Spring-like biomineralized hard structures : lessons from stomatopods

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NANYANG
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**SPRING-LIKE BIOMINERALIZED HARD STRUCTURES:
LESSONS FROM STOMATOPODS**

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Spring-Like Biomineralized Hard Structures: Lessons from Stomatopods

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Abstract

Stomatopods (mantis shrimps) are aggressive predators that use a pair of ultra-fast raptorial appendages to either strike or catch prey. This remarkable fast movement is driven by a power amplification system comprising components that must be able to repetitively store and release a high amount of elastic energy, a requirement that is best accomplished if some of their structural elements combine high stiffness with flexibility. An essential component of this system is the saddle, which is used to store a significant amount of elastic energy by bending prior to striking. During repetitive bending used for the storage of a considerable amount of elastic energy prior to their fatal strikes, the saddle requires to be robust enough to sustain mechanical stresses without failure.

This work comprises a comprehensive structure–chemical composition–property relationship study that sheds light on the microstructural and chemical designs of the saddle structure. Furthermore, to elucidate the role of the compositional variations in the saddle structure, quasi-static and dynamic mechanical properties of the saddle were investigated. To correlate the micro-scale chemo-structural and micro-mechanical properties with the functionality of the whole structure, finite element analysis was conducted.

MicroCT scans combined with electron microscopy imaging, Energy Dispersive Spectroscopy (EDS) mapping, as well as confocal Raman microscopy show that the saddle is a bi-material layer structure, with sharp changes in chemical composition and microstructure between the layers. The outer layer of the saddle is largely made of amorphous mineral phases, whereas the inner layer is predominantly an organic chitin/protein complex with a lower relative mineral content. The inorganic/organic phase distribution correlates with nano-mechanical mapping, with the outer (inorganic) layers being stiffer than the inner layers. With this design, regions of the saddle loaded in compression are primarily made of stiffer inorganic phases that can sustain higher compressive stresses, whereas regions loaded in tension contain a higher relative amount of biopolymeric components. Nanoscale Dynamic

Mechanical Analysis (NanoDMA) studies show that the outer layer plays the key role of storing the elastic energy, with a significantly higher storage modulus than the inner layer, while the inner layers mainly acts as a structural support that sustains tensile stresses. 3D finite element analysis (FEA) quantifies the stress level and provides visualization of the stress distribution in the saddle under compressive bending. Comparing the FEA results with the elastic-plastic properties of the saddle extracted from nanoindentation demonstrates that no plastic deformation occurs in the saddle during bending.

The thesis reveals that the saddle's chemical composition and microstructure have been spatially tuned to generate a stiff, yet flexible structure that is optimized for storage of elastic energy. This structure takes advantage of this spatial distribution and geometry to impose a neutral surface right at the interface of the two layers, and to generate a uniform stress distribution that likely enhances the resistance to mechanical fatigue. This comprehensive compositional, geometrical and mechanical study provides bio-inspired insights that could find wide usage in composite materials science.

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Table Captions

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Figure Captions

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Figure 5.1 The fractured cross sections of the merus parts. The uniform and compact structure of (A) the outer layer of the saddle differs from (B) the inner layer of the saddle, (C) the meral-V and (D) the merus which all possess a multi layer and fibrous structure.

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of the outer and the inner layers of the saddle as more logic way to compare the dynamic response of the two different layers illustrates much higher damping (dissipation) in the inner layers. **(D)** A schematic representation of the Kelvin-Voigt's model on the FESEM micrograph of the saddle cross-section. It illustrates that the spring-damper couple has a more efficient spring and a bigger damper in the outer layer and inner layer of the saddle, respectively.

- Figure 5.7 Construction of the 2D model of the saddle used for finite elements analysis.** **(A)** 20 FESEM micrographs stitched together of a longitudinal cross-section of the saddle used for the 2D modeling; red: outer layer, grey: inner layer. **(B)** The 2D model of the saddle “bi-layer” constructed in Abaqus. The mechanical properties of each layer were assigned according to the stress-strain nanoindentation values.
- Figure 5.8 The 2D finite elements analysis of the saddle.** The highly mineralized outer layer was under compression stresses while the inner layer was under tension stresses.
- Figure 5.9 Construction of the 3D model of the saddle for finite elements analysis.** **(A)** Contact regions (C1, C2), free edges (F1,F2), and transversal and sagittal planes from microCT images used to reconstruct the saddle in 3D. **(B)** Stacked microCT images of the cross-sections of the saddle in the transversal direction with fixed intervals. **(C)** A longitudinal cross-section of a saddle rendered from the microCT, used as a guide to construct the 3D model. **(D)** Process of the 3D model construction leading to **(E)** the final model in SolidWorks.
- Figure 5.10 Simplified 3D saddle model construction.** Extracted parabola fitted curves for the **(A)** longitudinal and **(B)** transversal cross-sections used for constructing the 3D model of the saddle. **(C)** 3D model of the saddle constructed in Solidworks, made of two layers and the fine meshed, which was used for running the modelings. **(D)** Load versus normalized displacement of the saddle under compression (refer to Section 4.3.3), highlighting the compressive load (30 N) used the finite element modeling. The load was selected such as to remain within the elastic region of the macroscopic tests.
- Figure 5.11 3D finite elements analysis results of a compressed saddle (external load of 30 N).** **(A)** Side view of the saddle. The maximum Von Mises stress (430 MPa)

on the saddle outer surface (center of the saddle) is well below the yield stress of the outer layer material. **(B)** Bottom view of the saddle showing the tensile stress distribution, with a maximum Von Mises stress of 230 MPa in the center of the saddle. **(C,D)** Cross-sections of the saddle illustrating that from the outer layer to the inner layer, the stress is maximum, reaches a minimum at the bi-layer interface, and then increases again in the innermost layer.

- Figure 5.12** **MicroCT tomography scan of a damaged saddle after a compression test.** **(A)** The outer layer (green color-filtered) indicates the presence of a uniform cracked pattern consisting of two series of cracks oriented at 45° from the loading axis. **(B)** In the inner layer, (blue color-filtered), no macro-cracks are observed. **(C)** Diagram showing the relative densities used for color filtration of the microCT scan of the saddle.
- Figure 6.1** MicroCT imaging of the mantis shrimp appendage and the contribution of the saddle as a spring to restore the elastic energy for its fatal strikes.
- Figure 6.2** SDS-PAGE results for different proteins in the saddle indicated by the red boxes with the corresponding molecular weights.
- Figure 6.3** MALDI-TOF spectrum for all proteins extracted from the saddle.
- Figure 6.4** Amino acid analysis of the proteins extracted from **(A)** the outer layer and **(B)** the inner layer of the saddle.

Abbreviations

2D	2-Dimentional
3D	3-Dimentional
AAA	Amino Acid Analysis
ACC	Amorphous Calcium Carbonate
ACP	Amorphous Calcium Phosphate
DMA	Dynamic Mechanical Analysis
EDS	Energy Dispersive X-ray Spectroscopy
FAP	Fluorapatite
FEA	Finite Element Analysis
FEM	Finite Element Modeling
FESEM	Field Emission Scanning Probe Microscope
FTIR	Fourier Transform Infrared Spectroscopy
LEI	Lower Secondary Electron Image
MicroCT	X-ray Micro Computed Tomography
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization-Time of Flight
SDS-PAGE	Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
TGA	Thermal Gravimetric Analysis
XRD	X-ray Diffraction

Chapter 1

Introduction

All biological materials have evolved over millions of years to optimize their mechanical properties in regard to their specific functions. The mantis shrimp raptorial appendage is no exception. This chapter briefly introduces this ambush predator and the features of its ultra-fast and powerful hunting appendage. The hypothesis that the key structure in the power amplification system of the appendage, the “saddle”, has tuned its stiffness and flexibility to act as an efficient spring is set along with the objectives of the whole study, which are principally the microstructural and mechanical characterization of the saddle.

1.1 Background

Living organisms have evolved to optimize their structural properties through millions of years to adapt to harsh various environmental conditions, and to meet their specific and often exclusive mechanical functions ^[1]. Among such remarkable adaption, notable examples of biocomposites with load-bearing functions include ^[2]:

- Protection and armor (*e.g.* mollusk shells, crustacean exoskeletons, and fish scales).
- Impact and fracture resistance (*e.g.* invertebrate claws and mantis shrimp dactyl club).
- Sharp and cutting tools (*e.g.* teeth, invertebrate mandibles, sucker rings, and squid beak).
- Lightweight exo/endoskeleton for structural stability and mobility (*e.g.* snail shells, invertebrate cuticles, and bone) ^[2].

These biocomposites are mainly assembled from very delicate and lightweight building blocks. These building blocks also have been formed very frequently among different taxa and from abundant elements in Nature, thus providing notable examples of convergent evolution. However, the key in the extraordinary mechanical properties of these biogenic materials lies in their arrangement in hierarchical structures going from the nano- to the macroscale. Knowledge gained by unraveling these complex hierarchical structures and their structure/function relationships can provide robust guidelines for designing of bio-inspired composites ^[3] with superior mechanical properties, *i.e.* elasticity, hardness, toughness, or fatigue resistance.

So far, the design principles in terms of structural, chemical and mechanical properties have been studied for many biological materials, including enamel ^[4], dentin ^[5], bone ^[4, 6], nacre ^[1, 7, 8] and other mollusk shells ^[9], sponge ^[10-13] and sponge spicules ^[14], conch shells ^[15-17], antler ^[18], sea urchin spines ^[19], corals ^[20], arthropods exoskeleton ^[21], chiton radula ^[22], sponge spicules ^[10-13], squid beak ^[23], sucker rings ^[11] and many other natural structures ^[1, 2, 24-27]. These named biological materials mostly belong to the marine species, which have passed millions of years of evolution ^[27].

Figure 1.1 Mantis shrimps (stomatopod) and their appendages. (A,C) Mantis shrimp spearer and (B,D) smasher along with images of their respective raptorial appendages.

The stomatopods are an ancient group of ambush predatory marine crustaceans^[28], which are equipped with a fascinating visual system consisting of 12 color receptors^[29-32] and very powerful and ultra-fast raptorial appendages^[33-36]. Based on the shape of their appendages, they are generally divided into two main categories: smashers and spearers (Figure 1.1). Smashers possess claws adapted to punch and shatter the hard-shell of preys, while the spearers use their barbed claws to grab fleshy moving animals^[36] (Figure 1.2). Common to both species is the fast velocity at which the appendages are deployed during a strike, although the smasher strikes quicker than the spearer, in the range of 14-23 m/s^[37] versus *ca.* 4.5-7 m/s for the spearer^[38]. The smashers can propel their claws at accelerations up to 10000 g, generating forces of up to 1000 times of their body weight^[33-35]. The energy required for these fatal and ultra-fast raptorial movements goes beyond the power that can be supplied by any known muscle, and thus in the initial explorations it was hypothesized that a potential elastic energy is stored in the extensor muscle and

apodeme ^[39]. Further studies have focused on the presence of a strong and effective spring-like structure, a hyperbolic paraboloid or saddle shape structure on the dorsal top of the merus segment as a discrete structure to store the energy for the strike ^[37], while additional studies revealed that an intriguing network of structures, including both the hard and soft tissues, located in the merus, works in concert to amplify power and speed ^[40, 41] (Figure 1.3). It has been also shown that much of the merus exoskeleton flexes to store energy as the meral-V rotates, ventral bars act as tape springs, and the saddle compresses and expands ^[40-42].

Figure 1.2 Mantis shrimps (Stomatopod) and their different hunting behaviours. (A) Spearer mantis shrimp impaling soft moving prey **(B)** Smasher mantis shrimp shattering hard shell prey. Credits: Roy Caldwell.

In stomatopods' raptorial appendages, the microstructure and chemo-mechanical characteristics of the dactyl club have been studied extensively ^[43-45]. It has been shown that the highly damage-tolerant composite in the club originates from the gradual transition from highly crystalline and oriented fluoroapatite (FAP) structure at the outer layer to fully amorphous and helicoidally-shaped chitinous structure in the inner layers ^[44]. However, little is known about the microstructural and chemo-mechanical features of the structural element responsible for the power amplification, such as the saddle.

In the power amplification system, the saddle is among the stiffest segment and has been proposed to play a significant role in storing a high amount of energy needed to

deliver a punch, with an identical mechanism both the spearer and the smasher ^[37, 39, 46]. As a hyperbolic-paraboloid structure located in the dorsal part of merus, the saddle is fixed in its longitudinal direction to the hard shell and is surrounded by a soft and flexible membrane. Although the color and size vary from species to species, the geometrical aspects of the saddle are identical.

