 5.9. Apparent crystallite size of apatite in the [002] direction at different maturation stages of the dactyl club. **A)** 2h, **B)** 1d, **C)** 2d, **D)** 1w, **E)** 2w and **F)** mature samples.

Figure 5.10. Apparent crystallite size of apatite in the [310] direction at different maturation stages of the dactyl club. **A)** 2h, **B)** 1d, **C)** 2d, **D)** 1w, **E)** 2w, and **F)** mature samples.

Figure 5.11. Ratio of the apparent crystallite size of apatite in the [002] and [310] directions at different maturation stages of the dactyl club. **A)** 2h, **B)** 1d, **C)** 2d, **D)** 1w, **E)** 2w and **F)** mature samples.

5.3.3 Apatite Fluorination with Club Development

Another useful measure from Rietveld refinement is the lattice parameters of the apatite crystal lattice. Apatite structures have a hexagonal Bravais lattice that with two variable lattice parameters: the axis lengths a and c . Figure 5.12 and 6.13 shows the a and c parameter maps for all the samples. Interestingly, the size of a varies considerably throughout each sample but the c parameter remains largely unchanged such that changes in unit cell volume is almost solely from the effect of a parameter attenuation. The measured values of c is close to pure FAP and hydroxyapatite (HAP), both having almost equivalent c sizes of 6.884 Å and 6.8814 Å, respectively [21]. Moreover, the size of a for pure FAP (a_F) and HAP (a_{OH}) has a larger difference of 9.367 Å and 9.418 Å, respectively [21]. The variation in the parameter sizes is due to the substitution of the OH^- site with F^- . F^- is smaller and more electronegative than OH^- leading to closer atomic distances between neighboring ions. This suggests apatite may have more variability as previously reported, with varying degrees of F^- and OH^- substitutions. Additionally, the further the club development the lesser the variance in a and the closer a is to pure FAP. This suggests that HAP maybe formed first, and later ionic substitutions takes place to increase FAP content. This aligns with a previous study by Amini *et al.* which concluded from *ab initio* atomic calculations that the impact region is more compatible with FAP than HAP because of the presence of sulphate groups that form epitaxial interfaces with FAP.

To verify if the chemical environment varies substantially with development, EDS hyperspectroscopy was performed with a FAP standard on the 1w and mature samples. Figure 5.14 and 6.15 show the EDS strip maps and line scans on the 1w and mature samples, respectively. A spatial resolution of around 1 µm was achieved in the maps. The Ca, P and F concentrations remain largely homogeneous through the impact region. This is further emphasized when P/Ca and F/Ca weight ratios (Figure 5.14D and 6.15D) were considered, suggesting that there was little variation in the ratio between P or F and Ca content (P/Ca and F/Ca) through the depth of the impact region in the mature samples. However, F/Ca ratio changes abruptly between the impact and the periodic regions. The P/Ca rises and dips while the opposite happens with F/Ca. In the 1w sample, the P concentration remained homogeneous throughout the length of the map, although the Ca and F concentrations decreased at the mineralization front around 250

μm into the sample. The F/Ca and P/Ca ratio did not vary much before the mineralization front. Comparing between the samples, the Ca and P weight percentages (wt. %) in the 1w sample was closer to the ideal apatite wt%. Moreover, the 1w P/Ca weight ratio on average was 0.55, which was greater than the mature sample average P/Ca ratio of 0.49. However, the ideal P/Ca weight ratio for pure FAP or HAP is around 0.46 and thus, the mature sample had a P/Ca ratio closer to pure apatite compared to the 1w sample, which was expected since the apatite crystallites should be fully developed in the mature sample. The lower than ideal P and Ca wt% in both samples were probably because in this EDS experiment the organic phases were not considered when calculating the wt%. Consequently, the lower P and Ca wt% in the mature sample compared to the 1w sample indicated that there may be more organic phases in the fully developed club compared to developing clubs.

However, there is little difference between the F/Ca ratios in the 1w and mature samples, suggesting that the amount of F remains constant throughout the development of the club. Therefore, F substitution during development may not be one of the factors for the decreasing a lattice parameter sizes with development. Interestingly, there was a broad spike in F/Ca near the mineralization front in the 1w sample. This could be attributed to an oval inclusion clearly visible in the F wt% map (Figure 5.14B). Another broad spike corresponds with a similar inclusion but deeper in at around $420\ \mu\text{m}$ from the surface at the club growth front. These inclusions may be remaining hemocyanin or soft cellular tissue that were not completely removed during sample preparation. Why F is present in high concentrations in these inclusions is currently unclear.

Figure 5.12. Maps of the lattice constant a . **A)** 2 hours, **B)** 1 day, **C)** 2 days, **D)** 1 week, **E)** 2 weeks, and **F)** mature samples.

Figure 5.13. Maps of the lattice constant c . **A)** 2 hours, **B)** 1 day, **C)** 2 days, **D)** 1 week, **E)** 2 weeks, and **F)** mature samples.

Figure 5.14. EDS on 1w sample. **A)** Backscatter electron image of a cross-section from the surface to the bulk of the impact region. The yellow box shows the location and dimensions of the EDS map. **B)** EDS maps of the box in **A** for elements Ca, P and F. **C)** The average element wt. % across the short axis of the maps in **B** plotted against the depth starting from the sample surface at 0 μm . **D)** The P and F to Ca ratios against the depth.

Figure 5.15. EDS on mature sample. **A)** Backscatter electron image of a cross-section from the surface to the bulk of the impact region. The yellow box shows the location and dimensions of the EDS map. **B)** EDS maps of the box in **A** for elements Ca, P and F. **C)** The average element wt. % across the short axis of the maps in **B** plotted against the depth starting from the sample surface at 0 μm. **D)** The P and F to Ca ratios against the depth.

5.3.4 Apatite Carbonation in the Dactyl Club

From the XRF maps, a lie between a_F and a_{OH} in most regions across all the samples. However, in several regions, the size of a was larger than that of a_{OH} . This is clearly observed in the 2h map, where in the top 10 μm of the sample $a > a_{OH}$. Sr substitution for Ca in apatite was not a possible cause because the c -axis increases in size with Sr substitution [21]. A recent study showed that the surface membrane is composed of apatite nanospheres with organics within the crystal lattices causing lattice strain [20]. However, the apatite lattice distortion observed is large and highly unlikely to be due to macrostrain alone. A study on biogenic aragonite with XRD measured macrostrains due to organics of about 0.001 in the c -axis of orthorhombic aragonite which translate

to about 0.006 Å which is much lower than the variations of around 0.1 Å in a observed in this study. The large a values could be related to other molecular substitutions within the apatite lattice like carbonate substitution. This hypothesis was tested with Raman and FTIR spectroscopy on the 1w, 2w and mature samples. There are two types of carbonated apatite (CAP): Type A carbonated apatite (A-CAP) and Type B carbonated apatite (B-CAP). CO₃ occupies the OH⁻ site in A-CAP and CO₃ occupies the PO₄⁻ site in B-CAP [21]. Table 5.2 summarizes the peak assignments for both Raman and FTIR data. Figures 5.16 and 5.17 show the Raman and FTIR spectra respectively, the locations where the spectra were obtained are shown in the optical microscope images on the left of the figures. High fluorescence was prevalent in the Raman spectra of the samples (i.e. 1w, 2w and mature). However, the main apatite bands were largely visible, especially from spectra taken nearer to the surface layer of the dactyl club. In our measurements, the PO₄ peaks were generally broad instead of sharp. The apatite P-O symmetric stretch ($\nu_{1,Ap}$) peak at 959 – 965 cm⁻¹ was the sharpest and the strongest apatite peak observed with Raman. While In FTIR, the $\nu_{1,Ap}$ peak is visible at 956 cm⁻¹ as a tiny shoulder. A strong peak was observed at around 432 – 435 cm⁻¹ which is assigned to apatite symmetric O-P-O bend ($\nu_{2,Ap}$). The peak at 586 – 589 cm⁻¹ can be attributed to the asymmetric O-P-O bend ($\nu_{4,Ap}$). These peaks do not vary much between samples. The $\nu_{2,Ap}$ and $\nu_{4,Ap}$ peaks were out of the range of the FTIR instrument and hence were not recorded with IR. The apatite asymmetric stretch ($\nu_{3,Ap}$) and carbonate symmetric stretch (ν_{1,CO_3}) peaks are superimposed. In Raman, this band is observed at 1065 – 1070 cm⁻¹. While in FTIR, one very strong peak at 1041 cm⁻¹ and a shoulder at 1097 cm⁻¹ were assigned to $\nu_{3,Ap}$ [22]; this IR band is the strongest band observed and engulfs the ν_{1,CO_3} peak completely. A shoulder is observed at 1102 – 1107 cm⁻¹ with Raman. According to Penel *et al.*, this band can be assigned to $\nu_{3,Ap}$ for A-CAP; however, this peak would likely be weak because OH⁻ substitution in CAP is generally low [23]. Moreover, the situation is further complicated again by the presence of a strong chitin peak at 1112 cm⁻¹ masking the $\nu_{3,Ap}$ band [24]. Raman and FTIR are complementary techniques as some vibration modes are observed in one but not another. An example is the carbonate out-of-plane bend (ν_{2,CO_3}), which is not Raman active but is IR active. With IR, a weak and broad band is observed at around 864 cm⁻¹, which is assigned to ν_{2,CO_3} [25]. The carbonate in-plane bend (ν_{4,CO_3}) is Raman active and was assigned to a weak and broad peak at around 710 cm⁻¹ in the 1w and mature samples [26]. The locations of the ν_{2,CO_3} and ν_{4,CO_3} peak suggest that they belong to amorphous

calcium carbonate [25]. Furthermore, only a single peak was observed for the ν_{4,CO_3} instead of doublets. Additionally, this peak is also superimposed with a chitin peak while the ν_{2,CO_3} may overlap with a HPO_4^{2-} band. The ν_{4,CO_3} peaks are IR active but were not recorded as they were out of range for the instrument. The low intensity of the bands at where carbonate is expected suggest that the apatite is not carbonated, and that carbonate is rather from amorphous calcium carbonate. However, a definitive conclusion is difficult because of the noise from fluorescence in Raman measurements. Furthermore, overlapping chitin peaks muddle the spectra, making carbonate determination challenging and non-trivial with vibrational spectroscopy. Therefore, due to the lack of evidence, the apatite in the dactyl club is likely not carbonated and hence the observed lattice parameter sizes cannot be explained by CO_3 substitution in the apatite lattice and the cause(s) is/are still elusive.

Table 5.2. Assignment of vibration modes observed with Raman and FTIR. Intensity: very strong (vs), strong (s), medium (m), weak (w), very weak (vw). High fluorescence obscures the Raman signal and FTIR signal is also noisy before smoothing. Hence, assignments for very weak signals may be noise observed and should be taken with a pinch of salt. Smoothing was done using a fast fourier transform filter with a symmetric window of size 5.

