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The Nanomechanics of Biomineralized Soft-Tissues and Organic Matrices

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Publication Date
2013

Peer reviewed|Thesis/dissertation
UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Nanomechanics of Biomineralized Soft-Tissues and Organic Matrices.

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Structural Engineering

by

Jiddu Bezares-Chávez

Committee in charge:

Professor Robert J. Asaro, Chair
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Professor Ratneshwar Lal
Professor Peter Novick
Professor Qiang Zhu

2013
The dissertation of Jiddu Bezares-Chávez is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2013
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature Page</td>
<td>iii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>Preface</td>
<td>ix</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xxii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>xxiii</td>
</tr>
<tr>
<td>Vita and Publications</td>
<td>xxv</td>
</tr>
<tr>
<td>Abstract of the Dissertation</td>
<td>xxvi</td>
</tr>
<tr>
<td>Chapter 1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 2 Experimental Methods</td>
<td>5</td>
</tr>
<tr>
<td>2.1 Shell Samples</td>
<td>5</td>
</tr>
<tr>
<td>2.2 Flat Pearl Preparation</td>
<td>5</td>
</tr>
<tr>
<td>2.3 Demineralization</td>
<td>6</td>
</tr>
<tr>
<td>2.3.1 EDTA</td>
<td>6</td>
</tr>
<tr>
<td>2.3.2 Ion-exchange Resin</td>
<td>6</td>
</tr>
<tr>
<td>2.4 Enzymatic and Alkaline Treatments</td>
<td>7</td>
</tr>
<tr>
<td>2.4.1 Trypsin</td>
<td>7</td>
</tr>
<tr>
<td>2.4.2 PNGase F</td>
<td>7</td>
</tr>
<tr>
<td>2.4.3 Proteinase K</td>
<td>7</td>
</tr>
<tr>
<td>2.4.4 Chitinase</td>
<td>8</td>
</tr>
<tr>
<td>2.4.5 Alkaline Peroxidation</td>
<td>8</td>
</tr>
<tr>
<td>2.5 Histochemical Labeling</td>
<td>8</td>
</tr>
<tr>
<td>2.5.1 Sample Preparation</td>
<td>8</td>
</tr>
<tr>
<td>2.5.2 Sample Drying and Mounting</td>
<td>9</td>
</tr>
<tr>
<td>2.5.3 Autofluorescence</td>
<td>9</td>
</tr>
<tr>
<td>2.5.4 Calcofluor White</td>
<td>9</td>
</tr>
<tr>
<td>2.5.5 Aminoacridone</td>
<td>10</td>
</tr>
<tr>
<td>2.5.6 Colloidal Iron</td>
<td>10</td>
</tr>
</tbody>
</table>
Preface

This Preface is written to acknowledge the submission of the PhD thesis of Jiddu Bazares-Chavez who passed away in mid March, 2013. He was, in fact, 2 weeks away from defending his thesis and at the time of his death was just over 90% completed with the writing. His thesis was based of 4 of his 6 published papers as described in the text of the thesis. As his advisor I, RJ Asaro, completed the thesis document by performing some editing, and completing several sections. In doing this no new research was done; the editing consisted of reporting on the work contained in the already published papers. As per University policy, permission has been obtained from the co-authors of these 4 papers to use the material in the thesis and this permission has been reported to the Graduate Council at UCSD.

Jiddu Bezares had performed at the highest level throughout his graduate studies at UCSD. His research has resulted in 6 major, full, papers published in first rate scientific journals such as 1) Journal of Structural Biology; 2) Journal of Theoretical and Applied Mechanics; and 3) Acta Materialia. These journals are all peer reviewed and the fact that he published 6 full peer reviewed papers in such prestigious journals even before completing the submission of his PhD thesis is absolutely extraordinary. I know of no other graduate student at UCSD in the SE department that has accomplished this scholarly feat. In the course of performing the research leading to these papers he was also instrumental in the development of a truly unique experimental facility; he worked directly with myself, his advisor, in designing novel
equipment to perform a variety of extremely difficult experimental measurements that were required to carry out the research that were the bases of his published papers. The laboratory he was key to the development of is absolutely unique to the UCSD campus and in fact has been used by researchers from 1) the Department of Nanotechnology; 2) the Department of Biology; 3) the MAE Department; and 4) the Department of Bioengineering, all at UCSD. Moreover, the assistance he offered in aiding these researchers has led to collaborations and submitted research proposals to the NIH and to NSF. Once again, I am unaware of such achievements by any other graduate student within the SE department at UCSD, or any other engineering department at UCSD.

Jiddu’s research was actually in, what most would judge to be, different areas; these included 1) the structure and structural performance of biological tissue and of biomineralized structures; 2) the stability and performance of nanotwinved fcc metals; and 3) the mechanics of the core structure of FRP composite materials. Jiddu, however, saw this as one field, namely the correlation of the structure and behavior of unique nanostructured materials and nanostructured structures. This took intellectual courage and far sight, again at a level most unusual for a graduate student. His work required skill in, _inter alia_, 1) biological histochemical and histochemical optical microscopy techniques; 2) unique types of nano-mechanical testing (now being copied by others at UCSD); 3) biochemical methods used to create specimens that could be tested by the unique methods developed within our laboratory; and 4) the design and development of unique experimental apparatus to carry out such experiments.

In my judgment I truly believe that the achievements outlined above truly warranted the awarding of a PhD to Jiddu Bezares-Chavez.

Robert Asaro, Advisor May 24, 2013
LIST OF FIGURES

Figure 1.1:  Examples of biomineralized structures. (a) Coccospheres made up of coccoliths. (b) Compact cortical bone. (c) The chambered nautilus. ................................................................. 2

Figure 1.2:  Schematic view of the red abalone. The shell consists of an outer prismatic layer and an inner layer of nacre. Note the tiled, brick-wall like structure with biopolymer layers that make up a matrix. These layers are the focus of most of the research reported on in this thesis. ................................................................. 3

Figure 2.1:  Figure of interlamellar layers mounted vertically in paraffin wax on a histology slide, and having been sliced at an angle of \(\sim 30-40\) degrees with a microtome. ................................................................. 14

Figure 2.2:  Figure a) depicts a thin flat nacre wafer sandwiched between “dog-bone” shaped templates; the wafer is held with tightened bolts passing through holes in the templates. b) Two finished “dog-bone” specimens after nacre has been ground off the edges, with thicknesses of \(\sim .5 \) mm. ................................................................. 17

Figure 2.3:  Experimental set up used within the Instron electro-mechanical tests system for performing tensile tests on the soft-tissue of the interlamellar layers. ................................................................. 18

Figure 2.4:  The outlines of prospective specimens to be cut out are drawn on the surface of a shell. The lengths of the samples are normal to the direction of shell growth. ................................................................. 19

Figure 2.5:  Figure (a) is an image of a typical undemineralized shell sample with its edge polished, used to estimate the number of il-layers; (b) the same sample with its ends embedded in epoxy, having undergone demineralization during which the calcitic region has fallen away. ................................................................. 20

Figure 2.6:  A drawing of a tensile test specimen with its ends embedded in epoxy, showing the dimensions used to calculate the cross sectional area of the combined il-layers in a sample. Interlamellar layer and tile lamellae thicknesses are taken to be 20 nm and \(\sim 500\) nm respectively. The insert is an SEM image (edge view) of nacre showing tile lamellae. ................................................................. 21
Figure 2.7: Schematic drawings of the embedding set-up. a) A rectangular shell section is placed centered and aligned in a 2-part Teflon mold. Resin is poured into the mold and allowed to set. b) The sample is extracted from the mold, and its other end embedded as well. The sample’s gauge length is \(~20-25\) mm.

Figure 2.8: a) A complete shell with the overall growth front and two outlines of rectangular tensile specimens to be cut out. Figure b) is of a demineralized tensile specimen where the black arrow indicates a section of a layer that does not span the entire gauge length. In c) the white arrow indicates a layer that is broken at its end; the black arrow indicates the shell growth front within this specimen. d) Edge view of a demineralized tensile specimen illustrating the general curvature of such specimens. e) Schematic of a tensile specimen seen from above and outlined in red as in (a), indicating how not all layers would be included within the entire gauge length due to the curvature of the growth front.

Figure 2.9: Photo of the tensile and relaxation test setup. Aluminum grips hold the specimen by its epoxy pucks using set-screws. An extensometer attached to aluminum grips measures displacement. The gauge length of the specimen is \(~25\) mm.

Figure 2.10: Schematic drawings describing the geometry of a Vicker’s microindenter tip. a) The half-angle between opposite faces of the tip is 68 deg. b) All four faces of the four-sided pyramid are shown where the angle between opposite sides is 136 deg. c) The surface area of a remaining imprint is calculated from the measured lengths of the imprint diagonals shown as dashed blue lines.

Figure 2.11: Figure describing the microindentation creep process. a) For a short dwell time (\(~5\) sec) the area of indentation is relatively small; b) - c) with increasing dwell time the size of the indentations increase as do the corresponding indentation diagonals used to measure hardness.

Figure 2.12: The three-sided pyramid geometry of a nanoindenter Berkovich tip is described. a) is an SEM image showing the front of a tip with its three faces and b) a schematic drawing where the large included angle of 142.3 deg between one of three edges and a face of the tip is shown.
Figure 2.13: A drawing showing the two directions along which tiles were indented in dry nacre; a) tiles were indented along their c-axes; b) tiles were indented along their edges.

Figure 2.14: Figure a) is a bright field image of a nacre flake indicating a region suitable for indentation (the blue circle at the upper right) and a region not suitable due to debris and tile terraces. b) A drawing showing a side view of a flake where the center would be suitable for testing due to being flat and free of debris.

Figure 2.15: The figure of a load-displacement (P-δ) curve used to calculate Young’s modulus by the Oliver and Pharr method. The slope of the initial portion of the unloading curve (S) describes a material’s elastic capacity to “rebound” during unloading. The slope is used to measure the Young’s modulus using equation 2.1 above.

Figure 2.16: A figure describing the kind of loading function that would be used to perform a load-controlled creep test; the function includes an initial loading segment, a hold at peak load, and a final unloading segment.

Figure 2.17: A figure describing the scanning capability of the nanoindenter used to obtain surface scans of samples and indentations. a) The nanoindenter tip can pivot about its base to “scan” a surface. b) The surface scan of a series of 5 indentations on a polycarbonate sample, where a Berkovich tip was used for indentation.

Figure 2.18: Drawings showing the effect of sample tilt on the symmetry of pile-up around a conospherical indenter. a) With the tip aligned normal to the surface pile-up is symmetric. The inner and outer edges would form concentric circles. b) With the sample tilted there would be greater pile-up around the left side of the tip. Viewed from above the edges of the pile-up would appear oblong and unaligned.

Figure 2.19: Schematic of the sample alignment set-up. The tripod consists of a steel plate with threaded bolts as legs and lock-nuts used to eliminate play between the bolts and plate. AFM pucks prevent sliding of the legs on the nanoindenter stage. An embedded sample is glued to the tripod, and it’s tilt adjusted by turning the tripod bolts.
Figure 2.20: The procedure used to precisely adjust the tilt of a sample. A typical nacre flake has a width in the range of $\sim 2$ mm as seen in a), however the region of interest may only contain a few tiles as seen in b) and have dimensions in the range of $\sim 10 \, \mu m^2$. Following an adjustment of the tripod’s tilt, it was essential that the sample be precisely placed at its previous location with the nacre flake having its previous orientation. The tilt of the surface of interest was adjusted to within .2 deg along both the x- and y- directions.

Figure 2.21: A figure describing the axes along which indentations were made on monolithic aragonite crystal. a) A crystal is shown with its c-axis aligned vertically; the c-axis is naturally aligned with the longer dimension of the crystal. b) The crystal was sectioned along the dotted square shown in a) with the exposed face corresponding to either the a- or b- axes. c) Indentations were made on the exposed face (along either the a- or b- axes), and along the c-axis as shown in b).

Figure 2.22: FEM model of nacre which includes the actual ratios of thickness and tile diameter to biopolymer layer thickness.

Figure 2.23: The finite element model of the nanoindentation process: isometric view (left), top view (middle), and B section view (right).

Figure 2.24: The standard linear solid model.

Figure 3.1: Figures (a) and (b) are SEM images of fractured nacre from H. rufescens illustrating tiles on nearly parallel lamella. The “terrace” consisting of one interlamellar layer of nacre is shown at higher magnification in (b), where the black arrow points to a central region discussed below. (c) Flat pearls grown on a glass slide inserted into the mantle of a live H. rufescens (described below). Note the “stack of coins” arrangement with a smaller tile nucleated at the top of each stack. (d) SEM image of a cross section of H. rufescens organic matrix, demineralized in EDTA, illustrating individual and apparently porous interlamellar layers.
Figure 3.2: Figures (a) and (b) are SEM images of demineralized organic framework in \textit{H. rufescens}. Note that the center of each tile imprint is more deeply etched; the apparent accumulation of organic material at the center. (c) A fluorescence micrograph of \textit{H. rufescens} stained with Calcofluore White following demineralization in EDTA. The insert is of \textit{N. pompilius} demineralized and stained in the same manner. (d) same as (c) except with demineralization by ion-exchange.

Figure 3.3: (a) Fluorescence images of \textit{H. rufescens} demineralized using EDTA, fixed, and stained with aminoacridone. (b) Unfixed \textit{H. rufescens} imaged after staining. Note the faint but distinct labeling at the center in the fixed sample, shown more clearly in the insert to (a).

Figure 3.4: Brightfield images of \textit{H. rufescens} stained with colloidal iron at pH in the range $1.8 \leq \text{pH} \leq 1.9$. (a) Demineralization performed using ion-exchange and fixation. (b) Demineralization performed using EDTA and without fixation. (c) \textit{H. rufescens} demineralized in EDTA, unfixed, and digested in trypsin + Proteinase K. (d) fluorescence image of the same material as in (c) but stained with CW.

Figure 3.5: Figures (a)-(c) are images obtained after demineralization with EDTA, without fixing, and labeled with WGA-gold. Strong labeling of the intertabular matrix is evident as is the appearance of a fibrous network (small arrow heads). (d) Fluorescence image of an EDTA demineralized sample, digested, with PNGase F, and stained with FITC-WGA.

Figure 3.6: Images (a)-(c) are AFM phase images of interlamellar layers obtained in tapping mode from \textit{H. rufescens} demineralized in EDTA, unfixed, and digested with (a) trypsin, (b,c) with trypsin + Proteinase K. (d) A phase image obtained in tapping mode of EDTA demineralized \textit{H. rufescens}, unfixed, but mounted on a histology slide in a near edge-on orientation.

Figure 3.7: (a,b) Images obtained from unfixed \textit{H. rufescens}, demineralized with EDTA, and labeled with polyclonal antibodies. (c) An image obtained on a sample identical to that used in (a,b) using aminoacridone staining shown for perspective. (d) An image of unfixed \textit{H. rufescens}, demineralized with EDTA, and labeled with pre-immune serum.
Figure 3.8: Figure (a) is an elevation profile obtained from a large scan of *H. rufescens*, demineralized in EDTA, undigested, and unfixed. (b) A topograph image accompanying (a) showing the elevations at the intertabular matrix. (c) A topograph similar to (b) from a different area.

Figure 3.9: (a) Quasi-static uni-axial tensile stress vs. strain response, at a nominal strain rate of $10^{-4}$sec$^{-1}$, at 20°C for nacre specimens of *H. rufescens*. (b) Same as (a) but for *N. pompilius* nacre. Tests conducted in-plane, *i.e.* parallel to the interlamellar nacre layers as per (Fig. 4.3b.).

Figure 4.1: Macromolecular “map” of the surface of an interlamellar layer; molecular types are indicated as found via the labeling methods described in Chapter 2. The intertabular matrix, and its molecular constituency, is also indicated.

Figure 4.2: Model paradigm for the growth process of nacre in *H. rufescens*.

Figure 4.3: (a) Shear lag model for the tensile stiffness of layered nacre. Tiles are illustrated as grey rectangles and the intertabular and interlamellar matrices in green. Note that the area fraction of organic matrix is exaggerated for illustration. (b) Same as (a) but now illustrating the separation of tiles from matrix at the tile ends and the “shear lag” process. (c) An illustration of simple shear of the interlamellar layer; this assumes the imposition of deformation gradient $\mathbf{F} = \mathbf{I} + \gamma \mathbf{sm}$. Chitin fibers are assumed to lie along directions inclined by angle $\phi$ to the interlamellar plane as illustrated in (d) of the figure. This leads to an estimated stretch of chitin fibers $\lambda^2 \approx 1 + \gamma \sin 2\phi$ and a normal strain in such fibers of $\varepsilon_n \approx 1/2\gamma \sin 2\phi$. The elastic energy stored within such fibers is then $\mathcal{E} = 1/2E_t\gamma^2\sin^2 2\phi$, where $E_t$ is the tensile modulus of chitin. Finally it’s assumed that the volume fraction of such fibers contributing to resistance is $f_e = \tilde{f}f$, where $f$ is the total volume fraction of chitin and $\tilde{f}$ the fraction of chitin fibers oriented to contribute to resistance in the direction of the applied shear stress. The model is completed by setting the elastic energy density stored in the fibers to that of a linear elastic solid with shear modulus $G$, *i.e.* set $\tilde{f} = f/8E_t\gamma^2\sin^2 2\phi = 1/2G\gamma^2$ to obtain $G = 1/4\tilde{f}fE_t\sin^2 2\phi$. 

xvi
Figure 5.1: Schematic diagram of the interlamellar layer structure according to Levi-Kalisman et al., (2001). Uni-axially aligned chitin fibers form a core within individual il-layers; the core is encased in layers of glycoproteins. ................................. 65

Figure 5.2: (a) The translucent material remaining after alkaline peroxidation has removed ~80 dry wt% of organic material. (b) Calcofluor White staining of il-layers after alkaline treatment indicates that the remaining material is chitin. ................................. 68

Figure 5.3: SEM image of sheets having undergone alkaline treatment. (a) Round 10-50 nm diameter holes are uniformly distributed over framework layers. (b) In some regions the holes appear larger and oblong with their long axes aligned, and take on the appearance of gaps between fibers. White lines at the top right of the image indicate the long axes of individual holes and are aligned over the surface of the folded il-layer. ................................. 69

Figure 5.4: SEM image of il-layers after alkaline and proteolytic treatments. (a) The sheets can appear to consist of densely packed uni-axially aligned fiber bundles. (b) Where sheets have been pulled apart laterally it becomes clear that they consist of uni-axially aligned and laterally bound single fibers. ................................. 70

Figure 5.5: SEM image showing (a) ~100 nm diameter round holes in il-layers having undergone alkaline treatment alone which are spaced ~2-3 µm apart. (b) A close up of these larger holes shows that their edges are not frayed or oblong but instead form closed rings. . . 70

Figure 5.6: SEM images of il-layers having undergone both alkaline and enzymatic treatments. (a) Fibers in four consecutive il-layers are lined up in parallel. The red line in the il-layer plane is normal to the shell’s growth direction. (b) Three chitin core sheets lie on top of each other with their respective fibers aligned. The red line in the il-layer plane is normal to the shell’s growth direction. . . . . . . . 71
Figure 6.1: SEM images of cleaved nacre having undergone a light alkaline treatment to remove surface protein. (a) Tile surfaces are covered with ∼10-50 nm diameter “mineral bridges” as described by Schaffer et al., (1997) which are not circular but are longer along one direction. The insert shows that the mineral bridges are aligned along the direction of their lengths. (b) The lighter regions at the center of tiles with diameters of ∼100 nm are the locations of the “major connections” described by Checa et al., (2011).

Figure 6.2: A schematic drawing of the tile nucleation and growth process showing the differences between mineral bridges and major connections, and the remaining surface texture following il-layer removal. a) The smaller mineral bridges (∼10-50 nm wide) grow into il-layer pores also (∼10-50 nm wide) but do not form continuous links between tiles. On the other hand major connections (∼100 nm wide) completely traverse il-layers forming wide continuous mineral connections between tiles. b) Tile surface texture remains after tile lamellae have been cleaved apart and il-layers removed; the imprint at the center of the tile marks the previous location of a major connection.

Figure 6.3: A drawing describing the angle formed by lines drawn diagonally across the points of connection between chitin fibers. From images such as (Figs. 5.4 and 5.6b) the angle φ is estimated to be 30 ± 5°.

Figure 6.4: A schematic drawing of chitin fiber alignment within an interlamellar layer according to two previously proposed models and the current observations. (a) Fibers are uni-axially aligned and no pores are located between them, Levi et al., (2001). (b) Fibers are randomly oriented with the spaces between fibers taking on the form of pores, Bezares et al., (2010). (c) Based on the current findings chitin fibers are uni-axially aligned, pores are formed by the gaps between fibers that are laterally connected only at certain points, and all fibers are oriented normal to the growth direction of the shell.

Figure 6.5: Models (a) and (b) representing either transversely isotropic (randomly oriented fibers) or orthotropic (aligned sets of fibers) structures, respectively. (c) A Kelvin viscoelastic model used by Bezares et al., (2010) fitted to their data on mechanical testing of il-layers.
Figure 7.1: AFM phase images obtained in tapping mode in a fully hydrated condition of demineralized framework. (a) Higher resolution scan of framework partially digested with trypsin and Proteinase K. Images reveal a fibrous core. (b) Lower resolution scan of framework tissue digested with trypsin only.

Figure 7.2: AFM topographical and phase images of demineralized framework. (a) - (d) are of biopolymer layers partially digested with trypsin and Proteinase K. (a,b) are a scan confined to a single tile imprint whereas (c,d) are from a scan spanning multiple tile imprints. White arrows indicate tile boundaries.

Figure 7.3: Quasi-static uni-axial stress vs. strain response of Fixed Dry (FD), Not-Fixed Dry (NFD), and Not-Fixed Wet (NFW) specimens. Tests were performed at a nominal strain rate of $10^{-4}sec^{-1}$, at $20^\circ C$. D is the percent of interlamellar layers damaged during sample preparation. This was estimated based on visual inspection of samples just prior to testing. E is the tensile modulus of an individual biopolymer layer.

Figure 7.4: Quasi-static uni-axial stress vs. strain response of Not-Fixed Wet (NFW) specimens. Tests were performed at a nominal strain rate of $10^{-4}sec^{-1}$, at $20^\circ C$. D is the percent of interlamellar layers damaged during sample preparation. This was estimated based on visual inspection of samples just prior to testing. E is the tensile modulus of an individual biopolymer layer as was done in connection with (Fig. 7.3).

Figure 7.5: Load-relaxation stress vs. time response of Not-Fixed Wet (NFW) specimens. Tests were performed at a nominal strain rate of $10^{-4}sec^{-1}$, at $20^\circ C$. D is the percent of interlamellar layers damaged during sample preparation. This was estimated based on visual inspection of samples just prior to testing. E is the tensile modulus of an individual biopolymer layer as was done in connection with (Fig. 7.3). The insert in the upper right corner illustrates the standard linear solid model used for analysis with its elastic and viscous elements.
Figure 7.6: Images of tensile specimens with different protein contents. a) As demineralized framework tissue; the thickness is essentially the same as for the mineralized shell sample. b) Following protein removal with Proteinase K the thickness is significantly reduced. c) Following protein removal with alkaline peroxidase and with almost all protein gone, the sample is entirely translucent.

Figure 7.7: Quasi-static uni-axial stress vs. strain, response of Not-Fixed Wet (NFW) specimens of *H. rufescens* as demineralized and subjected to enzymatic digestion with trypsin and Proteinase K for 18 hrs followed by digestion in chitinase for durations ranging from 48-72 hrs. Tests were performed at a nominal strain rate of $10^{-4} \text{sec}^{-1}$, at 20 °C.

Figure 7.8: Schematic of layer indicating normal and shear stresses described by the constitutive model.