Figure 1.3 Muscles and network of structures in the merus contributing to providing mantis shrimps with their fatal strike energy. (A) Muscles in the merus; extensor and flexor. (B) Merus segments; saddle, meral-V, and ventral bar. Adapted from Ref. [38, 47].

This thesis focuses on the saddle of the smasher mantis shrimp and is based on a holistic approach to comprehensively elucidate the structural features that play a key role in the remarkable capability of stomatopods to store a high amount of elastic energy in their power amplification system. The interplays between chemical composition, spatial distribution, microstructure, macroscopic geometrical features, and nano-mechanical properties of the saddle will be extensively presented.

1.2 Hypothesis

All elements located in the merus, which constitutes the mantis shrimp's power amplification system, synergistically contribute to storing a vast amount of potential elastic energy. In this thesis, the assumption is that the saddle has been designed to satisfy two principal criteria, namely being stiff enough to prevent local buckling and flexible enough to store a significant amount of elastic energy. Based on these assumptions, it is reasoned that the saddle possesses a multi-scale hierarchical organization with distinct properties allowing it to play its elastic energy storage role.

1.3 Objectives and Scope

In regard to the above-mentioned hypotheses, this dissertation aims to provide a comprehensive understanding of the hierarchical structure of the saddle. Nano-mechanical studies have also been conducted to evaluate the mechanical response at the micro-scale level. These complementary explorations will shed light on the correlations between the chemo-structural characteristics of each constituent and their mechanical functionalities. In order to achieve such a comprehensive description of the saddle, a wide variety of characterization techniques has been used: optical microscopy, field emission scanning electron microscopy (FESEM), X-ray diffraction (XRD), Raman spectroscopy, energy dispersive X-ray spectroscopy (EDS), Fourier transform infrared (FTIR) spectroscopy, thermal gravimetric analysis (TGA), amino acid analysis (AAA), and nanoindentation.

Following the micro-structural studies of its hierarchical structure, whole saddles were subjected to macroscopic compressive testing using a custom made universal micro-testing machine to assess their mechanical response under compression. Finite element analysis (FEA) was also carried out to model the saddle's structure under bending in order to study the stress distribution in the structure and to validate the experimental data.

To address the hypotheses, the overall objectives are as follow:

- [1] To uncover compositional and microstructural aspects of the stomatopod saddle at various length scales as a benchmark of a hierarchical structure capable of storing vast amount of elastic energy.
- [2] To evaluate the micro- and macro-mechanical properties of the saddle including the modulus, elastic-plastic behavior, and spring constant to provide both qualitative and quantitative information about the mechanical response.
- [3] To identify the conceptual design and micromechanical parameters that are necessary to generate an efficient spring-like structure from a rigid biomineralized exoskeleton.
- [4] To employ finite element modeling to visualize the saddle in compression/released mode and to quantify the stress distribution during energy storage.

The understanding of these underlying design principles could provide a roadmap to overcome and optimize the mechanical conflict between stiffness and flexibility.

1.4 Dissertation Organization

Chapter 1 gives an introduction to the stomatopod's power amplification system, with particular emphasis on the saddle. Chapter 2 presents the literature background on previous research on biological materials and their hierarchical organization. The stomatopod's appendage and its amplification system are introduced more elaborately, with an emphasis on the gaps in the current knowledge, which constitutes the basis of the next chapters. Chapter 3 provides the rationale and the methodologies of the experimental set-ups employed for the structural and mechanical characterization studies. A description of the finite element modeling is also provided in Chapter 3. Results from the microstructural and compositional studies are presented in Chapter 4. Chapter 5 comprises the micro- and macro-mechanical responses and validations from finite element modeling. Lastly, Chapter 6 provides a brief summary on the main findings of this work, and proposes future research directions.

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Chapter 2

Literature Survey

Nature has evolved numerous efficient design strategies to synthesize the complex biological materials to exhibit best properties for their functionality. In this chapter, some examples of these complex biogenic structures are presented and the approaches they take to optimize their hierarchical organization in order to meet specific mechanical requirements are reviewed. The main focus is on the mantis shrimp and its well-developed ballistic raptorial appendage. Accordingly, a comprehensive literature review on the previous studies conducted on the mantis shrimp hunting mechanism and feeding appendage is presented and the gaps in knowledge and open questions, which are principally the microstructural and mechanical features of the “saddle”, are set out.

2.1 Biological and Bioinspired Materials

Biological materials have evolved over millions of years and are optimized for their specific functions. Biological materials are made of several common components, but their uniqueness lies in the way such components are assembled together, often forming hierarchical structures with multifunctional purposes. Figure 2.1 represents a heptahedron inspired by Arzt, which summarizes the fundamentals features of biological materials ^[1, 2].

Figure 2.1 Fundamentals of biological materials. Re-drawn from Ref. [1].

Several biological materials are assembled as complex composites, frequently having exceptional mechanical properties to suit their specific roles and applications. Importantly, these complex composite materials are made from a limited number of elements, like C, N, O, H, Ca, P, S, and Si ^[3, 4], which form light and weak individual phases, but attain remarkably great strength when assembled to form building blocks and hierarchically-arranged structures ^[3]. The main building blocks to form biological materials are organic and mineral phases ^[1, 5]. Organics are divided into two main categories: proteins and polysaccharides. Proteins are essentially actually long-chain macromolecules composed of amino acid residues linked together via the peptide bond ^[1, 4, 6]. There are twenty-one

standard amino acids, which are divided into several sub-groups based on their physico-chemical properties: negatively or positively charged, polar uncharged, non-polar aliphatic, and special cases^[7]. The relative amount of each amino acids together with their primary sequences define different conformation of proteins, and consequently their distinctive properties^[4]. Collagen in bone and connective tissues (like tendon)^[3, 8-11], elastin in skin and artery walls^[12], and keratin in nails and hooves^[13] are well-known examples of structural (load-bearing) proteins. The other organic phases comprise polysaccharides, which are macromolecules consisting of long chains of monosaccharide units bound by glycosilic bonds. The two most abundant polysaccharides on Earth are cellulose (the main structural constituent of plants) and chitin (the dominant component in crustacean exoskeletons^[14, 15] and squid beaks^[16, 17]). Chitin, which is a polymer of N-acetyl glucosamine ((C₈H₁₃O₅N)_n), is mostly found to be wrapped with proteins in the form of nanofibrils^[14, 18]. From a mechanical point of view, most of the organic phases have a viscoelastic behavior^[19, 20] and their presence in biological materials results in flexibility of soft tissues and toughening of hard structures^[21, 22]. Minerals, which offer strength and load-bearing properties to the materials, are more abundantly found in “hard tissues” like enamel (85%)^[23], dentin (45%)^[24], nacre (95%)^[5, 25, 26], sponge spicules (89%)^[27-30], conch shell (99%)^[31-33], antler (36%)^[34] and cortical bone (40%)^[35, 36]. They can appear as either amorphous or crystalline structures; for instance, calcite (CaCO₃) (in mollusk shells^[37], sponge spicules^[38], and sea urchin spines^[39]), aragonite CaCO₃ (in conch and corals^[40]), amorphous CaCO₃ (in arthropods exoskeleton^[41]), hydroxyapatite Ca₁₀(PO₄)₆(OH)₂ (in enamel, dentine, and bone^[23, 35]), magnetite Fe₃O₄ (in chiton radula^[42]), and silica SiO₂.nH₂O (in sponge spicules^[27-30]) are some of the most prevalent biominerals in nature^[1, 4, 5, 43, 44]. The presence of mineral phases in biological structures normally gives rise to rigidity and load-bearing properties to the material^[45, 46]. Although most hard tissues are mineralized, some intriguing hard structures have been found, which are completely organic such as squid beak^[16], squid sucker ring teeth^[28], nails, horn and arthropod exoskeletons^[1].

Table 2.1 Biological materials studied over the last decades^[48], along with their biological functions and corresponding references.

Biological materials	Function	Ref
Antler	Tool	[34, 49]
Biosilica	Support	[30]
Black coral	Support	[50]
Bone (ortical/trabecular)	Support	[51]
Cephalopod beak	Tool	[52]
Chiton tooth	Tool	[42]
Conch	Protective armour	[32]
Crab claw (tip/inner)	Tool	[53]
Crustacean teeth	Tool	[54]
Dactyl club mantis shrimp	Tool	[55]
Deep-sea vent gastropod armor	Protective armour	[56]
Fish armour (ganoine)	Protective armour	[57]
Glycera jaw (outer/bulk)	Tool	[58]
Horse hoof	Tool	[59]
Human nail	Tool	[60, 61]
Human tooth (dentin)	Tool	[62, 63]
Human tooth (enamel)	Tool	[62, 63]
Insect cuticle	Support	[64, 65]
Lobster exoskeleton (outer/bulk)	Protective armour	[66]
Mussel thread (inside/coating)	Tool/Protection	[67]
Nacre	Protective armour	[68, 69]
Nereis jaw (with/without metal ions)	Tool	[70]
Sea Urchin tooth	Tool	[71]
Shark tooth (enameloid)	Tool	[72]
Spider fang	Tool	[73]
Squid sucker ring	Tool	[74]
Tucan beak (shell/foam)	Tool	[75]
Turtle shell	Protective armour	[76]

Over the last two decades, biological composites have been studied extensively. Table 1 outlines some of these biogenic materials and their related functions. The knowledge gained by how such materials are formed and assembled offers lessons and roadmaps to design high-performance composites^[3,47]. Accordingly, biomimicry holds great promises to overcome various challenges related to engineering, manufacturing, healthcare, and architecture. Working on biogenic materials gives us the opportunity to translate Nature's solutions into specific products for mechanically-challenging applications.

2.2 Hierarchically-Organized Structures

All biological materials are assembled from building blocks at the nano-scale that are intrinsically mechanically weak. However, the resulting mechanical properties of the assembled constituents at higher scales do not obey the well-known rule of mixture of composites (Figure 2.2). The hierarchical organization of the constituents endows the whole structure with superior mechanical properties^[77].

Figure 2.2 As exemplified by nacre and bone, the mechanical properties of natural composites exceed that of their constituents and the properties predicted by their homogenous mixtures. Re-drawn from Ref. [77].

Hierarchical structures in biological materials are responsible for the various and specific mechanical features, like resistance to damage or deformation^[78, 79], thus playing a substantial role in defining the properties of bulk material. A comprehensive insight into the effects of hierarchical features can assist the synthesis of new materials with desired physical properties tailored for special applications.

Biological materials science studies the hierarchical structure of natural materials and how they are assembled spanning from the smallest up to the larger length scales^[80]. The effect of the number of the hierarchical levels has been investigated and generalized with respect to the resulting mechanical properties of many bio-mineralized structures, such as bones^[81], nacre, enamel and dentine, conch, sponge spicules, and antler^[80]. It has been shown that the materials strength and toughness exhibit opposite trends as a function of the number of hierarchical levels (see Figure 2.3)^[82, 83].

Figure 2.3 (a) Schematic illustrating I, II, ..., to N levels of self-similar hierarchical structure assembled from a mixture of organic and inorganic materials. (b) Trends in materials strength and toughness as a function of the number of hierarchical levels. Adapted from Gao (2006)^[82, 83].

The hierarchical structure can be better illustrated by discussing typical examples of bone ^[81, 84], nacre ^[85] and lobster cuticle structure ^[41]. These structures comprise more than 5 hierarchical levels. Figure 2.4 illustrates the seven hierarchical levels of bone's structure. Starting from the molecular scale (~ 1 nm), three helices of α -collagen proteins are bundled together making up the larger structure, termed tropo-collagen and measuring about 300 nm in length. At the higher structural level, several tropo-collagens are assembled along with hydroxyapatite crystals to form mineralized collagen fibrils (~ 1 μ m in length). These fibrils are then stacked in oriented arrays (~ 10 μ m long), which in turn assemble into larger fiber patterns (~ 50 μ m dimension). Osteons are ultimately formed as fiber patterns are intermingled, and these are the larger building blocks comprising compact segments of bones ^[84].

Figure 2.4 Hierarchical structure of bone with the seven levels of hierarchy from macro- to nanoscale. The compact part of bone consists of osteons in the mm size range. Osteons are composed of a lamellar structure which has a fibrous structure. The fibers are made of collagen fibrils reinforced with minerals (hydroxyapatite). These building blocks are formed from single collagen molecules comprising three alpha-helical chains. Adapted from Ref. [81].