1-Week Raman	2-Week Raman	Mature Raman	IR	Assignment
432 s	433 s	435 s		$\nu_{2,Ap}$
	455 m			
		502 vw		chitin
586 m	589 s	586 m		$\nu_{4,Ap}$
	611 m			
713 m		708 vw		chitin + $\nu_{4,CO3}$
			864 w	$\nu_{2,CO3}$
884 w	874 w			HPO_4^{2-}
		896 vw		chitin
	944 m			
959 vs	965 vs	959 vs		$\nu_{1,Ap}$
1004 m	1005 s	1007 m		carotenoids
			1035 - 1041	
1035 s	1047 m	1035 m	vs	$\nu_{3,Ap}$
1065 w	1074 m	1071 m		$\nu_{3,Ap} + \nu_{4,ACC} + \nu_{1,B-CO3}$
			1097 s	$\nu_{3,Ap}$
1102 w	1107 vw	1107 w		chitin + $\nu_{1,A-CO3}$
1140 w		1145 w		chitin + $\nu_{1,B-CO3}$
	1159 m	1154 s		carotenoids + chitin + $\nu_{1,B-CO3}$
1208 vw		1204 vw		chitin
			1214 vw	
			1233 vw	Am III + $\nu_{3,Ap}$
1253 vw			1253 vw	chitin
	1269 vw	1261 w		
1326 s	1331 vw	1326 w	1329 vw	
			1340-1390	
1368 w	1384 vw	1374 w	w	
		1414 vw	1409 w	chitin + $\nu_{3,A/B-CO3}$
	1450 vw	1451 w	1445 w	chitin + $\nu_{3,A/B-CO3}$
			1506 vw	carotenoids
	1520 m		1522 vw	
			1540 vw	$\nu_{3,A-CO3}$
1566 m			1560 vw	Am II
			1585 w	chitin
1592 m			1604 w	Am I
	1620 w	1620 w	1630 vw	
	1661 w	1656 w	1650 vw	
			1668 vw

Figure 5.16. Raman spectroscopy measurements of dactyl clubs. **A)** Optical microscope image (left) of the mature club; red dots mark the location for the spectra shown on the intensity vs. wavenumber plots (right). Same for the other samples. **B)** 2w sample. **C)** 1w sample.

Figure 5.17. FTIR measurements of dactyl clubs. **A)** Optical microscope image of the mature club; open circles mark the location for the spectra shown in **B**. **B)** FTIR intensity vs. wavenumber plot. Inset: magnified image of the region in the red box showing the assignment of the peaks observed with FTIR.

5.3.5 Texture Analysis

Figure 5.18 show the crystal orientation or texture maps of the 1d and mature samples. FAP crystals in the impact surface layer form a ‘sandwich’ structure with three sublayers of differently oriented crystals as typically shown in Figure 5.18A. The apatite crystals in the first sublayer—immediately next to the surface was oriented—with its *c*-axis tangent to the surface, while the second sublayer was oriented in random directions and the third sublayer was parallel to the first. The crystal orientation in the impact surface layer is clearly differently from those in the impact region; this is clearly observed in the mature texture map shown in Figure 5.18B and the 1w texture map in Figure 5.8B. Previous synchrotron X-ray measurements showed that in the impact region the crystals are aligned normal to the surface of the club [2]. However, the WAXS data from this study shows that the texture in the impact region is more complex than apatite aligned towards the club surface. Figure 5.18B showed that the crystals are likely orientated following the chitin fiber arrangement which is generally

preferentially aligned towards the surface. Nevertheless, the texture is clearly different from that of the impact surface layer. The reason for the ‘sandwich’ texture observed in the impact surface is unclear. However, the impact surface region retained the orientation pattern from 1 day to 2 weeks of development, indicating that the mineralization patterns were ‘set in stone’ early in the club formation process. Mapping of the 3D texture using WAXS tensor tomography [27] can be performed to obtain a full picture of the ‘sandwich’ structure, which may offer greater clarity into the purpose of the impact surface layer. However, this is outside the scope of the present work.

Figure 5.18. Apatite (002) orientation plots for the **A)** 1 day and **B)** mature samples. The characteristic ‘sandwich’ structure of the impact surface layer can be seen in (A) where a sublayer with randomly orientated crystals is sandwiched between two layers with crystals orientated parallel to the surface; in contrast to the impact region (B) where the crystals are generally aligned perpendicular to the surface. 1w orientation plot can be found in Figure 5.8B.

5.3.6 Spatial-temporal distribution of Br in the dactyl club

Perhaps the most intriguing findings from the synchrotron measurements was the presence of Br in the dactyl club. Initially, this result was thought to be an artefact,

especially since Br was found at the surface of the dactyl club. Since, the artificial seawater in the laboratory aquarium, where the stomatopods were kept contain Br. However, Br was found in similar concentrations to the surface deeper within the dactyl club. Moreover, thorough rinsing and cleaning was performed on all the samples before sample preparation. Thus, the Br is unlikely to be an artefact. Figure 5.19 and 6.20 show the Ca and Br XRF maps from the synchrotron experiments. It is important to note that both the Ca and Br XRF maps are only normalized within each sample, and hence concentrations cannot be measured and directly compared between different maturation stages. This is especially so because the data were measured from two different beamlines and there was no common sample measured in both beamlines. Moreover, sample thickness affects the concentration measurements from XRF. Therefore, the data measured are best for observing the spatial distribution of elements, and only give a qualitative estimate of the amount of the elements. The Ca maps indicate roughly where there is material in the dactyl club since Ca is found in most regions of the club though in varying concentrations. Compared to Ca, XRF maps show that Br was concentrated into two bands, the first band is found at the surface of the club for all the samples except the 2w sample. Br was not present in the club surface of the 2w sample because it is likely that the surface impact layer was worn off from sample preparation as mentioned above. Br was observed in higher concentrations on the surface of the mature club, and less within the impact region other than at the microchannels. From the XRF data, the Br is likely found within the organic membrane that expands to form the initial template for the club. More brominated material is likely added to the club as it further develops since the Br was present in high concentrations in the impact surface of the 1w and mature samples. The second Br band was present at the growth front for the 2h, 1d and 2d clubs. While for the 1w and 2w samples, the second Br band was found in the middle of the club. The second Br band was not observed in the mature sample because it was likely out of the boundaries of the area mapped. Comparing location of the second Br band in the 1w sample with the 1w scale factor map (Figure 5.8B), it is obvious that the second band of Br coincides with the mineralization front. This is also the case for the 2w club (Figure 5.12E and 6.20F). Thus, it was hypothesized that Br is present within the proteins that regulate the biomineralization process.

Figure 5.19. Ca K α (8.639 keV) maps. **A)** Overview of week 1 sample (lower resolution scan), **B)** 2 hour, **C)** 1 day, **D)** 2 day, **E)** 1 week, **F)** 2 week **G)** Mature samples. Intensity is in arbitrary units and can only be compared within a sample and not between samples.

Figure 5.20. Br K α (11.924 keV) maps. **A)** Overview of week 1 sample (lower resolution scan), **B)** 2 hour, **C)** 1 day, **D)** 2 day, **E)** 1 week, **F)** 2 week, **G)** mature samples. Intensity is in arbitrary units and can only be compared within a sample and not between samples.

5.3.7 Identification of brominated club mineralization protein (CMP)

Proteomic studies were thus conducted on club mineralization protein-1 (CMP-1), the dominant protein regulating apatite mineralization of the dactyl club [6]. The results from the PRM method are displayed in Figure 5.21A and B, which consisted in spiking

SIL peptides into the wild type peptides and analyzing the data using the same method as above (top panels: precursors and bottom panels: corresponding fragment ions). Figure 5.21A shows the chromatograms from the non-brominated peptide (left panel: wild type peaks and right panel: SIL peaks) whereas Figure 5.21B shows the chromatograms from the brominated peptides (left panel: wild type peaks and right panel: SIL peaks). The chromatograms show that wild type fragment ions could be identified along fragments from the SIL peptides, thereby confirming the presence of brominated tyrosine Y₂₇₅ in CMP-1. Based on the peak areas of the precursor chromatograms, a higher fraction of peptides in the wild-type sample was non-brominated. Thus, Br is confirmed to be present in the dactyl club, at least within CMP-1. However, this begs the question, why is Br present in CMP-1? Bromination does not necessarily play a role in apatite biomineralization, although it will be an interesting follow up study. Br was found in several biotools [28], [29]. In the Nereis worm jaw, the presence of halogens, particularly Cl was correlated with an increase in hardness. This shown to be because Cl ions formed coordination bonds with Zn ions together with histidine residues [28]. The increase in hardness here is not due to the addition of transition metals into the biological material alone. Broomell *et al.*, showed that transition metals like Zn, Cu and Mn forming metal complexes with the proteins within the Nereis worm jaw led to an increase in hardness rather than raw increase of transition metal concentration [30]. Although Cl and Zn concentrations are correlated with hardness and elastic modulus, Birkedal *et al.* showed that other halogens, namely Br and I did not play a primary role in mechanical properties of the Nereis worm jaw [31], which in contrast to the dactyl club is unmineralized. In their study, they found that Br and I were localized at the surface of the jaw and suggested that they may instead contribute to chemical stability and protection from microbial activity. Results from the ICP measurements presented in the following section show that Br is present in trace amounts, and thus its efficacy in toughening the entire dactyl club is not plausible. A more likely explanation is that bromination would provide anti-microbial activity to CMP-1, which would help the dactyl club to develop with lesser interference from harmful pathogens, a protection which may be especially important immediately after molting when the animal is most vulnerable. Moreover, Br is also found on the surface of the dactyl club which is exposed to the environment and by extension countless potential pathogens.

Figure 5.21. Verification of bromination in CMP-1 by the parallel reaction monitoring (PRM) method. SIL peptides were spiked into the wild type CMP-1 sample, followed by identification of the precursor peptides and their fragmentation ions. **A)** Chromatograms with peaks corresponding to the non-brominated peptide precursor L274-R283 from CMNP-1 and their corresponding fragment ions (bottom). *Left:* peaks corresponding to the wild-type (“light”) peptide [L274-R283]_L and *right:* peaks corresponding to the SIL (“heavy”) peptide [L274-R283]_H. **B)** Chromatograms with peaks corresponding to the brominated peptide L274-R283 from CMP-1 (top) and their corresponding fragment ions (bottom). *Left:* peaks corresponding to the wild-type (“light”) peptide [L274-R283]_{L,Br} and *right:* peak corresponding to the SIL (“heavy”) peptide [L274-R283]_{H,Br}. For both brominated and non-brominated samples, wild-type precursors and their fragmentation ions could be identified in the wild-type samples, indicating that both brominated and non-brominated peptides are present in the native sample. Based on the precursor peak areas, a higher fraction of peptides was non-brominated.