Figure 8.1: (a)-(b) FEM model of nacre which includes the actual ratios of thickness and tile diameter to biopolymer layer thickness. The initial linear stress vs. strain response predicted by the simulations is shown in (c).

Figure 9.1: SEM images of a) the coherent nanograins comprising the intratubular *it* matrix in a tile; b) tile lamellae where the black arrows indicate the location of the interlamellar *il* matrix. Together the two form an *it-il* matrix.

Figure 9.2: Stiffness and hardness results obtained from nanoindents on nacre tiles and single crystal aragonite.

Figure 9.3: Nature of the indents made on single crystal aragonite.

Figure 9.4: Nature of the indents made on nacre tiles.

Figure 9.5: Typical load-displacement curves for (a) dry nacre, (b) heat treated nacre, (c) wet nacre, and (d) monolithic aragonite; note the load plateau at $\sim 230 \mu\text{N}$.

Figure 9.6: Young’s modulus vs. indentation depth for monolithic aragonite, heat treated nacre, dry nacre, and wet nacre.

Figure 9.7: Indentation profiles on: (a) dry nacre with symmetric pile-up; (b) wet nacre with blunted pile-up; (c) heat treated nacre with a compacted appearance and no pile-up; and (d) monolithic aragonite with uneven pile-up indicating anisotropy. A slip band is also visible on the left face of the indent associated with load plateaus in the P-δ curves.
Figure 9.8: Load relaxation curves for monolithic aragonite, heat treated nacre, dry nacre, and wet nacre. Note the greater amount of relaxation observed in wet nacre and the nearly absent observed in heat treated nacre.

Figure 10.1: FEM simulation of the monolithic aragonite indentation creep. The indentation depth is 55 nm.

Figure 10.2: A short-term simulation of nacre indentation creep. The indentation depth of indentation is 75 nm.

Figure 10.3: A long-term simulation of nacre indentation creep. The depth of indentation is 75 nm.

Figure 10.4: Contours of (a) strain $e_{33}$ and (b) strain $e_{11}$ of nacre indentation (B section view). The indentation depth is 75 nm.

Figure 10.5: Paradigm for the structure and performance of nacre. (a) “Brick-wall” like layered tile composite composed of high aspect ratio toughened ceramic CaCO$_3$ tiles within a biopolymer matrix; (b) the biopolymer matrix, which is itself viscoelastic, is composed of a chitin core within a hydrated protein matrix. Note that the tiles are themselves toughened by the incorporation of an intra-tile biopolymer network and protein inclusions.
LIST OF TABLES

Table 3.1: Results of chitin assays as per the protocol described in Section II.1. 55
ACKNOWLEDGEMENTS

First I would like to express my most sincere gratitude and affection to my entire family for their love, support, and encouragement throughout my entire university and graduate study. My mother and father have provided me with the sort of family structure that is necessary to enable a son or daughter to pursue such a challenging course as I did. For this and much more I most truly thank them. From my childhood my mother continually nurtured my interests in discovering and inventing; this propelled me into the world of science and engineering. My father was always a strong constructive and reinforcing presence that helped me to make key decisions that allowed me to successfully deal with the rigors of graduate school. To my sister and brother, I likewise give my deepest gratitude - they continuously provided humor and encouragement and at times vital help to me during these years.

I would like to give Dr. Robert Asaro a very special acknowledgement for his kind and patient guidance throughout my graduate study. He and I have come down quite a long path in performing entirely novel types of studies, publishing papers in leading international journals that some may think lay outside traditional Structural Engineering, and in the building of a completely new laboratory designed for the study of soft-tissue and cellular materials. The building of this unique facility will open the way for future students and researchers to perform new types of experimental probing of biological cellular and soft-tissue structures.

I would also like to give special acknowledgement to those with whom I had collaboration with during the course of this research, namely my co-authors, Dr. Maryln Hawley, Dr. Vlado Lubarda, Dr. Qiang Zhu, Dr. Zengli Peng, Dr. Peter Novick and, of course again my advisor Dr. Robert Asaro. They made performing this work a joy. These researchers rightly serve on my thesis committee but for Drs. Hawley and Peng due to distance.
Finally I would like to express gratitude to the administrative staff of the Structural Engineering Department at UCSD. They provided invaluable aid to me in working through the maze of administrative procedure and made it far easier for me to concentrate on my work without the burdens of bureaucracy.

The Thesis is organized around four published papers that have appeared in the Journal of Structural Biology and the Journal of Theoretical and Applied Mechanics. The body of the Thesis includes multiple references to the following articles in which the dissertation author was the primary investigator and author:


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ABSTRACT OF THE DISSERTATION

The Nanomechanics of Biomineralized Soft-Tissues and Organic Matrices.

by

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Doctor of Philosophy in Structural Engineering

University of California, San Diego, 2013

Professor Robert J. Asaro, Chair

The research reported on in this dissertation is concerned with the macro-molecular constitution and geometrical organization of the soft-tissue comprising the matrix of the nacreous portion of the shell of Haliotis rufescens, the Red abalone. Nacre is one of literally legions of intricate biomineralized structures that exist in nature and has long served as a paradigm for elegant and optimized structural design. Biomineralization involves, inter alia, the uptake and synthesis of elements
and compounds from the environment and their incorporation into highly optimized functional structures. Nacre has a structure described as a brick wall like with a matrix of biopolymer layers that are preformed and serve as a template into which nanocrystalline tiles of CaCO₃ precipitate. The matrix, or what are known as interlamellar layers, are of particular interest as they impart both toughness and strength to the composite ceramic nacre structure. The work first involved a histochemical mapping of the macromolecular structure of the interlamellar layers; this revealed the locations of proteins and functional molecular groups that serve as nucleation sites for the ceramic tiles. Parallel studies on the nacre of Nautilus pompilius, the Chambered Nautilus, revealed the generality of the findings. Of particular interest was determining both the content and layout of chitin within these layers. In fact it was determined that chitin was organized as a mostly uni-directional architecture of fibrils, with a certain fraction of fibrils laying at cross directions. Most remarkably, it was found that the fibrils possessed a very long range connectivity that spanned many tiles. This was determined by systematic atomic force (afm) and analytical optical histochemical microscopy. These findings were further verified by a unique form of mechanical testing whereby tensile testing was conducted on groups of interlamellar layers extracted from nacre. Mechanical testing led to a quantitative visco-elastic constitutive model for these layers and, in turn, to a complete mechanical/structural model for the complete nacre composite. Further verification was obtained via micro- and nano-indentation experiments which were modeled via detailed FEM numerical simulations. Nano-indentation also allowed a detailed assessment of the nano-structure and properties of the ceramic tiles which are best described as nano-scale composites composed of protein infiltrated CaCO₃ nano-grains within a biopolymer matrix. The role of water content, i.e. moisture content, was also determined via, in part experiments conducted on dehydrated nacre. These findings lead to a pathway for specifying optimal bio-mimicked or bio-inspired synthetic materials.
Chapter 1

Introduction

Biomineralization involves, *inter alia*, the uptake and synthesis of elements and compounds from the environment and their incorporation into highly optimized functional structures. The processes used are conducted under strict biological control and occur with a precision far exceeding human synthesis methods. Interestingly, the process has a quite special position in the life sciences since, unlike most other biological transformations and synthesis that leave no lasting effect, biomineralized structures such as bones, shells, teeth, *etc.* are firmly recorded in the fossil record. In fact, there is evidence that biomineralization has been going on upon the Earth for over 570 million years, and that the processes involved have been going on for 3500 million years - the Earth is approximately 4500 million years old. Examples of biomineralized structures are shown in Fig. 1.1: the example in Fig. 1.1a is of coccoliths, marine animals that form micron size structures assembled extracellularly from intracellular vesicles, containing intricate $\text{CaCO}_3$ components, that migrate to the cell membrane where they are released and incorporated into coccospheres (as shown). Figure 1.1b shows the structure of bone, *viz.* compact cortical bone, so-called due to its minimal gaps and open spaces. Bone structure such as this typically accounts for 80% of the bone content of the adult skeleton. Bone, unlike the coccolith
just described or other marine animal structures, is composed mainly of biopolymer matrix material. Finally, Fig. 1.1c shows the chambered nautilus (*Nautilus pompilius*) that possesses a truly magnificent structure that has fascinated humankind for millennia. The structure is, notably, remarkable in its functionality as well as its intrinsic beauty and pure symmetry. The structure of sea shells such as that of the red abalone (*Haliotis rufescens*) is likewise extraordinary and and its design is both structurally optimized and elegant in that such shells are synthesized by low energy methods, from common materials, and at plain atmospheric pressure. As it happens, the synthesis of biomineralized structures and the origins of their attractive functionality and properties are mediated by the soft-tissue within them. This is, indeed, the case in our study of *Haliotis rufescens*.

![Figure 1.1: Examples of biomineralized structures. (a) Coccospheres made up of coccoliths. (b) Compact cortical bone. (c) The chambered nautilus.](image)

The research carried out leading to this thesis has been concerned with 1) the macromolecular structure of the soft-tissue that provides the matrix of the shell of the abalone *Haliotis rufescens*; 2) the use of this information to construct specific models for the manner in which the shell structure is built; 3) the documentation of the nanocrystalline structure of the CaCO$_3$ tiles that are sandwiched within the soft-tissue; 4) a complete nanomechanical model for the entire shell (with a focus on the nacreous part); 5) the unique testing of extracted soft-tissue; and 6) testing and numerical modeling of the entire nacreous shell itself. The work reveals that the
biopolymer framework of the nacreous part of the shell of *Haliotis rufescens* both mediates its synthesis and its remarkable properties.

Figure 1.2 introduces *Haliotis rufescens* and it is the nacre portion that is the object of the research reported on herein. Inorganic and organic compounds, along with proteins, are secreted into the extrapallial space between the outer epithelium and the growing nacre. The outer prismatic calcitic layer is of a different structure and is not considered herein. Nacre, commonly referred to as *mother-of-pearl*, provides the overall structural integrity of the animal’s shell. Thus the focus of this research has been on the matrix of nacre, along with the nano-composite $CaCO_3$ tile structure that determines nacre’s properties.

**Figure 1.2**: Schematic view of the red abalone. The shell consists of an outer prismatic layer and an inner layer of nacre. Note the tiled, brick-wall like structure with biopolymer layers that make up a matrix. These layers are the focus of most of the research reported on in this thesis.

The work has involved a number of rather varied methods and steps. Documentation of the macromolecular structure was accomplished by using histochemical optical microscopy - in this, a wide range of chemical staining methods were used
to identify, essentially, the layout of macromolecular types and their likely roles in the synthesis of nacre. The crucial structural role of chitin was determined and the precise chitin architecture uncovered. In fact, it was found that chitin is arranged in a mostly uni-directional layup, but with a fraction of chitin fibrils laying at angles to this primary fibril direction. A specific mechanical model for the chitin structure was thereby proposed. Novel testing of the soft-tissue extracted from the shell, done for the first time in this work, revealed the soft-tissue’s remarkable long range connectivity; this led to a specific visco-elastic constitutive model for the soft tissue and provided a vital ingredient for a nano-mechanical model for nacre. This also provided a clear explanation for the long known exceptional mechanical properties of nacre and led to a new paradigm for future bio-inspired synthetic materials. Nanoindentation was performed to document the properties of nacre and this provided a direct pathway for validating the models. Finally, numerical finite element models were constructed and used to simulate the indentation tests themselves and the mechanical response of nacre.

This thesis is organized around four published papers that have appeared in the Journal of Structural Biology and the Journal of Theoretical and Applied Mechanics; these papers are those cited in Publications and authored by 1) Bezares, J., Asaro R.J. and Hawley, M. 2008, 2) Bezares, J., Asaro R.J. and Hawley, M. 2010, 3) Bezares, J., Peng, Z.L., Asaro, R.J. and Zhu, Q., 2011, and 4) Bezares, J., Asaro, R.J. and Lubarda, V.A. 2012. These co-authors have provided written permission, although not required by US Law, for the use of this published material. These four papers are adequately cited throughout the text wherever the material is used. The findings reported on herein provide a pathway to future optimized material’s design; such materials include novel synthetic bone, electro-magnetic films or plate-like structure and energy absorbing materials used as armor.
Chapter 2

Experimental Methods

2.1 Shell Samples

*Haliotis Rufescens* and *Nautilus pompilius* shells were obtained from The Abalone Farm Inc., Monterey, CA. Shells were obtained from animals that had been raised under conditions of constant temperature and diet to ensure that their shells did not develop the (∼100 nm) thick green protein layers which naturally occur due to seasonal changes in temperature and food supply. Shells were lightly sand blasted to clean their surfaces, and were stored dry at 20 °C.

2.2 Flat Pearl Preparation

Fifteen live *H. rufescens* animals were provided by the Abalone Farm Inc. and were kept in tanks with continuously running sea water at the Scripps Institution of Oceanography at UCSD. Flat pearls were prepared by inserting 5 mm diameter round glass slides under the mantle of live *H. rufescens* and allowing approximately three weeks for nacre to form. The slides were then removed and washed in dI water.
2.3 Demineralization

2.3.1 EDTA

Demineralization was performed by submerging shell samples in a solution of 1 M ethylenediaminetetraacetic acid (EDTA), pH 8.0 at 20 °C; the antiseptic cetylpyridinum chloride (CPC) was added at a concentration of 0.5%. Fixed organic framework samples were prepared by demineralizing nacre in a 4% formaldehyde solution in EDTA.

2.3.2 Ion-exchange Resin


Nacre fragments were placed in 10 mm long sections of dialysis tubing (MWCO 3500, 19 mm flat width, Fisher). The samples were submerged in 4 l of dI water containing 750 ml of pre-washed cation exchange resin (Dowex 50W×8 50-100 mesh, Sigma). A stir-bar was used to maintain the resin in constant suspension at 20 °C; the water was replaced once a day.

Fixed samples were placed in dialysis membranes containing a 4% formaldehyde, 0.5% CPC solution. These samples were allowed to demineralize in 50 ml conical tubes filled with 15 ml of the same exchange resin as above. The tubes were placed on a tilt platform which maintained the resin in suspension during decalcification; demineralization was complete after 4-5 weeks.
2.4 Enzymatic and Alkaline Treatments

Trypsin

Approximately 10 µg of wet organic framework were incubated for 24 hrs at 20 °C in 0.1 M ammonium bicarbonate buffer, pH 8.0, containing 1 mg/ml trypsin (Invitrogen). Samples were collected by centrifugation using a bench-top centrifuge, (Eppendorf).

PNGase F

N-Glycosidase F (New England Bio Labs, P0704S) is an amidase that cleaves between the innermost glucosamine and asparagine residues of numerous N-linked glycoproteins. Here it was used to degrade glycoproteins whose glucosamine groups might interfere with Calcofluor White or Wheat Germ Agglutinin labeling. One centimeter square sections of organic framework were separated into ∼1 µm thick sheets containing ∼10 interlamellar layers. The sheets were incubated in 1 µl of 10X glycoprotein denaturing buffer and dI water to make a total reaction volume of 750 µl. The samples and buffer were combined in 1.5 ml centrifuge tubes which were incubated in a water bath heated to 100 °C for 15 min. A total reaction volume of 20 ml was prepared by adding 2 µl of 10X G7 reaction buffer, 2 µl of 10% NP40, 2 µl N-Glycosidase F, and 14 µl of dI water. Samples were incubated in this solution for 2 hrs at 10 °C.

Proteinase K

Following Schaffer et al., (1997).

A 5 mM Hepes buffer solution, pH 7.5 was consistently used in the following protocol. Approximately 10 µg of fully hydrated biopolymer framework material were sonicated for 10 min in 1 ml of buffer. The samples were centrifuged at 12,000
rpm for 10 min in an Eppendorf bench-top centrifuge. The supernatant was removed and replaced with a .02% Proteinase K solution in the same buffer. The pellets were sonicated for 10 min and left to incubate for 2 hrs at 20 °C. The samples were then centrifuged at 12,000 rpm for 10 min, the supernatant removed and the pellets washed in the same buffer solution.

**Chitinase**

*Following Nudelman et al., (2008).*

Samples were incubated in 50 mM Phosphate Buffered Saline (PBS) pH 6.2, containing 8 units (U) of Chitinase (Sigma) at 35 °C for 72 hrs.

**Alkaline Peroxidation**

*Following Moses et al., (2006).*

Organic framework samples were submerged for 1 hr at 70 °C in a solution of 5% 10 N Sodium Hydroxide (NaOH), 10% concentrated hydrogen peroxide (H₂O₂), and 85% Milli-Q water. Samples were then thoroughly washed in dI water.

### 2.5 Histochemical Labeling

#### 2.5.1 Sample Preparation

Histochemical and immunohistochemical labeling procedures were performed on thin sheets of organic framework material containing ~1-20 interlamellar layers. The sheets were separated from bulk organic framework material using fine tweezers under a bench-top stereoscope (Olympus SZH Zoom Stereomicroscope).
2.5.2 Sample Drying and Mounting

Interlamellar layers were air dried on glass cover slips and mounted on glass microscope slides by first placing a drop of Entellan mounting medium (Merck) on the dried sample, and then inverting the cover slip onto the slide. The mounting medium was allowed to set prior to imaging. To avoid photobleaching due to ambient light fluorophore stained samples were prepared under low-light conditions and stored in the dark until just prior to imaging.

2.5.3 Autofluorescence

Biological structures may naturally emit light at wavelengths within the excitation and emission spectra of applied fluorophores. This can lead to images where unlabeled structures fluoresce alongside labeled structures. To account for autofluorescence, unlabeled samples were imaged using the fluorescence filter cubes required in the following fluorescence labeling protocols. For each filter the intensity of the microscope light source and the camera exposure time were adjusted to eliminate any fluorescence. Imaging labeled samples was performed while maintaining the same microscope and camera settings.

2.5.4 Calcofluor White

A 10% Potassium Hydroxide (KOH) reagent was prepared by dissolving 10 g of KOH in 90 ml of dI water to which 10 ml of glycerin were added. A second Calcofluor White (CW) reagent was prepared by dissolving 0.1 g of fluorescent brightener-28 (Sigma) in 100 ml of dI water under gentle heating. Interlamellar layer sheets, approximately 1-2 µm thick were mounted on slides. Two drops of each reagent were added to the samples. After 4 min, samples were rinsed in dI water, air dried and mounted on slides.
2.5.5 Aminoacridone


Interlamellar layers were incubated in solution of 1% 1-ethyl-3-(3 dimethylaminopropyl)-carbodiimide (EDC) in 20 mM PBS pH 4.5 at 20 °C. The samples were washed three times in 0.2 M borate buffer, pH 8.5, and incubated for 12 hrs in a solution of 1% Aminoacridone (Sigma) in the same buffer. Samples were air dried and mounted on slides.

2.5.6 Colloidal Iron

Working Solution Preparation

Following Pearse, (1968).

A 29% (w/v) stock colloidal iron solution was prepared by dissolving Ferric Chloride (FeCl₃) in boiling water. Complete dissolution was indicated by the water turning dark red at which point it was allowed to cool to 20 °C. To remove salts in the stock solution it was dialyzed three times for 24 hrs against a volume of dI water 5X that of the solution; the reagent was stored at 20 °C. A working colloidal iron solution, pH 1.8, was prepared consisting of dI water, glacial acetic acid, and stock colloidal iron solution in a ratio of (18:12:10).

Labeling Procedure

Following Nudelman et al. (2006).

Interlamellar layers were submerged in the working solution for 1 hr, rinsed thoroughly with 12% acetic acid, and incubated in a 1:1 solution of 5% Potassium
Ferrocyanide (K$_3$[Fe(CN)$_6$]) and 5% Hydrochloric Acid (HCl) for 20 min. Samples were then washed with dI water, air dried and mounted on slides.

### 2.5.7 FITC-WGA

*Following El Gueddari et al., (2002).*

Organic framework samples were incubated in a solution of 2% (w/v) bovine serum albumin (BSA) in PBS for 30 min at 20 °C. Samples were washed three times with PBS/Tween-20 and incubated for 1 hr in Fluorescein Isothiocyanate (FITC) conjugated to Wheat Germ Agglutinin (WGA) *i.e.* (FITC-WGA, Sigma), in (0.1 mg/ml PBS, 1% (w/v) BSA). Samples were washed three times with PBS/Tween-20, washed once in dI water, air dried and mounted on slides.

### 2.5.8 WGA-gold

**WGA-gold Complex Preparation**

*Following Geoghegan and Ackerman, (1997).*

Wheat Germ Agglutinin (WGA) is a lectin that specifically binds to sequences of three N-acetylglucosamine (GlcNAc) $\beta$-(1-4)- residues and thus has a very strong affinity to chitin. A solution was prepared containing 0.75 mg of WGA in 1 µl of dI water and 25 ml of colloidal gold (nominal size 10 nm) at pH 9.9. The solution was stirred for 3 min after which 1% polyethylene glycol (PEG) was added. After 5 min the reagent was centrifuged at 60,000 g in a Beckman ultracentrifuge for 1 hr at 4 °C. The supernatant was removed and the sedimented WGA conjugated-to-gold complexes (WGA-gold) resuspended to 5 ml in PBS, pH 8.0, containing 0.02% PEG.
Labeling Procedure

Approximately 1 mm thick fixed and unfixed demineralized nacre samples were sectioned into squares ~1 cm², and were incubated in WGA-gold for 30 min; WGA-gold was used at a dilution of 1:100 in PBS, pH 7.0. After labeling the samples were washed in six changes of PBS, pH 7.2.

Silver Intensification

Adapted from King et al., (1987).

WGA can be bound to colloidal gold particles with diameters no greater than 20 nm, a procedure typically used when labeling chitin in transmission electron microscope (TEM) protocols. However due to their small size the particles are not readily visible by scanning electron or optical microscopic techniques. The particles can however be coated with silver (enhanced) to increase their size, making them visible via SEM. Aurion R-Gent SE-EM (Aurion) silver enhancement reagent was prepared immediately before use following the Aurion kit instructions. Interlamellar layers treated with WGA gold complexes (i.e. the complexes have been bound to chitin), were rinsed in dI water three times and placed in the enhancing reagent for 4 min then washed in five changes of dI water. This increased the particle size to ~40 nm allowing them to be resolved via SEM.

Cytochemical Controls

The specificity to chitin of WGA-gold was determined by pre-incubating the complexes in N,N’,N”-triacetylchitotriose (no labeling was observed using these complexes). Another set of samples was incubated in unlabeled WGA, 0.5 mg/ml. (No change in the appearance of treated il-layers was observed from those untreated with unlabeled WGA.) Specificity to chitin was further determined by a comparative anal-
ysis using FITC-WGA (Molecular Probes) and CW which binds specifically to single β-1-3 and β-1-4 residues, (Maeda and Ishida, 1967; Peters and Latka, 1986; Lukes et al., 1993) as are found in Chitin and Cellulose. Of these two polysaccharides chitin is the only one which is labeled by both WGA and CW, and is the only one found in demineralized nacre.

### 2.6 Immunohistochemical Labeling

Polyclonal antibodies were generously supplied by Dr. Steve Weiner of the Weizmann Institute of Science, Rehovot, Israel.

Sheets containing multiple interlamellar layers were incubated in serum containing polyclonal antibodies for 1 hr. The serum was diluted 1:25 in PBS containing 0.25% w/v BSA to block non-specific binding. The samples were washed twice for 5 min with PBS containing Tween-20 (0.05% w/v). Samples were incubated for 40 min in the secondary antibody, Rhodamine conjugated goat-anti-rabbit (Jackson ImmunoResearch, diluted 1:100 in PBS). Prior to imaging samples were washed three times for 5 min with PBS and Tween-20 (0.05% w/v) to remove unbound antibodies. The samples were then rinsed in dI water, air dried and mounted on slides.

As a control, samples incubated in pre-immune serum were prepared as above.