Figure 2.5 Hierarchical structure of nacre (Abalone shell) with its five levels of hierarchy.

The basic building blocks are chitin chains and calcium carbonate (aragonite) crystals, which then assemble into chitin matrix and mineral bridges, respectively. Tiles and organic layers subsequently form mesolayers, which ultimately form the nacre shell of abalones. Adapted from Ref. [83].

Five levels of hierarchy can be observed for nacre making up, for instance, abalone shells, as illustrated in Figure 2.5. Long chains of chitin macromolecules bonded with proteins surround the aragonite (CaCO_3) single crystals that are then stacked to the next level of hierarchy, forming mineral bridges embedded within a chitin matrix^[69, 85]. These

structures further assemble to form the final meso-layers of nacre. The simultaneous presence of a soft organic part together with brittle aragonite crystals in the hierarchical structure gives rise to the superior mechanical properties in nacre ^[69, 86].

The third classical example is the carapace of lobster. This cuticle consists of three layers: epicuticle, exocuticle and endocuticle. The epicuticle, which is the outermost thin layer, is made of proteins and lipids acting as a barrier to prevent water loss. The other two layers have higher levels of hierarchy starting with chitin molecules bonded to proteins. These fibrils are bundled, twisted and stacked helicoidally. This helicoidal organization of fibers is called plywood or Bouligand structure ^[18, 87, 88].

Figure 2.6 Lobster cuticle hierarchical structure. (a) American lobster *Homarus americanus*. (b) SEM micrograph of fractured lobster cuticle. (c) Hierarchical structure of lobster cuticle starting from chitin molecules (polymer of N-acetyl-D-glucosamine). A bundle of chitin molecules are coated with proteins to form nanofibrils, which are then clustered to form chitin-protein fibers. These fibers are arranged horizontally. The chitin-protein fibers are horizontally twisted to form the typical plywood structure ^[89, 90].

The Bouligand (plywood) structure (see Figure 2.6) is the planar arrangement of parallel fibers stacked on top of each other, with the preferred orientation of each layer

gradually rotating from one layer to the next. The distance between two layers defines the stacking height (periodical distance) of a plywood structure. In lobster, the fibers are made of fibrils and the fibrils themselves are formed from chains of α -chitin crystallites, which are coated with proteins^[91, 92].

In crustaceans the cuticle is mostly mineralized with calcite, which is normally rich in magnesium, amorphous calcium carbonate, and a small amount of amorphous calcium phosphate^[93-97], giving rise to the outstanding mechanical properties of these biogenic composite materials. While similarities in cuticle architecture are observed between various species of crustaceans, significant variations are observed in terms of structure, composition and spatial phase distribution. Such physicochemical properties are natural adaptations that are tuned to meet the different environmental conditions which the species have to tolerate^[93-97].

The three examples of biological composites described in this section, (bone, nacre, and crustacean exoskeleton), as well as many other structures provide design guidelines for materials scientists. Biomimicry of these natural hierarchical structures have the potential to pave the way to develop novel materials with superior mechanical properties.

2.3 Mechanical Aspects of Biological Materials

Over the past 20 years many comprehensive studies have been conducted on the mechanical aspects of biological materials, especially focusing on their structure-property relationships. Wegst and Ashby have organized the major mechanical properties of biological materials in the form of charts and maps as convenient tools to predict the mechanical properties of other materials by concentrating a great amount of information into simple diagrams^[98, 99]. In these so-called “Ashby” plots, the Young modulus and strength can for instance be presented as a function of the density of the biocomposites. In contrast to metals and synthetic structural materials characterized by high densities, biological materials are lightweight (maximum density of 3 g/cm³), spanning five orders of magnitude in Young modulus from 0.001 to 100 GPa^[99].

Figure 2.7 A biological materials property map presenting strength versus density. The guidelines (dash lines) show different materials indices in tie in tension, beam in flexural and plate in flexural loading modes. Adapted from Ref. [99].

In these plots, material index guidelines are provided to facilitate the comparison, prediction or choosing the materials with particular mechanical properties. These mechanical properties are defined based on the loading modes they should sustain such as tension, flexure, energy absorption, failure and etc. Materials located in each guideline possess the same index accordingly ^[99]. Figure 2.7 presents a selection plot of biological materials presenting strength (σ_f) against density (ρ). The provided guidelines in the plot identify materials that have high strength and low weight. For instance, among the presented materials and considering the density, silk possess the highest strength.

The presented indices in this figure are:

$$M = \frac{\sigma_f}{E} \text{ (Under uniaxial load)}$$

$$M = \frac{\sigma_f^{\frac{1}{2}}}{E} \text{ (beam in flexure)}$$

$$M = \frac{\sigma_f^{\frac{2}{3}}}{E} \text{ (plate in flexure)}$$

Figure 2.8 A biological materials property map presenting Young's modulus versus strength. The guidelines (dash lines) are presented to identify materials with most elastic energy storage per unit volume and elastic hinges. Adapted from Ref. [99].

Other comprehensive compilations of these data relate to elastic modulus against strength of biological materials (Figure 2.8) ^[99]. In this plot the guidelines are materials indices for:

$$M = \frac{\sigma_f^2}{E} \text{ (Maximum elastic strain energy per unit volume; springs of minimum volume)}$$

$$M = \frac{\sigma_f}{E} \text{ (allow large, recoverable deformations; elastic hinges)}$$

In this plot map silk and cartilage possess the highest elastic strain energies per unit volume.

2.4 The Stomatopod

Mantis shrimp, also referred to as stomatopod (Figure 2.9), are aggressive marine crustaceans. They are also considered as living fossils ^[100, 101] since they date back to around 300 million years ago. They have had the same body structure for hundreds of millions of years, as evidenced by their fossil record (Figure 2.10) ^[102, 103]. This implies that the microstructure of their exoskeletons have been well-refined across many generations ^[104].

Figure 2.9 Mantis shrimp's anatomy. Credits for: Timmy Grohrock.

Figure 2.10 A proto-mantis shrimp fossil, *Daidal acanthocercus*, from 356-299 million years ago (Carboniferous period). Adapted from Natural History Museum website*.

* <http://www.nhm.ac.uk/nature-online/species-of-the-day/evolution/daidal-acanthocercus/>

These fossils reveal important steps in the evolution of the highly specialized modern mantis shrimp that catch preys with a pair of ballistic claws. Mantis shrimps inhabit the coral reefs all over the tropical and sub-tropical waters of the world ^[102, 105] and are active hunters ranging in size from 2 to 30 cm. They are well known for their incredible eyesight and fast appendages, with which they strike their preys with impact forces from 400 to 1500 N ^[106].

Over five hundred species of mantis shrimps have been identified, many of which appear in the Indo-West Pacific and Australia. Based on their lethal weapons, *e.g.*, claws or raptorial appendages, they are mainly categorized into two groups: spearers and smashers (Figure 2.11). Spearers have elongated barbed appendages that are used to impale fleshy fast-moving prey, whilst smashers possess a hammer shaped dactyl club suitable for punching and shattering hard shelled creatures or exoskeletons ^[107]. Mantis shrimps share several notable structures: highly complex eyes, ultra-tough clubs, impact-resistant telson armor, and their power amplification system located in their upper part of appendages.

Figure 2.11 The two types of stomatopods. (A) The spearer and (B) the smasher. Credits for: Roy Caldwell.

Mantis shrimps possess the most sophisticated visual system in the animal kingdom (Figure 2.12), superior even to high-tech military imaging softwares^[108-112]. Their eyes have twelve types of photoreceptors (in comparison the human's eye have only three color receptors)^[108, 109, 111, 112], with the added ability to detect circularly polarized light^[109, 110].

Figure 2.12 The satellite eyes of mantis shrimp, with their characteristic ability to move independently. Credits for: Roy Caldwell.

Another remarkable structure with which the mantis shrimp is equipped is its dactyl club^[107]. To perform mechanically-demanding tasks, stomatopods have evolved complex multi-scale structural and crystallo-chemical designs that endow their clubs with a remarkable damage tolerance. Notably, the clubs are made of distinct regions consisting of an outer impact layer, an inner softer region, and a nested region^[55]. The impact layer contains both crystalline and amorphous calcium phosphate. Textured crystalline Fluorapatite (FAP) is the dominant phase near the impact surface, whereas amorphous calcium phosphate (ACP) is more abundant near the interface with the inner region. The inner region is less mineralized and is comprised of partially mineralized chitin fibers decorated with amorphous calcium carbonate (ACC), which assemble into a helicoidal composite structure. Micro-channels running perpendicularly to the main club axis and

required for diffusion of inorganic ions and mineral deposition during club growth are also present in the inner layers ^[113]. Recent contact mechanics studies have shown that this microstructural design leads to distinct micro-mechanical responses in the layers ^[114], namely quasi-plasticity (Figure 2.13), high compressive yield strength in the outer layers and strain-hardening in the inner layers, which synergistically combine to endow the club with a remarkable impact tolerance ^[114].

Figure 2.13 Quasiplasticity and damage mechanism of the dactyl clubs under native blunt impact. (A) The outer layer club under native contact at low magnification showing a distinct plastically deformed region under the contact. (B) High magnification micrograph of the plastically deformed region with misaligned FAP crystallites. (C,D) High magnification micrographs of the aligned FAP crystallites in the elastic region in polished and fractured surface respectively. (E) Schematic illustration of FAP crystallites texture in plastic (green bars) and elastic regions (white bars). Adapted from Ref. [114].

2.4.1 The Stomatopod's Raptorial Appendage

The morphology of Stomatopods lethal weapons (Figure 2.14) has evolved to suit different functions. For instance, elongated and spiny appendages -found in spearers- are associated with burrow-hidden sit-and-wait predation of fleshy and evasive prey, like fish ^[115], whereas a bulbous hammer at the base of the dactyl -found in 'smashers'- is typically used for the smashing of hard shell creatures such as crabs, snails, and other mollusks ^[55, 116].

Figure 2.14 Different types of appendages found in various species of stomatopods, generally classified as smashers and spearers. Credits for: Roy Caldwell.

Mantis shrimp appendages are mainly composed of four segments, denoted as merus, carpus, propus, and dactyl for both species (see Figure 2.15).

Figure 2.15 Raptorial appendages of stomatopod spearer (A) and smasher (B) are composed of four segments: merus, carpus, propus, and dactyl.

2.4.2 Stomatopod's Hunting Mechanism

Regardless of the hunting mechanism, *e.g.* impaling or shattering, both species have in common the fast velocity at which the appendages are deployed during a strike. The smasher's club strikes quicker than the spearer's at velocities of 14-23 m/s ^[106] versus 4.5-7 m/s for the spearer, respectively ^[115]. Early work suggested that the potential elastic energy could be stored in the extensor muscle and apodeme ^[117]. Patek and co-workers ^[104, 116, 118-120] later affirmed that muscles alone could not supply the energy required to trigger these ultra-fast raptorial movements. They then further suggested that a structure in the raptorial appendage, called the "saddle" located on the dorsal top of the merus segment (see Figure 1.3), acts as a strong and effective spring, storing elastic energy for striking preys (Figure 2.16) ^[106].

Figure 2.16 The mechanics of a stomatopod's strike. (a) Compression and extension of the saddle captured with a high-speed camera. **(b)** Saddle compression and extension during strike. **(c)** Saddle is simulated as a spring; m: merus, v: meral-V, c: carpus, p: propus, d: dactyl. Adapted from Ref. [106].

Additional studies introduced another segment located distal–laterally in the merus, namely the meral-V, which is a stiff exoskeleton structure that contributes in storing the elastic energy. It was shown that as the extensor muscle contracts, the potential elastic energy is stored not only in the saddle but also in the meral-V (Figure 2.17) ^[106, 107, 121].

Figure 2.17 The coupled saddle and meral-V contribution in storing elastic energy. (A) Drawing depicting the lateral view of the merus segment (m) of the feeding appendage of mantis shrimp. The resting saddle (s) and meral-V (v) are presented in solid outline; the loaded saddle and meral-V are shown in a semi-transparent blue overlay. With the contraction of the extensor muscle, the saddle compresses to a more concaved shape and at the same time the meral-V rotates. The other mineralized segment, namely the ventral bar (vb), also extends along with this amplification network. **(B)** Medial view of the saddle in the merus. **(C)** An illustration diagram representing the flexion of the saddle and meral-V with orange spring symbols. Adapted from Ref. [107].