5.3.8 Chemical quantification of the dactyl club with ICP methods

As mentioned, the XRF data was at most qualitative. Therefore, ICP-OES and ICP-MS experiments were conducted to quantify chemical composition of the dactyl club. Table 5.3 shows the concentrations of Ca, P, Mg, Sr, S and Br of the samples measured with ICP. To the best of our knowledge, this is the first ICP measurement conducted on the stomatopod dactyl club. The Milli-Q H₂O sample showed little to none of the elements inspected except for P which has a concentration of 0.5 mg/L. The marine salt used vary considerably from the label provided by the supplier for some of the elements like Mg and S. Br is clearly detected in the dactyl club. The Br concentration in the club sample was 0.0457 mg/L after correcting for the amount present in the control. Table 5.4 shows the weight percentages of the elements in the dactyl club after correcting for the control. The measured elements made up about a quarter of the weight of the club, with Ca accounting for most of the weight and making up a fifth of the club's weight. In contrast, Br accounts for only 0.02% of the weight making it the least abundant among the elements measured by weight. The Br/Ca ratio is 11.7×10^{-4} which is 4.7 times larger than measured with XRF. Although, it is important to note that the synchrotron XRF measures the elemental concentration on a local scale unlike ICP which measures the elemental composition of the entire club.

Table 5.3. Elemental composition of dactyl club sample and controls. Ca, P, Mg, Sr and S concentrations were determined using ICP-OES, while Br concentration was measured with ICP-MS. Values in parentheses indicate the standard errors of the measurements. [†]Adapted from marine salt supplier label. [‡]Digested in 70% HNO₃ and 30% H₂O₂ solution at 90°C for 4 hours.

Sample	Ca (mg/L)	P (mg/L)	Mg (mg/L)	Sr (mg/L)	S (mg/L)	Br (mg/L)
Milli-Q H ₂ O	0.0	0.5	0.0	0.1	0.0	0.00
Salt Solution	372.6(0.3)	0.5	1594.8(3.2)	13.0	246.4(0.1)	103
Salt Solution [†]	420(20)	0.1	1340(50)	8.0(0.5)	900(50)	67.5(7.5)
Artificial Seawater	435.6(0.4)	1.8	1261.3(7.1)	14.8	496.1(2.5)	83.4
HNO ₃ Control [‡]	0.0	0.5	0.0	0.2	0.1	0.0439
MSDC [‡]	39.2(0.1)	8.6	1.8	0.7	0.4	0.0896

Table 5.4. Weight percentage of elements in the dactyl club. Elements measured composed around ¼ of the weight; the major element in terms of weight being Ca. Br is a trace element and makes up about 0.02% of the dactyl club by weight. *Measured with ICP-MS while the other elements were measured with ICP-OES.

Sample	Ca (wt%)	P (wt%)	Mg (wt%)	Sr (wt%)	S (wt%)	Br* (wt%)	Total (wt%)
MSDC	19.27	3.98	0.89	0.23	0.16	0.02	24.56

Figure 5.22. Schematic summary of the development of the dactyl club from the surface to the impact region. Bromine was found in the surface and at the mineralization front and a fraction of CMP-1 proteins were confirmed brominated at tyrosine 275. Apatite crystallites were discovered as early as 2 hours after molting. Apatite crystallites were larger and more isotropic in the impact surface than the impact region. Crystallites formed a unique ‘sandwich’ structure in the impact surface with randomly oriented crystallites sandwiched between two layers of crystallites aligned parallel to the surface. While the crystallites were aligned following the organic matrix in the impact region.

5.4 Conclusion

This study provided greater insight into the spatial-temporal arrangement and the size and shape of apatite in the dactyl club. Figure 5.22 illustrates the growth and development of the club, summarizing the main findings from this time evolution study. Apatite crystals were present within the club as early as 2 hours from molting. Crystallites were needle-like in shape with a smaller aspect ratio (more spherical) in the impact surface than the impact region. Lattice parameter from synchrotron X-ray mapping suggest that the apatite crystal chemistry changes with development, ultimately maturing into FAP. However, the details of the changes remain unclear. F⁻ substitution for OH⁻ and apatite carbonation hypotheses were tested, and the results showed that the variations in the *a* lattice parameter cannot be explained by both of these substitutions. Macrostrain may contribute to the lattice parameter changes but it is unlikely to be the only reason because the changes were large. Texture mapping revealed that the impact surface had a unique ‘sandwich’ structure which is very different from the texture in the impact region. The purpose for this unique texture in the impact surface is unclear and is a subject for further study. Most surprisingly was the discovery of Br within the surface of the dactyl club and at the mineralization front. Proteomic studies confirmed that some tyrosine residues of CMP-1 were brominated. CMP-1 is an important protein regulating apatite biomineralization in the dactyl club. Brominated proteins may have enhanced protection against chemical and enzymatic attacks, which may be important for successful apatite biomineralization in a marine environment teeming with microbes and other potential pathogens.

References

- [1] R. Wang and H. S. Gupta, “Deformation and Fracture Mechanisms of Bone and Nacre,” *Annu. Rev. Mater. Res.*, vol. 41, no. 1, pp. 41–73, Aug. 2011, doi: 10.1146/annurev-matsci-062910-095806.
- [2] J. C. Weaver *et al.*, “The Stomatopod Dactyl Club: A Formidable Damage-Tolerant Biological Hammer,” *Science*, vol. 336, no. 6086, p. 1275, Jun. 2012, doi: 10.1126/science.1218764.

- [3] S. Amini *et al.*, “Textured fluorapatite bonded to calcium sulphate strengthen stomatopod raptorial appendages,” *Nat. Commun.*, vol. 5, p. 3187, Jan. 2014, doi: 10.1038/ncomms4187.
- [4] S. Amini, M. Tadayon, S. Idapalapati, and A. Miserez, “The role of quasi-plasticity in the extreme contact damage tolerance of the stomatopod dactyl club,” *Nat. Mater.*, vol. 14, no. 9, pp. 943–950, Sep. 2015, doi: <http://dx.doi.org/10.1038/nmat4309>.
- [5] M. L. Reaka, “Molting in stomatopod crustaceans. I. Stages of the molt cycle, setagenesis, and morphology,” *J. Morphol.*, vol. 146, no. 1, pp. 55–80, May 1975, doi: 10.1002/jmor.1051460104.
- [6] S. Amini *et al.*, “A diecast mineralization process forms the tough mantis shrimp dactyl club,” *Proc. Natl. Acad. Sci.*, vol. 116, no. 18, pp. 8685–8692, Apr. 2019, doi: 10.1073/pnas.1816835116.
- [7] M. E. Birkbak, M. Guizar-Sicairos, M. Holler, and H. Birkedal, “Internal structure of sponge glass fiber revealed by ptychographic nanotomography,” *J. Struct. Biol.*, vol. 194, no. 1, pp. 124–128, Apr. 2016, doi: 10.1016/j.jsb.2016.02.006.
- [8] M. A. Marcus *et al.*, “Parrotfish Teeth: Stiff Biominerals Whose Microstructure Makes Them Tough and Abrasion-Resistant To Bite Stony Corals,” *ACS Nano*, vol. 11, no. 12, pp. 11856–11865, Dec. 2017, doi: 10.1021/acsnano.7b05044.
- [9] S. Amini, M. Tadayon, J. Q. I. Chua, and A. Miserez, “Multi-scale structural design and biomechanics of the pistol shrimp snapper claw,” *Acta Biomater.*, vol. 73, pp. 449–457, Jun. 2018, doi: 10.1016/j.actbio.2018.04.038.
- [10] A. B. Jensen, T. E. K. Christensen, and H. Birkedal, “Very large scale diffraction investigations facilitated by a Matrix-multiplication Facilitated Radial and Azimuthal Integration Algorithm - MatFRAIA,” *Unpublished*.
- [11] S. Frölich and H. Birkedal, “*MultiRef*: software platform for Rietveld refinement of multiple powder diffractograms from *in situ* , scanning or diffraction tomography experiments,” *J. Appl. Crystallogr.*, vol. 48, no. 6, pp. 2019–2025, Dec. 2015, doi: 10.1107/S1600576715020099.
- [12] J. Palle, T. E. K. Christensen, N. K. Wittig, T. Grünewald, and H. Birkedal, *Unpublished*.
- [13] A. Gourrier *et al.*, “Scanning small-angle X-ray scattering analysis of the size and organization of the mineral nanoparticles in fluorotic bone using a stack of

- cards model,” *J. Appl. Crystallogr.*, vol. 43, no. 6, pp. 1385–1392, Dec. 2010, doi: 10.1107/S0021889810035090.
- [14] C. G. Ryan, D. R. Cousens, S. H. Sie, W. L. Griffin, G. F. Suter, and E. Clayton, “Quantitative pixe microanalysis of geological material using the CSIRO proton microprobe,” *Nucl. Instrum. Methods Phys. Res. Sect. B Beam Interact. Mater. At.*, vol. 47, no. 1, pp. 55–71, Mar. 1990, doi: 10.1016/0168-583X(90)90047-X.
- [15] C. G. Ryan *et al.*, “The Maia 384 detector array in a nuclear microprobe: A platform for high definition PIXE elemental imaging,” *Nucl. Instrum. Methods Phys. Res. Sect. B Beam Interact. Mater. At.*, vol. 268, no. 11–12, pp. 1899–1902, Jun. 2010, doi: 10.1016/j.nimb.2010.02.052.
- [16] V. A. Solé, E. Papillon, M. Cotte, Ph. Walter, and J. Susini, “A multiplatform code for the analysis of energy-dispersive X-ray fluorescence spectra,” *Spectrochim. Acta Part B At. Spectrosc.*, vol. 62, no. 1, pp. 63–68, Jan. 2007, doi: 10.1016/j.sab.2006.12.002.
- [17] J. M. Hughes, M. Cameron, and K. D. Crowley, “Structural variations in natural F, OH, and Cl apatites,” *Am. Mineral.*, vol. 74, no. 7–8, pp. 870–876, Aug. 1989.
- [18] S. Gallien, E. Duriez, C. Crone, M. Kellmann, T. Moehring, and B. Domon, “Targeted Proteomic Quantification on Quadrupole-Orbitrap Mass Spectrometer,” *Mol. Cell. Proteomics*, vol. 11, no. 12, pp. 1709–1723, Dec. 2012, doi: 10.1074/mcp.O112.019802.
- [19] N. A. Yaraghi *et al.*, “A Sinusoidally Architected Helicoidal Biocomposite,” *Adv. Mater.*, vol. 28, no. 32, pp. 6835–6844, Aug. 2016, doi: 10.1002/adma.201600786.
- [20] W. Huang *et al.*, “A natural impact-resistant bicontinuous composite nanoparticle coating,” *Nat. Mater.*, Aug. 2020, doi: 10.1038/s41563-020-0768-7.
- [21] J. C. Elliott, *Structure and Chemistry of the Apatites and Other Calcium Orthophosphates.*, 1st ed. Amsterdam: Elsevier Science, 1994.
- [22] V. M. Bhatnagar, “IR-spectra of fluorapatite and fluorochlorapatite,” *Experientia*, vol. 23, no. 1, pp. 10–12, Jan. 1967, doi: 10.1007/BF02142236.
- [23] G. Penel, G. Leroy, C. Rey, and E. Bres, “MicroRaman Spectral Study of the PO₄ and CO₃ Vibrational Modes in Synthetic and Biological Apatites,” *Calcif. Tissue Int.*, vol. 63, no. 6, pp. 475–481, Dec. 1998, doi: 10.1007/s002239900561.