### 2.7 AFM Scans of Inclined Interlamellar Layers

A section of EDTA extracted framework tissue was mounted, edge-on, on a histology slide and sliced in a microtome so that approximately 1-2 μm of material stood nearly upright, i.e. nearly orthogonal to the glass slide. The sections were inclined to the vertical by ~30-40° as depicted in (Fig. 2.1).
Figure 2.1: Figure of interlamellar layers mounted vertically in paraffin wax on a histology slide, and having been sliced at an angle of \(\sim 30-40\) degrees with a microtome.

2.8 Microscopy

2.8.1 Optical Microscopy

Samples were observed using a Nikon Eclipse 80i optical microscope equipped with a Photometrics CoolSNAPez digital camera. Brightfield and differential interference contrast images were taken with this microscope. Calcofluor White and Aminoacridone labeled samples were viewed using a 11003 V3 filter set, while samples labeled with FITC and Rhodamine where viewed using a 41001 filter set (Chroma).

Brightfield images of nacre flakes were taken using an Nikon Ti inverted microscope; (to be discussed in the nanoindentation section).

2.8.2 Scanning Electron Microscopy (SEM)

The SEM images in Figures 3.1 and 3.2 were taken under the guidance of Evelyn York at the Scripps Institution of Oceanography at UCSD. All other SEM images were taken with the assistance of Ryan Anderson at the NANO3 facility, Atkinson Hall, UCSD.
Sample Dehydration and Drying

Samples were dehydrated in a graded series of 40:60:80:90:100:100 ethanol in dI water for 20 min per change. Dehydrated samples were critical point dried (CPD) with liquid carbon dioxide using a Tousimis Autosamdri 815B supercritical-point dryer. Samples were mounted with carbon tabs and sputter coated with 10-15 nm of Iridium using a Denton Desk IV Sputter Coater. Imaging was performed using a Philips XL30 ESEM operated at 10 kV.

2.8.3 Atomic Force Microscopy (AFM)

AFM imaging was performed by Marilyn Hawley of the Materials Science and Technology Division at the Los Alamos National Laboratory, Los Alamos, NM.

Fluid cell imaging was performed using a Veeco Metrology Multimode AFM with Nanoscope IIIa controllers. Hydrated samples were imaged using a fluid cell holder with an o-ring designed to prevent liquid from spilling onto the AFM scanner. Samples mounted on AFM pucks were held in place using the o-ring seal. 500 nm²-20 µm² scans of interlamellar layer regions were obtained. For the imaging of dry samples a Nanoscope Digital D3000 AFM was used.

2.9 Chitin Assays

The following assays were performed by Dr. Rafael Sentandreu and Dr. Luis Castillo at the University of Valencia, Valencia, Spain.

This method was based on the evaluation of glucosamine content found in organic framework samples. 1 mg of tissue (dry weight) was resuspended in 1 ml of 6 M HCl and hydrolyzed at 100 °C for 17 hrs, together with N-acetylglucosamine
(GlcNAc) as a standard. Samples were then dried and resuspended in 1 ml of dI water. The quantity of glucosamine released by the hydrolysis of 100 ml of this material was determined as follows. An equal volume of 4% (v/v) acetylacetone in 1.5 M Sodium Carbonate (Na$_2$CO$_3$) was added, and the preparation heated to 100 °C for 20 min. Samples were then diluted with 700 µl of 96% ethanol and incubated for 1 hr at 23 °C, after which 100 ml of Ehrlich’s reagent (1.6 g/ml p-dimethylaminobenzaldehyde, 6 M HCl, 50% ethanol) were added. Samples were incubated for 15 min at 65 °C before absorbance was read on a spectrophotometer at 520 nm.

2.10 Tensile Testing of Dry Nacre

2.10.1 Sample Preparation

Shells were sand blasted to remove their outer calcitic layers leaving only their inner iridescent nacre. Regions with minimal curvature were sectioned out using a pneumatic grinder equipped with an abrasive cut off wheel. The sections were thinned via polishing to a final thickness of ∼.5 mm using 60-800 grit SiC paper on a Struers TegraPol-21 polisher. During the polishing procedure the samples were continuously washed under a stream of tap water. The polished samples were held between stainless steel templates, pictured in (Fig. 2.2) while a pneumatic grinder with an abrasive die was used to remove shell extending beyond the templates. Finished specimens are are called “dog-bone” specimens due to having a dog-bone shape. Figure 2.2b shows two finished dog-bone specimens.

2.10.2 Testing Procedure

Tensile testing was carried out with an Instron (# 5586) electro-mechanical test system; displacement was measured from the cross-head displacement of the
device. An extensometer was not used due to the fragility of the small and very thin tensile specimens. Nevertheless, the Young’s modulus of dry nacre obtained in this manner was consistent with literature values. Aluminum “grips” showing in (Fig. 2.3a), were machined which could house the ends of dog-bone specimens. Each end of a specimen was held in the space formed between the two parts of a “grip” (Fig. 2.3b). Rubber pads were inserted in one of the “pieces” to prevent the sample from slipping out of plane. These grips were themselves held an pulled on by the Instron machine’s pneumatic grips.

2.11 Tensile Testing of Framework Tissue

2.11.1 Sample Preparation

Rectangular shell sections approximately 50 mm long, 10 mm wide and 1-2 mm thick, were cut from H. rufescens shells with their lengths perpendicular to the direction of shell growth as shown in (Fig. 2.4). Alternatively the orientation of the specimen lengths could be said to be parallel to the growth front of the shell; the
growth front is indicated in (Fig. 2.4) by a red line. In the figure the outlines of samples to be cut-out have been drawn on the shell. The edges of the samples were polished to ensure uniformity of the sample widths and to clearly expose the interface between nacreous and calcitic layers. The precise dimensions of the nacreous region of each sample were documented from images taken with a Photometrics Cool-SNAPez digital camera mounted on a bench top stereoscope (Olympus SZH Zoom Stereomicroscope). Before demineralization a typical sample contained portions of the shell’s prismatic layer which was readily removed during the demineralization process leaving only the tissue associated with the nacreous layers. Figure 2.5a is of a polished sample used to estimate the number of il-layers, while Fig. 2.5b is of the same but now demineralized sample where only the tissue associated with the nacreous region remains. From SEM images the average thickness of individual tile lamellae was estimated to be $\sim 500 \pm 20$ nm. The thickness of il-layers has been inde-
pendently measured (Currey, (1977); Jackson et al., (1988); Sarikaya et al., (1990)), and was taken here to be 20 nm. From these two measurements and the thickness of the nacreous layers an estimate of the number of tile lamellae and thus il-layers, could be made. Sample dimensions used to estimate the cross sectional area of organic framework layers, later used to calculate stresses from tensile test data, are indicated in (Fig. 2.6).

Figure 2.4: The outlines of prospective specimens to be cut out are drawn on the surface of a shell. The lengths of the samples are normal to the direction of shell growth.

2.11.2 Embedding and Demineralization

The ends of dry polished samples such as the one in (Fig. 2.5a) were encapsulated in Epofix epoxy resin (Struers) as follows. A two part Teflon mold was prepared with one part having oblong holes through its thickness and the second being a piece of Teflon sheet, as shown in (Fig. 2.7a); the two parts were clamped tightly together. Tensile specimens prepared as described above were placed at the
center of holes and aligned vertically as shown in (Fig. 2.7a). EpoFix resin was poured into the holes and allowed to cure for 24 hrs. Samples were extracted and the embedding procedure repeated on their other ends as shown in (Fig. 2.7b). Care was taken to ensure that the flat faces of the epoxy “plugs” on both “gripping” ends were aligned and that specimens were centered within both plugs. The length of the exposed region of a sample between the two epoxy pucks will be referred to as the 

\textit{gauge length}; these varied from $\sim 20$-$25$ mm. Once embedded, the samples were immersed in EDTA until the exposed region of the shell was fully demineralized. A typical demineralized sample is shown in (Fig. 2.5b).

2.11.3 Biochemical Treatments

Demineralized tensile specimens were subjected to the following seven treatments intended to alter the structure of their protein and polysaccharide components.
Figure 2.6: A drawing of a tensile test specimen with its ends embedded in epoxy, showing the dimensions used to calculate the cross sectional area of the combined il-layers in a sample. Interlamellar layer and tile lamellae thicknesses are taken to be 20 nm and ∼500 nm respectively. The insert is an SEM image (edge view) of nacre showing tile lamellae.

Figure 2.7: Schematic drawings of the embedding set-up. a) A rectangular shell section is placed centered and aligned in a 2-part Teflon mold. Resin is poured into the mold and allowed to set. b) The sample is extracted from the mold, and its other end embedded as well. The sample’s gauge length is ∼20-25 mm.

This was an attempt to elicit changes in framework layer mechanical response which could readily be interpreted in terms of expected structural alterations due to the treatments. (The following degradation protocols were previously described in the Enzymatic and Alkaline Treatments section.)
1) To induce the rapid and severe cross-linking of framework protein samples were fixed for 2 hrs in 2.5% glutaraldehyde in 100 mM PBS, pH 7.0; these samples will be referred to as Fixed-Wet (FW).

2) To observe the effects of dehydration on samples fixed as in 1) above, samples were additionally dehydrated via an ethanol series (see SEM sample dehydration) and critical-point dried; these samples will be referred to as Fixed-Dry (FD).

3) Fully hydrated samples having undergone no other treatment will be referred to as Not-Fixed-Wet (NFW).

4) Similarly, samples having undergone no other treatment besides dehydration and drying will be referred to as Not-Fixed-Dry (NFD).

5) To investigate the effect of partial protein removal, some samples were incubated in Proteinase K for 24 hrs. During testing these samples were maintained fully hydrated by the addition of dI water with a micropipette. Samples prepared in this manner will be referred to as Proteinase K (PK).

6) Following the removal of protein as described in 5) chitin was enzymatically degraded with Chitinase. The samples were then rinsed in dI water and maintained fully hydrated throughout the rest of the testing procedure; these samples will be referred to as Proteinase K-Chitinase (PK-CH).

7) To investigate the effect of near complete protein removal some samples were subjected to the alkaline peroxidation procedure. These samples will be referred to as Alkaline Peroxidase (AP).

Due to the lengths of these tensile specimens, critical-point drying was performed using a Polaron KE3000 drier where samples were housed in a cylindrical chamber having a 30 mm internal diameter and 82 mm length, during drying. Drying was performed under the guidance of Evelyn York at the Scripps Institution of Oceanography at UCSD.
2.11.4 Specimen Damage and Fiber Engagement

Specimens prepared as just described suffered from several forms of “damage” where damage denotes the fact that not all the il-layers contained in a section of shell would support tensile forces. Layers that could support force in a tensile specimen are referred to as being engaged. Several reasons for damage were identified, i.e. non-engagement, that include the following,

1) curvature of the shell growth front,
2) specimen and layer curvature,
3) layer damage or rupture during demineralization, and
4) non-uniform specimen thickness.

Precise estimates of the expected non-engagement of layers for each specimen were not possible by visual inspection, but overall bounds were established as will be discussed next.

The “damage” caused by the general curvature of the growth front is described by (Figs. 2.8a-c). Figure 2.8a shows a complete shell where both the overall growth front and outlines of two tensile specimens to be cut out of the shell are indicated; the inserts to the right indicate growth front outlines in such extracted specimens. A result of growth front curvature is that not all layers span the full gauge length of the specimens; an example of a layer that terminates within the gauge length is indicated by the black arrow in (Fig. 2.8b). Sections of such layers were easily removed by gently probing them with a spatula. In addition, layers were seen to be broken at their ends such as indicated by the white arrow in (Fig. 2.8c). The curved growth front associated with this layer is also outlined by the black arrow. It was estimated that between 10-20% of all layers would be non-engaging for these reasons. The effects of specimen and layer curvature are explained by (Figs. 2.8d,e). Figure 2.8d is a photo of the edge of a specimen prior to testing. The general curvature of the specimen, and thus of the layers within it, is evident.

When rectangular specimens are cut out of a shell the result is that not all il-
Figure 2.8: a) A complete shell with the overall growth front and two outlines of rectangular tensile specimens to be cut out. Figure b) is of a demineralized tensile specimen where the black arrow indicates a section of a layer that does not span the entire gauge length. In c) the white arrow indicates a layer that is broken at its end; the black arrow indicates the shell growth front within this specimen. d) Edge view of a demineralized tensile specimen illustrating the general curvature of such specimens. e) Schematic of a tensile specimen seen from above and outlined in red as in (a), indicating how not all layers would be included within the entire gauge length due to the curvature of the growth front.

Layers are fully incorporated in the specimen as illustrated in (Fig. 2.8e). For example those layers illustrated by broken lines in (Fig. 2.8e) would not span the entire gauge length and thus would not support tensile force, i.e. would not be engaged. The examination of multiple specimens suggested the range of 10-20% for such non-engaged layers. Layer damage was investigated by SEM inspection of demineralized tensile specimens. Most such damage was located at the epoxy/biopolymer layer interface. The fraction of layers so damaged is estimated to be in the range 10-25%. Only layers not included in the category under the growth front curvature heading are included in this category. Finally, it was noted that although care was taken to prepare specimens of uniform thickness there was some variation that was estimated to be in the range ±5%. Thus another 5-10% loss in fully engaged layers is expected from this. In total it was expected that the percentage of non-engaged layers would constitute 35-75% of all layers in any given specimen.
2.11.5 Testing Procedure

Tensile testing was carried out in a desktop Instron (# 5586) electro-mechanical test system. The gripping and extension set up is shown in (Fig. 2.9). Axial strain was monitored using an extensometer (Epsilon, SK-1023) mounted on aluminum grips. It was noted that the force required to extend the extensometer was negligible. In addition, the stiffness of the gripping fixtures was at least 1000X that of the specimens and thus the extensometer extension was interpreted as that of the specimen’s gauge length. Tests were carried out under displacement control to induce a constant strain rate or under load control to induce constant stress.

![Figure 2.9](image_url)

*Figure 2.9: Photo of the tensile and relaxation test setup. Aluminum grips hold the specimen by its epoxy pucks using set-screws. An extensometer attached to aluminum grips measures displacement. The gauge length of the specimen is \( \sim 25 \) mm.*
Figure 2.10: Schematic drawings describing the geometry of a Vicker’s microindenter tip. a) The half-angle between opposite faces of the tip is 68 deg. b) All four faces of the four-sided pyramid are shown where the angle between opposite sides is 136 deg. c) The surface area of a remaining imprint is calculated from the measured lengths of the imprint diagonals shown as dashed blue lines.

2.12 Microindentation Creep Testing of Aragonite

Microindentation tests were performed using a Buehler Micromet 5124 microindenter. A diamond Vickers indenter tip was used for all experiments; it’s geometry is described in (Fig. 2.10). The tip has the shape of a four-sided pyramid with an included angle of 68 degrees i.e. the angle between opposing faces (see Fig. 2.10a). Hardness is defined as the ratio of applied force to surface-area of contact (F/A), where F is measured in kilo-grams force (kgf). The area of contact can be calculated from the diagonals of a resulting indentation, indicated by dashed blue lines in (Fig. 2.10c). From the average length ‘d’ of the indentation diagonals the area can be calculated via the following relation, \( A = \frac{d^2}{2\sin(138^\circ/2)} \).

Microindentation creep tests were performed on monolithic aragonite to test its time-dependent response. A 9.8 \( \mu \)N force was applied at a rate of 1 mN/sec. Images of the resulting indentations were captured using a CCD camera mounted directly on the microindenter. Buehler’s Omnimet software was used to measure the lengths of the indentation diagonals from which the surface area of indentation was calculated. Time-dependent response was measured by varying the dwell time of the indenter tip on the sample from 5-900 sec. By recording the increase in indentation area size with respect to dwell time, the time-dependent drop in hardness
was calculated. Figures 2.11a-c describe the microindentation creep process where with increasing dwell time the size of indentations increase. The increasing diagonal lengths are used to measure the decrease in sample hardness.

Figure 2.11: Figure describing the microindentation creep process. a) For a short dwell time (∼5 sec) the area of indentation is relatively small; b) - c) with increasing dwell time the size of the indentations increase as do the corresponding indentation diagonals used to measure hardness.

2.13 Nanoindentation

2.13.1 Indentation Using a Berkovich Tip

Figure 2.12: The three-sided pyramid geometry of a nanoindenter Berkovich tip is described. a) is an SEM image showing the front of a tip with its three faces and b) a schematic drawing where the large included angle of 142.3 deg between one of three edges and a face of the tip is shown.
Dry Nacre

Indentations were performed along the two directions shown in (Fig. 2.13). Indentations were made either along the c-axis of the tiles i.e. on the top surfaces of the tiles, or along the edges of tiles. The later may be referred to as the Edge-On direction.

Figure 2.13: A drawing showing the two directions along which tiles were indented in dry nacre; a) tiles were indented along their c-axes; b) tiles were indented along their edges.

Indentation along the c-axis of tiles.

Strips of shell were cut out using a cut-off wheel and cleaved along their thickness using a hammer whereby thin translucent nacre flakes were released. The thinnest flakes were inspected under an optical microscope where individual tiles could be resolved. Flat regions containing planes with hundreds of tiles were identified and selected for indentation testing. A region on a flake suitable for indentation is shown in (Fig. 2.14a) where single tile lamellae span ~100 µm². Flakes were glued to AFM pucks and mounted on the nanoindenter stage.

Indentation along the edges of tiles.

Pieces of dry shell were embedded in EpoFix resin (Struers); the pieces were
aligned with their cross sections exposed after embedding. The samples were wet-ground using 800 grit SiC paper, polished using a .04 μm colloidal silica suspension, washed with dI water and air dried.

Figure 2.14: Figure a) is a bright field image of a nacre flake indicating a regions suitable for indentation (the blue circle at the upper right) and a region not suitable due to debris and tile terraces. b) A drawing showing a side view of a flake where the center would be suitable for testing due to being flat and free of debris.

Heat Treated Nacre

Dry nacre flakes were glued onto AFM pucks and placed in an oven for 12 hrs at 200 °C. During heating the Super Glue bonding the flakes to the AFM pucks was burnt off. The head of a pin was used to push additional glue underneath the flake to ensure it was fully supported against the puck.

Wet Nacre

Dry nacre flakes were glued onto AFM pucks and submerged in dI water for 3 days; samples were maintained moist during testing by the application of dI water with a micropipette.
Monolithic Aragonite

An aragonite crystal was purchased from a mineral supplier (Beads, Crystals and More, Encinitas, CA). The crystal was sectioned using a SiC cut-off wheel, polished using standard metallographic procedures, and etched for 5 min in 0.5 M EDTA pH 8.0. Crystal sections were mounted on AFM pucks for placement and alignment in the nanoindenter. The monolithic crystal naturally forms with its longer dimension along its crystallographic c-axis (001). The indenter tip was aligned with the c-axis of the crystal to within \(0.5 \pm 0.1^\circ\). Values of hardness obtained from indentations in this manner were in agreement with the known hardness of aragonite crystal of \(\sim 6.5\) GPa. Indentations placed on other faces of the crystal rendered values of hardness in agreement with literature values for those faces \(\sim 4.6\) GPa (\(\bar{1}10, \bar{1}30\)), (see Kearney et al. (2006)).

2.13.2 Testing Procedure

A Ubi 1 Nanomechanical test instrument (Hysitron, Inc., Minneapolis, MN) with a diamond Berkovich tip having a 100 nm radius of curvature was used to perform tests where the Young’s modulus \(E\) and hardness \(H\) of samples were determined. A trapezoidal load function with a 5 sec hold under load control was used to determine the reduced modulus for contact depths ranging between 25 nm and 300 nm. The Young’s modulus was determined from the load \((P)\) vs. indentation depth \((\delta)\) curves using the following method of Oliver and Pharr, (2004). From a \(P-\delta\) curve (see Fig. 2.15) the reduced modulus \(E_r\) is determined from the initial slope \((S)\) of the unloading portion of the curve, and the area of elastic contact. The contact area is determined from the indentation depth and the known geometry of the tip. For each tip a calibration was performed to obtain a relationship between the surface area of the tip and its length. The reduced modulus \(E_r\) obtained as described above is related to the Poisson’s ratio \(\nu_0\) and the Young’s modulus \(E_0\) of the specimen by
the following relation,

\[ \frac{1}{E_r} = \left( 1 - \nu_0^2 \right) / E_0 + \left( 1 - \nu_i^2 \right) / E_i, \]  

(2.1)

where \( \nu_i = 0.07 \) and \( E_i = 1141 \) GPa are the Poisson’s ratio and the Young’s modulus of the diamond indenter.

![Load-Displacement Curve](image)

**Figure 2.15**: The figure of a load-displacement (P-\( \delta \)) curve used to calculate Young’s modulus by the Oliver and Pharr method. The slope of the initial portion of the unloading curve (S) describes a material’s elastic capacity to “rebound” during unloading. The slope is used to measure the Young’s modulus using equation 2.1 above.

Displacement controlled relaxation tests were performed using a trapezoidal loading function with a 40 sec hold at peak, and a maximum displacement of 75 nm, (see Fig. 2.16). Drift was monitored for 40 sec before performing each indentation, and the displacement was held for 180 sec between tests to allow the system to stabilize. All tests were performed at 20 °C on an active isolation table in an environmental chamber.

### 2.13.3 Surface Scan Acquisition

Surfaces can be scanned using the nanoindenter by rastering a tip across a sample and measuring elevation changes. Height contours obtained in this manner
**Figure 2.16:** A figure describing the kind of loading function that would be used to perform a load-controlled creep test; the function includes an initial loading segment, a hold at peak load, and a final unloading segment.

can be combined to form 3-dimensional topographs. The interpretation of indentation data along with these images can provide important information about the mechanisms underlying a material’s deformation. Figure 2.17a is of a Berkovich nanoindenter tip over an aragonite sample; b) is a topograph obtained by scanning the surface of an indented polycarbonate sample with a Berkovich tip.

**Figure 2.17:** A figure describing the scanning capability of the nanoindenter used to obtain surface scans of samples and indentations. a) The nanoindenter tip can pivot about its base to “scan” a surface. b) The surface scan of a series of 5 indentations on a polycarbonate sample, where a Berkovich tip was used for indentation.
2.13.4 Precision Contact Surface Alignment

Tripod Fixture

The following technique was developed to demonstrate that tiles in nacre are isotropic. In principle, the deformation of an isotropic half-space due to indentation with a symmetric tip, should result in a symmetric deformation pattern. A conospherical tip was used in this experiment where the shape of the material “pile-up” forming around the indenter tip was used as the observed deformation pattern. Pile-up however is also dependent on the tip being precisely aligned with the c-axis of the tile. Figure 2.18 describes how asymmetric contact due to a tilted surface would lead to asymmetric pile-up. To ensure that contact was symmetric, the following procedure was used.

Figure 2.18: Drawings showing the effect of sample tilt on the symmetry of pile-up around a conospherical indenter. a) With the tip aligned normal to the surface pile-up is symmetric. The inner and outer edges would form concentric circles. b) With the sample tilted there would be greater pile-up around the left side of the tip. Viewed from above the edges of the pile-up would appear oblong and unaligned.

A miniature tripod was built which could be fixed rigidly to the nanoindenter stage. A diagram of the tripod fixture is presented in (Fig. 2.19). Fine-pitch bolts (10-40) were used as legs with their tips polished to remove any extending threads. Sliding of the tripod legs on the surface of the nanoindenter stage was prevented.
in the following manner. AFM pucks with holes drilled through their centers were Super Glued to the stage; the hole diameters were just slightly larger than those of the leg tips. The tripod leg tips were placed into the AFM puck holes to prevent the legs from moving during the adjustment of the tripod’s tilt. The legs traversed a 1/4 in. steel plate on which samples were glued with Super Glue. Lock nuts between the bolt heads and plate were necessarily tightened to eliminate play. Gradations were marked on the heads of bolts to keep track of their rotation. Sample tilt could be adjusted to within \(0.2 \pm 0.01^\circ\) along both the x and y directions.