Further complementary studies revealed that the strike is driven by a complex power amplification structure (Figure 2.18) located in the merus and comprising hard, soft, and connective tissues that all work in concert to amplify the power and the speed of the strike^[107, 121].

Figure 2.18 A schematic presentation of a power amplification system with its major components, such as the engine, amplifier, and tool. Mantis shrimp's appendage with its dactyl club compared to the strike by boxing glove (A) and a hammer (B). The engine can be represented by a muscle, the amplifier by a spring or a latch, and the end tool by the boxing glove or the hammer. (C) All functional modules contributing to the power amplification system are depicted on a microCT image of a peacock mantis shrimp raptorial appendage. Adapted from Ref. [119].

Expanding on the hunting mechanism more elaborately, similar to all other crustaceans, the mantis shrimp controls the movement of its appendages with two opposed muscles, which are connected to the carpus. They are both located in the merus, and are not connected to the saddle. The ambush predator slowly contracts the large extensor muscle in the merus, while the smaller flexor muscles in the merus brace the two sclerites as a latching structure, thus preventing the movement of the dactyl club^[117, 122, 123]. When the extensor muscle is fully contracted, the saddle is compressed, and the meral-V rotates while the ventral bar expands like a tape spring. At this stage, the entire merus exoskeleton flexes. When the target is close enough to the animal, the flexor

muscle expands and consequently releases the sclerites, causing a rapid propulsion of the propus and the dactyl outward toward the prey ^[107, 117, 118, 121-123] (Figure 2.19). During the strike, all the flexing exoskeletal structures (saddle, meral-V, ventral bar and the whole merus), muscles, connective tissues and apodemes return to their initial resting mode, releasing the stored potential energy into an ultra-fast and powerful strike ^[107, 118, 120].

Figure 2.19 A schematic diagram depicting the amplification system units status and the merus condition before and after the strike. (A) Ready-to-strike mantis shrimp appendage has a fully contracted extensor muscle, a loaded saddle, proximally rotated meral-V, and extended ventral bar. (B) During the strike the saddle and ventral bar extend back to their resting state and the meral-V drives the appendage toward the target. Adapted from Ref. [120].

2.5 Scope of This Project

Among the structures contributing to the power amplification system of the mantis shrimp's strike, the saddle is the stiffest structure that plays a crucial role in restoring a high amount of energy both in spearers and smashers ^[106, 117, 120]. With a hyperbolic-paraboloid structure, which constitutes a particular geometry with two opposite curvatures, the saddle is described as the first mineralized structure acting like a spring. Patek *et al.* have investigated this power amplification system, emphasizing the role of the saddle. However, a detailed materials analysis and microstructural study of the saddle has so far not been undertaken. Moreover, the actual degree of mineralization of the saddle remains yet to be revealed.

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Chapter 3

Experimental Methodology

Regarding to the objectives of this thesis, provided in Chapter 2, a wide range of experimental approaches have been employed to study the saddle component of merus in the raptorial appendage of mantis shrimp. In this chapter, all the characterization techniques including their principals and theories and also the sample preparation requirements for each are introduced. These techniques are mainly used for microscopy and geometrical characterization, elemental and structural characterization, chemical characterization, and mechanical characterization. Nanoindentation studies, compression experiment, and finite element modeling are used to study the mechanical responses of the saddle.

3.1 Rationale for the selection of Methods/Materials

A range of sample preparation and characterization methods has been used in the project to address questions raised within the scopes of this thesis work. In this chapter, these methods will be described in detail and presented in five main sections: (i) sample preparation, (ii) microscopy and geometrical characterization, (iii) elemental and structural characterization, (iv) chemical characterization, and (v) mechanical characterization. The latter has been further subdivided into nanoindentation studies, compression experiment, and finite element modeling.

3.2 Sample preparation

Fresh spearer (*Harpiosquilla harpax*) and smasher mantis shrimps (*Odontodactylus scyllarus*) were obtained from local commercial sources in Singapore. The spearer types were collected from local seafood stores and the smasher types were purchased from local aquarium stores. The samples from both species were either kept at -80 °C freezer for appendage's analyses or alive in the Biological and Biomimetic Materials Laboratory (BBML) tanks. It should be also noted that all the experiments were conducted on both spearer and smasher mantis shrimp saddles and in case of different results, the species have been named in each analysis. According to the requirements of the experiments, different sample preparation approaches were adopted for different experiments. Table 3.1 presents a brief overview of the sample preparation methods associated with specific experiments.

A surgery blade was used under a stereomicroscope (Carl Zeiss SteREO Discovery.V8) to dissect saddles from other body segments. The dissection was precisely performed to avoid the presence of any other hard or soft tissue left around the saddles. These excess tissues could indeed affect the quality and the interpretation of the microCT data and the compression tests. The samples were washed with deionized water several times to remove all residual salts and organic debris. For mounting and polishing purposes, the saddles were then impregnated with epoxy resin (EpoFix Kit, Struers, Denmark) in mounting dishes based on required directions. The cold mounting epoxy resin was prepared by mixing both resin and hardener in 15:2 volume ratios and was stirred for 2-3

minutes in a fume hood before pouring it into the mounting dish. To ensure a proper bonding between the samples and the resin, and to remove all the bubbles forming within the epoxy, the dish was later placed in vacuum chamber for around 1 hour. The hardening time was 12 hours and the peak temperature was 40 °C. A well-fixed sample is a critical requirement for nanoindentation studies, especially in hydrated conditions.

To obtain access to the required cross sectional plane of the samples, they were cut using a diamond blade cutter (IsoMet 4000, Buehler, USA) at 2000 rpm. Later, the surface of the samples was grinded and polished in different steps using abrasive SiC papers (grades 1000, 2400, and 4000). Final polishing was performed on cotton using 1 µm diamond paste and 40 nm colloidal silica suspensions. To remove diamond paste and wash away debris, polished samples were cleaned in ultrasonic bath for 5 minutes in 5 to 1 volume ratio of water and ethanol between each polishing step. The polished samples were used for optical microscopy, EDS, Raman and nanoindentation studies (Figures 3.1 and 3.2). To provide a better visualization of structural features of the saddle, some samples were fractured in liquid nitrogen to prevent surface damages that the polishing process may produce or to investigate the microstructural features using FESEM.

Table 3.1 An overview of the sample preparation methods required for each characterization technique used in this thesis, with the corresponding sections.

Characterization	Technique	Sample preparation method	Section
Microscopical and Geometrical	Optical Microscopy	Mounted and polished samples	3.3.1
	FESEM	Fractured samples; mounted and polished samples	3.3.2
	MicroCT	Dissected samples; full saddle or full merus	3.3.3
Elemental and Structural	EDS	Mounted and polished samples	3.4.1
	XRD	Powdered samples	3.4.2
	Raman Spectroscopy	Mounted and polished samples	3.4.3
Biochemical Characterization	TGA	Fresh saddle, separated inner and outer layer	3.5.1
	FTIR	Demineralized and deproteinized sample	3.5.2
	SDS-PAGE	Extracted protein	3.5.3
	MALDI-TOF	Extracted protein	3.5.4
	AAA	Fresh saddle	3.5.5
Mechanical	Nanoindentation	Mounted and polished samples	3.6.4 to 3.6.6
	NanoDMA	Mounted and polished samples	3.6.7
	Micro-Mechanical Testing	Dissected samples; full saddles	3.7

Figure 3.1 *Harpiosquilla harpax* mantis shrimp and sample preparation procedures. **(A)** Spearer mantis shrimp provided from local seafood stores. **(B)** Dissected appendage. **(C)** Merus exoskeleton after removing the muscles and fleshy tissues, washed with DI water and ready for dissecting the saddle under the stereomicroscope. **(D)** Dissected saddle. **(E)** Resin embedded saddles cut longitudinally and transversally and polished ready for nanoindentation, Raman, EDS and other studies.

Figure 3.2 Dissected merus, sectioned and polished sample. The whole merus impregnated in the epoxy resin and cut in different sections to have access to all parts of merus exoskeleton, meral-V, ventral bar, and saddle.

3.3 Microscopy and Geometrical Characterization

Initially, different microscopy methods were utilized to study the micro-structural features of the saddle. Optical and scanning electron microscopies (SEM) were deployed to observe the samples at different magnifications. Macroscopic geometrical aspects were also investigated using microscopy and microCT techniques.

3.3.1 Optical Microscopy

Optical microscopy was used to analyze the cross-sectional structural features of the sample using a Zeiss A1 microscope equipped with 2.5X, 10X, 20X, 50X and 100X objective lenses, Differential Interference Contrast (DIC) filter, and polarized light. Bright field and polarized light modes were used to visualize the overall structure and birefringence properties of the samples, respectively. The birefringence properties of the samples were probed by rotation of the microscope stage at 0°, 45° and 90° degrees.

3.3.2 Field Emission Scanning Electron Microscopy

In order to probe structural features and geometrical aspects of the different layers of the saddle, a field emission scanning electron microscope (FESEM) 7600 F (JEOL, Country) was used. To prevent surface charging, the samples were coated with platinum for 60 s at 20 Amp to deposit 8-10 nm of conductive coating. Low secondary electron image (LEI) detector as well as 5 kV applied voltage was used to improve image quality and to further prevent surface charging.

3.3.3 X-ray Micro Computed Tomography (MicroCT)

X-ray micro computed tomography was used to provide 3D geometrical and structural specification of the saddle at the micro scale. Both separated saddle and complete merus were used to measure the overall dimension as well as the layers distribution and related thickness along the samples. The outputs were used to reconstruct

a 3D map of the sample for subsequent finite element modeling. The different layers were differentiated according to the extracted relative density spectrum.

An inspeXio SMX-90CT Plus (Shimadzu, Japan) with a 90 kV voltage and 0.008 and 0.012 mm/pix voxel size was used for the dissected saddle and merus, respectively. The 3D model was rendered using VGStudio Max software.

3.4 Elemental and Structural Characterization

In order to gain information about the chemo-structural properties and the elemental distribution along the saddle, several characterization techniques were used, including EDS, XRD, and Raman spectroscopy.

3.4.1 Energy dispersive X-ray spectroscopy (EDS)

To perform elemental analysis on the saddle of mantis shrimp, Energy dispersive X-ray spectroscopy (EDS) measurements were conducted. EDS spectroscopy was performed on polished and carbon-coated samples to obtain elemental distribution in points and imaging mode in the layers of the saddle. Preliminary experiments were performed using a FESEM 7600 F (JEOL, USA) equipped with INCA energy dispersive spectrometers with a detector area of 12 mm². Complimentary elemental mapping was performed at the Wyss Institute for Biologically Inspired Engineering at Harvard University using a Tescan Vega (Czech Republic) SEM equipped with two diametrically opposed energy dispersive spectrometers with individual detector areas of 30 mm² (Bruker XFlash 5030). The accelerator voltage for EDS studies was set to 20 keV.

3.4.2 X-ray powder diffraction (XRD)

In order to analyze the crystal structure and chemical composition of the saddle, X-ray diffraction analysis was performed using a XRD Bruker D8. Due to sample size limitations -the minimum cross section area required for XRD experiment is 1 mm x 1mm - several saddles were grinded in the mortar in liquid nitrogen and the powder was

flattened on a glass slide for optimal preparation of the required surface area (around 1 cm x 1 cm). The XRD spectra were recorded at 0° to 120°.

3.4.3 Raman spectroscopy

Structural features of the saddle samples were probed in single point mode or maps using Raman spectroscopy.

Raman spectrometry of embedded and polished cross-sections was acquired with a confocal Raman microscope (alpha300, WITec, Ulm, Germany) equipped with 488 and 532 nm laser sources and Nikon or Zeiss objective lenses with different numeric aperture (NA). In order to increase the lateral resolution (*ca.* $0.61 \lambda/\text{NA}$), higher numerical aperture and lower wavelength are desirable. Initial Raman spectroscopy studies were performed along a saddle cross-section in three different points located from inner to outer layer. Each Raman spectrum was acquired for 90 s using 0.5 s integration time. For Raman imaging, the data were acquired with a polarized confocal Raman microscope (alpha300, WITec) using 0.3 s integration time and 1 μm spatial resolution. The WITec Project Plus 2.08 was used for filtering, spectrum processing such as average and baseline subtractions, and analysis. Raman microscopy images were generated by plotting the intensity of the Gaussian fit of the signal corresponding to the wavenumber ranges of each phase. All the Raman studies were collected in the range of 400-3500 cm^{-1} to cover the whole range for organic and inorganic components.