- [24] A. Zając, J. Hanuza, M. Wandas, and L. Dymińska, “Determination of N-acetylation degree in chitosan using Raman spectroscopy,” *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.*, vol. 134, pp. 114–120, Jan. 2015, doi: 10.1016/j.saa.2014.06.071.
- [25] A. Grunenwald, C. Keyser, A. M. Sautereau, E. Crubézy, B. Ludes, and C. Drouet, “Revisiting carbonate quantification in apatite (bio)minerals: a validated FTIR methodology,” *J. Archaeol. Sci.*, vol. 49, pp. 134–141, Sep. 2014, doi: 10.1016/j.jas.2014.05.004.
- [26] A. Awonusi, M. D. Morris, and M. M. J. Tecklenburg, “Carbonate Assignment and Calibration in the Raman Spectrum of Apatite,” *Calcif. Tissue Int.*, vol. 81, no. 1, pp. 46–52, Jul. 2007, doi: 10.1007/s00223-007-9034-0.
- [27] T. A. Grünewald *et al.*, “Mapping the 3D orientation of nanocrystals and nanostructures in human bone: Indications of novel structural features,” *Sci. Adv.*, vol. 6, no. 24, p. eaba4171, Jun. 2020, doi: 10.1126/sciadv.aba4171.
- [28] H. C. Lichtenegger *et al.*, “Zinc and mechanical prowess in the jaws of Nereis, a marine worm,” *Proc. Natl. Acad. Sci.*, vol. 100, no. 16, pp. 9144–9149, Aug. 2003, doi: 10.1073/pnas.1632658100.
- [29] B. W. Cribb, A. Rathmell, R. Charters, R. Rasch, H. Huang, and I. R. Tibbetts, “Structure, composition and properties of naturally occurring non-calcified crustacean cuticle,” *Arthropod Struct. Dev.*, vol. 38, no. 3, pp. 173–178, May 2009, doi: 10.1016/j.asd.2008.11.002.
- [30] C. C. Broomell, F. W. Zok, and J. H. Waite, “Role of transition metals in sclerotization of biological tissue,” *Acta Biomater.*, vol. 4, no. 6, pp. 2045–2051, Nov. 2008, doi: 10.1016/j.actbio.2008.06.017.
- [31] H. Birkedal *et al.*, “Halogenated Veneers: Protein Cross-Linking and Halogenation in the Jaws of Nereis, a Marine Polychaete Worm,” *ChemBioChem*, vol. 7, no. 9, pp. 1392–1399, Sep. 2006, doi: 10.1002/cbic.200600160.

Chapter 6

Additional Studies

This chapter presents other research work undertaken on the stomatopod dactyl club, which did not warrant a full results chapter. Two studies are covered which address the nanocrystal rotation mechanism in the impact region of the dactyl club, and high-resolution 3D imaging of the dactyl club. The work presented here are ongoing and provide direction for further study into the stomatopod dactyl club.

6.1 Mechanism of Quasiplasticity in Stomatopod Dactyl Club

6.1.1 Introduction

A previous study [1] showed using synchrotron diffraction that the mineral phase within the impact region of the mantis shrimp dactyl club was preferentially oriented with their (002) plane normal to the surface. This anisotropy enhanced the mechanical properties of the club along the striking direction of the club. Amini *et al.* observed using SEM that the nanocrystals were rotated in response to loading on the surface, dissipating the energy and preventing brittle failure of the impact region of the club [2]. In this study, the mechanism for nanocrystal rotation was explored. The objectives were to derive a physical model to relate the nanocrystal rotation to the force or stress applied on the dactyl club. Synchrotron μ XRD was used in this study because it can map large areas with high resolution. Moreover, XRD is a standard technique used for crystallography.

6.1.2 Materials and Methods

6.1.2.1 μ XRD Sample Preparation

Single loading events were conducted on a custom uniaxial mechanical tester. The tips used to depress the surface of the clubs with this setup were tungsten carbide balls with diameters around 350 μ m. Tips were attached to a straight cylindrical shaft using cyanoacrylate glue. Cyclic loading events were performed using a Hysitron TI-950 nanoindenter equipped with a Multi-range Nanoprobe (MRNP) and a 50 μ m cono-spherical tip. The sample surfaces were kept hydrated with a tissue wet with DI water before indentation. Experimental loads were calculated to encompass the full range of the stress-strain behaviour observed in the indentation stress-strain graph of the dactyl club conducted in a previous study (Figure 6.1C) [2]. For both single and cyclic loading tests, the model used in calculations were a flat surface depressed with a perfect sphere; which allows the use of Hertzian contact mechanics to describe the indentation. Figure 6.1A shows one of the single loading samples, a flow diagram is used to illustrate the calculation process for the 17.5 N load. The desired stress value is read off the stress strain graph (Figure 6.1C) and the corresponding load found using Equation 2.7 and

2.8. The same process was repeated to calculate the rest of the loads which is listed in Table 6.1. For cyclic loading tests, care was taken to ensure that the spherical depth of the cono-spherical tip was not exceeded. Indents in the elastic regime were placed beside their plastic counterparts so that they can be found later. In many cases, the indents produced by the nanoindenter are invisible to the naked eye. Hence, markings were drawn with a fine marker under a stereo-microscope to indicate where the indents are and to avoid them during cutting. The nanoindenter provided some advantages compared with the custom mechanical tester, including selecting indentation sites using a microscope as well as accurate and automatic load control.

General protocol for cutting was as follows: Rough cutting was performed on an Isomet 1000/4000 Precision Saw with a Struers 50A15 alumina cut-off wheel. Cutting speeds of 200 and 250 RPM were used with feed rate of 4 mm/min. Feed rate cannot be precisely controlled on Isomet 1000 but were managed by hand with assistance of a counterbalance weight. Fine cutting was done where minimal material removal was required; for example, when cutting samples for the synchrotron μ XRD experiment. The protocol used for polishing all samples was as follows: Plane grinding was carried out on Metken Forcipol 1V at 250 RPM under running water using silica paper of 1000 and 1200 Grit paper. Fine grinding was carried out at 150 RPM using 2500 and 4000 Grit paper. A damp polishing cloth impregnated with 40 nm Silica colloidal solution was used for final polishing on Struers RotoPol-35. Samples were rotated by 180° within each step and 90° between steps. Each step is completed when marks from the previous step has been removed. Samples were washed in running water between every grinding and polishing step, this is critical to prevent abrasives and debris from the previous step affecting the current step. Samples were sonicated in DI water for 2 mins to remove any silica nanoparticles remaining on the samples. After indentation, the samples were cut about 0.5 – 1 mm away from the indentation sites and directly mounted on its cross-section on a metal disc for grinding and polishing. Special care was taken to avoid grinding pass the indents and keeping the ground surface parallel to the indents – if there were multiple indents on one slice. The indents were regularly viewed using a stereo-microscope throughout the polishing process. Final polishing was performed using the colloidal solution when the sample is polished to the edge of the indent.

Table 6.1. Indentation details of the samples measured during the two trips to the ALS: The type of loading, maximum indentation loads (P), their corresponding stresses (σ), and the expected deformation state areas surrounding the indents. Control samples were also measured during each trip: pristine (non-damaged) and native damaged samples. *Sample indented at three different areas with the same load for 1, 10 and 100 cycles and X-ray mapping was done for each area. ** μ XRD maps taken from the areas on the left (L), bottom (B) and right (R) of the native damage.

	Sample	Type	P (N)	σ (GPa)	Deformation State
First Trip to ALS	SL1	Single loading	7	3.3	Elastic
	SL2	Single loading	12	4.0	Elastic
	SL3	Single loading	17.5	4.5	Elastic
	SL4	Single loading	25	5.1	Plastic
	SL5	Single loading	32	5.6	Plastic
	SL6	Single loading	36	5.8	Plastic
	SL7	Single loading	43	6.1	Plastic
	SL8	Single loading	53	6.6	Plastic
	SL9	Single loading	65	7.0	Plastic
	P1	Pristine	-	-	Undeformed
	N1	Native damaged	-	-	Plastic
	N2	Native damaged	-	-	Plastic
Second Trip to ALS	ML1*	Multiple loading	0.05	1.6	Elastic
	ML2*	Multiple loading	0.6	3.7	Elastic
	ML3*	Multiple loading	4	7.0	Plastic
	P2	Pristine	-	-	Undeformed
	N3L**	Native damaged	-	-	Plastic
	N3B**	Native damaged	-	-	Plastic
	N3R**	Native damaged	-	-	Plastic

Figure 6.1. **A)** Optical microscopy image of single loaded sample club. **B)** Sagittal cross-section club in **A**, box enclosing the 17.5 N indent. **C)** Indentation stress strain curve from [2] used to calculate the loads using Equation 2.7 and 2.8, which is printed above **A**. **D)** SEM image of the 17.5 N indent – taken in LEI mode at 500 $\times$ magnification; scale bar is 10 μm .

6.1.2.2 μXRD Mapping

μXRD experiments were performed to quantify the degree of misorientation of the FAP crystals during a damage event. Four types of damage regions were analyzed; (i) No damage – pristine, (ii) native damage – from natural use, (iii) single loading event, and (iv) cyclic loading event. These experiments were conducted at the 12.3.2 beamline at the Advanced Light Source (ALS) of the Lawrence Berkeley National Laboratory (LBNL), USA. A schematic of the 12.3.2 beamline is given in Figure 6.2. Conventional Bragg diffraction geometry was used with the 2θ angle set to 50° and a sample-to-detector distance of around 160 mm. The samples were attached to the stage using double-sided tape. An optical video camera was used to locate the visible indents. Lasers were used together with the video camera to position the indents directly under the X-ray beam. X-ray fluorescence mapping of a large area was used to confirm the beam was aimed at the indents. The region directly below the indents were irradiated and the diffracted X-rays were collected with a resolution up to 5 μm in the horizontal

axis and 8 μm in the vertical axis. White (polychromatic) X-rays were used for fluorescence mapping due to higher beam intensity and this was done by moving the mono-chromator away from the beam path. Monochromatic X-rays were used for the diffraction experiments. The beam energy was adjusted to around 8 keV for the monochromatic beam. Beam exposure times were 60 s and 30 s per spot for the X-ray fluorescence and diffraction maps, respectively.

Figure 6.2. Schematic layout of the ALS beamline 12.3.2 with a superbend dipole magnet source. The beamline acceptance is 0.2 mrad (horizontal and vertical). The M1 toroid mirror focuses the source onto a virtual object. This intermediate image is demagnified by a ratio of 8:1 and 16:1 in horizontal and vertical, respectively, by a pair of KB mirrors. A four-bounce Si(111) monochromator can optionally be brought into the beam path for monochromatic microdiffraction on nanosized grains [3], [4].