**Figure 2.19:** Schematic of the sample alignment set-up. The tripod consists of a steel plate with threaded bolts as legs and lock-nuts used to eliminate play between the bolts and plate. AFM pucks prevent sliding of the legs on the nanoindenter stage. An embedded sample is glued to the tripod, and it’s tilt adjusted by turning the tripod bolts.

**Tilt Correction Procedure**

Tilt was measured by obtaining 3-dimensional scans of sample surfaces using the nanoindenter’s scanning capabilities. Adjustments were made by loosening the lock-nuts, turning the bolt legs and re-tightening the lock-nuts to remove play. To precisely adjust the tripod’s tilt it was necessary to remove it from the nanoindenter chamber. Within a nacre flake, that might have dimensions (length, width) in the mm range, a suitable area for indentation might only include 4-6 tiles, as described
in (Fig. 2.20). Tiles had to be smooth and free of debris as well as large enough to accommodate a deep enough indentation that would lead to sufficient “pile-up” to obtain a clear surface scan. Between tilt adjustments the position and orientation of a sample was maintained the same by setting the tripod feet in holes drilled in AFM pucks. Tilt was adjusted until it was less than .2° along either the x- or y- axes.

![Diagram](image)

**Figure 2.20:** The procedure used to precisely adjust the tilt of a sample. A typical nacre flake has a width in the range of ∼2 mm as seen in a), however the region of interest may only contain a few tiles as seen in b) and have dimensions in the range of ∼10 μm². Following an adjustment of the tripod’s tilt, it was essential that the sample be precisely placed at its previous location with the nacre flake having its previous orientation. The tilt of the surface of interest was adjusted to within .2 deg along both the x- and y- directions.

**Dry Nacre**

Samples were glued to AFM pucks using Super Glue. The pucks were then glued to the tripod stage with tile c-axes along the axis of indentation as depicted in (Fig. 2.19).

**Monolithic Aragonite**

A single monolithic aragonite crystal was sectioned into two parts using a Secotom-10 (Struers) sectioning machine and embedded in EpoFix resin. Crystal surfaces were ground using 800 grit SiC paper and polished using a .04 μm colloidal silica suspension on a Struers TegraPol-21 polisher. The exposed surface of one of the
embedded sections corresponded to the c-axis of the crystal while the exposed surface of the other section was oriented along either the a- or b- crystallographic axes, but not the c-axis. Figure 2.21 describes the orientations along which indentations were made on the crystal.

Figure 2.21: A figure describing the axes along which indentations were made on monolithic aragonite crystal. a) A crystal is shown with its c-axis aligned vertically; the c-axis is naturally aligned with the longer dimension of the crystal. b) The crystal was sectioned along the dotted square shown in a) with the exposed face corresponding to either the a- or b- axes. c) Indentations were made on the exposed face (along either the a- or b- axes), and along the c-axis as shown in b).

2.14 Numerical Simulations

All numerical simulations presented in this dissertation were performed by Dr. Zhangli Peng while a graduate student at the University of California, San Diego.

2.14.1 Uni-axial Tensile Testing of Dry Nacre

A finite element model was developed using ABAQUS Explicit (ABAQUS Inc., Providence, RI) to simulate the tensile testing of the “dog-bone” nacre specimens shown in (Fig. 2.2b). The relative dimensions regarding the assumed hexagonal tile thickness and diameter to the thickness of the interlamellar layers was accounted
for. It was further assumed, for the small strains imposed, that at the interface between the CaCO$_3$ tiles and organic framework layers, both were strongly bound together. Nonlinear effects associated with intertabular layer separation or interlamellar sliding were not included. The CaCO$_3$ tiles were assumed to be isotropic with a Poisson’s ratio of $\nu = 0.3$ based on nanoindentation tests performed on dry nacre. The Young’s modulus of single tiles was assumed to be $E_c = 100$ GPa, based on the findings of Vincent, (1982). The Young’s modulus and in-plane shear modulus of interlamellar layers were initially assumed to be $E = 0.98$ GPa and $G_p = 0.3$ GPa based on the estimates of Bezares et al., (2008).

![Figure 2.22: FEM model of nacre which includes the actual ratios of thickness and tile diameter to biopolymer layer thickness.](image)

2.14.2 Nanoindentation of Dry Nacre and Aragonite

Finite Element Model

A finite element model was developed using ABAQUS Explicit (ABAQUS Inc., Providence, RI) to simulate the time-dependent processes associated with the nanoindentation of nacre and aragonite along their c-axes. For computational efficiency, the symmetry of both the Berkovich indenter and an idealized mineral tile
were exploited and only 1/6 of the system was modeled, (Fig. 2.23a). Surface A was allowed to slide tangentially due to the weak constraint from surrounding tiles and organic material at intertabular boundaries, the boundaries formed by the lateral abutment of tiles. The bottom of the lowest tile was fully fixed. Other boundary conditions were specified based on the symmetry described in (Fig. 2.23).

To make sure that the computational domain along the c-axis was sufficiently large (i.e. there were enough layers were included to ensure the fully fixed boundary condition at the lowest surface was sufficiently accurate), numerical tests were undertaken to ensure that during indentation simulations the deformation of the layers furthest from the indenter was negligible. From numerical tests it was found that by using five mineral layers and five biopolymer layers the fully-fixed condition was essentially satisfied. Near the contact region the computational mesh was refined for greater resolution. The model employed 167228 C3H8R elements (i.e. uniformly reduced integration brick elements) with the mesh being refined near the contact region. The Berkovich indenter was assumed rigid with an inclined face angle $\beta$ of 24.7 degrees and an apex angle $\gamma$ of 77.1 degrees. The mineral tiles were idealized as perfect hexagons with edge lengths of 2.5 $\mu$m and thicknesses of 600 nm. A 20 nm biopolymer layer thickness was used. These parameters were based on SEM images and literature values. Contact between the indenter and tile surface was assumed to be hard and frictionless.

A similar finite element model was developed to extract to the viscoplastic properties from indentation creep experiments on monolithic aragonite; the same mesh described above for dry nacre was used (Fig. 2.23). In this case the biopolymer layers were replaced by aragonite and fully fixed boundary conditions were prescribed at surfaces A and B but not at the contact surface (see Fig. 2.23a).
Figure 2.23: The finite element model of the nanoindentation process: isometric view (left), top view (middle), and B section view (right).

Constitutive Relations

*Monolithic Aragonite*

Both tiles and monolithic aragonite were assumed to behave as viscoplastic materials, with constitutive properties modeled using the rate-dependent plasticity overstress model of Perzyna, (1971), i.e.

\[
\dot{\varepsilon}_p = \frac{\sigma_Y}{\eta_p} \left( \frac{\sigma}{\sigma_Y} - 1 \right)^n,
\]

where \( \dot{\varepsilon}_p \) is the equivalent plastic strain rate, \( n \) is the rate-sensitivity exponent, \( \sigma_Y \) is the yield stress, \( \eta_p \) is the plastic viscosity, and \( \sigma \) the Cauchy stress. Plastic strain takes place when the yield condition \( \sigma > \sigma_Y \) is satisfied, which is incorporated via the Macaulay bracket \( \langle f \rangle = f \) if \( f > 0 \), and \( \langle f \rangle = 0 \) if \( f \leq 0 \). For simplicity, the rate-sensitivity exponent \( n \) was set to unity so that Eq. (2.2) became

\[
\dot{\varepsilon}_p = \frac{\langle \sigma - \sigma_Y \rangle}{\eta_p}.
\]

It was noted that setting \( n = 1 \) in Eq. (2.2) led to results that matched experiments closely.
Interlamellar layers were modeled using the standard linear solid model shown in (Fig. 2.24), i.e.

$$\sigma = \int_0^t E(t - t') \frac{de}{dt'} dt', \quad (2.4)$$

where $E(t) = E_0 + E_1 \exp(-t/\tau)$ is the instantaneous Young’s modulus, $E_0$ the Young’s modulus, $\tau = \eta/E_1$ the characteristic time, $\eta$ the viscosity, $\sigma$ the Cauchy stress, and $e$ the engineering strain.

**Figure 2.24:** The standard linear solid model.
Chapter 3

Results: Framework Biochemical Structure

3.1 Introduction

Biomineralization is a well regulated process within living organisms, and involves control over, \textit{inter alia}, the morphology of typically nano-composite structures, crystal nucleation and growth, crystallographic texture, and polymorph type (\textit{e.g.} Lowenstam and Weiner, (1989); Simkiss and Wilbur, (1989); Baeuerlein, (2000)). In mollusk shells, among a wide array of other cases, mineralization appears to occur within a preformed 3-dimensional organic framework which acts as the template that provides the above mentioned control (\textit{e.g.} Bevelander and Nahahara, (1969); Wada, (1972); Schaffer \textit{et al.}, (1997); Nudelman \textit{et al.}, (2006); Bezares \textit{et al.}, (2008)). The organic framework thus mediates the growth, \textit{i.e.} the “fabrication”, of the mineralized nano-composite and, as it happens, is also key to what is seen to be a rather excellent array of mechanical properties of the shell (\textit{e.g.} Sarikaya \textit{et al.}, (1992); Evans \textit{et al.}, (2001); Bezares \textit{et al.}, (2010,11,12)). It is, accordingly, vital to understand the structure of the organic framework and in particular its macromolecular
layout so that critical features such as the crystal nucleation site and the chemical/structural morphology that control mechanical behavior may be understood in a more quantified manner.

The biopolymer framework has previously been studied primarily through optical microscopy. Crenshaw and Ristedt, (1976) took a unique approach to studying the macromolecular structure in that they attempted a mapping of the framework’s components using histochemical light microscopy. They used the fact that once demineralized, the interlamellar matrix reveals the outlines of the tiles, and mapped the location of sulfates, carboxylates, and calcium binding sites within them. Figure 3.2a is a SEM image of tile outlines on an il-layer. Wada, (1980) later confirmed the presence of high concentrations of sulfur in the central region, thus suggesting that the nucleation site was located there. Nudelman et al., (2006) pursued the histochemical approach and identified four different zones, as defined by the molecular constituency, on the framework surface. Crenshaw and Ristedt’s, (1976) and Nudelman et al.’s (2006) work was performed on the cephalopod Nautilus pompilius, whereas the latter performed comparative study on the bivalve Atrina rigida. Here the approach is extended, but for the case of the gastropod Haliotis rufescens, with comparative study on Nautilus pompilius so as to obtain a more comprehensive understanding of the commonality and variances among different members of the mollusk group. The general findings of Nudelman et al., (2006) are confirmed here, but now for Haliotis rufescens.

Falini et al., (1996) and Gotliv et al., (2003) isolated acidic macromolecules from extracted interlamellar layers and showed that they helped nucleate aragonite when introduced into an in vitro assembly of (Loligo) β-chitin and (Bombyx mori) silk fibroin. Gotliv et al., (2003) isolated their matrix molecules from A. rigida and Nudelman et al., (2006) showed positive results on both A. rigida and N. pompilius using polyclonal antibodies raised against these same molecules. Here the same immunohistochemical procedure is applied to label the demineralized interlamel-
lar/intertabular matrix of *H. rufescens* to explore the possibility that these mineral nucleating macromolecules may be present, and thereby aid in locating potential aragonite nucleating sites.

There have been reports of chitin in the framework of several mollusks other than *H. rufescens* (Weiner *et al.*, (1980,83); Poulicek, (1983)). Until the results of assays reported herein, no truly definitive and quantitative evidence for chitin had been documented for *H. rufescens*. Findings concerning chitin provide an important, and previously not understood picture of the organic framework that has specific implications to the mechanical and structural performance of the shell.

AFM and histochemical fluorescence microscopy results to be presented, map the macromolecular structure including chitin in interlamellar layer tile imprints of organic framework tissue extracted from *H. rufescens* nacre. These results are then used to confirm and extend models for nacre growth and for developing an approach to modeling the shell’s mechanical properties. This provides the path to biomimetics and bio-duplication of synthetic materials.

### 3.2 Results

#### 3.2.1 SEM Observations

The following SEM observations are briefly described most particularly to provide perspective on the structure of nacre in *H. rufescens*. Figures 3.1a,b show SEM images of the tile cross-section of a fractured shell that illustrate the interdigitated nacre “brick-wall” like structure. Additional description of the structure, including the interlamellar *il* and intertabular *it* layers may be seen in the images and are described in the caption. Interlamellar regions are identified by the horizontal white arrows in (Fig. 3.1b); the intertabular regions are identified by the vertical white arrows. The black arrow indicates a central region observed in all tiles. Figure
3.1d shows an image of individual demineralized interlamellar layers of the EDTA extracted organic matrix. Such images were obtained from critical-point dried matrix tissue and show il-layer thicknesses in the range of 80-100 nm; in the shell the interlamellar layers are 20 nm thick as just noted, the difference being due to loss in confinement and swelling of the extracted material. The layers appear porous in (Fig. 3.1d) and this will be further elucidated when AFM results are shown later. Figure 3.1c shows the growth of “flat pearls” grown on the surface of glass slides inserted under the mantle of live H. rufescens; such observations will be referred to
in the discussion of nacre growth.

### 3.2.2 Histochemical Labeling

**Calcofluor White**

Calcofluor White (CW) is a fluorophore that binds to glycans and fibrillar polysaccharides such as chitin (e.g. Herth, (1980); Ramaswamy *et al.*, (1997)). Interlamellar layers were stained and imaged after demineralization in EDTA or by ion-exchange resin. In both cases strong binding was detected as shown in (Figs. 3.2c,d). For perspective, however, SEM images of demineralized nacre taken normal to the interlamellar plane, (Figs. 3.2a,b), are first presented. The intertabular ma-

![Figure 3.2](image)

**Figure 3.2:** Figures (a) and (b) are SEM images of demineralized organic framework in *H. rufescens*. Note that the center of each tile imprint is more deeply etched; the apparent accumulation of organic material at the center. (c) A fluorescence micrograph of *H. rufescens* stained with Calcofluore White following demineralization in EDTA. The insert is of *N. pompilius* demineralized and stained in the same manner. (d) same as (c) except with demineralization by ion-exchange.
trix, surrounding the tiles, is clearly revealed as is the appearance of an evidently organic ring-like structure organized around what were the tiles’ centers. Etching, in the form of “pits”, occurs throughout the surface of the interlamellar layers. The interlamellar layers at locations of the tile centers typically etch deeply. Later in (Figs. 3.8a,b) it is shown that indeed the central regions of the demineralized interlamellar layers are depressed relative to the intertabular matrix and the region between it and the center region. The ring-like structure will be discussed in chapters 5 and 6 in connection with a chitin structural core found within il-layers.

Figures 3.2c,d show the effect of CW staining on *H. rufescens* and *N. pompilius*, respectively; the insert in (Fig. 3.2c) is also an image obtained from *N. pompilius*. Strong binding of CW is observed at the intertabular matrix in both cases and, at a lesser intensity, at the very center of the tiles. The center illumination is not in the form of a ring-like structure, but rather appears as a quite localized area of CW binding. At the intertabular boundaries, staining of the matrix often appears in the form of a “double-layer” as, for example, in (Fig. 3.2c).

**Aminoacridone**

The *in vitro* studies of Addadi *et al.*, (1987) showed that together carboxylates and sulfates may help induce calcite crystal nucleation where the crystals have a definite orientation. Aminoacridone is a fluorescent compound that binds to carboxyl groups in the presence of carbodiimide (EDC). Here, il-layers extracted using EDTA were imaged in both fixed and unfixed states as shown in (Figs. 3.3a,b) respectively. As the samples are observed in epifluorescence, labeling appears white in the images. The unfixed samples showed labeling in the intertabular matrix, but little or no labeling was observed within the tile imprints and, in particular, at the center of the tiles, (Fig. 3.3). On the other hand for fixed samples, (Fig. 3.3a), labeling was observed albeit faint (see insert and the white arrow head). The central regions typically appeared dark, and in fact black in the unfixed samples. Very low levels of
Figure 3.3: (a) Fluorescence images of *H. rufescens* demineralized using EDTA, fixed, and stained with aminoacridone. (b) Unfixed *H. rufescens* imaged after staining. Note the faint but distinct labeling at the center in the fixed sample, shown more clearly in the insert to (a).

fluorescence were detected in regions between the center and the intertabular regions.

**Colloidal Iron**

Sulfates are known to strongly bind calcium and thus are of interest for understanding the tile nucleation process. Staining with colloidal iron is commonly performed to label acidic polysaccharides *via* the binding of iron to charged acidic groups, including carboxylates and sulfates. To distinguish the labeling of sulfates from carboxylates, staining is done at a pH low enough to protonate the carboxylates. Thus the staining was performed at a pH of 1.8, so that only sulfates were labeled. Strong labeling was observed within ring-like structures in the center of the tile imprints, as clearly seen in both (Figs. 3.4a,b). In fact, the stained ring-like structures are most pronounced in *H. rufescens* demineralized in EDTA and unfixed. At the intertabular matrix there is the near absence of labeling.

Samples were also demineralized and digested with trypsin + Proteinase K, stained and imaged. Figures 3.4c,d show results following staining with colloidal iron and CW, respectively. Labeling by colloidal iron was essentially eradicated after digestion indicating that the macromolecules responsible for colloidal iron binding
Figure 3.4: Brightfield images of *H. rufescens* stained with colloidal iron at pH in the range $1.8 \leq \text{pH} \leq 1.9$. (a) Demineralization performed using ion-exchange and fixation. (b) Demineralization performed using EDTA and without fixation. (c) *H. rufescens* demineralized in EDTA, unfixed, and digested in trypsin + Proteinase K. (d) fluorescence image of the same material as in (c) but stained with CW.

were removed. On the other hand, labeling of the intertabular regions and in the center regions by CW was hardly affected.

**FITC-WGA and WGA-gold**

Figures 3.5a-c show SEM images *H. rufescens* obtained after demineralization with EDTA and labeled with WGA-gold; (Fig. 3.5d) is a fluorescence image of il-layers labeled with FITC-WGA after demineralization in EDTA and digestion with PNGase F. PNGase F was used to degrade glycoproteins entrapped in the organic matrix which could interfere with labeling of the Glucosamine resides found in Chitin. Distinct staining is evident on the intertabular matrix with heavy accumulations of
colloidal gold seen outlining, in many cases, complete intertabular boundaries. Labeling of fibrous structures is also seen within the tile imprints as is most evident in (Fig. 3.5c) (see the white arrow). The diameters of the fibers were only approximately determined and appear to lie in the range of \(\sim 30\) nm. FITC-WGA staining, shown in (Fig. 3.5d), reveals again the strong labeling of the intertabular matrix and the pattern of projection of structure emanating from it. In (Figs. 3.6), and especially in (Figs. 3.6b-c), are presented AFM images revealing more clearly defined fibrous structures where the fiber diameters are in the range of 5-10 nm. Results presented in chapter 5, indicate that without digestion, or with partial digestion, there is additional organic matter attached to the fibers explaining the differences in

![Figure 3.5](image)

**Figure 3.5:** Figures (a)-(c) are images obtained after demineralization with EDTA, without fixing, and labeled with WGA-gold. Strong labeling of the intertabular matrix is evident as is the appearance of a fibrous network (small arrow heads). (d) Fluorescence image of an EDTA demineralized sample, digested, with PNGase F, and stained with FITC-WGA.
diameter observed here. In fact it is only after severe protein removal via combined alkaline and Proteinase K treatments that fibers are clearly exposed with diameters also in the range of 5-10 nm.

**Figure 3.6:** Images (a)-(c) are AFM phase images of interlamellar layers obtained in tapping mode from *H. rufescens* demineralized in EDTA, unfixed, and digested with (a) trypsin, (b,c) with trypsin + Proteinase K. (d) A phase image obtained in tapping mode of EDTA demineralized *H. rufescens*, unfixed, but mounted on a histology slide in a near edge-on orientation.
3.2.3 Immunohistochemical Labeling

As described in the introduction, acidic proteins containing high concentrations of aspartic and/or glutamic acids are an integral part of the organic matrix, and may help nucleate aragonite crystals in in vitro assemblies of silk and chitin, Falini et al., (1996). Figures 3.7a and b show that in H. rufescens labeling is quite sharp and highly localized.

Figures 3.7a,b show that in H. rufescens labeling is quite sharp and highly localized. Labeling is distinct at the intertabular matrix, and perhaps sharpest at the “vertex junctions” of the tile imprints. Labeling is also seen at the imprint centers as is especially clear in (Fig. 3.7b). Figure 3.7d shows a typical image from a sample treated with pre-immune serum alone where no staining is detected. Figure 3.7c is an image obtained with aminoacridone staining as shown earlier in (Fig. 3.3), that is shown here for perspective. The patterns of staining are quite similar suggesting co-location of molecules rich in carboxylates and aragonite nucleating macromolecules.

3.2.4 AFM Analysis

Both high and low resolution scans, in tapping mode, were performed over areas ranging from 500 X 500 nm up to 30 X 30 µm, the latter covering the range of the bright field and fluorescence images. Scans were made on specimens fixed and unfixed and with various degrees of protein digestion and/or staining. The images reveal fibrous structures characterized by at least two distinct length scales, the smaller characterized by fibers, or fiber bundles, and the larger by an open network that creates pore-like openings. Figure 3.8 shows two typical scans over a larger area for an unfixed and undigested specimen of H. rufescens stained with CW.

Figure 3.8b shows a topograph image while (Fig. 3.8a) shows an elevation profile of that region. As in the histochemical images shown above, these large scans
Figure 3.7: (a,b) Images obtained from unfixed *H. rufescens*, demineralized with EDTA, and labeled with polyclonal antibodies. (c) An image obtained on a sample identical to that used in (a,b) using aminoacridone staining shown for perspective. (d) An image of unfixed *H. rufescens*, demineralized with EDTA, and labeled with pre-immune serum.

reveal the outlines of tiles on the surface of the organic interlamellar layers. The central regions are depressed relative to the surrounding tile imprint as noted by the two central arrows. This depressed region is collocated with the central regions described in 3.2a,c. What is also notable from the height profile of (Fig. 3.8a) is that the top edges of the intertabular layers are not flat but contain height differences of the order of 50 nm. As also noted above, the tile imprint centers are depressed, and the scans show these depressions are nearly 100 nm. The structure at the imprint centers is discussed below.

Figure 3.6 are phase images of *H. rufescens* all obtained in tapping mode under fully hydrated conditions in a wet cell. Figure 3.6a-c shows a progression of higher resolution scans of unfixed specimens digested with trypsin or trypsin +
Figure 3.8: Figure (a) is an elevation profile obtained from a large scan of *H. rufescens*, demineralized in EDTA, undigested, and unfixed. (b) A topograph image accompanying (a) showing the elevations at the intertabular matrix. (c) A topograph similar to (b) from a different area.

Proteinase K. The lowest resolution scan of (Fig. 3.6a), of a specimen that was digested in trypsin only, shows what appears to be a “porous” structure. Closer inspection, however, reveals embedded fibers (e.g. at the white arrow) and the fact that the layers contain a core of fibers. The fibrous core is more clearly revealed in (Fig. 3.6b) which is of a specimen digested in trypsin + Proteinase K. Individual fibers are visible, and that they are embedded in less organized organic material is evident. Thus the interlamellar layers are not “porous” *per se* but are composed of a fibrous core, or network, that is embedded in and sandwiched between less organized organic material. Individual fibers appear to have diameters in the range 5-10 nm and run for lengths of at least 1 µm, although to date their length distribution has
not been quantitatively determined. Figure 3.6c shows a higher resolution scan which provides additional evidence of the fibrous network and suggests that the fibers may indeed be organized in nearly parallel bundles. In both fixed and unfixed samples there are examples where the fibers appear to be so organized, and examples that suggest a more random orientation.