3.5 Biochemical characterization

A range of biochemical characterization techniques was used to analyze and quantify each phase present in the sample. The physico-chemical characterization of the chitin layer was conducted by FTIR together with the amino acid analysis of the proteins to provide a thorough understanding into the organic composition of the saddle.

3.5.1 Thermal Gravimetric Analysis (TGA) and Gravimetric Assay

In order to quantify the water, mineral and organic content of the saddle, two different techniques were employed, namely gravimetric assay and thermal gravimetric analysis (TGA). The first approach involved weighing the initial fresh samples, followed by freeze-drying and re-measuring the sample weight to evaluate the water content in the sample. Subsequently, experimental samples were treated in 0.1 M EDTA (ethylenediaminetetraacetate) for 7 days, weighed once more after freeze-drying to calculate the mineral content ^[1-3]. To selectively dissolve the proteins (but not chitin), two methods were used. The first method was used to demineralize the samples by first grounding them in liquid N₂ and then dissolving them in 8 M UREA and in 5% acetic acid solutions with a homogenizer. The samples were then centrifuged at 14000 rpm for 5 min to separate the supernatant from the non-dissolved solid residues. For the second method, the demineralized samples were soaked in an alkaline peroxide solution (mixture of H₂O, 30% H₂O₂, and 10 N NaOH, with volume percentages of 92.5%, 5%, 2.5%, respectively) at 70° C until the samples were completely depigmented, according to a procedure described in Ref. [4]. The solid insoluble fraction after the alkaline peroxidation procedure was rinsed several times with DI water and weighted after freeze-drying to quantify the weight percentage of chitin and protein.

Thermogravimetric analysis (TGA) was also carried out on a freshly dissected saddle, from which the outer and inner layers were carefully separated from each other. Measurements were conducted using a TGA TA Instrument Q500 with a mass resolution of 0.1 µg. The experiment was performed with a linear heating rate of 5.00 °C min⁻¹ in an inert atmosphere from ambient temperature to 900 °C.

3.5.2 Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared (FTIR) spectroscopy was used to evaluate the organic composition of the saddle, mainly the crystalline structure of the polysaccharide ^[5]. The transmission mode of Bruker FTIR Vertex 70 model was used for analysing demineralized samples, as well as the soluble and insoluble fractions of the simple

gravimetric assay in the range of 4000 to 800 cm^{-1} with a resolution of 4 cm^{-1} . The spectra were analyzed using the Bruker OPUS 6.0 software.

3.5.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to investigate the proteins molecular weight of the samples, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed.

The supernatant of the sample dissolved in 8 M urea in 5% acetic acid was collected after being centrifuged at 14000 rpm for 5 min. The samples were first mixed at 1:1 ratio with 2X Laemmli buffer (Bio-rad, California, USA) and boiled for 5 min. They were then run into a gel (4% stacking gel and 10% resolving gel) by SDS-PAGE performed at 10 mA and 250 V. The gel was subsequently stained with Coomassie Blue R-250 (AppliChem, Germany) to reveal protein bands.

3.5.4 Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectroscopy (MALDI-TOF)

The extracted proteins were also analyzed by Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectroscopy (MALDI-TOF) to determine their molecular weights. The measurement was done in a Krato Axima Performance MALDI-TOF mass spectrometer (Kratos-Shimadzu Biotech, UK). 10 mg/ml sinapinic acid in 50:50 solution of water (0.1 wt% trifluoroacetic acid):acetonitrile was used as the matrix. The sample was prepared using the “sandwich” method ^[6]. 1 μl matrix was first loaded onto the MALDI-TOF sample plate. Once the matrix was dried, 1 μl sample was loaded on top of the matrix and another 1 μl matrix was loaded on top. At last, the whole dried sandwich was used to run the experiment. The linear mode was used to record the spectra, with 500 laser shots per sample at a power of 110 system units.

3.5.5 Amino Acid Analysis (AAA)

In order to identify the relative amount of each amino acid in the proteins of the saddle, amino acid analysis (AAA) was employed.

Prior to AAA, each sample, both the solid residue and the supernatant, was acid-hydrolysed in 6 M HCl with 5% phenol as an anti-oxidant *in vacuo*. The hydrolysis was conducted at 110 °C for 24 hours. The hydrolysates were flash-evaporated using a speed vacuum (ScanVac Scan speed 32, USA), washing them several times with DI water and methanol in 1000 rpm and 52 °C. The products were then re-suspended in 300 µl of sample dilution buffer (SYKAM, Germany). The samples were centrifuged at 14 rpm for 5 min. AAA was conducted using a ninhydrin-based detection with post-column derivatization (Sykam S 433 AAA). The elution time of the peaks was calibrated with a standard amino acid solution. For chitin elution, glucosamine (the hydrolysis product of chitin and chitosan) was used as a standard.

3.6 Mechanical Analysis: Nanoindentation Studies

Mechanical responses of the saddle were investigated both at the micro- and the macro-scales. In order to probe the mechanical properties of the distinct layers of the saddle at the micro-scale, nanoindentation studies were performed on the finely polished cross-sectional surface of the samples. The experiments were done using a Triboindenter TI-950 (Hysitron, MN, US). Based on the required mechanical properties, two series of experiments were performed according to the Oliver-Pharr method (Section 3.6.1) and Hertzian contact theory (Section 3.6.2), respectively. The first theory was used to extract the elastic modulus and hardness of the different layers of the sample (in both high resolution line scan and mapping modes) in hydrated (Figure 3.3) and dry conditions. These parameters were calculated from loading-unloading curves extracted from indentation studies using a cube corner tip and a 2D standard transducer. A maximum load of 1000 μN using a loading function 5s–2s–5s (loading–holding–unloading) was used for the experiments. The tip was calibrated using a fused quartz standard sample ($E=69.6 \text{ GPa} \pm 10\%$).

Hertzian contact theory was used to extract the elastic-plastic response of the different layers calculated from partial loading – fully unloading indentation curves using a 10 μm cono-spherical fluid cell indentation tip and a 30 mN maximal force transducer. The load was applied in 70 cycles from 50 μN to 15 mN in both dry and hydrated conditions. The extracted contact depths and loads were used to calculate the indentation stress-strain curves.

Figure 3.3 Schematic image of the test setup for nanoindentation studies in hydrated mode. The submerged polished sample embedded in epoxy resin is placed inside the dish. A fluid cell tip is used to perform the indents on the sample.

3.6.1 Oliver-Pharr theory

Depth sensing indentation studies were performed using the Oliver-Pharr method ^[7], which is used for a wide range of indentation tip geometries to extract materials elastic modulus and hardness ranging from the KPa to GPa^[8, 9]. The elastic response of the unloading section of the loading-unloading curve, the maximum applied load and the calculated tip area function were used to measure elastic modulus and hardness of the samples. The interval of the indents in both high-resolution line scan and mapping were 10 μm and 8 μm , respectively.

Figure 3.4 A schematic indentation loading unloading curve using a sharp tip. By increasing the applied load from the initial contact point, the tip penetrates in the sample. Loading continues up to the maximum load set by the user. Over unloading, the sample recovers elastically and the overall plastic deformation records when the contact load returns to zero.

The initial segment of the unloading curve is used to calculate the stiffness and the contact depth of the sample (Figures 3.4 and 3.5) as follows:

Figure 3.5 A schematic of indentation residual impression and its related loading-unloading curve. The unloading curve is used to extract the stiffness (S), contact depth (h_c), and contact area (A_c).

$$E = \frac{dP}{dh} \frac{1}{2} \frac{\sqrt{\pi}}{\sqrt{A}} \quad (\text{Eq. 3.1})$$

and

$$H = \frac{P}{A} \quad (\text{Eq. 3.2})$$

where $\frac{dP}{dh}$ is the slope of the unloading curve, A is the contact area and P is the maximum applied load. The tip area can be calculated by curve fitting of the extracted data from indentation curves using a standard sample with known elastic modulus (refer to Section 3.6.3).

A loading-unloading curve with no residual impression depth represents a fully elastic response of the sample (Figure 3.6). In these cases, Oliver-Pharr method is not applicable.

Figure 3.6 Fully elastic and elastic-plastic behavior of a material under indentation. For a fully elastic response, the loading and unloading curves almost overlap, whereas for the elastic-plastic response, the unloading curve follows a different path, with a residual indentation depth observed after a loading/unloading cycle.

3.6.2 Hertzian contact theory

In his seminal work, Hertz demonstrated that the indentation contact radius (a) is related to the applied load (P), the indenter radius (R) and the elastic properties of the material ^[10] (Eq. 3.3). This theory has been used since the late 1980s to probe elastic properties of materials under contact load. In this method, blunt tips (mainly a rigid spherical indenter) are used to impose a mostly elastic response to the sample, rather than to plastically deform it (which occurs with sharp tips due to stress localization). Figure 3.7 shows a schematic of a Hertzian contact.

Figure 3.7 A schematic Hertzian contact. $h_{\max}$ is the total depth of penetration under the indentation load P , h_a is the depth of the circle of contact from the specimen free surface, h_c is the distance from the bottom of the contact to the contact depth circle, a is the radius of the contact circle, and R is the radius of the indenter.

According to the Hertzian theory:

$$a^3 = \frac{3PR}{4E} \quad (\text{Eq. 3.3})$$

For rigid indenter and non-rigid specimens:

$$h = \delta = \frac{a^2}{R} \quad (\text{Eq. 3.4})$$

where h is the contact depth and δ is the distance of mutual approach. Then, the load as a function of contact depth can be expressed as:

$$P = \frac{4}{3}ER^{\frac{1}{2}}h^{\frac{3}{2}} \quad (\text{Eq. 3.5})$$

Figure 3.8 A schematic Hertzian curve fitting on a loading-unloading indentation curve. During loading, the initial response is elastic and is followed by plastic yielding, which can be detected as the curve deviates from the ideal Hertzian solution. The elastic response is used to calculate the elastic modulus using Eq. 3.5. Upon unloading, the response is fully elastic and can be extracted by a linear fitted curve. The residual impression is due to yielding occurring when the load exceeds the elastic regime.

3.6.3 Calibration, tip selection and load functions

In order to calibrate the contact area of the indentation tips, standard fused quartz ($E = 69.6 \pm 10\%$ GPa) and polycarbonate ($E = 3.2 \pm 10\%$ GPa) samples were used for shallow and deep indents, respectively. According to the required mechanical parameters (high resolution E modulus mappings and indentation stress-strain curves), two different indentation tips were used for the experiments. A cube corner tip (apex radius 50-70 nm) was used for high-resolution line and area scans. For indentation stress-strain studies, a cono-spherical fluid cell with 10 μm nominal radius was used to meet the Hertzian contact criteria, and to cover both elastic and plastic indentation responses.

To calculate the elastic modulus, one of the key parameters to consider is the contact area (Eq. 3.1 and 3.2), which is a function of the contact depth. The contact area can be fitted with a mathematical function of the contact depth (h_c) as follows:

$$A = C_0 h_c^2 + C_1 h_c + C_2 h_c^{\frac{1}{2}} + C_3 h_c^{\frac{1}{4}} + C_4 h_c^{\frac{1}{8}} + C_5 h_c^{\frac{1}{16}} \quad (\text{Eq. 3.6})$$

where the first term represents the ideal area function of the indenter (2.598 for a cube corner and 24.5 for a Berkovich probe).

Figure 3.9 Schematic of multiple curve plot of calibration indents and extracted data (contact depths and areas) used for tip area calibration. The exact load range will depend on which type of probe is being used and by the calibration limits set by the user. The extracted contact depths define the range of calibration.

3.6.4 Elastic modulus and hardness mapping

In order to probe the elastic modulus and hardness of the cross-section of the saddle, high-resolution nanoindentation mapping (interval steps 8 μm, overall area 80 μm x 380 μm) was performed in dry and hydrated conditions. The experiment was implemented using a Triboindenter TI-950 (Hysitron, MN, US) equipped with a 2D standard transducer and a fluid-cell cube corner tip. A 5-2-5 s loading function with maximum load 1000 μN and 400 μN (for dry and hydrated conditions, respectively) in automation mode. The collected data were mapped using XYZ plot software (Hysitron, MN, US).