6.1.2.3 EBSD

For EBSD measurements, freshly molted dactyl club were mounted, cut and polished following the same protocol as for the μXRD samples. Additional sample preparation, different conditions and instruments were used to find an experimental setup that produced Kikuchi patterns from the dactyl club. A dactyl club sample is further polished

with Ar ion cross-section polishing before EBSD measurement. A second sample was prepared and carbon-coated. Combinations of 15 kV and 20 kV accelerating voltages and 12 and 14 probe current settings on a JEOL 7600F FESEM were used. The same sample was later coated with 5 nm gold and tested with the same combinations. A third sample was prepared and etched with 5 M Guanidine Triocyanide. Guanidine Triocyanide is commonly used to denature proteins. A few drops were placed on the sample surface for around 20 mins and the sample left to dry overnight before carbon coating and EBSD measurement. The third sample was tested on a newer JEOL FESEM model (JEOL 7800F). The third sample was also tested using another JEOL 7800F equipped with a CMOS EBSD detector which is the fastest and most sensitive detector available to the author at the time of experiment. A fourth uncoated sample was tested with the same setup as well.

6.1.3 Results

μ XRD was used to study the crystal orientation of FAP nanocrystals in the impact region of the stomatopod dactyl club. Table 6.1 lists the samples tested at the synchrotron. The samples are divided into two main groups: those that went through a single indentation loading cycle and those that went through multiple indentation loading cycles. Within the two groups, the samples are divided into those that are elastically deformed, those that are plastically deformed, and the control samples (pristine and native damaged). The region below the indents were rastered with the X-ray probe to generate X-ray diffraction maps. The coordinates of the coloured squares represent the spatial distribution of the scan points within the map. The colour indicates the highest intensity ($I_{\max}$) recorded at the (002) plane while the black line denotes the corresponding angular position ($\chi_{\max}$) – refer to section 3.4.2 for more details. The position of the black line can be understood as the FAP crystal orientation (ϕ) at that scan point (i.e. $\phi = \chi_{\max}$). A quick inspection of the single loaded sample maps (Figure 6.4) shows that ϕ remained fairly constant throughout the region scanned while the intensity varied more. Pristine and native damaged samples were used as control and their XRD maps (Figure 6.3) showed little difference with the single loaded samples. Only the largest and smallest loaded samples are shown in Figure 6.4 – SL9 (65 N) and SL1 (7 N) samples respectively. Figure 6.5 shows the multiple loaded XRD maps of the 600 mN (elastic regime) and 4000 mN (plastic regime) samples. Each map covered

the area below three indentations of the same load but different number of loading cycles. For example, the 600 mN map contains the area below the 1, 10 and 100 cycles indents. Hence, a small region below each indent was selected and analyzed. Selected diffraction patterns for analysis are indicated by the coloured boxes. As with the single loaded samples, a visual comparison of the boxed regions showed little differences between them.

Statistical analysis was performed on the data from the single and multiple loading cycle samples and the results are presented in Figure 6.6. The mean ϕ for single loaded samples did not change much between samples and no trend was thus observed from the mean ϕ . The standard deviation showed larger variance between samples but no clear trend as well. The standard deviation dropped between SL1 and SL2 (12.5 N) but increased after till the SL6 (36 N) sample where it dropped again to a lower value in SL7 and SL8 (43 and 53 N) (Figure 7.6A). There was a larger difference in the intensity values between the single loaded samples. The intensity mean showed a clear decreasing trend between samples SL3 to SL9 with load (17.5 N to 65 N) (Figure 6.6C). It should be noted that the pristine sample showed the largest intensity and highest variance out of all the tested samples. Figure 6.6C compared the ϕ between the multiple loading cycle samples. Unlike the single loading cycle samples, the samples that were loaded multiple times showed differences in ϕ between different loads but little differences within the same load. Even though the X-ray map for the 50 mN sample contains data from three indents with different numbers of loading cycles because of the size of the indents, only a few scan points would contain data from regions affected by the stress field generated by the indent. Therefore, data for the 50 mN sample was not considered. The mean of ϕ and $I_{\max}$ for the 4000 mN sample remained almost constant between different loading cycles, while there is a slight change in ϕ and $I_{\max}$ means for the ten cycle 600 mN sample (Figure 6.6C and D). $I_{\max}$ decreased sharply between the 600 mN and 4000 mN samples which is similar to the results of single loading cycle samples. A single native damaged sample was mapped in three locations; the region to the left (N3L), the right (N3R) and directly beneath (N3B) the damage were mapped. The ϕ was observed to be significantly different, indicating that the preferred orientation of FAP crystals is different between the mapped regions (Figure 6.6C). The mean $I_{\max}$ and standard deviation between the N3L and N3R samples were similar. However, the means and standard deviations of both samples are significantly

larger than the N3B sample. EBSD was another method used to determine the crystalline texture. Different loading conditions were not applied to samples tested with EBSD. Unfortunately, all configurations mentioned in the previous section did not produce any Kikuchi bands. This is unexpected because the dactyl club is most definitely mineralized.

Figure 6.3. μ XRD maps of a **A)** pristine (undamaged) (P1), and a **B)** native damage (N2) region. The black lines and the coloured squares indicate the mean FAP crystal orientation (ϕ) and the mean maximum intensity (I_{max}) using the 002 diffraction peak measured at the respective (X,Y) coordinates

Figure 6.4. μ XRD maps from area next to single-loading indents. **A)** In the elastic regime; 7 N load (SL1). **B)** In the plastic regime; 65 N load (SL9). The black lines and the coloured squares indicate the mean FAP crystal orientation (ϕ) and the mean maximum intensity (I_{max}) using the 002 diffraction peak measured at the respective (X,Y) coordinates

Figure 6.5. μ XRD maps from area next to multiple-loading indents. **A)** In the elastic regime; 600 mN load (ML2) **B)** In the plastic regime; 4000 mN load (ML3). Coloured boxes enclose the data used for statistical analyses presented in Figure 6.6. One cycle (Red), ten cycles (Yellow), hundred cycles (Blue). The black lines and the coloured squares indicate the mean FAP crystal orientation (ϕ) and the mean maximum intensity ($I_{\max}$) using the 002 diffraction peak measured at the respective (X,Y) coordinates.

6.1.4 Discussion and Conclusion

According to the μ XRD results, the average crystal orientation in the region surrounding a damage did not change, whether the damage was artificially induced through indentation or by natural impact. However, this could be due to the highly penetrating nature of X-rays. The incident X-rays travels around 20 μm into the sample at each spot, so ϕ would be the average orientation of all FAP crystals over the entire 20 μm depth. And hence, ϕ could be similar to the ϕ from undamaged regions. Therefore, the intensity of the diffracted beam was explored as another way of comparison. The intensity of single loading cycle samples decreased with increasing load. A lower maximum intensity and broader peak suggest that crystals are more randomly orientated. Further studies to analyze the possible effect of the number of loading cycles was performed. Multiple loading cycle samples showed little difference in their mean crystal orientation as well as the intensity of the diffracted beam indicating that the number of loading cycles did not induce rotation of FAP crystals within the sample. However, similar to single loading cycle samples, a decreasing trend in intensity was observed for all the loading cycles. Moreover, attenuation in mean I_{max} was observed from different regions within the same native damage sample (i.e. N3L, N3B and N3R). The average I_{max} may be indirectly related to the degree of crystal misorientation in the region. As the results from synchrotron measurements proved inconclusive, EBSD was explored as another method to study the texture of the dactyl club. Initial trials with different sample preparation and on different instruments were unable to detect any Kikuchi lines. This is unexpected because of the mineral nature of the impact region. High flatness is necessary for EBSD methods which is achieved on the ion polished sample. And different beam conditions and coatings were tested with the second sample. Even etching away organic material to ensure that the minerals are exposed on the surface did not yield any positive results. Finally, a better detector on a newer FESEM system was used. However, no Kikuchi lines were once again observed on both coated and uncoated samples with this setup. The spatial resolution of EBSD is affected by the atomic number (Z). For aluminium which has a lower Z compared to other metals like platinum and iron, grain sizes of up to 50 nm can be resolved [5]. Moreover, spatial resolution decreases three-fold when the detector is tilted 70°C with respect to the electron beam which is the case for these experiments. This decrease of spatial

resolution is because of the increase of the interaction volume of the electron beam [5]. The apatite in the dactyl club is composed of elements with lower Z than aluminium. The size of the nanocrystallites were smaller than 200 nm based on the synchrotron XRD measurements and on data from previous studies [2], [6]–[8]. Therefore, the sample could be below the resolution for conventional EBSD, and Kikuchi patterns were not observed in the impact region of the club. Moreover, a recent study (published after these experiments were conducted) observed the presence of organics within and between the apatite nanocrystallites [8]. These amorphous zones and defects within the crystallites, compounded with the lower electron dose used for these experiments—because of sample charging and to avoid beam damage—lead to low EBSD signal-to-noise ratios. Thus, the background noise could be high enough to mask the signal leading to no Kikuchi patterns recorded at the detector. Finally, it is possible that the electron beam caused beam damage which would lead to the loss of the pattern although this is unlikely since the apatite crystals should be stable with the low accelerating voltages of 5, 10 keV used. Therefore, EBSD does not seem to be an effective method to map the crystal structure orientation on the impact region of the dactyl club and other methods should be explored. In summary, both synchrotron μ XRD and EBSD yielded inconclusive results, so there remains more to be done to adequately address the hypothesis that FAP crystals rotate in response to mechanical stress within the impact region of the stomatopod dactyl club and to derive a physical model relating stress to the degree of misorientation.

Figure 6.6. Comparison between degree of misorientation in A) single-loading samples and B) multiple-loading samples. C) Comparison between signal intensity in single-loading samples and D) multiple-loading samples.

6.2 Ptychographic X-ray Computed Tomography (PXCT)

6.2.1 Introduction

Micro-computed tomography (micro-CT) is a common technique applied to obtain 3D structural information of biological structures including the stomatopod dactyl club [1], [7]. However, the resolution attainable from conventional micro-CT equipment is limited to several microns [9]. With synchrotron radiation, submicron X-ray spot sizes are attainable enabling higher resolution tomograms to be collected. Moreover, the high brilliance of synchrotron X-rays enable for fast scanning of samples. Phase contrast imaging is used because it has better contrast for lighter elements compared to absorption contrast imaging [10]. Additionally, phase contrast imaging does not require absorption of X-rays for contrast, thus higher energy X-rays can be used which should lead to lesser X-ray absorption and lower radiation damage to the stomatopod dactyl club [10]. Instead of using X-ray optics to recover and magnify the X-ray image, a lenseless approach is adopted. The attraction of lenseless imaging methods is the high resolution achievable. In conventional imaging, resolution scales with the size of the lens, while in lenseless imaging the resolution is proportional to sample-to-detector distance and detector size. Compared to large X-ray lens with minimal aberrations a large detector is more affordable and easier to create. However, the phase problem must be solved when using lenseless imaging methods. This can be achieved using ptychography which is explained in greater detail in section 3.4.3. With ptychographic X-ray computed tomography (PXCT) 3D resolutions up to 90 nm were achieved in sponge glass fiber which are close to two orders of magnitude smaller than resolution lengths achievable by conventional micro-CT [11].