Figure 3.6d shows a phase image from a scan taken in a completely different orientation. Here a section of EDTA demineralized, unfixed and undigested, framework tissue was first mounted, edge-on, on a histology slide and sliced in a microtome so that approximately 1-2 µm of tissue stood nearly upright, i.e. nearly orthogonal to the glass slide; the orientation was much like that shown in the SEM image of (Fig. 3.1d). The sections were, though, inclined to the vertical by about 30-40 °. Specimens of this type were fully hydrated and scanned in tapping mode in a wet cell. The individual layers are evident and are hydrated so that their thickness is estimated (correcting for the angle of inclination) at ~80-120 nm. In the shell, the interlamellar layers appear to be in the range ~30 nm in thickness, but demineralized tissue in water maintains a thickness of roughly that of the intact shell, i.e. it hydrates and swells by factors of approximately 3-4. Fibers are visible even in such undigested sections, and in fact on sections such as these appear to be arranged in an organized network. With reference to the labeling of chitin with WGA-gold as shown in (Fig. 3.5) it is possible that the finer resolution of the fibers in the AFM images is due to residual amounts of organic matter surrounding the chitin fibers without, or with partial, digestion as used. In addition, the SEM specimens were sputter coated and this would also contribute to their appearing somewhat thicker.

3.2.5 Chitin Content

Chitin assays were performed on demineralized framework material after demineralization in EDTA in unfixed conditions. The results are summarized in Table
Table 3.1: Results of chitin assays as per the protocol described in Section II.1.

<table>
<thead>
<tr>
<th>Condition of tissue</th>
<th>dry wt%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue in water</td>
<td>6.4%</td>
</tr>
<tr>
<td>Digested with trypsin</td>
<td>6.5%</td>
</tr>
<tr>
<td>Digested with trypsin + Proteinase K</td>
<td>6.9%</td>
</tr>
</tbody>
</table>

3.2.6 Mechanical Properties

Quasi-static tensile tests were carried out on both *H. rufescens* and *N. pompilius*; results are shown in (Fig. 3.9). The Young’s modulus for *H. rufescens* and *N. pompilius* are found to be $E = 70 \pm 5$ GPa and $E = 60 \pm 6$ GPa respectively.

![Figure 3.9](image-url)
Chapter 4

Discussion: Framework
Biochemical Structure

4.1 Chitin Content

The results are consistent with the expectation that with digestion the weight fraction of chitin is measured to increase. As the digestion methods were not aggressive and were designed to remove only modest amounts of protein from the top layers, the modest increases in chitin weight fraction observed are expected. Moreover, the level of chitin is found to be higher than reported in other studies (e.g. Poulicek, 1983), but certainly consistent with the levels reported by Goffinet and Jeuniaux, (1979) for other mollusks. The assays are also consistent with the significant amounts of chitin observed via AFM and histochemical staining with FITC-WGA. Chitin assays can be prone to slight overestimates due to the fact that it is known that several proteins within the framework are glycosylated and that the glycosyl moieties of some of them may contain glucosamine residues. Given the consistencies as just noted, however, such overestimates, if any, are quite modest.
4.2 Macromolecular Mapping

A summary of the results just presented is shown in (Fig. 4.1). At least five distinct zones characterized by populations of macromolecular constituency are mapped. Chitin is indeed present and forms a structural core that underlies the interlamellar framework and is a major, if not the major, constituent of the intertabular matrix. AFM height profiles show that the central regions are depressed relative to the surrounding tile imprint. This depressed region is collocated with the central regions described in (Fig. 3.2b) where large holes perforate the layers. These results suggest that the localized CW and WGA labeling at the central regions is due to chitin being particularly exposed at those locations. Exposure would result from the tearing of an interlamellar layer as a nucleating tile grows up and through it. Chitin is also found to be present in high concentration in the intertabular matrix (Figs. 3.2c-d). At the center of an interlamellar layer is a region rich in carboxylates and aragonite nucleating proteins (Figs. 3.3 and 3.7). Surrounding the center is an annulus rich in acidic sulfates as seen in (Figs. 3.4a,b). The intertabular matrix is also rich in carboxylates and sulfates consistent with the findings of Nudelman et al., (2006) for *N. pompilius* and *A. rigida*.

As for the organization of chitin, the AFM images indicate that the fibers are often arranged in bundles (Fig. 3.6c) and span lengths of at least 1 µm (Figs. 3.6a,b). They form the structural core of interlamellar layers that eventually becomes a major constituency of the intertabular matrix. These particular images suggest that bundles take on an apparently random orientation. In Chapters 5 and 6 it will be shown that the fibers are in fact well organized, with minor implications to the constitutive model presented here.
Figure 4.1: Macromolecular “map” of the surface of an interlamellar layer; molecular types are indicated as found via the labeling methods described in Chapter 2. The intertabular matrix, and its molecular constituency, is also indicated.

4.3 Implications for Nacre Nucleation and Growth

Figure 4.2 summarizes findings of a possible growth mechanism for nacre in *H. rufescens* based on the organic template model, described most recently by Addadi *et al.*, (2006). Of the growth paradigms proposed over the years, the data supports a scenario whereby tiles grow through the interlamellar layers thereby forming “stacks” that are readily reproduced as “flat pearls” as shown in (Fig. 3.1c). As tiles grow laterally, *i.e.* within a layer, and water is displaced, both chitin and silk-like protein are accumulated within the intertabular matrix. This accounts for the chitin concentrating at the intertabular regions as labeled in (Figs. 3.5a-c). Both chitin and protein are also accumulated within the interlamellar layers between the tiles and chitin core.

Figure 4.2: Model paradigm for the growth process of nacre in *H. rufescens*. 
De novo nucleation is believed to occur at sites that result in the central regions being stained by aminoacridone, colloidal iron, and polyclonal aragonite nucleating antibodies. Here concentrations of negatively charged sulfates and carboxylates concentrate Ca\(^{+2}\). Such sites are indicated in red in (Fig. 4.2). Interlamellar layers are not so much porous *per se*, but rather that they are built on a fibrous network *i.e.* a core to be discussed in the following chapter. Of course, the model also allows for the possibility of calcium carbonate precursors, such as the (chemically unstable) amorphous precursors discussed by Watabe *et al.*, (1976), that are formed elsewhere and transported to the sites of tile growth. Such a possible scenario has also been discussed recently by Addadi *et al.*, (2006).

As tiles grow through the layers to form a new tile on the higher layer, the “mineral bridges” that form absorb the macromolecules that are part of the site. Organic material extends some distance into the thickness of the tile. This helps explain the more rapid dissolution of mineral at the tile centers during demineralization and the center structures seen in etched tiles or on demineralized layers (*e.g.* Figs. 3.2a,b). It also explains the origin of a protein intracrystalline matrix that exist in all tiles.

### 4.4 Mechanical Properties and Modeling

#### 4.4.1 Mechanical Properties

Quasi-static tensile tests were carried out on both *H. rufescens* and *N. pompeiius*; results are shown in (Fig. 3.9). Eq. (4.1) was used to extract the interlamellar layer shear modulus \(G_p\) based on measured values of the tensile modulus of nacre \(E\). The volume fraction of interlamellar matrix was taken to be \(g = t/h\), where \(t\) is the matrix layer thickness and \(h\) the tile thickness. As it happens, \(g \approx 0.06 - 0.066\) which is fortuitously close to \(f\) the fraction of chitin in the organic matrix.
For *H. rufescens* it’s found that $E = 70\pm 5$ GPa. If it’s assumed that $E_c = 100$ GPa, and $g = 0.063$, it’s found that $G_p = 0.41$ GPa. The assumed modulus $E_c = 100$ GPa is based on values quoted for intact monolithic CaCO$_3$ (see for example Gao *et al.*, 2006). However as was noted in connection with (Figs. 3.2a,b) as examples, ceramic tiles are infiltrated with organic material that would tend to reduce their stiffness. If $E_c$ is assumed to be less than 100 GPa, the estimate for $G_p$ will be somewhat larger. Nonetheless, the agreement between the estimate of $G_p = 0.41$ GPa and the model estimate of $G_p \approx 0.3$ GPa is encouraging. For *N. pompilius*, it’s found $E = 60 \pm 6$ GPa, and with $\rho = 15$ and $g = 0.063$, a value of $G_p = 0.31$ GPa is obtained, in quite good agreement with the model estimate.

More refined estimates of the elastic, or possibly viscoelastic, response of nacre tablets would take into account the intracrystalline matrix and the true nature of the tablets as coherent nanocrystalline aggregates, *e.g.* as noted in the Introduction with references to Rousseau *et al.*, (2005) and Oaki and Imai, (2005). It is easy to see, as noted in the Introduction, that this would lead to reductions in tablet stiffness of at least 10% which would place the estimates of overall nacre stiffness, that use the model estimate of interlamellar stiffness, in even better accord with experiments as shown in (Fig. 3.9).

### 4.4.2 An Interlamellar Layer Constitutive Model

The results and extractions of material properties from tensile data such as shown in (Fig. 3.9) below can be analyzed using the model for layered nacre shown in (Fig. 4.3). The model is based on a familiar assumption that load is transferred from the organic matrix to the ceramic tiles by interfacial shear, *i.e.* by “shear lag”. Analysis of (Figs. 4.3a,b) yields an approximate expression (*e.g.* Gao, (2006); Gao *et al.*, (2003)) for the effective (elastic) shear modulus, $G_p$, of the interlamellar matrix

$$\frac{1}{E} = \frac{4g}{G_p(1 - g)^2\rho^2} + \frac{1}{(1 - g)E_c}. \quad (4.1)$$
In eq. (4.1), $G_p$ is the interlamellar shear modulus to be inferred from the tensile tests, assumed to be linear elastic as in an expression $\tau = G_p \gamma$ with $\tau$ and $\gamma$ defined in (Fig. 4.3c); $E$ is the measured tensile modulus of nacre tested in the configuration of (Fig. 4.3b); $g$ is the volume fraction of interlamellar matrix; $\rho$ is the aspect ratio (i.e. the ratio of diameter to thickness) of the ceramic tiles; and $E_c$ is the modulus of the CaCO$_3$ tiles. To extract estimates for $G_p$ from the analysis given in (Fig. 4.3c), it is noted that $f$, the fraction of chitin in the interlamellar matrix, as estimated from the chitin assays is $f \sim 0.064 - 0.069$, with an average value of $\langle f \rangle = 0.066$. If next it is assumed that roughly one half the fibers are collinear with the direction of shear and one half are in the shear direction, then $\tilde{f} = 0.25$. Finally, the geometrical factor, $\sin^2 2\phi$ is estimated as $\langle \sin^2 2\phi \rangle|\pi/4 = 0.5$. From these assumptions and estimates the following is obtained

$$G_p \approx \frac{1}{32} f E_f.$$  

(4.2)

Taking as an estimate for $E_f$, $E_f = 137$ GPa (e.g. Nishino et al., 1999), then $G_p \approx 0.3$ GPa. The above estimate contains several competing sources of approximation. For one, the contributing, and most likely viscoelastic, stiffness of the protein has been ignored. On the other hand, a maximal effect of chitin was assumed in contributing to the stiffness. Nonetheless, the estimate is thought to be of the correct order of magnitude and most likely within a factor of two of the actual value.

### 4.5 Summary and Conclusions

The understanding of the molecular structure of the organic framework of *H. rufescens* nacre has been advanced in this study. By combining SEM, histochemical microscopy, and AFM scanning probe microscopy, a detailed model for nacre’s nucleation and growth process has been provided. Conclusions are, on the one hand, consistent in essential content with those of Nudelman et al., (2006) who studied *N. pompilius* and *A. rigida*, yet provide additional perspective on the structural makeup...
Figure 4.3: (a) Shear lag model for the tensile stiffness of layered nacre. Tiles are illustrated as grey rectangles and the intertabular and interlamellar matrices in green. Note that the area fraction of organic matrix is exaggerated for illustration. (b) Same as (a) but now illustrating the separation of tiles from matrix at the tile ends and the “shear lag” process. (c) An illustration of simple shear of the interlamellar layer; this assumes the imposition of deformation gradient $\mathbf{F} = \mathbf{I} + \gamma \mathbf{sm}$. Chitin fibers are assumed to lie along directions inclined by angle $\phi$ to the interlamellar plane as illustrated in (d) of the figure. This leads to an estimated stretch of chitin fibers $\lambda^2 \approx 1 + \gamma \sin 2\phi$ and a normal strain in such fibers of $e_n \approx 1/2 \gamma \sin 2\phi$. The elastic energy stored within such fibers is then $\mathcal{E} = 1/2 E_t \gamma^2 \sin^2 2\phi$, where $E_t$ is the tensile modulus of chitin. Finally it’s assumed that the volume fraction of such fibers contributing to resistance is $f_e = \tilde{f} f$, where $f$ is the total volume fraction of chitin and $\tilde{f}$ the fraction of chitin fibers oriented to contribute to resistance in the direction of the applied shear stress. The model is completed by setting the elastic energy density stored in the fibers to that of a linear elastic solid with shear modulus $G$, i.e. set $\tilde{f} = f/8 E_t \gamma^2 \sin^2 2\phi = 1/2 G \gamma^2$ to obtain $G = 1/4 \tilde{f} f E_t \sin^2 2\phi$.

of the nacre organic framework. The distribution and role of chitin, in particular, has been revealed with much more clarity, with additional resolution of chitin’s distribution within the framework.

As for mechanical properties, these results provide fresh and valuable insight for assessing molecular structural models of the framework. In Chapters 7 and 8
the documentation of the static and dynamic response of framework layers will be presented. These results are of great value since as it will be shown the organic framework is indeed viscoelastic with the dynamic response being intimately linked to molecular structure (e.g. Doi and Edwards, (1986)).

In the following chapter the documentation of chitin fiber architecture within single interlamellar layers, and their orientation will be presented. Chapter 6 will discuss the implications of these findings to interlamellar layer and shell mechanical response.
Chapter 5

Results: Framework Architecture

5.1 Introduction

Nacre’s toughness is three orders of magnitude greater than that of its mineral phase (Jackson et al., (1988); Gao et al., (2003)) largely due to the crack blunting and deflecting capabilities of the interlamellar layers, making the study of their structure of principle importance.

The biochemical structure and amino acid composition of il-layers which form a 3-dimensional framework have been characterized (Gregoire, (1957,72); Crenshaw and Ristedt, (1976); Nudelman et al., (2006); Cariolou and Morse, (1988); Bezares et al., (2008)). Il-layers consist of 75-80 dry wt% aspartic acid rich glycoproteins Addadi and Weiner, (1985) and chitin (Peters, (1972); Goffinet and Jeaniaux, (1979); Weiner and Traub, (1980); Poulicek, (1983); Weiss et al., (2002)) which in *H. rufescens* amounts to $\sim6.4$ dry wt% Bezares et al., (2008). The predominant model for il-layer structure is based on TEM observations of il-layer fragments (Watabe, (1965); Weiner, (1979); Weiner and Traub, (1980); Nakahara, (1979,83); Levi-Kalisman et al., (2001)) and is depicted in (Fig. 5.1). It consists of an electron-lucent core of parallel $\beta$-chitin fibers sandwiched between layers of aspartic acid rich macromolecules.
To date observations of chitin fiber alignment within il-layers have only been made using fragments of material and thus nothing has been said about the potential alignment of fibers across numerous il-layers or alignment with respect to the growth direction of the shell. Alignment between chitin fibers and the crystallographic axes of single tiles has been found in the nacres of numerous mollusks (Weiner and Traub, 1980; Weiner et al., 1983). Of significance to this study is that in the class cephalopoda, specifically in N. rupertus, chitin fiber axes were aligned “along the direction of bilateral symmetry” of the shell, that is, aligned normal to the growth direction of the shell. A similar fiber-growth direction alignment can be inferred by combining the findings of Wada, 1958 that tile b-axes in the bivalve P. martensii are normal to the growth direction of the shell with the results of Weiner et al., 1983 that in the same species chitin fibers are aligned with the b-axes of tiles. The current findings indicate that in a third class of mollusks gastropoda, specifically in H. rufescens, chitin fibers are also aligned normal to the growth direction. Moreover this alignment is found over all il-layers imaged where the native orientation of il-layers has been maintained, and across areas covering square millimeters!

**Figure 5.1:** Schematic diagram of the interlamellar layer structure according to Levi-Kalisman et al., 2001. Uni-axially aligned chitin fibers form a core within individual il-layers; the core is encased in layers of glycoproteins.

The structure of il-layers, specifically, in H. rufescens, has been imaged via AFM following the degradation of matrix layers with various proteases (Schaffer et al., 1997; Bezares et al., 2008). Schaffer et al., 1997 uncovered evidence of apparently randomly oriented fibers ∼10 nm wide between which 5-50 nm diameter
pores were formed. The fibers were suggested to be chitin or unidentified protein but without differentiation or verification. A model for tile nucleation and growth across il-layers was presented where the pores were sites through which growing aragonite could traverse il-layers in what were termed “mineral bridges”. Using AFM imaging, coupled with various histochemical techniques and following similar proteolytic treatments of il-layers Bezares et al., (2008) came to the conclusion that the fibers were indeed chitin with a far more organized structure. The current findings extend those of Bezares et al., (2008), and are in accordance with the conceptual model of Levi-Kalisman et al., (2001). Here it is found that after both alkaline and proteolytic degradation of il-layer protein, a core of aligned chitin fibers is exposed with pores being due to gaps formed between fibers which partially retain some lateral connectivity.

From a mechanical standpoint the issue of fiber orientation is of great significance in biological structures in particular when properties such as material stiffness and relaxation times are measured using uni-axial tensile tests, Wainwright et al., (1976). Such tests were performed by Bezares et al., (2010) on il-layers extracted from the nacre of H. rufescens. These results will be presented in chapter 7. In brief, the viscoelastic response of il-layers was characterized by fitting a linear Kelvin model to relaxation data, which rendered parameters of $E_0 = (0.668 \pm 0.088)$ GPa, $E_1 = (0.311 \pm 0.092)$ GPa, and $\eta = \tau E_1 = (42 \pm 0.37 \times 10^6)$ Pa·sec, where $\tau$ is the relaxation time constant. It was also found that chitin fibers were the major contributor to il-layer stiffness verifying the previous suggestion by Weiner et al., (1983) that the chitin core might serve a mechanical function as the structural framework of il-layers. The significance of the connectivity between individual chitin fibers, essential for the transfer of load across samples which included thousands of tile imprints, was briefly discussed but not specifically investigated. Here the issue of fiber-fiber connectivity and its effect on the mechanical response of il-layers is addressed. The current finding that chitin fibers are uni-axially aligned indicates that the elastic
and viscoelastic properties of il-layers should be orthotropic. The alignment might also have a measurable effect on the stiffness and toughness of nacre at the macro scale due to the key role of chitin in providing il-layer stiffness and the importance of il-layers to the mechanical response of nacre.

In what follows a series of SEM images documenting the successive removal of protein from il-layers by alkaline and proteolytic treatments, which expose a structural core of uni-axially aligned chitin fibers, are presented. The growth of mineral bridges through the chitin core specifically and the porosity of il-layers are discussed in terms of these new findings. It is proposed that the alignment of chitin fibers normal to the shell growth direction as is presented herein for the gastropod *H. rufescens* may also be found in other classes of mollusks. In Chapter 7, the implications of fiber alignment within single il-layers to the mechanical response of nacre will be discussed in terms of a more complete constitutive model for interlamellar layers.

### 5.2 Results

#### 5.2.1 Chitin Content

The alkaline treatment of bulk demineralized biopolymer framework material leaves completely translucent samples as shown in (Fig. 5.2a) where an estimated 80 dry wt% of organic material has been removed from the as-demineralized material. Epifluorescence imaging of translucent il-layers stained with Calcofluor White show strong fluorescence of entire sheets as in (Fig. 5.2b).
Figure 5.2: (a) The translucent material remaining after alkaline peroxidation has removed \(~80\) dry wt\% of organic material. (b) Calcofluor White staining of il-layers after alkaline treatment indicates that the remaining material is chitin.

5.2.2 Chitin Core Structure

Following Alkaline Treatment

Following the partial removal of protein by alkaline treatment alone, il-layers appear perforated with holes generally appearing round with diameters in the range of 10-50 nm as shown in (Fig. 5.3a). The size and distribution of these holes over regions that in some cases span square millimeters for the most part are fairly uniform. In some regions of such samples the holes take on an oblong shape with their longer axes aligned as evident in (Fig. 5.3b). A greater degree of protein removal appears to have taken place in these regions where the holes look more like gaps between parallel fiber-like structures.

Following Alkaline and Proteinase K Treatments

The additional removal of protein with Proteinase K reveals entire sheets of uni-axially aligned and densely packed fibers as evident in (Fig. 5.4a). In regions where sheets are slightly pulled apart laterally it is seen that the fiber bundles consist of \(~10\) nm diameter fibers bound to each other laterally but clearly uni-axially aligned. What were previously oblong holes now are clearly seen to be gaps between
fibers which still maintain some lateral connectivity as seen in (Fig. 5.4b).

Larger, and round, rather than oblong holes consistently of $\sim 100$ nm in diameter appear in some regions and are approximately 2-3 $\mu$m apart though it is difficult to judge their precise spacing due to the sheets being folded. Along the borders of the holes, fibers do not appear frayed but seem to form continuous rings as evident in (Fig. 5.5).

**Fiber Alignment**

Images of multiple sheets from numerous il-layers show that fibers are co-aligned across multiple sheets. Having taken into consideration the direction of the samples with respect to the growth direction of the shell it is found that the bundles in all layers are normal to the growth direction as noted in (Fig. 5.6).
Figure 5.4: SEM image of il-layers after alkaline and proteolytic treatments. (a) The sheets can appear to consist of densely packed uni-axially aligned fiber bundles. (b) Where sheets have been pulled apart laterally it becomes clear that they consist of uni-axially aligned and laterally bound single fibers.

Figure 5.5: SEM image showing (a) ~100 nm diameter round holes in il-layers having undergone alkaline treatment alone which are spaced ~2-3 µm apart. (b) A close up of these larger holes shows that their edges are not frayed or oblong but instead form closed rings.
Figure 5.6: SEM images of il-layers having undergone both alkaline and enzymatic treatments. (a) Fibers in four consecutive il-layers are lined up in parallel. The red line in the il-layer plane is normal to the shell’s growth direction. (b) Three chitin core sheets lie on top of each other with their respective fibers aligned. The red line in the il-layer plane is normal to the shell’s growth direction.
Chapter 6

Discussion: Framework Architecture

6.1 Identification of a Chitin Core Within Interlamellar Layers

It has been estimated that approximately 75-80 dry wt% of the organic matrix comprising il-layers consists of acidic glycoproteins, Addadi and Weiner, (1985). Alkaline peroxidase treatment of il-layers removes $\sim$80 dry wt% of organic material and leaves behind il-layers which strongly fluoresce under Calcofluor White staining. Calcofluor White preferentially labels $\beta$-1-4 polysaccharides such as chitin indicating that the remaining material is in fact chitin, in particular considering the amount of protein removed. The translucent appearance of alkaline treated samples (see Fig. 5.2a) is quite similar to that of the chitin framework exposed in jumbo squid beaks having undergone the same alkaline peroxidase treatment, Miserez et al., (2008). To clearly expose individual chitin fibers, as seen in (Fig. 5.4b), requires the successive alkaline and enzymatic degradation of protein in il-layers. Individual fibers are not as clearly visible in samples having undergone alkaline treatment alone (Fig.5.3a). It
was previously noted from AFM images of protein removal by enzymatic treatments alone which included both trypsin and Proteinase K where insufficient to remove all organic material around individual fibers. It has also been noted after alkaline hydrolysis alone protein again remains bound to chitin Zents et al., (2001). The finding here that it takes both enzymatic and alkaline treatments in succession could suggest that il-layers might include proteins that play two distinct roles. Some proteins might play a strictly biochemical role being involved in tile nucleation and growth as described in the last chapter. The second group of proteins, perhaps even a single protein, might serve a structural role as the binding between chitin fibers; this would be vital considering the significance of fiber-fiber connectivity to chitin core mechanical response. This will be discussed in detail in Chapters 7 and 8.