3.6.5 Partial Loading-Unloadings and Stress-Strain Curves

In order to evaluate the elastic plastic response of the samples and whether the saddle undergoes yielding and plastic deformation under compressive forces, indentation stress-strain curves of the sample were measured. Although the stress-strain curves are normally extracted from tensile testing, nanoindentation technique was used due to the sample size limitations. To calibrate the 10 μm cono-spherical tip, a wide range of forces (50 μN to 12000 μN , for 125 indents) were applied to polycarbonate and fused quartz standard samples, allowing to cover 35 to 1700 nm of contact depth range. Using the extracted stiffness (S) values and known Young's moduli E of polycarbonate and fused quartz, the contact radii were calculated from:

$$a = \frac{S}{2E} \quad (\text{Eq. 3.7})$$

$a-h_c$ values were subsequently plotted and fitted with the following expression:

$$a = 13.879 \times (h_c + 68.00775)^{0.78572} \quad (\text{Eq. 3.8})$$

The tip radius R at each contact depth can then be calculated as:

$$\frac{a}{R} = \frac{1}{\sqrt{(\tan^{-2} \theta + 1)}} \quad (\text{Eq. 3.9})$$

where $a = f(h_c)$ is the contact radius, and θ is the tangent angle.

The indentation stress-strain points for each cycle was finally calculated using:

$$\varepsilon = \frac{a}{R} \quad (\text{Eq. 3.10})$$

$$\sigma = P_m = \frac{P}{\pi a^2} \quad (\text{Eq. 3.11})$$

To collect the required data for the above-mentioned equations, a 70 cycles loading-unloading test was performed on the sample under Hertzian contact loads. The experiment was performed using a Triboindenter TI-950 (Hysitron, MN, US) equipped with a 1D standard transducer (maximum load) and a fluid-cell cono-spherical tip (radius 10 μm). The tip was calibrated using standard fused quartz and polycarbonate samples for

required depth ranges. The measured contact depths and applied loads were used to extract the indentation stress-strain curves.

3.6.6 Energy Expenditure Index

Energy expenditure index, symbolized by ψ , is a parameter that describes the relative elastic-inelastic property of a material under stress and inelastic strains. It is calculated as the ratio of the dissipated energy to the total input energy. The energy index is a normalized term, representative of the amount of absorption and recovered energy.

In nanoindentation studies, the area under the loading curve in a load-displacement curve (loading-unloading) corresponds to the total energy (U_t) (Figure 3.10, purple), and the area under the unloading curve indicates the recovered energy (U_e) (Figure 3.10, green). Their difference corresponds to the work spent in irreversible processes, including permanent deformation, protein unfolding or untwisting, and cracking, and it is expressed as the dissipated energy during indentation, U_d (Figure 3.10, blue) ^[11].

$$U_t = U_d + U_e \quad (\text{Eq. 3.12})$$

and

$$\Psi = (U_d/U_t) \times 100 \quad (\text{Eq. 3.13})$$

where U_t corresponds to the total energy spent, U_d is the dissipated energy, U_e is the recovered energy and Ψ is the energy index.

Figure 3.10 Schematic diagram of a typical elastic-plastic deformation during an indentation cycle. The dissipated energy (U_d) during indentation is illustrated by the blue area, delimited by the loading and unloading curves. U_e (green area) is the amount of recovered energy due to the elastic behavior of the material. The purple lines defines the area corresponding to the total energy (U_t).

The energy expenditure index (Ψ) of the cross-sectional sample of the saddle was measured on the sample in dry and hydrated conditions. The experiments were performed using a Triboindenter TI-950 (Hysitron, MN, US) equipped with a 2D standard transducer and a fluid-cell cube corner tip. A 500 μN maximum load (for both dry and hydrated state) in automation mode (interval steps 8 μm , overall area 80 $\mu\text{m} \times 400 \mu\text{m}$) was set for the experiment. The collected data were analyzed using OriginPro 9.0 and mapped using XYZ plot software (Hysitron, MN, US).

3.6.7 Nanoscale Dynamic Mechanical Analysis (NanoDMA)

Nanoscale Dynamic Mechanical Analysis (NanoDMA) is a very recent and powerful technique that is used to probe nanoscale dynamic properties of materials. By measuring the resulting load amplitude, displacement amplitude and the phase shift during the test (Figure 3.11), the *CMX* control algorithms equipped in the nanoDMA instrument can

provide the continuous calculation of the loss modulus, storage modulus and $\tan(\delta)$ as a function of indentation contact depth, time, and frequency.

Figure 3.11 Schematic illustration of the origin of resultant phase lag and deformation in nanoDMA when a sinusoidal stress to strain load is applied.

In this experiment, the indenter probe applies a quasi-static force with a small oscillation (oscillatory force) to the sample. The transducer that is connected to the sample detects the dynamic responses from the force-displacement signals. The response and signals are processed leading to stiffness and damping properties measurements. These results give valuable insights into a material's elastic response (storage modulus) or its tendency to absorb or dissipate energy (damping, $\tan \delta$). The complex modulus, E^* , which is the sum storage plus the loss modulus is defined in Eq. 3.14 as:

$$E^* = E' + iE'' \quad (\text{Eq. 3.14})$$

where E' is the storage modulus (the in-phase (real) component which is a measure of the elastic response of the material) and E'' is the loss modulus (the out-phase, imaginary component), which is a criterion to measure the viscous property of materials.

The phase lag between the force and displacement waves (stress and strain) is named δ , and the $\tan \delta$ that is the ratio of the loss to the storage modulus, which is often called damping.

The *CMX* algorithm, which is an add-in to the nanoDMA, is able to accurately calculate these mentioned terms using the Kelvin-Voigt model:

$$E'' = \frac{\omega C_s \sqrt{\pi}}{2\sqrt{A_c}} \quad (\text{Eq. 3.15})$$

$$E' = \frac{K_s C_s \sqrt{\pi}}{2\sqrt{A_c}} \quad (\text{Eq. 3.16})$$

$$\tan \delta = \frac{E''}{E'} = \frac{\omega C_s}{K_s} \quad (\text{Eq. 3.17})$$

where ω is the frequency, C_s is the compliance, K_s is the stiffness of the sample, and A_c is the contact depth.

The DMA experiments were done using a Triboindenter TI-950 (Hysitron, MN, US) equipped with a DMA transducer and a cube corner tip. A *CMX* loading function with a maximum load of 1000 μN and a frequency of 220 Hz was applied to the samples polished down to 0.04 μm in dry and hydrated conditions.

3.6.8 Sample preparation and test procedure

Due to the nature of the indentation experiments, the surface roughness has a significant effect on the measured values (Figure 3.12). Hence, in order to minimize the surface roughness effects, the sample surface must be very smooth and finely polished.

Figure 3.12 Schematic illustration of an inappropriate sample surface contact with a conical tip due to the surface roughness.

In order to prevent this situation to happen, the samples were finely polished to 1 μm using diamond paste and finally to 40 nm using colloidal silica suspension.

3.7 Mechanical Analysis: Compression

In order to identify the mechanical response of saddles under compressive loads and to measure their spring constant, a customized micro-tension-compression tester was used. The machine was equipped with a 450 N LSB200 loadcell (Futek, USA) with 0.1 N resolution and a 5 cm linear variable differential transformer (S series, Solartron, UK) with 1micron resolution (Figure 3.13). SENSIT test and measurement software were used to record the load-displacement data. The load cell and LVDT were calibrated by their respective manufacturers. Fresh samples were longitudinally fixed between the grips of the testing rig with super glue and were immersed in a water container for 30 minutes to keep them hydrated. The load-displacement data was recorded using real-time data loggers. The load was increased gradually at a rate of 2 N/s to the immersed samples until failure. The linear part of the load-displacement curves were used to calculate the saddle spring constant of the samples. Transversal expansion measurements were obtained using a 1 mm resolution digital micrometer (series 293-230 Mitutoyo, Japan), with the data recorded on the computer using a USB-ITN cable (Mitutoyo, Japan).

Figure 3.13 Customized micro testing machine used for compressing the saddle for stiffness determination. (A) An overview of the machine and its load and displacements gauges, and (B) a fixed saddle immersed in water during the experiment.

3.7.1 Data analysis

To calculate the saddles' stiffness, the load-displacement curve were plotted using OriginPro 9 software and the linear part of the curves was used to calculate the stiffness of the samples. StatPlus was used for statistical evaluations of the results, *i.e.* mean values, *p*-values, standard errors and deviations.

3.8 Mechanical Analysis: Finite Element Modeling (FEM)

In order to further analyze the mechanical response in the samples during a loading/release cycle, finite element modeling was used to assess the distribution of load and strain in the individual layers of the saddle during compressive loading.

3.8.1 2D Finite Element Modeling

Initially, a 2D planar model in longitudinal direction was constructed to model the saddle. To acquire precise geometrical shape and dimensions, FESEM micrographs of the saddle in the longitudinal cross sections were used. The finite element software Abaqus/Explicit 6.15 under plane-strain conditions was used to analyze the stress distribution. The data collected from nanoindentation studies used as input data for the constitutive response of each layer. The constitutive laws for each layer obtained by

fitting the experimental Hertzian stress-strain curves in hydrated conditions. For the outer layer with the experimentally-obtained elastic-plastic behavior, J2 plasticity with isotropic hardening behavior was used. For the inner layer, a strain stiffening hyper elastic behavior (Ogden constitutive model with $n = 3$) was employed.

3.8.2 3D Finite Element Modeling

Since the saddle has a complex geometrical structure with opposite curvatures, 2D modeling is not sufficient to provide rational results of the saddle's response under compression. Moreover, the results on the amount of stresses are not reliable either. Hence, to improve the results of stress distribution, a 3D model was generated with SolidWorks simulations software (SolidWorks 2014).

To create a more realistic 3D structure of the saddle in SolidWorks, microCT scans of the cross sectional views of the saddle at specific intervals were first used. For a more comprehensive model, cross sections were later curve-fitted to obtain the paraboloid equations in both longitudinal and transverse cross sections. The size and geometrical parameters of the model were obtained from averaging the actual saddles' shapes and dimensions of a large number of saddles ($n = 24$).

Each section of the saddle was assigned with materials properties acquired from the nanoindentation experiments.

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Chapter 4

Geometrical, Microstructural and Chemical Characterizations^{*}

The appendage of mantis shrimps exhibit an impressive set of characteristics adapted for providing high velocity impacts, with storing of elastic energy within the saddle part. Based on the functionality of the saddle they should possess excellent elasticity and exhibit remarkable extensibility that is fully, yet quickly, reversible. These properties highly depend on the chemo-microstructure and the spatial distribution of the building blocks. The high-resolution chemical and microstructural mapping and the nanomechanical analysis reveal that the saddle possess a bi-layer structure that is spatially tuned to combine the flexibility and stiffness to both act as an efficient spring and prevent the probability of the local buckling.

^{*} This section has been published and front-covered substantially as “M. Tadayan, *et al.*, *The Mantis Shrimp Saddle: A Biological Spring Combining Stiffness and Flexibility*, Adv. Funct. Mater., 2015, 25, 6437-6447”.

4.1 Introduction

In regard to the literature survey on the mantis shrimp power amplification system, previous studies have revealed the various sources of storing-energy mechanisms during a striking cycle, including flexing of the merus exoskeleton to restore the energy, rotation of the meral-V, flexing of the ventral bars acting as tape springs, and the compression and expansion of the saddle ^[1-3]. While a detailed picture of the kinematic mechanisms involved during energy storage and release is currently still being refined, there is evidence that among these coupled structures the saddle is among the stiffest and plays a central role in storage of elastic energy, with a mechanism that remains highly similar between the smasher and the spearer species ^[4-6].

In order to maximize the impact energy, the saddle must store a high amount of elastic energy, *i.e.* its spring constant must be high and it must exhibit substantial elastic flexibility. Ideally, this requires stiff building blocks that are capable of being elastically deformed; yet not too brittle in order to prevent internal damage during deformation, which would seriously undermines repetitive usage. This provides an “engineering” challenge because high stiffness and flexibility are generally viewed as mutually exclusive properties of structural materials, including biological materials ^[7-9]. According to large-scale microCT observations ^[2], the saddle has been suggested to be partially mineralized, which would fit the first requirement since mineralized phases are stiffer than biopolymers. At the same time, minerals are more brittle and much less extensible than most unmineralized structures, which would thus fail the second requirement ^[10]. However, a detailed materials analysis and microstructural study of the saddle structure has so far not been undertaken, such that the actual degree of mineralization of the saddle remains an open question.