6.2.2 Material and Methods

6.2.2.1 Sample Preparation

A tiny sample with minimal damage was required for the PXCT. The ideal sample would be a cylinder less than 50 μm in length with its cross-section as circular as possible. Mounting the sample in resin should be avoided to preserve the original structure of the club. Hence, a custom method was used to prepare the samples. The

club was cut using a diamond cut-off wheel into slices about 1 mm thick. The slices were sandwiched between sandpaper and thinned to around 60 μm , with the final step using 4000 Grit paper. Thin rectangular rods of about cut from the polished slices using a scalpel by hand under a stereomicroscope. Each rod was about 200 μm long were taken from the impact surface, impact region and periodic region. Although a circular cross-section is ideal for tomography, 3D reconstructions of samples with quadrilateral cross-section were achievable with tens of nanometers resolution [12].

6.2.2.2 PXCT Experiment

PXCT was conducted at the cSAXS Beamline at the Swiss Light Source (SLS) in Paul Scherrer's Institute (PSI) in Villigen, Switzerland. Figure 6.7A illustrates the experimental set up for PXCT. The samples were mounted on an OMNY Pin holder [13] along its long axis (Figure 6.7B). Projections of the sample were captured at different angles following the Golden Ratio scanning method [14]. This method is used because it allows for continuous scanning until the desired 3D resolution is achieved. Each projection is made by many overlapping scans arranged according to the fermat spiral, which is advantageous compared to rectangular or concentric patterns because it requires less time to scan an area [15]. More importantly the randomized scan pattern avoids visual artefacts often observed in the rectangular scanning pattern and the scans are more evenly spaced compared to concentric scanning pattern. An iterative algorithm developed by the SLS scientists was used to reconstruct the tomogram slices which was stacked and processed using ImageJ-based FIJI software package [16] to obtain cross-section images and 3D models of the internal structure of the impact region at the three different regions mentioned above.

Figure 6.7. A) Schematic of the SLS cSAXS beamline/setup used in PXCT experiment [12]. C) OMNY Pin used with PXCT setup; samples were mounted upright on the tip of the pin [13].

6.2.3 Results

The impact and periodic region of the dactyl club was imaged using PXCT. Three regions were studied the impact surface, the impact region and the periodic region. Figure 6.8 shows the results of the PXCT experiment. Scans were performed using the Golden Ratio projection sequence, which has the major advantage of allowing continuous scanning. Continuous scanning is useful when the sample has not been studied before and the number of projections to achieve the desired resolution as well as the stability of the sample under X-ray is not known, which is the case for the dactyl club. The impact surface is highly mineralized as previously reported [1] and hence, is highly stable under the X-ray beam and imaging is only limited by time. A 27 hours scan of the impact surface yielded a voxel resolution of 51 nm. The voxel resolution of the bulk impact region and the periodic region were 71 nm and 65 nm respectively. The intensity of each voxel is proportional to the electron density within the voxels. Unfortunately, the signal from PXCT comes from a series of interference patterns recorded from overlapping areas rather than from characteristic X-rays or X-ray diffraction, so it does not provide compositional and crystallographic information. And

therefore, 3D elemental or mineral maps were not collected. Nonetheless, brighter voxels contain heavier elements and atoms which are closely packed. From the image contrast, it is obvious that the impact surface and impact region (Figure 6.8A and B) have higher density than the periodic region (Figure 6.8C). The 3D model of the impact surface showed many microchannels which are about 94 nm at the bottom and about 69 nm on average near the surface (Figure 6.8A and D). The channels do not travel in a straight pattern but with a twisting pattern as shown in the 3D reconstructed image in Figure 6.9. The number of identified microchannels decreases by around three times towards the surface of the club, from around 1088 to 365. The average size of the microchannels in the bulk impact region increased to around 113 nm in size (Figure 6.8B). The number of microchannels did not increase as compared to the microchannel. This is probably due to the large inclusion found in the impact region. The inclusion was not observed in SEM or optically before in previous dactyl club samples. Interestingly, the region around the inclusion is brighter than the rest of the image, indicating a higher electron density within the region. A brighter ring followed by a darker ring, surrounded the microchannels in the bulk impact region, creating a pattern similar to leopard spots. PXCT of the periodic region showed a highly fibrous network, which is expected since the periodic region is made up of chitin fibers. Although the calculated resolution is better than the sample from the impact region, the tomograms are blurry likely because of radiation damage. The microchannels present in the periodic region are visibly larger than those in the impact region, measuring around 171 μm on average (Figure 6.8C). The number of microchannels observed was 506 which is about half of the number in the lower end of the impact surface.

Figure 6.8. X-ray Ptychographic tomograms. **A)** Cross-sections of the impact surface; inset showing the microchannels within the impact region. **B)** The impact region; inset showing microchannels which resemble osteons. **C)** The periodic region. **D)** Image of the club closer to the surface than **A**; inset illustrating a lower density of microchannels closer to the surface.

Figure 6.9. 3D stack of tomogram slices reconstructed from PXCT data. The 3D model was generated using FIJI software.

6.2.4 Discussion and Conclusion

This study is the first 3D spatial imaging of the stomatopod dactyl club at nanometer resolution. The club has a highly complex structure that is optimized by nature as a hunting appendage for hard-shell marine creatures like mollusks and bivalves. Common features present in all three regions are the microchannels. They are commonly observed in crustacean cuticles [17] and previous work has shown they are present within the stomatopod dactyl club as well [2]. Microchannels serve as pathways for transport of ions and proteins necessary for the growth and development of

crustacean cuticle, in this case the dactyl club. Amini *et al.* found that microchannels also serve to toughen the club through densification under load [2]. The size of the microchannels decreases closer towards the surface. This behavior probably optimizes the transportation of nutrients to the club. Miniaturization may also serve to improve the mechanical properties of the material similar to the effect of grain size on a metal. Hence, the natural reduction of microchannel size towards the surface of the club may be linked to the mechanical gradient observed by Weaver *et al.* [1]. Similar deductions may be made of the spatial density of microchannels, which increases from the periodic to the impact region. However, the density of microchannels decrease in the impact surface. This can be explained by a weaker need for microchannels at the surface as the surface layer serves as a barrier to prevent exchange of fluids between the mantis shrimp and the surround seawater. Furthermore, the effect of the surface membrane on the mechanical properties is usually considered as negligible. Bone osteon-like patterns found in the bulk impact region has not been observed in literature before. Half of the toughness in cortical bone has been attributed to crack deflection at the interface of osteons. These osteon-like patterns in the impact region could also serve to deflect cracks and hence toughening the club [18]. The inclusion observed in the bulk impact region has not been observed previously, and is likely to be an artefact. The region enclosing the inclusion has higher mineral content than the rest of the sample. Additionally, the inclusion is within a highly compact and mineralized portion of the club. Hence, it is unlikely that the inclusion was introduced during sample preparation and it was more likely to have been created during club formation. Stomatopods, especially the large males, are prone to develop shell rot with age and this inclusion could be an instance of the effect of shell rot in the mantis shrimp club. Further analysis, would be required to support this hypothesis.

References

- [1] J. C. Weaver *et al.*, “The Stomatopod Dactyl Club: A Formidable Damage-Tolerant Biological Hammer,” *Science*, vol. 336, no. 6086, p. 1275, Jun. 2012, doi: 10.1126/science.1218764.
- [2] S. Amini, M. Tadayon, S. Idapalapati, and A. Miserez, “The role of quasi-plasticity in the extreme contact damage tolerance of the stomatopod dactyl club,” *Nat. Mater.*, vol. 14, no. 9, pp. 943–950, Sep. 2015, doi: <http://dx.doi.org/10.1038/nmat4309>.
- [3] N. Tamura and P. U. P. A. Gilbert, “X-Ray Microdiffraction of Biominerals,” in *Methods in Enzymology*, vol. 532, Elsevier, 2013, pp. 501–531.
- [4] M. Kunz *et al.*, “A dedicated superbend x-ray microdiffraction beamline for materials, geo-, and environmental sciences at the advanced light source,” *Review of Scientific Instruments*, vol. 80, no. 3, p. 035108, Mar. 2009, doi: 10.1063/1.3096295.
- [5] G. C. Sneddon, P. W. Trimby, and J. M. Cairney, “Transmission Kikuchi diffraction in a scanning electron microscope: A review,” *Materials Science and Engineering: R: Reports*, vol. 110, pp. 1–12, Dec. 2016, doi: 10.1016/j.mser.2016.10.001.
- [6] S. Amini *et al.*, “Textured fluorapatite bonded to calcium sulphate strengthen stomatopod raptorial appendages,” *Nature Communications*, vol. 5, p. 3187, Jan. 2014, doi: 10.1038/ncomms4187.
- [7] N. A. Yaraghi *et al.*, “A Sinusoidally Architected Helicoidal Biocomposite,” *Adv. Mater.*, vol. 28, no. 32, pp. 6835–6844, Aug. 2016, doi: 10.1002/adma.201600786.
- [8] W. Huang *et al.*, “A natural impact-resistant bicontinuous composite nanoparticle coating,” *Nature Materials*, Aug. 2020, doi: 10.1038/s41563-020-0768-7.
- [9] J. E. Elkhoury, R. Shankar, and T. S. Ramakrishnan, “Resolution and Limitations of X-Ray Micro-CT with Applications to Sandstones and Limestones,” *Transp. Porous Media*, vol. 129, no. 1, pp. 413–425, Aug. 2019, doi: 10.1007/s11242-019-01275-1.
- [10] P. Willmott, *An Introduction to Synchrotron Radiation: Techniques and Applications*, 1st ed. Wiley, 2011.