6.2 Porous Structure of Interlamellar Layers

Previous AFM images of il-layers extracted from H. rufescens nacre by demineralization and proteolitically treated show a porous appearance where pores are approximately round and with diameters of ∼50 nm (Schaffer et al., (1997); Bezares et al., (2008)); these AFM images however span only a few microns. SEM imaging of alkaline peroxidase treated il-layers provide much sharper images, making clear the size and distribution of pores which are 10-50 nm wide, in agreement with the findings of the aforementioned AFM studies (see also Fig. 5.3a). With the further removal of protein by Proteinase K treatment ∼10 nm diameter chitin fibers are exposed which are connected laterally at points ∼200 nm apart (Fig. 5.4b). What previously appeared to be pores are in fact aligned gaps between these fibers and are no longer round but oblong in shape with shorter and longer axes having lengths of approximately 10 nm and 200 nm, respectively. That in some cases, such as in (Fig. 5.4a), the fibers are densely packed together, hiding the pores, may be an artifact of the drying process during which pores may have collapsed due to capillary forces.
The randomly orientated fibers previously imaged via AFM are now entirely gone with the complete removal of protein Bezares et al., (2008), suggesting that some of the fibers in those studies might have been protein.

### 6.3 “Mineral Bridges” and “Major Connections”

Tile surfaces are covered with nodules (Fig. 6.1a) which have been called “mineral bridges” as it was proposed that they formed continuous mineral connections between tiles across il-layers, Schaffer et al., (1997). Mineral bridges have an approximate size of 10-50 nm and are longer along one direction than the other as in (Fig. 6.1a), where the insert shows that their lengths also have a certain amount of alignment similar to what is found in il-layer pores (Fig. 5.3b). Recent SEM imaging of nacent tiles growing in gastropod nacre has revealed that mineral bridges result from mineral growing into il-layer pores but not fully traversing them to form continuous connections, (see Fig. 6 in Checa et al., (2011)). Tiles do have a central protein rich region which can be exposed by alkaline treatment (Fig. 6.1b). These regions are the nucleation points on pre-existing tiles where the mineral for a new growing tile first traverses the il-layer above it, upon which the new tile grows (Fig. 6.2a). The protein rich regions have a diameter of \( \sim 100 \) nm. To differentiate between true tile-tile connections and mineral bridges true connections have now been called “major connections”, Checa et al., (2011). A comparison between the number and size of il-layer pores i.e. 10-50 nm as in (Fig. 5.3a) and the number and size of mineral bridges on tiles (Fig. 6.1a) shows that they are similar in size and distribution, supporting the finding that pores serve as locations into which mineral grows during tile formation, Checa et al., (2011). The larger holes in (Fig. 5.5), with \( \sim 100 \) nm diameters, spaced at least 2-3 \( \mu m \) apart closely match those of major connections. These holes are associated with the chitin exposure discussed in Chapter 4 which led to preferential labeling at tile-imprint centers with CW and FITC-WGA. These
Figure 6.1: SEM images of cleaved nacre having undergone a light alkaline treatment to remove surface protein. (a) Tile surfaces are covered with $\sim$10-50 nm diameter “mineral bridges” as described by Schaffer et al., (1997) which are not circular but are longer along one direction. The insert shows that the mineral bridges are aligned along the direction of their lengths. (b) The lighter regions at the center of tiles with diameters of $\sim$100 nm are the locations of the “major connections” described by Checa et al., (2011).

holes are most likely the points of traversal of growing tiles through the il-layer chitin core.

6.4 Fiber Orientation in Intact Nacre

The surface texture of tiles may alternatively be interpreted as being the remaining imprints of il-layers after they have been removed as in (Fig. 6.1) where an alkaline treatment was used. A schematic drawing of the resulting surface texture is shown in (Fig. 6.2b). Tile surface texture reveals that within intact nacre, fibers are not pressed together in sheets as in (Fig. 5.4a) but are in fact configured with gaps and the fiber-fiber connectivity that leaves pores with dimensions and arrangement close to what is seen in (Fig. 5.3b).

Organic framework tissue can deform substantially after being extracted by demineralization as for example in (Fig. 5.4a) where fibers are closely packed in one region and spread apart in another. Deforming il-layers extracted from demineralized
**Figure 6.2:** A schematic drawing of the tile nucleation and growth process showing the differences between mineral bridges and major connections, and the remaining surface texture following il-layer removal. a) The smaller mineral bridges (∼10-50 nm wide) grow into il-layer pores also (∼10-50 nm wide) but do not form continuous links between tiles. On the other hand major connections (∼100 nm wide) completely traverse il-layers forming wide continuous mineral connections between tiles. b) Tile surface texture remains after tile lamellae have been cleaved apart and il-layers removed; the imprint at the center of the tile marks the previous location of a major connection.

nacre would likely result in layer “damage” in the form of pore coalescence or fiber breakage. An analysis of extracted il-layers undergoing significant deformation would require some consideration of these kinds of effects. Within intact nacre however il-layers are completely confined by surrounding mineral. From il-layer imprints on tiles such as in (Fig. 5.4 and Fig. 5.6b) an estimation of fiber orientation within intact nacre can be made. Measuring the angle $\phi$ as shown in (Fig. 6.3), between lines diagonally across the points of lateral connection between fibers results in $\phi = 30 \pm 5^\circ$.

**Figure 6.3:** A drawing describing the angle formed by lines drawn diagonally across the points of connection between chitin fibers. From images such as (Figs. 5.4 and 5.6b) the angle $\phi$ is estimated to be $30 \pm 5^\circ$. 

76
6.5 Chitin Fiber Orientation with Respect to the Direction of Shell Growth

In the nacre of two classes of mollusks, bivalves and cephalopods, chitin fiber b-axes have been found to lie normal to the growth direction of the shell within il-layers (Wada, (1958); Weiner et al., (1983)). X-ray and electron diffraction techniques were used in those studies, which involved imaging small fragments of il-layers extracted from pieces of shell. As such, the alignment of chitin fibers with respect to the direction of shell growth, and the alignment of fibers in multiple consecutive il-layers, could not have been investigated. These two limitations would have been encountered in the Cryo-TEM work of Levi-Kalisman et al., (2001), as well, since il-layer fragment suspensions were used in their study. In the present study, SEM imaging following alkaline and proteolytic treatments has permitted chitin fiber alignment to be studied over large areas compared to previous XRD, ED, and TEM studies. Furthermore the orientation of individual il-layers is maintained (Fig. 5.6) which is not possible when using il-layer sections or fragment suspensions because layers lose their initial and relative orientations. Beginning with large samples of demineralized nacre, where the growth direction of the shell was first noted, it is found that chitin fiber alignment in all layers is normal to the direction of shell growth, adding now gastropods to the classes of mollusks where chitin fibers are aligned in this manner. Considering the manner of shell growth in all classes of mollusks whereby tiles are added at the edge of the shell in rows parallel to the edge (i.e. normal to the direction of shell growth), it would seem natural for the chitin core to also grow at the shell edge by the addition of fibers in the same parallel manner. It’s suspected that the alignment of fibers normal to the direction of shell growth observed in this study for the gastropod *H. rufescens* is to be found in most if not all classes of mollusks. A summary of the current observations of chitin fiber alignment within single il-layers is presented in (Fig. 6.4) below, where they are compared and
contrasted to previous chitin core structural models.

Figure 6.4: A schematic drawing of chitin fiber alignment within an interlamellar layer according to two previously proposed models and the current observations. (a) Fibers are uni-axially aligned and no pores are located between them, Levi *et al.*, (2001). (b) Fibers are randomly oriented with the spaces between fibers taking on the form of pores, Bezares *et al.*, (2010). (c) Based on the current findings chitin fibers are uni-axially aligned, pores are formed by the gaps between fibers that are laterally connected only at certain points, and all fibers are oriented normal to the growth direction of the shell.

6.5.1 Implications for Mechanical Response

Considering that chitin fibers are oriented normal to the direction of shell growth, and in view of the finding by Bezares *et al.*, (2010) that chitin is the principle contributor to the stiffness of il-layers, (to be discussed in chapters 7 and 8), it is to be expected that the organic framework has a greater tensile stiffness in the direction normal to that of shell growth. In Bezares *et al.*, (2010) the structural integrity of il-layers was entirely lost following the degradation of chitin with chitinase, while the stiffness was hardly affected by the removal of most but not all protein with Proteinase K alone. An il-layer placed in uni-axial tension along a direction normal to the fiber direction would be placing the il-layer protein matrix in tension without loading chitin fibers; this is akin to testing an il-layer where chitin has been removed but protein remains. This suggests that the stiffness of il-layers tested in tension in a direction transverse to the chitin fiber direction, i.e., along the direction of shell growth, should be vanishingly small.
Being that single tiles in “brick-wall” like tile lamellae do not have a preferred orientation and that individual tiles behave as isotropic solids, Bezares et al., (2011) suggested that tile lamellae and thus nacre could be considered as transversely-isotropic. Interlamellar layers shown in this study to be orthotropic, with chitin fibers uni-axially aligned, make nacre a layered composite of alternating isotropic and orthotropic lamina, in which all orthotropic layers (il-layers) are co-aligned. As such nacre as a whole might exhibit an orthotropic response. For example, a mode-I type crack propagating along the growth direction of the shell, in a plane normal to il-layers, should place chitin fibers in tension, while a mode-I crack running normal to the growth direction and normal to il-layers should split the chitin fibers. The contribution of chitin fiber alignment to this kind of response has yet to be quantified.

6.6 Framework for an Interlamellar Layer Viscoelastic Model

Based on the initial findings of Bezares et al., (2010), and on the novel findings reported on herein, models are proposed for the biopolymer il-layers as sketched in (Figs. 6.5a,b). Figure 6.5a is implies that the structural chitin fiber network

![Figure 6.5](image)

**Figure 6.5:** Models (a) and (b) representing either transversely isotropic (randomly oriented fibers) or orthotropic (aligned sets of fibers) structures, respectively. (c) A Kelvin viscoelastic model used by Bezares et al., (2010) fitted to their data on mechanical testing of il-layers.

is composed of, essentially, in-plane extended and connected, randomly oriented,
fibers. Note that the fibers should be seen as extended whereas the figure indicates only their orientation; this was the scenario envisioned by Bezares et al., (2010) and used in their analysis. Figure 6.5b, on the other hand, depicts long extended fibers, but with aligned orientation. This is orthotropic in the plane as discussed above. If the fibers are aligned, the angle $\phi$ would be small, with $\phi \to 0$ if the fibers are perfectly aligned. The detailed development of viscoelastic constitutive models for both structural motifs is underway.

The material constitutive response has been confirmed by Bezares et al., (2010) to be viscoelastic. To be specific they successfully fit a linear Kelvin model shown in (Fig. 6.5c) to their relaxation data, finding that $E_0 = (0.668 \pm 0.088) \text{ GPa}$, $E_1 = (0.311 \pm 0.092) \text{ GPa}$, and $\eta = \tau E_1 = (42 \pm 0.37 \times 10^6) \text{ Pa} \cdot \text{sec}$, where $\tau$ is the relaxation time constant.

### 6.7 Summary and Conclusions

Direct evidence has been provided via SEM imaging that a core of chitin fibers exists within il-layers of gastropod nacre arranged in parallel not only within individual layers but in all layers and, quite surprisingly, normal to the growth direction of the shell. The parallel arrangement within single il-layers fully supports the currently accepted il-layer structure model suggested by Levi-Kalisman et al., (2001). Exposing the chitin core requires protein removal by consecutive alkaline and enzymatic treatments. Closed circular holes with diameters of $\sim 100$ nm are frequently found with a center to center spacing that suggests that these may be the locations where major connections (i.e., mineral bridges) traverse single il-layers by spreading apart rather than tearing individual fibers. The findings of fiber alignment have a number of important implications to the mechanical properties of nacre as it is hypothesized that the stiffness of single il-layers should be orthotropic, with the stiffness transverse to the fiber orientation being vanishingly small in comparison to that
along the fiber length. To ascertain this would require performing a comprehensive investigation as outlined in Bezares et al., (2010), where samples would be tested in tension along the direction of shell growth; this could be achieved using a more sensitive experimental approach such as is described in Opdahl and Somorjai, (2001) for PDMS films, but this is beyond the present scope and is left for future investigation. The findings of chitin fiber alignment in relation to growth direction for gastropod nacre are consistent with previous similar findings for bivalve and cephalopod nacres, which suggests that this may be the case for other classes of mollusks. This should be investigated by a comparative study using the alkaline and proteolytic treatments described herein, which would require the SEM imaging of chitin fiber orientation over large length scales. Successful modelling remains an open area of investigation of great importance for bio-duplication and novel materials development.
Chapter 7

Results: Framework Mechanical Response

7.1 Introduction

It is of vital importance to document, analyze, and model the properties of the biopolymer matrix if a quantitative understanding of nacre’s composite mechanical performance is to be possible. To date the matrix (i.e. the framework) behavior has been only qualitatively (and in an ad-hoc manner) modeled or indirectly (and partially) extracted from simple mechanical tests on intact mollusk shell pieces. Thus little information is known about the actual mechanical response of the framework itself even though such information would have distinct implications for framework structure and connectivity, including connectivity imparted by chitin-protein interactions. Here a first attempt has been undertaken to provide a direct experimental probe of the framework’s response to mechanical loading and to formulate a viscoelastic model based on the data that is adequate for quantitative analysis of intact nacre response to quasi-static monotonic and cyclic forces.

The results are interpreted within the perspective of what is known biochemi-
cally about the framework’s molecular make up, such as for example that it contains a chitin core. The results reveal quite interesting and surprising insights into the framework behavior and provide sufficient quantitative data to formulate and calibrare a viscoelastic model. It is found that the chitin core does indeed appear to dominate the mechanical response and that the response is weakly viscoelastic and basically of an elastic nature. For example, relaxation time constants of order $\tau = 140 \pm 4$ sec from in-plane relaxation tests on completely demineralized and macroscopic sections of biopolymer framework are extracted. What is possibly surprising about such results is that the sections had a span that included 2000-3000 ceramic nacre tiles indicating that the framework is connected with a load-bearing capacity across multiple nacre tiles. The results are consistent with the view that the core has a continuous chitin network where chitin constitutes approximately 6% of the framework volume. Conclusions concerning the role of chitin as the major structural constituent of the biopolymer framework are supported by additional studies following enzymatic digestion of protein and protein plus chitin.

Of particular importance to the present study are the reports of chitin in the biopolymer frameworks of a number of mollusks, including *Haliotis rufescens* (Weiner *et al.*, (1980,83); Goffinet and Jeuniaux, (1979); Zentz *et al.*, (2001); Weiss *et al.*, (2006), and Bezares *et al.*, (2008)). Bezares *et al.*, (2008) succeeded in obtaining the first quantitative analysis in *H. rufescens* with the result that in the extracted framework the chitin content was in the range 6.4-6.9 dry wt%, consistent with the chitin contents reported by Goffinet and Jeuniaux, (1979) for other mollusk species. The assays cited so far report chitin content in wt% whereas for structural analysis estimates in vol% are needed. To date reports of stereology or TEM tomography studies of chitin within the framework of any mollusk have not been provided. This is addressed below. It should be noted, however, that in the intact shell nacre of *Haliotis rufescens* observed in this study the interlamellar layers are $\sim$20 nm thick. Also, Bezares *et al.*, (2008) show AFM images that identify chitin fibers with a
thickness of \( \sim 8 \text{ nm} \) and themselves layered so that within a 20 nm thick il-layer the chitin core is at least 16 nm in total thickness. Such information is vital for stereological estimates of vol%.

The current focus is on the elastic and viscoelastic behavior of the protein matrix/chitin fiber composite structure comprising interlamellar layers. Bezares et al., (2008) made estimates of the in-plane shear stiffness of interlamellar layers, which here is extended to the more general elastic, possibly viscoelastic, tensile response. Bezares et al., (2008) formulated a model for the structural response based on the notion that the chitin core provides the main structural reinforcement. Based on tensile tests conducted on intact shell sections, they estimated the in-plane modulus, \( G_p \), to be \( G_p \approx 0.3 \text{ GPa} \). This was additionally justified by a composite model. Their reasoning is followed here, where new tests are performed on the biopolymer framework itself to find the tensile modulus to be \( E \approx 0.98 \text{ GPa} \). The findings imply a strong degree of connectivity of the chitin core within the interlamellar plane, over spans that range over many tile imprints.

7.2 Results

7.2.1 AFM Observations and Analysis

Both high and low resolution scans, in tapping mode, were performed over areas ranging from 500 nm x 500 nm to 4 \( \mu \text{m} \) x 4 \( \mu \text{m} \). Images revealed the layered structure of demineralized tissues and the fibrous nature of the chitin core. In particular, imaging across the cross section of sections of the demineralized organic framework as it existed within the gauge sections of the tensile specimens described in the experimental methods was first performed. Figures 7.1 are phase images, all obtained in tapping mode, under fully hydrated conditions in a wet cell. Figures 7.1a-c show a progression of lower resolution scans of unfixed specimens digested
with trypsin or trypsin + Proteinase K. The highest resolution scan of (Fig. 7.1a), of a specimen that was digested in trypsin + Proteinase K, shows what appears to be a “porous” structure. Closer inspection, however, reveals embedded fibers (e.g. at the white arrow) and the fact that the layers contain a core of fibers. The fibrous core is also clearly revealed in (Fig. 7.1b) which is of a specimen digested in trypsin only. Individual fibers are visible, and that they are embedded in less organized organic material is evident. Thus the interlamellar layers are not “porous” per se but are composed of a fibrous core, or network, that is embedded in and sandwiched between less organized organic material. Individual fibers appear to have diameters in the range 5-10 nm and run for lengths of at least 1 µm, although to date their length distribution has not been quantitatively determined.

Fibers are delineated by their somewhat brighter shade, which in these phase images corresponds to their higher hardness as compared to the surrounding material. Quantitative stereology was performed to estimate the fiber volume fraction assuming the following. As noted above, the fibers are seen to have an average diameter of 8 nm and to be arranged in multiple layers within each interlaminar layer (see Fig. 8d of Bezares et al., (2008)). Thus at least 16 nm of the total thickness of 20 nm of an interlaminar layer, as in the intact shell, is composed of fibrous chitin. Areal stereology on 12 images of the resolution of (Fig. 7.1a) and 15 images with the resolution of (Fig. 7.1b) led to the estimate of area fraction of fibers in the range 7.7 ± 1%. Given that the “thickness fraction” of fibers is approximately 16/20 = 0.8, it was estimated that the volume fraction of fibers is in the range 0.8(7.7 ± 1)%, or 6.1 ± 0.8%.

The phase image of (Fig. 7.2d) indicates that the fibrous core indeed appears to continuously span across tile boundaries. Both the images contained in (Figs. 7.2a,b and 7.2c,d) also indicate a rather randomly oriented fibrous structure. The interpretation of these observations are consistent with the mechanical response reported on next.
Figure 7.1: AFM phase images obtained in tapping mode in a fully hydrated condition of demineralized framework. (a) Higher resolution scan of framework partially digested with trypsin and Proteinase K. Images reveal a fibrous core. (b) Lower resolution scan of framework tissue digested with trypsin only.

The general picture that emerges for the structure of the interlamellar layers is that of a nearly planar structural chitin core, with a 2-dimensional lay up, with fiber orientations that are uniformly distributed over spans that encompass multiple tiles. The core is sandwiched between, and infiltrated by, a less organized but highly functional mostly protein hydrogel. The macromolecular makeup of the proteins has been explored and mapped (e.g. Crenshaw and Ristedt, (1976); Nudelman et al., (2006); Bezares et al., (2008)), but is believed to contribute in only a minor way to the layer’s mechanical properties.

7.2.2 Elastic Response

Quasi-static tensile tests were performed on specimens prepared in all conditions described in Section 2.K.3; results for selected specimens are shown in (Fig. 7.3). Samples were tested at a strain rate of $10^{-4}sec^{-1}$, at 20 °C. In most cases a small, but finite, amount of axial stretching was required before the specimens bore measurable forces. This was attributed to the straightening of the overall bundle of
Figure 7.2: AFM topographical and phase images of demineralized framework. (a) - (d) are of biopolymer layers partially digested with trypsin and Proteinase K. (a,b) are a scan confined to a single tile imprint whereas (c,d) are from a scan spanning multiple tile imprints. White arrows indicate tile boundaries.

layers within the gauge section. Most specimens displayed a remarkable ability to sustain significant forces despite damage done to individual layers during the demineralization process. Thus it was noted that many of the individual layers remained intact and allowed meaningful properties to be extracted. The displacements associated with this straightening were subtracted from the total displacements and the net displacement used to compute the axial strain. The data was then compiled and plotted as described below. Axial strain was measured via the extensometer as seen
HR BioPolymer Tension

Figure 7.3: Quasi-static uni-axial stress vs. strain response of Fixed Dry (FD), Not-Fixed Dry (NFD), and Not-Fixed Wet (NFW) specimens. Tests were performed at a nominal strain rate of $10^{-4} \text{sec}^{-1}$, at 20 °C. D is the percent of interlamellar layers damaged during sample preparation. This was estimated based on visual inspection of samples just prior to testing. E is the tensile modulus of an individual biopolymer layer.

in (Fig. 2.9). Axial stress was computed by first estimating the number of layers contained in each sample from the measured overall specimen thickness and then by dividing the total axial force by the number of layers times the measured average interlamellar layer thickness, $t = 20 \text{ nm}$, and the sample width; these dimensions are detailed in (Fig. 2.6). This simple analysis assumes that the axial force is born essentially by the interlamellar layers. The stress vs. strain curves were initially nearly linear so that estimates of the axial modulus, E, was relatively straightforward. The elastic modulus was estimated and various values are listed on (Fig. 7.3) adjacent to the selected curves. For the sample data shown, these values covered a range $0.143 \text{ GPa} \leq E \leq 0.44 \text{ GPa}$. It was noted that the so extracted values did not correlate...
with the condition of the specimens, and thus the variations in modulus where attributed to the degree of non-engagement of some individual layers within the overall bundles. Non-engagement, it was envisioned, was due to either pre-damage of some individual layers or to the fact that some layers are longer than others and are not initially engaged as noted in Section 2.K.5. The process whereby longer layers may be engaged at larger strains is discussed below. Non-engagement, quantified by the parameter D, is designated alongside several of the curves by the symbol D in (Fig. 7.3), with D estimated in via following model analysis.

The SEM observations of Bezares et al., (2012) illustrate that over a span of an individual tile, and certainly over the span of multiple tiles, chitin fibers and fiber bundles take on what is approximately a uni-axial orientation as described in (Fig. 6.3). Moreover, the observations suggest that chitin fibers are in a strict planar arrangement. Thus a model of a roughly 2-dimensional uni-axial fibrous network was adopted to describe the composite stiffness. The stiffness was attributed to the chitin alone. Such a model, for example as found in Courtney, (2000) would estimate the composite stiffness as

\[ E \approx \kappa f E_f, \]  

(7.1)

where \( \kappa \) is a numerical factor empirically estimated at \( \kappa \approx 0.2 \), \( f \) is the volume fraction of chitin, and \( E_f \) is the axial modulus of chitin. Chitin assays presented in chapter 3, provide the estimated value \( f \approx 0.06 \). Based on (Nishino et al., (1999) and Steinbuchel and Rhee, (2005)) \( E_f \) is taken to be 80 GPa. With this, the composite stiffness is estimated to be

\[ E \approx .98 \text{ GPa}. \]  

(7.2)

On this basis it’s estimated that for the stiffest sample data shown in (Fig. 7.3), for instance, \( D = 1 - 0.44/0.98 = 0.46 \), or \( D \approx 54\% \). Other such estimates are listed on (Fig. 7.3). In this way it’s estimated that \( 54\% \leq D \leq 85\% \). There were indeed samples with greater degrees of pre-damage, but these bore nearly vanishingly small forces and were disregarded. To judge that such degrees of initial non-engagement
were reasonable, an attempt to directly observe layer damage was made as described next.