In this chapter it is aimed to elucidate this apparent dichotomy by characterizing the chemical composition and spatial distribution of the constitutive phases of the saddle. Such studies have previously not been conducted and thus the details of the mineral content and their spatial distribution will provide a better understanding of the saddle functionality.

4.2 Experimental Methods

Based on the aim of this chapter, a comprehensive characterization of the saddle, the major element contributing in storing substantial amount of elastic energy for ballistic strikes of mantis shrimp, was done. The characterization was mainly focused on the compositional and microstructural features and subsequently the hierarchical organization of the saddle. Later relevant geometrical investigations and mechanical techniques were deployed to analyze the mechanical responses as well.

4.2.1 Research Specimens (Saddle)

The saddles of the fresh specimens of *Harpiosquilla harpax* (Figure 4.3) and *Odontodactylus scyllarus* (Figure 4.4) were used in this chapter. All samples were prepared as described in Section 3.2.

4.2.2 Microstructural Imaging and Characterization

In order to image the microstructure and the geometrical aspects of the saddle, FESEM and microCT techniques were used, respectively. The merus and saddle images were captured using an EOS 700D camera equipped with an EF 100 Macro lens (Canon, Japan). EDS technique was deployed to study the elemental distribution. For further details see Sections 3.3.2, 3.3.3, and 3.4.1.

4.2.3 Biochemical Characterization and Gravimetric Assay

In order to characterize the biochemical properties and calculate the organic/inorganic ratio and the water content of samples, some protocols and approaches were employed such as UREA treatment, alkaline peroxidation, and the TGA technique. Figure 4.1 shows the appearance of the spearer mantis shrimp saddle after each treatment. FTIR and AAA were performed on both the soluble and insoluble fractions to further analyse the chitin and proteins. Further details are provided in Sections 3.5.1, 3.5.2, and 3.5.5.

Figure 4.1 The saddle samples used in this study. (A) A fresh sample dissected from the merus segment. (B) Demineralized saddle with 0.1M EDTA for 7 days. (C) Demineralized and deproteinized saddle by 0.1M EDTA and alkaline peroxidation, respectively.

4.2.4 Raman Spectroscopy and Imaging

Initial Raman spectroscopy studies were performed along a saddle cross-section in three different points located in the innermost, inner, and outer layer. Each Raman spectrum was acquired for 90 s using 0.5 s integration time. For Raman imaging a 532 nm laser source and a 20X Nikon objective lens with 0.4 numeric aperture (NA) and the lateral resolution of ca. $0.61 \lambda/\text{NA}$ were used and finally the data was acquired using 0.3 s integration time and 1 μm spatial resolution. The images were generated by plotting the intensity of the Gaussian fit of the signal corresponding to the wavenumber ranges of phosphate ($930\text{-}990 \text{ cm}^{-1}$), carbonate ($1060\text{-}1100 \text{ cm}^{-1}$), and organic matrix ($2810\text{-}2980 \text{ cm}^{-1}$). The details for the device and other settings are provided in Section 3.4.3.

4.2.5 Nanoindentation

Indentation studies were conducted on embedded and finely polished samples using a cube corner tip (50-70nm) diamond fluid cell tip. Indentation mapping was done over an area of $80 \times 380 \mu\text{m}$ that included the entire width ($380 \mu\text{m}$) of the saddle's cross section and the indentation spacing was 8 μm in both dried and hydrated conditions. A 1 μN set point force was used to accurately detect the samples' surfaces. The details and theories behind this experiment are further described in Section 3.6.1 and 3.6.4.

4.2.6 Compression Studies

To measure the stiffness of whole saddle samples, a custom-made micro uniaxial testing machine was used to compress the saddle in hydrated condition. See Figure 3.13 and refer to Section 3.7 for further details of the test setup.

4.2.7 Data analysis and plotting

Statistical analysis and data plotting were done using the Statplus and OriginPro 9.1 software. A total number of 48 saddle samples were tested in the entire study of this chapter.

4.3 Results and Discussion

As a hyperbolic-paraboloid structure located in the dorsal part of the merus, the saddle is fixed along its longitudinal direction to the hard shell of the merus, and is flanked with a soft and flexible membrane on its sides (Figure 4.2 and 4.3). Although the colour and size vary from species to species, the geometrical characteristics of the saddle remain essentially identical in all species ^[1, 5].

Figure 4.2 Photographs of *Harpiosquilla harpax* stomatopod's feeding appendage. (A) Merus in the upper part of the appendage. (B) Medial view of the saddle in the merus. (C) Top view of the merus, the saddle, and the rigid contact regions. (D) The saddle and meral-V positions in the merus from the lateral view. (C1, C2) and (F1, F2) indicate the contact points and free edges, respectively.

Figure 4.3 Photographs of *Odontodactylus scyllarus* stomatopod's feeding appendage. (A) Merus in the upper part of the appendage. (B) Medial view of the saddle in the merus. (C) Dorsal view of the merus, the saddle, and the rigid contact regions. (D) The lateral view showing the position of meral-V in the merus.

4.3.1 Macroscopic features and saddle microstructure

Macro-photo images displayed in Figure 4.2 and 4.3 illustrate the characteristic hyperbolic-paraboloid shape of the saddle, which is elongated along the longitudinal axis of the body. The saddles' sizes varied from 6.7 ± 1.5 mm in length and 5.9 ± 1.3 mm in width ($n = 25$). In order to visualize the overall mineral distribution in the structure, a whole saddle structure was probed by microCT. The resulting 3D scan is shown in Figure

4.4A, where the location of the fixed points C1 and C2 is also highlighted. Cross-section images were then generated from the 3D scan across mutually perpendicular planes, namely the sagittal (C1-C2) and the transversal (F1-F2) planes, as illustrated in Figure 4.4B. In these sections, the phase contrast was obtained by filtering the CT scan with the mass density, such that light regions are color-coded in blue while denser regions appear red. This density contrast clearly indicates that the saddle is comprised of two distinct layers: an outer, denser layer, and an inner layer with a lower mass density. While the overall thickness of the saddle remains constant ($346 \pm 58 \text{ } \mu\text{m}$; $n = 25$), the relative thickness of both layers is not uniform across the saddle. The inner layer expands towards the external edges, whereas the thickness of the outer layer gradually decreases away from the center of the saddle and eventually vanishes at the free edges. This feature is observed for both cross-sectional planes. Furthermore, a very thin layer was observed on the free surface of the saddle (Figure 4.4C). As described later, this thin layer is unmineralized and is suggested to serve as a waterproof protective layer, very similar to the role of waxy epicuticle in arthropod cuticles ^[11].

Figure 4.4 MicroCT scan of a saddle of spearer mantis shrimp. **(A)** MicroCT image of the whole saddle (in green) illustrating its position in the merus. C1 and C2 points are fixed points through which the compressive force is transmitted in the native saddle. **(B)** Mutually perpendicular cross-sections planes subsequently used to visualize mineral distribution. C1-C2 is the sagittal plane and F1-F2 the transversal plane. **(C)** MicroCT cross-sections as defined in (B) with filtered density distribution. The outer layer is denser (highly mineralized, red), whereas the inner layer is less dense (weakly mineralized, blue). Towards the edges of the saddle, the outer (highly mineralized) layer vanishes. The thin green region corresponds to the waxy, waterproof coating. **(D)** Density filtering colour specification for the images in part (B) and (C).

Figure 4.5 FESEM micrographs of a spearer mantis shrimp saddle. **(A)** Low-magnification sagittal, and **(B)** transversal cross-sections of a whole saddle (C1, C2, F1, and F2 are defined as in Figures 4.2 and 4.4) each obtained by stitching 20 individual FESEM images. The red highlighted region indicates the outer highly mineralized layer. **(C)** Polished and urea-etched transverse cross-section near the center of the saddle, showing microstructural distinctions between each layer. λ is the periodic distance of the plywood structure in the inner layers. **(D)** Decrease of the outer layer thickness close to the contact regions. **(E)** Fractured surface of the saddle. **(F)** Higher magnification micrograph of the outer layer illustrating the presence of microtubular elements in the compact outer surface. **(G)** Higher magnification of the inner layer, showing the presence of sub-micron fibrous elements arranged in a helicoidal “Bouligand” pattern.

Low-magnification images of sagittal and transverse cross-sections, consisting of 20 FESEM micrographs assembled together, are depicted in Figure 4.5A-B, where the outer (denser) layer is highlighted in red for clarity. A higher magnification micrograph near the central region and encompassing the full thickness reveals that both layers are microstructurally distinct. The outer layer is more compact and exhibits a smoother overall appearance. As detected on the CT scans, it is thicker in the middle, becomes thinner towards the edges (Figure 4.5D), and finally disappears near the contacts points. The inner layer, on the other hand, exhibits a periodic sheet-like structure, with the thickness of the sheets (as represented by their periodic wavelength, λ) gradually decreasing towards the innermost region of the saddle. Fractured cross-sectional micrographs of the saddle are depicted in Figure 4.5E (low magnification image) and Figures 4.5F-G (higher magnification images) and provide additional information about the layers' microstructures. The outer layer (Figure 4.5F) contains compact microtubules generally oriented perpendicular to the free surface. In contrast, micro-fibrils are stacked into sheets in the inner layer. The micro-fibril orientation in the sheets is parallel to the free surface and this preferred orientation gradually varies between sheets. These sub-layers thus exhibit the classical “Bouligand-like” twisted plywood structure previously observed in the stomatopod's dactyl clubs^[12] and in other crustacean exoskeletons^[13-15].

4.3.2 Chemical composition and spatial distribution

Inorganic and organic spatial distribution

A low magnification FESEM micrograph of a saddle cross-section is depicted in Figure 4.6A. Raman spectra profiles and chemical images were subsequently acquired at various points along this cross-section, which were located either in the outer or in the inner layers. Raman spectra (Figure 4.6B) reveal significant differences between the layers, especially with regard to the relative intensities of the main bands. In the outer layers, four bands are observed at wavenumbers of 963 cm⁻¹, 1085 cm⁻¹, 1600-1700 cm⁻¹, and 2860-2940 cm⁻¹, which can be attributed to phosphate, carbonate, amide I (proteins), and C-H stretching of organic matrix (mainly chitin), respectively^[16], with the phosphate and carbonate bands being clearly the most intense. Two other sharp peaks at 1156 cm⁻¹

and 1520 cm^{-1} are attributed to carotenoid, an organic pigment responsible for the green or orange color of the saddle ^[16]. The broad bands (with full width at half maximum (FWHM) around 30 rel cm^{-1}) associated with the phosphate and carbonate vibrations imply the amorphous nature of these phases, thus corresponding to ACP and ACC respectively ^[17]. Figure 4.7 represents the phosphate band FWHM map on the saddle outer layer, for instance. The position of ACC Raman peak suggests around 30% Magnesium substitution ^[18, 19], whereas the ACP peak position indicates also the presence of the fluorine ions in the mineral phase. In the inner layers, the intensities of mineral phase bands are much smaller and similar to those arising from the organic matrix. Here, the peaks in the ranges $1600\text{ to }1700\text{ cm}^{-1}$ and $2800\text{ to }3000\text{ cm}^{-1}$ correspond to proteins and organic matrix in general, respectively, whose relative abundance are clearly higher in the inner layers of the saddle ^[16].

Visualization of the spatial distribution of these phases was achieved by imaging the major inorganic (ACP and ACC) and organic bands (Amide I and chitin) by polarized confocal Raman microscopy, as shown in Figure 4.6C. The images distinctly indicate that mineral phases are mainly localized in the outer layer, while the inner layers are predominantly organic. This pattern was corroborated by treating the saddle with 0.1 M EDTA for 12 hours and by comparing FESEM images of the sample before and after treatment (Figure 4.4D). EDTA treatment led to complete removal of the outer layer. The inner layer, on the other hand, remained mostly intact, although there was some shrinkage.

Figure 4.6 Raman spectra profiles and confocal imaging of a saddle cross-section. (A) FESEM of a saddle displaying the point locations for Raman spectra. **(B)** Raman spectra in the range of $800\text{--}3200\text{cm}^{-1}$ showing the major bands attributed to phosphate, carbonate, and the organic phase. **(C)** High resolution Raman images normalized and filtered for bands at $930\text{--}990\text{ cm}^{-1}$ (phosphate), $1060\text{--}1100\text{ cm}^{-1}$ (carbonate), and $2810\text{--}2980\text{ cm}^{-1}$ (organic matrix) **(D)** FESEM micrograph of a saddle cross-section before (left) and after (right) incubation in 0.1 M EDTA for 12 hours. EDTA incubation led to full removal of the outer layer and shrinkage of the inner layer.