- [11] M. E. Birkbak, M. Guizar-Sicairos, M. Holler, and H. Birkedal, “Internal structure of sponge glass fiber revealed by ptychographic nanotomography,” *J. Struct. Biol.*, vol. 194, no. 1, pp. 124–128, Apr. 2016, doi: 10.1016/j.jsb.2016.02.006.
- [12] M. Holler *et al.*, “X-ray ptychographic computed tomography at 16 nm isotropic 3D resolution,” *Sci. Rep.*, vol. 4, no. 1, p. 3857, May 2015, doi: 10.1038/srep03857.
- [13] M. Holler *et al.*, “OMNY PIN—A versatile sample holder for tomographic measurements at room and cryogenic temperatures,” *Rev. Sci. Instrum.*, vol. 88, no. 11, p. 113701, Nov. 2017, doi: 10.1063/1.4996092.
- [14] A. Kaestner, B. Münch, P. Trtik, and L. Butler, “Spatiotemporal computed tomography of dynamic processes,” *Opt. Eng.*, vol. 50, no. 12, p. 123201, Dec. 2011, doi: 10.1117/1.3660298.
- [15] X. Huang, H. Yan, R. Harder, Y. Hwu, I. K. Robinson, and Y. S. Chu, “Optimization of overlap uniformness for ptychography,” *Opt. Express*, vol. 22, no. 10, p. 12634, May 2014, doi: 10.1364/OE.22.012634.
- [16] J. Schindelin, I. Arganda-Carreras, and E. Frise, “Fiji: an open-source platform for biological-image analysis,” *Nat. Methods*, vol. 9, no. 7, pp. 676–682, 2012, doi: 10.1038/nmeth.2019.
- [17] R. Roer and R. Dillaman, “The Structure and Calcification of the Crustacean Cuticle,” *Am. Zool.*, vol. 24, no. 4, pp. 893–909, Nov. 1984, doi: 10.1093/icb/24.4.893.
- [18] R. Wang and H. S. Gupta, “Deformation and Fracture Mechanisms of Bone and Nacre,” *Annu. Rev. Mater. Res.*, vol. 41, no. 1, pp. 41–73, Aug. 2011, doi: 10.1146/annurev-matsci-062910-095806.

Chapter 7

Conclusions and Recommendations

This final chapter joins the threads of the studies performed in this thesis. The implications of the results and their contributions to the research community are covered. Opportunities for future work and their benefits are presented and discussed.

7.1 General discussion

7.1.1 Fracture toughness determination in hard biological materials

For the first time, chevron-notched microcantilevers were used to evaluate the fracture toughness of the stomatopod dactyl club. The fracture properties of biological materials are commonly assessed using indentation methods because it is simple to apply and it can measure small samples. This has been the method of choice for the stomatopod dactyl club where conventional methods cannot be used due to size constrain. However, fracture toughness measured by indentation may not be accurate and have been criticized [1]–[4]; the details of which have been covered elsewhere in the introduction and literature review chapters of this thesis. With microcantilevers, the fracture toughness of the dactyl club was probed using conventional fracture mechanic methods. This affirms the reliability of fracture toughness values obtained from these measurements. Both LEFM and EPFM were used to assess the fracture toughness of the impact region. LEFM greatly underestimated the fracture toughness of the impact region suggesting that the primary mechanism for fracture toughening in the impact region is by plastic dissipation which is captured with EPFM. Fracture toughness of the impact region was also assessed in different orientations using microcantilevers. It was hypothesized that the fracture toughness would vary with orientation like hardness and elastic modulus. Fracture toughness was expected to decrease the further off from the surface normal it was measured. However, on average fracture toughness was significantly different with orientation suggesting that fracture toughness at the microscale may be isotropic instead of anisotropic like other mechanical properties. Overall, the successful application of chevron-notch microcantilevers for fracture toughness measurement on the stomatopod dactyl club opens the door for accurate micro-fracture toughness measurements in other hard mineralized tissues. Fracture toughness can be evaluated in different directions with this method as well. This is valuable to characterize structural biological materials since these are often characterized by anisotropic mechanical properties to fulfil their specific functional needs.

7.1.2 Time study of stomatopod dactyl club development

In this study, stomatopod dactyl club samples were taken from six different time points after molting, and high-resolution WAXS and XRF maps of their cross-sections were obtained synchrotron X-ray experiments. Apatite was found to be present in the dactyl club as early as 2 hours from molting. A repeating theme from the X-ray diffraction results was the large differences between the crystal size and orientation in the surface impact and impact regions. The shape of the apatite crystallites was more isotropic in the surface of the clubs while in the impact regions they had larger aspect ratios of more than 3:1. Texture mapping revealed that the impact surface also had vastly different texture than compared to the impact region. Results from lattice parameter measurements seem to suggest that the apatite chemistry does not remain constant throughout the club's growth; rather it is likely that ionic substitutions occur. The first hypothesis was hydroxyapatite was deposited on the organic matrix and later converted to FAP through F^- substitution. Carbonation was also suspect to be a cause for lattice parameter shifts since it is not uncommon for apatite found in biological systems to be carbonated. However, further studies with EDS, Raman and FTIR suggested that both F^- substitution for OH^- and apatite carbonation are not present in the dactyl club during development. Although the exact cause for the lattice parameter changes is unclear, the most plausible explanation based on all spectroscopic data is the presence of amorphous calcium carbonate. Synchrotron XRF measurements uncovered for the first time the presence of Br in the dactyl club. Br was shown to be present in trace amounts and incorporated as brominated tyrosine in CMP-1, a major protein regulating apatite biomineralization in the dactyl club. The purpose of Br in CMP-1 is uncertain although it is suspected to provide protection against chemical and microbial attacks, which may be important for rapid and successful apatite mineralization post molt, when the creature is in its most vulnerable state. Overall, this study provided a deeper understanding of the spatial-temporal distribution and chemistry of apatite in dactyl clubs during its development after molting. However, as more was uncovered about the dactyl club more questions arose as well. The potential for novel ultra-tough biomimetic composites motivated much study on the mature dactyl club and most of the novel strategies employed has been uncovered already. However, there is much territory left to be explored on the growth and development of the dactyl club and other biological materials for the matter.

7.2 Future Work

7.2.1 Extending fracture toughness methods to other biological materials

Interestingly, the fracture toughness values from the microcantilever method were close to those determined using indentation fracture and Weibull analysis [5], suggesting that plastic dissipation could be determined reasonably using indentation and Weibull analysis. It would be interesting to test if this indentation-based method would be reliable when tested on other hard biological materials. On a similar note, the chevron-notch microcantilever method used in this study can be applied to study the fracture toughness of other hard mineralized biological materials like bone and the results compared with large scale measurements conducted using conventional methods to understand more about the fracture behavior of biological materials at the microscale.

Since microcantilevers assess the fracture toughness at the microscale and even nanoscale, the fracture toughness measured may not be representative of the entire impact region. This is especially true for biological materials, which often possess hierarchical ordering of structures and mechanical gradients. Therefore, questions for further studies are how does the fracture toughness of the dactyl club change when measured at larger length scales of 100 microns or larger? And does fracture toughness show orientation dependence at larger scales? While FIB excels in producing micron to submicron size samples with damage zones as small as several nanometers, it is not suited to prepare the large microcantilevers required to test this hypothesis because of the extremely long cutting times required. Instead, a better alternative may be to use femtosecond lasers. Femtosecond lasers are able to cut a large microcantilevers in less time than the FIB would take to prepare a single chevron-notch microcantilever used in Chapter 4. The only drawback is the larger damage zone sizes of 2-6 μm [6]. The damage zone is acceptable given the size of the microcantilever. However, the notch and guidelines should still be machined with FIB since a sharp notch would provide more accurate fracture toughness values. Therefore, femtosecond lasers can be used to prepare microcantilevers for testing the fracture toughness of the impact region on a larger scale.

7.2.2 Further exploration into dactyl club biomineralization

The results from the time study on the stomatopod dactyl club left many questions for further investigations. The cause of lattice parameter shifts remain unclear. Thus, future research can be conducted to determine the different factors causing the lattice parameter to change with time. Substitution of F^- for OH^- post mineralization was rejected as a possible factor based on EDS experiments. EDS is a semi-quantitative technique and it cannot provide information on molecular bonding. Other techniques like X-ray photoelectron spectroscopy (XPS) or X-ray atomic near edge spectroscopy (XANES) should provide more conclusive results. Since both XPS and XANES can provide details on the oxidation state of the elements and the coordination bonding between elements in the dactyl club. Moreover, XPS is a quantitative technique [7]. Thus, the composition of apatite within the dactyl club at different stages of its development can be accurately determined. An advantage of XANES over XPS is that it does not require a high vacuum environment and it can be performed along with other X-ray techniques at synchrotron beamlines.

Time studies on the dactyl club revealed that Br was present on the surface as well as at the mineralization front. Moreover, brominated tyrosine was identified in CMP-1. The proposed purpose of Br in the dactyl club is to protect it from chemical and microbial attacks, especially after molting when the stomatopod is most vulnerable. This hypothesis can be tested in future studies with readily available anti-microbial assays. Whether Br plays a role in biomineralization and if that role is a direct or an indirect one is an interesting subject for further research. This can be determined through *in vitro* studies. Moreover, the pathway for bioapatite mineralization in stomatopod dactyl club can be further studied. Often multiple proteins are involved in the biomineralization process and identification of other proteins is already underway.

7.2.3 A model for nanocrystal rotation in the impact region

Results from μ XRD and EBSD experiments were not fully conclusive. Nanocrystal rotation in response to stress could not be ascertained from μ XRD data. Maximum intensity was used as a proxy for crystal orientation and a weak relationship between

intensity and load was observed. This is hardly definitely proof of nanocrystal rotation in the impact region. Moreover, no Kikuchi bands were observed with EBSD, which is surprising since the dactyl club is clearly heavily mineralized based on many previous studies. Therefore, further research could use other crystallography methods to verify nanocrystal rotation in response to load or stress and to create a physical model of the nanocrystal rotation mechanism. From this model, the degree of misorientation could be related to the load or stress applied and the contribution of this mechanism to the fracture toughness could be evaluated. A deeper understanding of this phenomenon may inspire new tough ceramic engineering composites.

7.2.4 High resolution elemental and diffraction imaging

PXCT experiments generated tomograms with 3D resolutions down to 51 nm in the stomatopod dactyl club. This has not been done for the dactyl club before this. However, PXCT fell short of providing the element and mineral apatite distribution in 3D. Therefore, follow up studies can focus on obtaining nanoscale compositional and crystallographic data on the dactyl club in 3D. Bragg ptychography can be used to obtain the X-ray diffraction data and by extension the 3D crystallography information [8]. Elemental 3D resolution from XRF tomography is still limited to hundreds of nanometers and hence for more precise mapping it maybe more suitable to use other techniques like FIB tomography with X-ray photoelectron spectroscopy (XPS).