### 7.2.3 Failure Mechanisms and Sample Damage

Additional insight into the process of non-engagement and engagement at larger strains was obtained by stretching the specimens to larger tensile strains. A typical response of hydrated specimens is shown in (Fig. 7.4); the inserts within the figure are meant to aid in visualizing the process of individual layer rupture and engagement of initially longer layers.

![Quasi-static uni-axial stress vs. strain response of Not-Fixed Wet (NFW) specimens](image)

**Figure 7.4:** Quasi-static uni-axial stress vs. strain response of Not-Fixed Wet (NFW) specimens. Tests were performed at a nominal strain rate of $10^{-4} \text{sec}^{-1}$, at 20 °C. D is the percent of interlamellar layers damaged during sample preparation. This was estimated based on visual inspection of samples just prior to testing. E is the tensile modulus of an individual biopolymer layer as was done in connection with (Fig. 7.3).

As already noted, the initial stress vs. strain response was nearly linear as
was shown in (Fig. 7.3). At larger strains load drops were observed followed by reloading segments of the stress vs. strain curve. Often, but not always, the initial reloading slope was quite similar to the initial slope, as is the case shown in (Fig. 7.4). The modulus, computed as described above, is listed along with the estimated degree of non-engagement associated with these segments. At even larger strains, however, the slopes of the reloading segments of the stress vs. strain plots were lower and typically continued to decrease in magnitude as the specimen progressed to complete failure via rupturing. This phenomenology was interpreted as follows. Insert I, in (Fig. 7.4), schematically illustrates the initial situation where certain layers are taut, i.e. engaged, and others are non-engaged either because they are damaged, i.e. broken, or so much longer than the shorter ones as to render them initially non-engaged. Continued stretching leads to the rupture of the most highly stretched layers and load drops whereupon slightly longer layers are now engaged followed by load increases. This leads to the schematic scenario of insert II. Here some initially engaged layers are ruptured and initially longer ones are then engaged. Thus the overall stiffness remains similar. Upon further rupturing of engaged layers, the overall stiffness must decrease due to the exhaustion of available layers through continued rupture. This behavior is not consistent with intra-protein events such as domain unfolding, as observed during the stretching of titin or spectrin for example, that would tend to produce a more periodic “saw tooth” like response (e.g. Rief et al., (1997,99); Law et al., (2003); Zhu and Asaro, (2008)). Reloading segments that have similar slopes and peak stresses to the initial segment are rare and, when they occur at all, are confined to the segment immediately following the initial loading segment. Instead the behavior is consistent with the scenario outlined in analyzing (Fig. 7.3) and depicted in the inserts to (Fig. 7.4). Thus although these results so far do not definitely establish it, they are consistent with the structural scenario outlined above and with an interlamellar layer modulus of $E \approx 98$ GPa. Additional perspective is provided by the study of time-dependent response as described next.
7.2.4 Viscoelastic Response

Layer stiffness is associated with a chitin framework that would display an essentially elastic response. To provide further confirmation of this, load relaxation after an initial straining to a fixed tensile strain was followed and analyzed using a standard linear viscoelastic model as shown in the insert within (Fig. 7.5). In fact, it was found that the simple standard linear model (Doi and Edwards, 1986) provided an adequate fit. Relaxation time constants, \( \tau = \eta/E_1 \), were found in the range, \( \tau = 140 \pm 4 \) sec. The fitted moduli, also defined in the insert, are listed in (Fig. 7.5) adjacent to selected curves. Scaled moduli, \( E_0 \) and \( E_1 \), were computed by

\[
\begin{align*}
R_1 & : E = .148 \text{ GPa} \\
R_2 & : E = .21 \text{ GPa} \\
R_3 & : E = .276 \text{ GPa} \\
R_4 & : E = .202 \text{ GPa} \\
R_5 & : E = .195 \text{ GPa} \\
\end{align*}
\]

\[
\begin{align*}
E_0 & = 0.114 \text{ GPa} \\
E_1 & = 0.034 \text{ GPa} \\
E_0 & = 0.134 \text{ GPa} \\
E_1 & = 0.061 \text{ GPa} \\
E_0 & = 0.134 \text{ GPa} \\
E_1 & = 0.061 \text{ GPa} \\
E_0 & = 0.034 \text{ GPa} \\
E_1 & = 0.061 \text{ GPa} \\
E_0 & = 0.114 \text{ GPa} \\
E_1 & = 0.034 \text{ GPa} \\
E_0 & = 0.114 \text{ GPa} \\
E_1 & = 0.034 \text{ GPa} \\
E_0 & = 0.114 \text{ GPa} \\
E_1 & = 0.034 \text{ GPa} \\
E_0 & = 0.577 \text{ GPa} \\
E_1 & = 0.403 \text{ GPa} \\
E_0 & = 0.134 \text{ GPa} \\
E_1 & = 0.061 \text{ GPa} \\
E_0 & = 0.134 \text{ GPa} \\
E_1 & = 0.061 \text{ GPa} \\
E_0 & = 0.134 \text{ GPa} \\
E_1 & = 0.061 \text{ GPa} \\
E_0 & = 0.134 \text{ GPa} \\
E_1 & = 0.061 \text{ GPa} \\
E_0 & = 0.134 \text{ GPa} \\
E_1 & = 0.061 \text{ GPa} \\
E_0 & = 0.577 \text{ GPa} \\
E_1 & = 0.403 \text{ GPa} \\
E_0 & = 0.134 \text{ GPa} \\
E_1 & = 0.061 \text{ GPa} \\
E_0 & = 0.134 \text{ GPa} \\
E_1 & = 0.061 \text{ GPa} \\
E_0 & = 0.134 \text{ GPa} \\
E_1 & = 0.061 \text{ GPa} \\
\end{align*}
\]

**Figure 7.5**: Load-relaxation stress vs. time response of Not-Fixed Wet (NFW) specimens. Tests were performed at a nominal strain rate of \( 10^{-4} \text{sec}^{-1} \), at 20 °C. D is the percent of interlamellar layers damaged during sample preparation. This was estimated based on visual inspection of samples just prior to testing. E is the tensile modulus of an individual biopolymer layer as was done in connection with (Fig. 7.3). The insert in the upper right corner illustrates the standard linear solid model used for analysis with its elastic and viscous elements.
multiplying $E_0$ and $E_1$ by the ratios of the ideal composite layer with a modulus given by eq. (7.2), 0.98 GPa, to the particular modulus of each specimen, i.e. by $0.98 \text{ GPa} / (E_0 + E_1) \text{ GPa}$. These were found to lie within the range $E_0 = 0.668 \pm 0.088 \text{ GPa}$ and $E_1 = 0.311 \pm 0.092 \text{ GPa}$. This leads to an estimate of viscosity $\eta = 42 \pm 0.37 \times 10^6 \text{ kPa-s}$. This range for $\eta$ certainly suggests an essentially elastic response. It’s noted that the analysis of visco-elastic behavior is similar to that recently employed by, for example, Nagayama et al., (2007) on smooth muscle cells (SMC’s). They found relaxation times in the range $141 \text{ s} \leq \tau \leq 384 \text{ s}$ in rat aortic SMC’s. For such cells, however, the viscosity is much lower due to the much lower elastic moduli $E_0$ and $E_1$. On the other hand, the tissue of the framework is not as stiff as higher chitin content structures such as the squid beaks studied by Miserez et al., 2008.

### 7.2.5 Response Following Framework Layer Degradation

Effects of enzymatic digestion with trypsin plus Proteinase K or alkaline peroxidase was found to depend on the extent and choice of such treatments. Trypsin plus Proteinase K digestion was found to remove significant amounts of protein, as seen, for example, in (Fig. 7.6b). Measurements of protein weight loss were estimated by assuming that 80 dry wt% of the total biopolymer weight was protein, as the earlier amino acid assays had established. Following digestion with trypsin plus Proteinase K for 18 hrs, for example, it was found that between a minimum of 28.4 dry wt% to as high as 88.5 dry wt% of protein was lost. Removing protein, in fact, was found to reveal the fibrous core that it is believed is mainly responsible for the structural integrity of the interlamellar layers. Severe digestive treatments such as performed using alkaline peroxidase, as described in Section 2.D, removed $\sim 92$ dry wt% of the protein and produced tissue samples lacking any measurable structural integrity. This is consistent with the view that the connectivity of the chitin network is due to protein-chitin association, so that when nearly all protein is lost the
structural connectivity is also lost.

Figure 7.6: Images of tensile specimens with different protein contents. a) As demineralized framework tissue; the thickness is essentially the same as for the mineralized shell sample. b) Following protein removal with Proteinase K the thickness is significantly reduced. c) Following protein removal with alkaline peroxidase and with almost all protein gone, the sample is entirely translucent.

Digestion with trypsin plus Proteinase K alone had no measurable effect on stiffness as noted earlier for both stress relaxation and quasi-static tensile response. Treatments with chitinase, however, produced very significant degradation in stiffness. This provides further evidence for the view that it is the chitin that provides the structural integrity of the interlamellar layers in *H. rufescens*.

7.2.6 An Interlamellar Layer Constitutive Model

The analysis of the results of tensile testing and relaxation tests lead us to propose the constitutive relation, based on a standard linear solid model for a viscoelastic material, *viz.*

\[
\sigma + \tau \dot{\sigma} = \bar{E}_0 \epsilon + \eta \left( \frac{\bar{E}_0 + \bar{E}_1}{\bar{E}_1} \right) \dot{\epsilon},
\]

\[
\tau = \frac{\eta}{\bar{E}_1},
\]

(7.3)
Figure 7.7: Quasi-static uni-axial stress vs. strain, response of Not-Fixed Wet (NFW) specimens of *H. rufescens* as demineralized and subjected to enzymatic digestion with trypsin and Proteinase K for 18 hrs followed by digestion in chitinase for durations ranging from 48-72 hrs. Tests were performed at a nominal strain rate of $10^{-4} \text{sec}^{-1}$, at 20 °C.

where the parameters are described in the insert to (Fig. 7.5). In eq. (7.3) $\tau$ is the relaxation time, $\eta$ the viscosity, and $E_0$ and $E_1$ the elastic element moduli. Superposed dots indicate a partial time derivative, $\partial/\partial t$. The constitutive law is expressed in the usual 1-dimensional form where $\sigma$ and $\epsilon$ are the axial stress and strain, respectively. The results for $\bar{E}_0$, $\bar{E}_1$, and $\tau$ were given above and for summary they are listed as

$$
\bar{E}_0 = 0.668 \pm 0.088 \text{GPa} \\
\bar{E}_1 = 0.311 \pm 0.092 \text{GPa} \\
\tau = 140 \pm 4 \text{s.}
$$

(7.4)

It should be noted, however, that the relation in eq. (7.3) is meant to apply to in-plane stress components as illustrated in (Fig. 7.8). Transverse normal stresses such as shown as $\sigma_2$ may well behave in a very different manner.
Figure 7.8: Schematic of layer indicating normal and shear stresses described by the constitutive model.
Chapter 8

Discussion: Framework Mechanical Response

8.1 Organic Framework Mechanical Response

In this study, for the first time the constitutive properties of the biopolymer framework in a mollusk shell, *viz.* that of *Haliotis rufescens* have been directly documented. Other attempts have been either indirect such as what was presented in Chapter 4, (2008) where the shear stiffness was extracted from measurements of the tensile response of intact shell sections *via* an analytic shear-lag model, or entirely qualitative such as the AFM probes of Mohanty *et al.*, (2008) that only demonstrated that the layers are strongly bound to the ceramic tiles. Here however specimens, of macroscopic dimensions, comprised entirely of demineralized biopolymer framework have been tested. These findings show that the framework is structurally connected, *via* the chitin core network, over distances that span multiple tiles. The framework is essentially elastic in that its viscoelastic response is characterized by a rather high viscosity, \( \eta = 42 \pm 0.37 \times 10^6 \) kPa·s and long relaxation times, \( \tau \approx 140 \) s. The instantaneous in-plane modulus is found to be approximately \( E \approx 0.98 \) GPa.
Although the deduction of this modulus is somewhat circumstantial, as it is based on the simple composite model of eq. (7.2), the consistency with the totality of data along with the structural basis obtained from chemical assays and AFM analysis provide credibility for this proposal. The biopolymer layers are, therefore, much more than a mere adhesive, but rather a roughly planar structural member with both significant in-plane shear and tensile stiffness.

The proposal here that it is a chitin network that provides the structural integrity of il-layers is supported by the observations that enzymatic digestion with trypsin plus Proteinase K had little or no effect on mechanical response whereas digestion with chitinase led to a nearly complete loss of structural integrity. Moreover, although there are as yet to date no comparative studies of the biopolymer framework of mollusks, it should be noted that these results on viscoelastic response are generally consistent with those reported for chitosan films by Lopez da Silva and Santos, (2007) and with the elastic response reported for chitin extracted from crab shells by Niwa et al., (1998). In fact, when reinterpreted within the framework of the linear viscoelastic model of eqs. (7.3) it is found that the time constants found by Lopez da Silva and Santos, (2007) for chitosan films would be in the range $40 \text{ sec} \lesssim \tau \lesssim 50 \text{ sec}$ as compared to the finding of $\tau = 140 \pm 4 \text{ sec}$ for the interlamellar layers of $H. \ rufescens$. That the measured time constants are approximately three times as large indicates the somewhat larger importance of a viscous constituent. This could be due to the protein involved in the connectivity of chitin within the chitin core as seen in (Fig. 5.3) and in particular in (Fig. 5.4b). This would suggest load transfer among chitin fibers \textit{via} a \textit{“shear lag” mode}. If such a model is further confirmed it would readily lead directly to quantitative modeling that might then be validated \textit{via} experiments as were proposed in the concluding remarks in Chapter 5.

In this manner it may be appreciated how the sort of mechanical measurements and analysis reported on herein are directly coupled to biochemical measurements and analysis.
Additional consistency is obtained via the simulation of the overall structural response of intact nacre. A finite element model was accordingly developed as shown in (Figs. 8.1a,b). The relative dimensions regarding the assumed hexagonal tile thickness and diameter to the thickness of the biopolymer layers was accounted for in this model. It was further assumed, for the small strains that were imposed, that the interface between the CaCO$_3$ tiles and the interlamellar and intertabular layers were strongly bound as suggested by the qualitative AFM probes conducted by Mohanty et al. (2008). Accordingly, for the purposes of this study, the nonlinear effects associated with intertabular layer separation or interlamellar sliding as studied by Evans et al. (2001) were not investigated. Moreover, any sort of “interlayer” between the ceramic tiles and the biopolymer layers as described, for example, by Nassif et al. (2005) as amorphous layers in their studies of the abalone Haliotis laevigata were not included in the model. Probing of the Haliotis rufescens nacre via nanoindentation did not, in fact, reveal any such layers. Nanoindentation test results will be presented in Chapter 9, which show that single CaCO$_3$ tiles are isotropic with a Poisson’s ratio of $\nu = 0.3$. In the following model, the Young’s modulus of a single tile was assumed to be $E_c = 100$ GPa (Vincent 1982). Nanoindentation results presented in Chapter 9 will require that this value be adjusted to $E_c = 90$ GPa. A more sophisticated numerical model, which takes into account both the elastic and viscoplastic responses of single tiles, and an adjusted Young’s modulus of 90 GPa, will be presented in Chapter 10. In the present model, for the biopolymer layers initial estimates of $E = 0.98$ GPa and Bezares et al.’s (2008) estimate for the in-plane shear modulus $G_p = 0.3$ GPa were made but it was immediately noted that, if isotropy is assumed, these estimates would just violate the elastic stability requirement (see e.g. Asaro and Lubarda 2006)

$$-1 < \nu = \left( \frac{E}{2G_p} - 1 \right) < \frac{1}{2}, \quad (8.1)$$

since this yields $\nu \approx 0.63$. Accordingly the adjustment $G_p \rightarrow 0.327$ GPa, was made which is entirely within the limits of accuracy of Bezares et al.’s (2008) original
estimates. This yielded $\nu \approx 0.498$ which satisfies the stability requirement of eq. (8.1). This, in turn, leads to the deduction that the biopolymer layers are nearly incompressible. With this adjustment the resulting computed modulus for nacre was extracted from the predicted stress vs. strain curve, shown in (Fig. 8.1c), as $E = 80$ GPa, which is in excellent agreement with Bezares et al.’s (2008), which is also $E_{\text{exp}} \approx 80$ GPa. In passing it should be noted that the finite element model shown in (Figs. 8.1a,b) is readily extended to include all the features noted above such as interface separation or the presence of interlayers. Further studies may, indeed, be directed at exploring the associated non-linear effects as well as the phenomenology of structural degradation caused by extreme mechanical loading.

In connection with (Fig. 7.3) it was recalled that the results for elastic modulus did not correlate with the states of hydration. This too was believed to be consistent with the proposal that the chitin core imparts an essentially elastic con-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fem_model.png}
\caption{(a)-(b) FEM model of nacre which includes the actual ratios of thickness and tile diameter to biopolymer layer thickness. The initial linear stress vs. strain response predicted by the simulations is shown in (c).}
\end{figure}
stitutive character to the interlamellar and most likely the intertabular layers. It was noted, however, that the progressive failure and engagement process described earlier was observed only in hydrated specimens.

These results have provided a clear description of a viscoelastic constitutive relation for the organic framework of nacre in *Haliotis rufescens*. Here it is proposed that the structural integrity of the framework is primarily due to the chitin core that appears to be connected over distances spanning many ceramic tiles. This picture raises questions as to the manner of this connectivity which are as yet unexplained such as the mechanisms of load transfer between fibers. The SEM observations and discussion presented in Chapters 5 6 would suggest that this could be by shear lag involving a possible fiber-fiber “bonding” protein. The relaxation and creep tests both indicate that the demineralized framework is indeed capable of supporting significant and sustained in-plane force without rupture.

In Chapters 6 and 7 which follow, nanoindentation test results of nacre under various states of hydration and protein removal will be presented. The initial goal of such testing was to investigate the mechanical response of interlamellar layers within intact nacre and to try to relate these to what has just been reported. The significant role of the intratabular organic matrix, first documented by Watabe, (1963), on the elastic and viscoplastic properties of individual tiles will be discussed. Also presented will be quantitative results which for the first time show that tiles are isotropic. To sort out the contributions of nacre’s various organic and mineral components to its composite viscoelastic response, a numerical model will be described. The model takes into account the elastic and time dependant properties of both the interlamellar layers and tiles.
Chapter 9

Results: Tile Structure and Mechanical Response

9.1 Introduction

Nacre tiles often respond, via diffraction, as single crystalline tablets but are known to contain an organic intratabular it matrix, readily seen in (Fig. 9.1a). Recently, for example, Rousseau et al., (2005) performed AFM imaging, in tapping mode, and TEM dark field imaging of nacre tablets in the oyster Pinctada maxima and provided evidence for a continuous intratabular matrix surrounding coherent nanograins that comprise individual tablets. Their results suggest, among other things, a pathway for modeling the mechanical response of nacre that is used below in suggesting a preliminary model for the tiles themselves. Likewise, Oaki and Imai, (2005) describe a hierarchical structure of nacre in the pearl oyster Pinctada fucata in which individual tiles are seen to be composed of nano-scale “building blocks” (i.e. nano-crystals) surrounded by an organic matrix. These observations provided additional detail for the existence of organic material within nacre’s tiles noted much earlier by Watabe, (1963). The AFM images shown by Rousseau et al., (2005) can
be used to demonstrate that the stiffness of individual tiles should be less than that of monolithic CaCO$_3$ and in fact just on the basis of a simple rule of mixtures should be on the order of at least 10% less. This estimate is based on the apparent thickness of the intratabular matrix as seen, for example, in the phase image of (Fig. 3b) of Rousseau et al., (2005). This fact is used in the modeling described below. At the same time, the interlamellar $il$ layers, indicated with black arrows in (Fig. 9.1b), would impart increased toughness to the structure via the energy absorptive capability of, what in Chapters 7 and 8 was demonstrated to be a viscoelastic matrix. The intratabular matrix and interlamellar layers found within and between aragonite tiles will together be referred to as the $it-il$ matrix. As it happens, it will be shown that intact nacre (i.e. nacre within a dry shell) is well modeled as being viscoelastic as such, but with values of the constitutive parameters being somewhat different as explained below.

![Image](image_url)

**Figure 9.1:** SEM images of a) the coherent nanograins comprising the intratabular $it$ matrix in a tile; b) tile lamellae where the black arrows indicate the location of the interlamellar $il$ matrix. Together the two form an $it-il$ matrix.

There are, as yet, a number of reports describing various aspects of the mechanical behavior of nacre. Most specifically, and relevant to the present work, are those using nanoindentation to probe mechanical response (see e.g. Bruet et al., (2005) - Li et al., (2004), see also Currey, (1977) involving a study not involving
nanoindentation). Taken together these reports have shed much light on various aspects of the phenomenology of deformation of nacre, while at the same time often presenting conflicting conclusions and perspectives as to the key aspects of nacre that mediate its properties. A brief survey follows.

In an early study Currey, (1977) conducted experiments on nacre extracted from a variety of gastropods, cephalopods, and bivalves. Currey provided important evidence for inelastic deformation that he described as “plastic”, and also made mention of a possible viscoelastic response. Particular constitutive models or material parameters however were not presented as would be needed for numerical model simulations of complete nacre response.

Kearney et al., (2006) performed nanoindentation on monolithic aragonite and demonstrated a plastic response. They described patterns of pileups around indents and showed that to adequately describe them a crystal plasticity model was required. In fact they employed the viscoplastic crystal plasticity model of Peirce et al., (1983). The precise viscoplastic kinetic parameters were, however, not detailed. The perspective in their report suggests that aragonite is the ceramic constituent of many biological mineralized structures, in particular nacre.

Prior to this, Bruet et al., (2005) performed nanoindentation tests on nacre obtained from Trochus niloticus and found evidence for the plastic response of the ceramic tiles. Computational simulations were performed on the composite nacre where interlamellar layers were modeled as nonlinear elastic (not viscoelastic) and the tiles as essentially aragonite. Precise values of elastic-plastic constitutive parameters were not provided in their report, and no mention was made of viscoplastic behavior.

Huang and Li, (2009) performed both characterization, via x-ray diffraction, and mechanical testing of nacre subjected to heat treatments involving temperatures of 500 °C and 1000 °C. They also reported drops in hardness and Elastic modulus. Aragonite undergoes a phase transformation to calcite at 420 °C. Whether the changes in hardness and modulus were due to burning off the it-il matrix or changes
in crystal structure, could not have been made. Findings here indicate that already at 200 °C the il-\(i\)-it matrix is burn off and that tiles exhibit an entirely different response from intact nacre which could be described as “quasi-plastic”. Huang and Li, (2009) do report that nacre platelets are composed of nano-sized particles, yet the tiles exhibit single crystal diffraction patterns as noted above.

The existence of water within dry nacre was investigated via PA-FTIR by Mohanty et al., (2006) who suggested that it exists in “bulk” form. It was also speculated that due to organic material nacre as well as single tiles might exhibit a viscoelastic response. Precise values of viscoelastic constitutive parameters were not provided in their report, and no mention was made of viscoplastic behavior. Verma et al., (2007) later provided strong evidence for water, though now as chemisorbed molecules at tile/il-layer interfaces, being found in nacre. They state that water “may facilitate viscoelastic behavior of the aragonite”, though again no specific constitutive parameters were reported. Moreover tiles were assumed to be essentially aragonite crystals with no mention being made of their nanograin structure.

Barthelat and Espinosa, (2003) describe nacre tiles as composed of nanocrystallites, studied time-dependent response of nacre and concluded that nacre displayed a viscoelastic response. No specific model or analysis was presented but they attributed the time dependent response to water content within the nacre structure. As far as the ceramic tiles were concerned, it was suggested that the elastic properties of single tiles were essentially those of monolithic aragonite. Barthelat et al., (2006) followed this work with nanoindentation studies on nacre which was modeled as an elastic composite with the \(i\)-\(t\)-il matrix being linear elastic.

Li et al., (2004) performed \textit{in situ} dynamic AFM imaging of dog-bone shaped nacre specimens undergoing tensile and 3-point bending tests. It was concluded that the \(i\)-\(t\)-matrix under tension imparts tiles with a viscoelastic/viscoplastic response through the “rubber-band” like binding of nanograins. Specific constitutive parameters were however not measured. Furthermore their samples were prepared by wet
grinding and polishing. As will be presented, hydration leads to significant alterations in tile structure and time-dependent response. It should be noted that sample surfaces, on which nanograins were imaged via AFM, would have incurred damage due to grinding and polishing. Hydration and damage were not accounted for in their study of the plastic deformation and time-dependent response of tiles.

Likewise Mohanty et al., (2008) performed AFM tests of the biopolymer layers within nacre and reported a time-dependent response that could well be interpreted as viscoelastic. Mohanty et al., (2008) also based their findings on what are reported to be single protein AFM pulling tests. Aside from the fact that the presented force vs. displacement curve they present displays major characteristic differences to those reported, and substantiated, for molecules such as titan (Rief et al., (1997)), RNA (Liphardt et al., (2001)), and spectrin (Rief et al., (1999); Law et al., (2003)), no specific proteins were identified in their work. Moreover, since it is also now known that the structural integrity of the nacre matrix is strongly mediated by its chitin core, it is unclear how relevant such information would be. Chitin does not display such saw tooth like force vs. displacement response of a domain unfolding protein like titan or spectrin (see e.g. Bezares et al., (2010)). At least one protein, viz. Lustrin A, that contains a foldable domain structure has been isolated from the organic matrix of nacre, Shen et al., (1997). However, the report of the AFM pulling experiments of Mohanty et al., (2008) does not mention how, or if, this protein was isolated and/or selectively adhered to. Again, it is vital to understand that since the structural integrity of nacre’s organic matrix is due to its chitin network, it is unlikely that the isolated response of a protein such as Lustrin A would be relevant with respect to the overall mechanical response of nacre per se. What can be said is that evidence for a time-dependent response was found but its causes, e.g. viscoplastic response of the tiles, viscoelastic response of the matrix, moisture content in the biopolymer matrix, and full characterization remained unclear. AFM molecular pulling experiments have been performed by others, e.g. Smith et al., (1999) on material lying within the
organic matrix of nacre from *Haliotis rufescens*. Their emphasis, however, was more on the role of proteins in contributing to adhesion, clearly of importance *vis-à-vis* the composite’s internal binding.

Taken together, the general view outlined to date is that nacre is composed of a layered brick-wall like tiled structure encased within a biopolymer matrix. The tiles, are themselves nano-scale composites composed of crystallographically aligned nanosized grains separated by an organic matrix. The aragonite ceramic within the tiles displays a plastic response, and presumably viscoplastic. Thus, although there have been repeated claims that the response of nacre tiles is unaffected by the biopolymer structure within them, the findings here are that the ceramic/biopolymer composite tiles cannot be effectively modeled using the properties of monolithic aragonite. What the findings also suggest is that nacre’s moisture content also contributes to its time-dependent response. What is then required is a way of identifying, and sorting out, the effects of the various potential contributions to the overall viscoelastic behavior.

The essential focus here is on correlating the behavior of nacre in intact shells stored at room temperature. Such nacre is referred to as *dry nacre*. To gain additional perspective tests were performed on nacre that was hydrated by immersion in water for extended periods of time - this is referred to in what follows as *wet nacre*. Nacre subjected to heat treatment at elevated temperatures sufficiently high as to remove protein in the *it* and *id* layers but not induce a phase transformation of the mineral was also tested and will be referred to as *heat treated* nacre. Finally, nanoin dentation tests were performed on monolithic aragonite to compare and contrast its response with that of single tiles. The results indicate a response that is entirely different in stark contrast to previous reports. A detailed FEM analysis is then presented which focuses on *dry nacre* as it was found that this leads to more definitive results when compared to experiments. In wet nacre the behavior was found to be extremely sensitive to the precise conditions of hydration (*e.g.* to the duration of
hydration) which made definitive property characterization challenging.

The following section is a presentation of experimental results. These results are then duplicated numerically, from which the constitutive properties of the structural components are extracted and the detailed responses of these components predicted. Based on these a model is proposed explaining the origins of the viscoelasticity of the system and a new paradigm for potential synthesis of bio-duplicated composites.

9.2 Results

9.2.1 Isotropic Response

In order to provide a quantitative baseline for comparing the response of the nanocrystalline composite nacre tiles to monolithic single crystalline aragonite, nanoindentation was performed on both. Results are shown in Figs. 9.2 - 9.4. Figs. 9.2a,c and 9.2b,d show the results for extracted stiffness, and for hardness, for nano-composite nacre tiles and single crystal aragonite, respectfully. Note that for the nacre tiles indents were made on the tile faces (C-axis) and on the tile edges (Edge On) and for the aragonite perpendicular to the (001) and (110) crystal faces. Note also that the results for stiffness and hardness are obtained via isotropic elastic analysis and thus for single crystal aragonite should be taken as illustrative, yet still quantitative and revealing real trends. As expected, single crystal aragonite displays both a stiffness and hardness exceeding that of the composite, polymer containing nacre tiles.

Afm like scans of the indentations are shown in Figs. 9.3 and 9.4 for indents made on single crystal aragonite and nacre tiles, respectfully. As discussed be Bezares et al. (2010), the pile-ups surrounding indents made on pure aragonite display clear signs of plastic deformation occurring via crystallographic slip; those surrounding
nacre tile indents show signs of more isotropic inelastic deformation.

### 9.2.2 Elastic Response - Young’s Modulus

Figure 9.5 shows typical load-displacement curves, obtained from nanoindentation tests, that were used in this study to calculate the Young’s modulus of dry, wet, and heat treated nacre, as well as monolithic aragonite.

The Young’s modulus of dry nacre at a 75 nm indentation depth was 70 GPa. Figure 9.6 shows the variation in the reduced modulus of aragonite, dry nacre, heat treated nacre, and wet nacre, up to indentation depths of 300 nm. The Young’s modulus of Aragonite at a 55 nm indentation depth was 114 GPa. The Young’s modulus and hardness of nacre and monolithic aragonite, as determined from nanoindentation tests, varies with indentation depth due to the indentation size effect described by Nix et al., (1998). With increasing depth, there is an initial exponential decrease in
modulus, followed by an eventual taper to a constant value. In monolithic aragonite the modulus begins to taper at $\sim 300$ nm, eventually reaching a constant value of $\sim 80$ GPa. In the finite element model to be presented, the Young’s modulus of aragonite crystal at a 55 nm indentation depth was derived from this collection of results and taken to be 114 GPa. In a similar manner the Young’s modulus of a single nacre tile at an indentation depth of 75 nm was assumed to be 70 GPa.

In particular, it is noted that scatter in the graph for wet (i.e. hydrated) nacre is too great for accurate determination of the modulus.

9.2.3 Plastic Response - Nanoindentation Surface Scans

Typical indentation surface scans are shown in (Fig. 9.7). Figure 9.7a shows a typical scan of an indent performed on dry nacre. Pileups are clearly visible

Figure 9.3: Nature of the indents made on single crystal aragonite.
surrounding the indentation that are symmetrical. The topography of indentations in wet nacre differed from that of dry nacre in that the edges of the indentations appeared “blunted” and swollen. Indentations in heat treated nacre do not resemble either the dry or wet cases (Fig. 9.7c). Indentations in heat treated tiles display sharp clearly defined edges and no pile-up. Indentations in monolithic aragonite as seen in (Fig. 9.7d), displayed anisotropic pileups. This is indeed expected for an indentation made on an anisotropic elastic-plastic material such as ductile crystal. These results suggest that: 1) nacre tiles can be described as viscoplastic solids but, unlike monolithic aragonite, are isotropic and not anisotropic single crystals; 2) after heat treatment, and with the concomitant loss in intra-tile protein, tiles behave as a quasi-plastic porous material; and 3) in a fully hydrated state, *i.e.* following an extended exposure to water, tiles behave as ductile isotropic solids, but with a much reduced hardness as compared to dry nacre.

**Figure 9.4:** Nature of the indents made on nacre tiles.
Figure 9.5: Typical load-displacement curves for (a) dry nacre, (b) heat treated nacre, (c) wet nacre, and (d) monolithic aragonite; note the load plateau at \( \sim 230 \mu N \).

### 9.2.4 Time-Dependent Response

#### Load Relaxation

Relaxation tests performed on a fused quartz standard, which should not exhibit relaxation, resulted in displacement overshoots during loading followed by an initial rapid decrease in load within the first 3 sec of the tests such as shown in (Fig. 9.8). Results from this initial period were thus excluded from the analysis. An imposed 75 nm displacement on a fused quartz standard resulted in no drop in load following the initial 3 sec period. The viscoplastic behavior of the biomineral tablets in nacre was tested in comparison to the viscoplastic behavior seen in monolithic
Figure 9.6: Young’s modulus vs. indentation depth for monolithic aragonite, heat treated nacre, dry nacre, and wet nacre.

aragonite. Relaxation tests performed on monolithic aragonite showed that the mineral exhibits a long-term viscoplastic response to indentation loading as seen in (Fig. 9.8). Relaxation was greatest in wet nacre followed by dry nacre, and least in heat treated nacre. Heat treated nacre exhibited even less relaxation than aragonite. The effect of hydration of the organic framework material correlates with time-dependent response, where relaxation is most pronounced in wet nacre, followed by dry nacre and least in heat treated nacre.
Figure 9.7: Indentation profiles on: (a) dry nacre with symmetric pile-up; (b) wet nacre with blunted pile-up; (c) heat treated nacre with a compacted appearance and no pile-up; and (d) monolithic aragonite with uneven pile-up indicating anisotropy. A slip band is also visible on the left face of the indent associated with load plateaus in the P-δ curves.
Figure 9.8: Load relaxation curves for monolithic aragonite, heat treated nacre, *dry nacre*, and *wet nacre*. Note the greater amount of relaxation observed in *wet nacre* and the nearly absent observed in heat treated nacre.
Chapter 10

Discussion: Tile Structure and Mechanical Response

10.1 Isotropic Response

10.2 Elastic Response - Young’s Modulus

Values of 70, 80, and 100 GPa have been used in previous models of nacre as the Young’s modulus of the biomineral (Wang et al., (2001); Barthelat et al., (2005); Gao et al., (2003)).

Simulations of 55 nm deep indentations in aragonite crystal along its c-axis and 75 nm indentations in dry nacre along the out-of-plane direction of a single tile were performed. As seen in (Fig. 9.8), the Young’s moduli in these two cases are around 114 GPa and 70 GPa, respectively. The Young’s modulus of single tiles, which contain protein inclusions, falls between those of nacre and monolithic aragonite. Biopolymer layers in nacre make it more compliant than single tiles. The tiles however contain protein inclusions not found in aragonite, thus they are more compliant than the monolithic crystal. Stereographic measurements from SEM im-
ages of deproteinized as well as heat treated nacre indicate the void fraction in single
tiles to be approximately 20%. Thus the modulus of a single tile is taken as 90
GPa, which is a 20% reduction in the modulus of aragonite. It should be noted, in
particular, that scatter in the graph for wet (i.e. hydrated) nacre is too great for
accurate determination of the modulus. This is probably due to the complicated and
inhomogeneous structure of the biomineral tablets, which contain protein inclusions
that undoubtedly swell after hydration, leading to the softening of the mineral.

This is characteristic of the quasi-plastic response observed in contact tests on
sintered porous ceramics; Latella, (1997). As with porous ceramics, the subsurface
(crushing of voids in tiles likely prevents the development of the sub-surface hydro-
static stress states required to induce the upwards flow of material which around an
indenter tip is described as pile-up. The indents in heat treated nacre appear very
different from those presented by Huang and Li, (2009) who also performed nanoin-
dentation tests on heat treated nacre, though at lower loads. Their scans show more
blunted indentations with surrounding pile-up similar to what is found here in wet
nacre. Their samples were prepared following wet metalographic polishing proce-
dures which would have resulted in the extensive washing and thus hydration of
their samples as well as softening due to tile damage brought on by the abrasive
process. These issues were avoided in this study by exposing tiles by the cleavage of
nacre as described in the Experimental Procedures.

10.3 Numerical Simulations

Finite element simulations were performed for indentations on monolithic
aragonite and dry nacre. It was found that both viscoplastic and viscoelastic param-
eters were needed for accurate fitting of the time dependent responses.

By numerically duplicating the creep curves for aragonite (Fig. 10.1), it was
estimated that the Young’s modulus of monolithic aragonite $E_{\text{aragonite}} \approx 114$ GPa,
the yield stress $\sigma_Y \approx 10$ GPa, and the plastic viscosity $\eta_p \approx 5000$ GPa·sec.

![Graph showing FEM simulation of the monolithic aragonite indentation creep. The indentation depth is 55 nm.](image)

**Figure 10.1:** FEM simulation of the monolithic aragonite indentation creep. The indentation depth is 55 nm.

Similarly, simulations were carried out of the indentation of dry nacre. Since the tiles are known to include a certain amount of biopolymer, Watabe, (1963), they are expected to be more compliant than monolithic aragonite. Based on SEM images, it was estimated that the inclusion ratio of the mineral tiles is about 80% so that the stiffness reduction is approximately 20%. As was previously shown from indentation tests using a conospherical indenter, the tiles exhibit an isotropic response; a Poisson’s ratio $\nu = 0.3$ was used in the simulations. In the following simulations the Young’s modulus $E_{tile} \approx 90$ GPa, the yield stress $\sigma_Y \approx 8$ GPa, and the plastic viscosity $\eta_p = 5000$ GPa·sec were assumed.

Close inspection of the creep curves for dry nacre show that there are short and long term time-dependent responses with characteristic times of $\sim1$ sec, and $\sim50$ sec respectively. The short term response may be due to the motion of entrapped water molecules adsorbed to the mineral at protein-mineral interfaces. As noted in the introduction, water was located at these regions in dry nacre by Verma et al.,
Water may also exist in the form of “clusters” within individual tiles as described in the aforementioned article, however due to its confinement by surrounding mineral, it is unlikely to contribute significantly to the time-dependent response of dry nacre. This however would not like be the case for wet nacre as will later be explained. The speculation is that the long term time-dependent behavior make be due to the deformation of the biopolymer framework itself. To identify the short term and long term viscoelastic properties of the biopolymer, a short term simulation with $E_0 = 0.1 \, \text{GPa}$, $E_1 = 0.88 \, \text{GPa}$, $\tau = 0.7 \, \text{sec}$ (Fig. 10.2) and a long term simulation with $E_0 = 0.015 \, \text{GPa}$, $E_1 = 0.135 \, \text{GPa}$, $\tau = 45 \, \text{sec}$ (Fig. 10.2) were performed. In both cases it was assumed $\nu = 0.3$. With these parameters, both the short term and the long term responses were accurately reproduced. This further leads to the proposal of a novel paradigm for the nacre composite discussed in the Summary and Conclusions section.

Figure 10.2: A short-term simulation of nacre indentation creep. The indentation depth of indentation is 75 nm.

The results show that numerous biopolymer layers but (especially the one nearest the indented surface) contribute to the overall deformability of nacre during indentation. It was found that for an indentation depth of 75 nm, by using the set of
Figure 10.3: A long-term simulation of nacre indentation creep. The depth of indentation is 75 nm.

parameters in the long term simulation, the maximum thickness changes of the first mineral tile and the first biopolymer layer were 49.1 nm and 11.6 nm, respectively. The total thickness change of all other layers was 14.3 nm. In (Fig. 10.4) are plotted contours of the logarithmic strains $e_{33}$ (in the thickness direction) and $e_{11}$ (in-plane strain). $e_{33}$ displays a large negative value (-0.87) at the first biopolymer layer, suggesting that the nacre was significantly compressed in that area. Figure 10.4b shows that the first biopolymer layer is also slightly stretched ($e_{11} > 0$), but its strain $e_{11}$ is much smaller than $e_{33}$. In the second biopolymer layer, $e_{11}$ has significantly decreased. Previous studies by Mohanty et al., (2007) have demonstrated that nacre exhibits a time-dependent response, and have suggested that multiple biopolymer layers significantly contribute to that response. The analysis based on measured relaxation curves, quantifies the response of multiple layers and incorporates that response in the assessment of the overall time-dependent response of nacre. For both monolithic aragonite and nacre indentations, the finite element simulations underestimated the experimentally observed height of the pileup. This characteristic is consistent with other numerical models (e.g. Bruet et al., (2005)). It does not however have a significant effect on the accuracy of the prediction of nacre viscoelasticity.
Figure 10.4: Contours of (a) strain $e_{33}$ and (b) strain $e_{11}$ of nacre indentation (B section view). The indentation depth is 75 nm.

Incidently, the detailed pileup geometry of monolithic aragonite can more accurately be simulated through a crystal plasticity constitutive model developed by Peirce et al., (1983). Such detail was not a focus of the present study.

10.4 Time-Dependent Response

The view is that layer stiffness is associated with a chitin framework that would display an essentially elastic response. To provide further confirmation of this, load relaxation after an initial straining to a fixed tensile strain was followed and analyzed using a standard linear viscoelastic model as shown in the insert within (Fig. 2.24). In fact, it was found that the simple standard linear model (Doi and Edwards, 1986) provided an adequate fit. Relaxation time constants, $\tau = \eta/E_1$, were found in the range, $\tau = 140 \pm 4$ sec. The fitted moduli, also defined in the insert, are listed in (Fig. 10.4) adjacent to selected curves. Scaled moduli, $\bar{E}_0$ and
$E_1$, were computed by multiplying $E_0$ and $E_1$ by the ratios of the ideal composite layer with a modulus given by eq. (8.1), 0.98 GPa, to the particular modulus of each specimen, i.e. by $0.98 \text{ GPa} / (E_0 + E_1) \text{ GPa}$. These were found to lie within the range $E_0 = 0.668 \pm 0.088 \text{ GPa}$ and $E_1 = 0.311 \pm 0.092 \text{ GPa}$. This leads to an estimate of viscosity $\eta = 42 \pm 0.37 \times 10^6 \text{ kPa}\cdot\text{sec}$. This range for $\eta$ certainly suggests an essentially elastic response. It’s noted that the analysis of viscoelastic behavior is similar to that recently employed by, for example, Nagayama et al., (2007) on smooth muscle cells (SMC’s). They found relaxation times in the range $141\text{s} \leq \tau \leq 384\text{s}$ in rat aortic SMC’s. For such cells, however, the viscosity is much lower due to the much lower elastic moduli $E_0$ and $E_1$. On the other hand, the tissue of the framework is not as stiff as higher chitin content structures such as the squid beaks studied by Miserez et al., (2008).

To further confirm the structural integrity of the tissue in the tensile samples, creep tests at various levels of constant applied stress were conducted. Strain vs. time was monitored over times ranging up to 900 sec. The results were entirely consistent with those obtained from the relaxation tests. Since the creep tests yielded no additional constitutive data, they are not shown.

### 10.5 Summary and Conclusions

These results have provided evidence for the general picture of nacre’s constituency, and have shed new light on the relationship between its make-up and its performance as a structural composite material. Based upon these findings, it is useful to propose a novel model, or paradigm, for such a layered composite as shown in (Fig. 10.5). Toughness is obtained firstly by virtue of the large tile aspect ratio, viz. a tile diameter/thickness ratio that lies in the range 10-20, (e.g. Gao et al., (2003,06)), and by the fact that the interdigitated brick-like tile layers are encased within a soft, ductile biopolymer matrix. The large tile aspect ratio provides effi-
Figure 10.5: Paradigm for the structure and performance of nacre. (a) “Brick-wall” like layered tile composite composed of high aspect ratio toughened ceramic CaCO$_3$ tiles within a biopolymer matrix; (b) the biopolymer matrix, which is itself viscoelastic, is composed of a chitin core within a hydrated protein matrix. Note that the tiles are themselves toughened by the incorporation of an intra-tile biopolymer network and protein inclusions.

cient load transfer and the biopolymer layers provide, *inter alia*, for crack deflection mechanisms. Moreover, the tiles themselves are not fragile monolithic CaCO$_3$, but are actually nanocrystalline with embedded proteinaceous material, as indicated in the tile drawn at the upper left corner of (Fig. 10.5b). Thus the tiles themselves are toughened ceramics with a stiffness and hardness clearly reduced as compared to those of monolithic aragonite. As noted in the background discussion, there has been considerable suggestion that monolithic aragonite can serve as a model for nacre tiles. These results suggest quite the contrary, from the standpoints of elastic and viscoplastic response, and fracture resistance, *inter alia*. The biopolymer matrix is viscoelastic and is characterized by a large elongation to failure (> 3%) as demonstrated by Bezares *et al.*, (2010). A point of note is that these strains are not unusual for a biopolymer due to the protein dependent chitin fiber connectivity within the
biopolymer framework. Still another feature of nacre is its moisture/water content, also indicated in (Fig. 10.5). This provides an additional contribution to the overall time-dependent constitutive response of nacre and for still another mechanism of energy dissipation during deformation. It isn’t so much that the biopolymer layers of nacre behave as a tough sponges per se, since the water, i.e. moisture, content of the it and il layers is undoubtedly low; thus describing the moisture effects to a liquid water phase per se is most likely misleading. Yet it is likely that the presence of moisture/water within these layers does indeed provide for an additional energy dissipative mechanism that contributes substantially to nacre’s toughness.

The paradigm depicted in (Fig. 10.5) is intended to provide a basis for developing new pathways for biomimetics, i.e. for bio-duplication of novel synthetic composite materials. A key to this would be to sandwich high aspect ratio toughened ceramic tiles with moisture/water containing viscoelastic tissue (e.g. polymer). Perhaps not emphasized in the literature, to date, it is required as shown herein that the ceramic tiles themselves need be toughened. Monolithic CaCO$_3$ ceramic tiles in nacre would not have produced a composite with the attractive strength-toughness properties now well attributed to naturally synthesized nacre.
Chapter 11

Conclusion

The results of the research reported on herein have led to a number of novel and vital insights into both the structure and mechanical/structural performance of the abalone of *Haliotis rufescens*. For one, optical histochemical microscopy has provided a detailed picture of the macromolecular structure of the so-called interlamellar layers that, in turn, provide the template into which the aragonitic ceramic tiles grow. Nucleation sights for tile growth were uncovered along with an early glimpse of the chitin network that has been shown to provide the stiffness and strength of the interlamellar layers. Analytical optical microscopy uncovered the remarkable long-range connectivity of chitin fibrils. It was further found that chitin fibrils were organized with a mostly uni-directional architecture - with a minor percentage of fibrils lying in cross directions. This picture was consistent with earlier atomic force microscopy (afm) that is also reported on herein. These results led to the detailed specification of an anisotropic visco-elastic constitutive model also described. The role of the water content, *i.e.* the moisture content, of the interlamellar layers was made far more clear, in particular with respect to the role of moisture content in enhancing energy absorption.

Future work, motivated by these findings, could include attempts to reminer-
alize the demineralized interlamellar framework. This could lead to a truly novel class of synthetic materials. In addition, the results presented herein provide a pathway for bio-duplicated synthetic materials that should possess enhanced energy absorbing characteristics. This pathway is made specific due to the findings of the the role of the visco-elastic interlamellar layered framework and of the nanostructured tiled ceramic. Finally, the novel techniques developed during this research can be applied to study of a legion of biological materials for the purpose of determining their mechanical properties. For example, the idea of being able to test macroscopic sections of demineralized tissue is truly novel to this research.
Bibliography