References

- [1] J. J. Kruzic, D. K. Kim, K. J. Koester, and R. O. Ritchie, “Indentation techniques for evaluating the fracture toughness of biomaterials and hard tissues,” *Nanoindentation Biol. Mater.*, vol. 2, no. 4, pp. 384–395, Aug. 2009, doi: 10.1016/j.jmbbm.2008.10.008.
- [2] P. Ostojic and R. McPherson, “A review of indentation fracture theory: its development, principles and limitations,” *Int. J. Fract.*, vol. 33, no. 4, pp. 297–312, Apr. 1987, doi: 10.1007/BF00044418.
- [3] G. R. Anstis, P. Chantikul, B. R. Lawn, and D. B. Marshall, “A Critical Evaluation of Indentation Techniques for Measuring Fracture Toughness: I, Direct Crack Measurements,” *J. Am. Ceram. Soc.*, vol. 64, no. 9, pp. 533–538, Sep. 1981, doi: 10.1111/j.1151-2916.1981.tb10320.x.
- [4] P. F. Cesar *et al.*, “ADM guidance—Ceramics: Fracture toughness testing and method selection,” *Dent. Mater.*, vol. 33, no. 6, pp. 575–584, Jun. 2017, doi: 10.1016/j.dental.2017.03.006.
- [5] S. Amini *et al.*, “Textured fluorapatite bonded to calcium sulphate strengthen stomatopod raptorial appendages,” *Nat. Commun.*, vol. 5, p. 3187, Jan. 2014, doi: 10.1038/ncomms4187.
- [6] B. Girard, D. Yu, M. R. Armstrong, B. C. Wilson, C. M. L. Clokie, and R. J. D. Miller, “Effects of femtosecond laser irradiation on osseous tissues,” *Lasers Surg. Med.*, vol. 39, no. 3, pp. 273–285, Mar. 2007, doi: 10.1002/lsm.20466.
- [7] M. C. Burrell, “Chemical Analysis, Electron Spectroscopy,” in *Encyclopedia of Materials: Science and Technology*, Elsevier, 2001, pp. 1142–1149.
- [8] F. Mastropietro *et al.*, “Revealing crystalline domains in a mollusc shell single-crystalline prism,” *Nat. Mater.*, vol. 16, no. 9, pp. 946–952, Sep. 2017, doi: 10.1038/nmat4937.

Appendix A³

Westergaard introduced a complex stress function (Z) that is a solution to stress field for an infinite plate with a central crack (Figure R2). The stress field is represented by:

$$\sigma_{xx} = \operatorname{Re} Z - y \operatorname{Im} Z' \quad (\text{A1})$$

$$\sigma_{yy} = \operatorname{Re} Z + y \operatorname{Im} Z' \quad (\text{A2})$$

$$\tau_{xy} = -y \operatorname{Re} Z' \quad (\text{A3})$$

Z and its derivative, Z' are given by equations A4 and A5 below.

$$Z(z) = \frac{\sigma_{\infty}}{\sqrt{1 - \left(\frac{a}{z}\right)^2}} \quad (\text{A4})$$

$$Z'(z) = \frac{-\sigma_{\infty} a^2}{\left(z^2 \left(1 - \left(\frac{a}{z}\right)^2\right)\right)^{\frac{3}{2}}} \quad (\text{A5})$$

Figure A2. Crack in an infinite plate.

³ The majority of this section is summarized from the Linear Elastic Fracture Mechanics chapter in the book by T.L. Anderson [1] and from ref. [2].

Where σ_∞ is the external/far-field stress, a is the crack length and z is the complex number $x + iy$. Irwin showed that the Westergaard stress function can be simplified in the vicinity of crack tip by substituting $z = a + re^{i\theta}$ into Z (Figure R2). Importantly, near the crack tip $a \gg r$, which is the key relationship used to simplify Equation A6.

$$Z(z) = \frac{\sigma_\infty}{\sqrt{1 - \left(\frac{a}{a + re^{i\theta}}\right)^2}} \quad (\text{A6})$$

Simple rearranging of the denominator yields the following:

$$Z(z) = \frac{\sigma_\infty}{\sqrt{\frac{2are^{i\theta} + r^2e^{i2\theta}}{a^2 + 2are^{i\theta} + r^2e^{i2\theta}}}}} \quad (\text{A7})$$

Since, near the crack tip $a \gg r$, $a^2 \gg ar \gg r^2$, and so A7 can be simplified to:

$$Z(z) = \frac{\sigma_\infty}{\sqrt{\frac{2are^{i\theta}}{a^2}}} \quad (\text{A8})$$

Which can be further simplified to:

$$Z(z) = \sigma_\infty \sqrt{\frac{a}{2r}} e^{-i\frac{\theta}{2}} \quad (\text{A9})$$

Using Euler's identity, $e^{i\alpha} = \cos \alpha + i \sin \alpha$, A9 becomes:

$$Z(z) = \sigma_\infty \sqrt{\frac{a}{2r}} \left(\cos \frac{\theta}{2} - i \sin \frac{\theta}{2} \right) \quad (\text{A10})$$

And since A1-3 only requires the real part of Z , A10 is simplified to:

$$\text{Re } Z(z) = \sigma_\infty \sqrt{\frac{a}{2r}} \cos \frac{\theta}{2} \quad (\text{A11})$$

Substituting $z = a + re^{i\theta}$ into Z' and simplifying yields the following equation:

$$Z'(z) = \frac{-\sigma_{\infty} a^2}{\left((a + r e^{i\theta})^2 \left(\frac{2a r e^{i\theta} + r^2 e^{i2\theta}}{a^2 + 2a r e^{i\theta} + r^2 e^{i2\theta}} \right) \right)^{\frac{3}{2}}} \quad (\text{A12})$$

Z' can be simplified by neglecting the negligible terms near the crack tip according to $a^2 \gg ar \gg r^2$:

$$Z'(z) = \frac{-\sigma_{\infty} a^2}{\left(a^2 \left(\frac{2a r e^{i\theta}}{a^2} \right) \right)^{\frac{3}{2}}} \quad (\text{A13})$$

Further simplification and applying Euler's identity yields:

$$Z'(z) = \frac{\sigma_{\infty}}{2r} \sqrt{\frac{a}{2r}} \left(\cos \frac{3\theta}{2} - i \sin \frac{3\theta}{2} \right) \quad (\text{A14})$$

Since only the imaginary part of Z' is present in A1-3:

$$\text{Im } Z'(z) = \frac{\sigma_{\infty}}{2r} \sqrt{\frac{a}{2r}} \sin \frac{3\theta}{2} \quad (\text{A15})$$

Substituting A11 and A15 into A1 gives:

$$\sigma_{xx} = \sigma_{\infty} \sqrt{\frac{a}{2r}} \cos \frac{\theta}{2} - y \frac{\sigma_{\infty}}{2r} \sqrt{\frac{a}{2r}} \sin \frac{3\theta}{2} \quad (\text{A16})$$

Which can be simplified to:

$$\sigma_{xx} = \sigma_{\infty} \sqrt{\frac{a}{2r}} \left(\cos \frac{\theta}{2} - \frac{y}{2r} \sin \frac{3\theta}{2} \right) \quad (\text{A16})$$

Substituting $y = r \sin \theta$:

$$\sigma_{xx} = \sigma_{\infty} \sqrt{\frac{a}{2r}} \left(\cos \frac{\theta}{2} - \frac{1}{2} \sin \theta \sin \frac{3\theta}{2} \right) \quad (\text{A16})$$

A16 can be further simplified by using the trigonometric identity, $\frac{1}{2} \sin \theta = \cos \frac{\theta}{2} \sin \frac{\theta}{2}$:

$$\sigma_{xx} = \frac{\sigma_{\infty} \sqrt{a}}{\sqrt{2r}} \cos \frac{\theta}{2} \left(1 - \sin \frac{\theta}{2} \sin \frac{3\theta}{2} \right) \quad (\text{A16})$$

Repeating the process for σ_{yy} and τ_{xy} the following equations are obtained.

$$\sigma_{yy} = \frac{\sigma_{\infty} \sqrt{a}}{\sqrt{2r}} \cos \frac{\theta}{2} \left(1 + \sin \frac{\theta}{2} \sin \frac{3\theta}{2} \right) \quad (\text{A17})$$

$$\tau_{xy} = \frac{\sigma_{\infty} \sqrt{a}}{\sqrt{2r}} \cos \frac{\theta}{2} \sin \frac{\theta}{2} \cos \frac{3\theta}{2} \quad (\text{A18})$$

Two observations can be inferred from A16-18. Firstly, the magnitude of the stress field increases in proportion to $\sqrt{r}$ creating a singularity near the crack tip. And secondly, $\sigma_{\infty} \sqrt{a}$ is a common term in all three component stresses and defines the conditions near the crack tip. $\sigma_{\infty} \sqrt{a}$ is defined as the stress intensity factor, K . Knowing K the component stresses—and by extension, strains and displacements—for any combination of r and θ can be determined. Thus, K is another parameter, along with the energy release rate, G , which can describe the fracture properties of linear-elastic materials. For a centre crack in an infinite plate (Figure R2), G is given by:

$$G = \frac{\pi \sigma_{\infty}^2 a}{E} \quad (\text{A19})$$

So, K and G can be related by the expression:

$$G = \frac{\pi K^2}{E} \quad (\text{A20})$$

It was popular although not necessary to include the term $\sqrt{\pi}$ into the definition for K so that the equation A20 would be cleaner as shown below.

$$K = \sigma_{\infty} \sqrt{\pi a} \quad (\text{A21})$$

$$G = \frac{K^2}{E} \quad (\text{A22})$$

Here the K is shown to be related to G only for a centre crack in an infinite plate. However, Irwin [3] has shown that this relationship applies to all configurations. Substituting the $\sqrt{\pi/\pi}$ into equations A16-18, the component stresses become:

$$\sigma_{xx} = \frac{\sigma_{\infty}\sqrt{\pi a}}{\sqrt{2\pi r}} \cos \frac{\theta}{2} \left(1 - \sin \frac{\theta}{2} \sin \frac{3\theta}{2} \right) \quad (A23)$$

$$\sigma_{yy} = \frac{\sigma_{\infty}\sqrt{\pi a}}{\sqrt{2\pi r}} \cos \frac{\theta}{2} \left(1 + \sin \frac{\theta}{2} \sin \frac{3\theta}{2} \right) \quad (A24)$$

$$\tau_{xy} = \frac{\sigma_{\infty}\sqrt{\pi a}}{\sqrt{2\pi r}} \cos \frac{\theta}{2} \sin \frac{\theta}{2} \cos \frac{3\theta}{2} \quad (A25)$$

Therefore, through the Westergaard approach the stress field near the crack tip was shown to be connected to the global boundary conditions making K a more practical parameter to measure the fracture property of linear-elastic materials. However, it is important to note that the equations for the stress field shown here is applicable only for an infinite plate with a centre crack and the Westergaard approach is only useful for some cracked configurations. Moreover, an exact solution is not obtained through this approach because the problem was simplified with the approximation of $a \gg r$. Nevertheless, the solutions were accurate near the crack tip which is most important since it is the crack tip conditions that controls if the crack propagates. Additionally, the Westergaard approach led to the definition of the stress intensity factor which is one of the most important parameters in fracture mechanics.

References

- [1] T. L. Anderson, *Fracture mechanics: fundamentals and applications*, 2nd ed. Boca Raton: CRC Press, 1995.
- [2] "Stress Intensity Factor." Fracture Mechanics.
<https://www.fracturemechanics.org/sif.html> (accessed Jul 14, 2021).
- [3] G.R. Irwin, "Analysis of Stresses and Strains near the End of a Crack Traversing a Solid," *Journal of Applied Mechanics*, vol. 24, pp. 361-364, 1957.