Figure 4.7 The phosphate Raman band full width at half maximum shows that the phosphate is in amorphous phase in the saddle. (A) The Raman map region on FESEM micrograph of the saddle. (B) The FWHM of around 30 rel cm⁻¹ of the phosphate imply that the ACP is the stable phase in the outer layer.

By rotating the polarization of the incident laser light from 0 to 90 degrees the anisotropy of chitin/protein network was clearly observed (Figure 4.6C, green) ^[20].

Next, elemental analysis of a saddle cross-section was conducted using energy X-ray Dispersive Spectroscopy (EDS). Both point spectra on different regions and elemental mapping of a whole section were obtained (Figure 4.8A,B). Elemental mapping shows high contents of calcium (Ca) and phosphorous (P) in the outer layer, in agreement with the presence of calcium carbonate and calcium phosphate detected by Raman spectroscopy. Another salient feature of the outer layer is its enrichment in magnesium (Mg). Mg has been established to stabilize amorphous phases in biominerals ^[21, 22], which is again fully consistent with the amorphous state of these phases as suggested by Raman spectroscopy. On the other hand these elements are much less abundant in the inner layer,

which is instead enriched in carbon (C). Point spectra (Figure 4.8C) further indicate that the decrease in Ca, P, and Mg is gradual from the outer to the inner layer. Taken together, Raman and EDS data unambiguously show that the saddle outer layer is mineralized with ACP and ACC, whereas the degree of mineralization decreases in the inner layer, which is predominantly organic. To assess the relative weight fraction of organic and inorganic phases in each layer as well as their water content, the individual layers were carefully separated and subjected to Thermal Gravimetric Analysis (TGA) (Figure 4.9 and 4.10). In the outer layer, the water content was 14 wt. %, whereas for the inner layer the water content was 31 wt. %. In the dry state (namely after removing the weight contribution from water), the mineral to organic weight ratio was 84:16 for the outer layer and 50:50 for the inner layer (Table 4.1).

Figure 4.8 EDS elemental maps of a sectioned and polished spearer mantis shrimp saddle. (A) Optical micrograph revealing the layers and presenting the region and points for EDS maps and spectra, respectively. (B) High resolution elemental maps of calcium (Ca, yellow), phosphorous (P, green), and magnesium (Mg, magenta), In each map, carbon (C) content is shown in red. (C) Point spectra acquired in different positions along a saddle cross-section, showing both in major (top) and minor (bottom) elements.

Figure 4.9 Thermo gravimetric analysis (TGA) of the whole fresh saddle, the outer, and the inner layer. TGA results for (A) the whole, (B) the outer, and (C) the inner layer of the saddle. Water removal is completed at 200 °C, organic material degrades between 200 and 560 °C, and the decarboxylation of ACC phases happens above 560 °C ^[23].

Table 4.1 Water, mineral and organic content of the outer and inner layer of the saddle as obtained from TGA measurements.

	Water Weight % in fresh sample	Mineral Weight % in dry sample	Organic Weight % in dry sample
Whole Sample	23	67	33
Outer Layer	14	84	16
Inner Layer	31	50	50

Organic Composition

In order to elucidate the chemical composition of the organic phases present in the saddle, Fourier Transform Infrared (FTIR) spectroscopy and quantitative Ninhydrin-based Amino Acid Analysis (AAA) ^[24] measurements were conducted on a demineralized saddle (Figure 4.10A,i). The main bands of FTIR spectra correspond to Amide I and II and to polysaccharides, consistent with the presence of proteins chitin respectively. Zoom-in on the polysaccharide band (Figure 4.10B) and comparison with α - and β -chitin from the literature ^[25] allows us to conclude that α -chitin is the polymorphic chitin phase of the saddle.

The demineralized saddle was further treated in a solution of 5% acetic acid/8M urea and the soluble fraction analyzed by FTIR. The spectrum (Figure 4.10A, ii) shows that the intensity of chitin peaks nearly vanishes, indicating that proteins and chitin can be separated by this method. AAA profiles of a demineralized saddle are displayed in Figure 4.10C. Comparing the whole saddle with a glucosamine (GA) standard –the hydrolysis product of chitin– indicates that GA has the same elution time as Histidine (His), which would preclude accurate amino acid quantification given the high abundance of chitin in the saddle. Therefore quantitative AAA was conducted on a demineralized saddle that was subsequently incubated in 5% acetic acid/8M urea (soluble fraction) since this

treatment selectively separates chitin from proteins (Figure 4.10A,ii). Treatment with alkali peroxide (mixture of H₂O, 30% H₂O₂, and 10N NaOH, with volume percentages of

Figure 4.10 Biochemical analysis of saddle. (A) Fourier transform infrared (FTIR) spectra of saddle before and after chitin/protein separation. (B) Zoom-in in the 1750-900 cm⁻¹ region of the insoluble (protein-free) fraction. Doublet peaks (1660 and 1624 cm⁻¹) in the Amide I region as well as the 1022 cm⁻¹ peak are characteristics of α -chitin^[25]. (C) Amino acid analysis of (i) the whole saddle, (ii) a glucosamine (GA) standard corresponding to the hydrolysis product of chitosan, (iii) the insoluble fraction after 5% acetic acid/8M urea treatment, (iv) the insoluble fraction of alkali peroxidation treatment, and (v) the soluble fraction after 5% acetic acid/8M urea treatment. The inset in (B) is a FESEM micrograph after both EDTA and alkaline peroxidation treatments (selective dissolution of both minerals and proteins), showing the general structure of chitin fibrils.

92.5%, 5%, and 2.5%, respectively) was also used as an alternative method to separate proteins from chitin and identical results were obtained. A representative AAA spectrum of the protein hydrolysate following chitin removal is shown in Figure 4.10C (spectrum v), with the quantitative analysis presented in Table 4.2. The data indicate an amino acid

composition dominated by hydrophobic residues, notably Alanine (Ala), Glycine (Gly), and Valine (Val), which together account for 35 mol. pct. of the total amino acid composition. Another noticeable feature pertains to the relatively high abundance of acid residues, with aspartic acid (Asx) and glutamic acid (Glu) combining to ~ 17 mol. pct. of the total amino acid composition. Acidic proteins are well-known to play an important role in biomineralization processes ^[26, 27]. For instance phosphorylated proteins such as osteopontin in bone are important to regulate apatite mineralization during bone formation ^[28]. Similarly the Pif shell matrix protein has been shown to regulate calcium carbonate crystallization during nacre formation ^[29], and it is suggested that similar proteins account for the abundance of acidic residues detected in proteins extracted from the saddle. FESEM observations of the saddle structure were also conducted following EDTA and alkaline peroxidation treatments, after which only chitin remains (Figure 4.10B, inset and Figure 4.11B,C). This micrograph demonstrates that the residual chitin phase has an overall microstructure that is very similar to that prior to selective dissolution (Figure 4.5), suggesting that chitin forms the skeleton assembling into the helicoidal bouligand structure.

Table 4.2 Amino acid composition of the proteins in the saddle in mole percent.

Amino Acid	Mole percent (%)	Amino Acid	Mole percent (%)
Ala	14.5	Thr	4.7
Gly	10.1	Leu	4.4
Val	9.5	Phe	4.3
Asx	8.8	Tyr	3.9
Glu	8.1	Ile	3.7
Arg	6.4	Lys	3.6
Pro	5.5	His	3.5
Ser	4.9	Others	4.1

Figure 4.11 (A) Organic and mineral composition of the whole saddle obtained from simple gravimetric assay experiments. (B,C) FESEM micrograph after both EDTA and alkaline peroxidation treatments (selective dissolution of both minerals and proteins), showing the general structure of chitin fibrils.

4.3.3 Micromechanical properties and macroscopic mechanical response

Mechanical properties mapping of the saddle for both species were obtained by nanoindentation in both dry and wet conditions. These measurements show that the two layers exhibit significant differences, with the outer layer in dry conditions having a

higher elastic modulus ($E_{\text{dry}} = 33.7 \pm 0.3$ GPa) and hardness ($H_{\text{dry}} = 1.31 \pm 0.03$ GPa) than the inner layer ($E_{\text{dry}} = 10.1 \pm 0.1$ GPa; $H_{\text{dry}} = 64 \pm 9$ MPa) (Figure 4.12A). Both values sharply drop at the interface, but in contrast to the dactyl club^[30, 31] or many other biotools^[32], the mechanical response remains essentially homogenous within the individual layers. The mechanical contrast between the two layers is amplified under hydrated conditions (Figure 4.12A,B). Thus for the outer layer, the modulus drops 6-fold from dry to hydrated conditions ($E_{\text{hydrated}} = 5.02 \pm 0.02$ GPa), whereas the drop is 11-fold for the inner layer ($E_{\text{hydrated}} = 0.92 \pm 0.06$ GPa). These trends are fully consistent with the higher content of chitin/protein organic complex in the inner layer, since the mechanical response of biopolymers is well-established to be more susceptible to hydration than heavily mineralized phases^[10, 32]. It should also be mentioned that the mechanical properties of the outermost, waxy coating is orders of magnitude lower than the other layers, which is consistent with a water barrier role^[33] as opposed to a structural role. Comparisons between the two species are shown in Figure 4.13.

Figure 4.12 Mechanical properties of the spearer mantis shrimp saddle obtained by nanoindentation. (A) High-resolution elastic modulus (E) and Hardness (H) mapping of a saddle cross-section in dry (left) and hydrated (right) conditions. **(B)** Average E values for the outer and the inner layers in both dry and hydrated conditions. The decrease in these properties under hydrated conditions is more marked for the inner (11-fold) than for the outer (6-fold) layer.

Figure 4.13 Nanomechanical studies of mantis shrimp's saddle bi-material in dry and hydrated condition. (A) Elastic modulus and (B) hardness bar charts of dry and hydrated saddle showing that the mechanical properties in spearer and smasher samples are not statistically different (* for each pair, p -value > 0.1).

p -level	p -level	p -level	p -level	p -level	p -level	p -level	p -level
0.49623	0.96411	0.52572	0.4436	0.85633	0.48448	0.66883	0.63422

In the native merus, the compressive forces generated by the extensor muscle^[1, 2] are transmitted to the saddle in the longitudinal direction (Figure 4.2C) through the contact points C1 and C2. In order to study the saddle's macroscopic response to native compression when the animal is storing energy prior to its strike, the saddles were subjected to compressive testing using a custom made universal micro-testing machine. The saddle was fixed at both contact points (C1 and C2) and the load was applied longitudinally (Figure 4.14A). The axial displacement was measured with a Linear Variable Displacement Transducer (LVDT) and the transverse displacement along the

F1-F2 direction (Figure 4.14A) was simultaneously monitored at regular load intervals using a digital micrometer. As the saddle was longitudinally compressed, it concomitantly expands laterally, leading to an overall flattening of the structure as schematically illustrated in Figure 4.14B. Representative curves are shown in Figure 4.14C, which display the longitudinal force versus the normalized displacements in both the longitudinal and the transverse direction. The average spring constant was found to be 143.6 ± 31.8 N/mm (Figure 4.14D) and no statistical differences was observed for the spring constant as a function of size within the range of saddle size tested ($p > 0.1$, Figure 4.15). Similarly, there was no statistical difference in spring constant between the spearer and the smasher species. The relative data scattering in these graphs can be correlated to the different levels of mineralization in the saddles due to unknown molting cycles of the mantis shrimps sacrificed. For a given load, the lateral expansion was found to be about 2.5-fold greater than the longitudinal contraction. In terms of stress distribution, an imposed bending along the sagittal plane (C1-C2) leads to compressive stresses in the outer layers and to tensile stresses in the inner layers for most the of the saddle cross-sections (Figure 4.14E). However, the saddle geometry imposes an overall more complex loading state: since the saddle extremities along the transversal plane (F1-F2) expand (Figure 4.14F), the outer layers near these extremities must also be under tensile stress. Based on the compression tests on hydrated samples, it is suggested that the saddle can be compressed up to 2-3% in the longitudinal direction without any damage or failure. The stored elastic potential energy E_p in the saddle can be simply calculated from the area under the load-displacement δ graph (Figure 8D) or using the simple equation $E_p = \frac{1}{2}k\delta^2$. Considering the maximum compression taking place at 25-40 N of force and a spring constant k of 143.6 ± 31.8 N/mm, the elastic potential energy stored in the saddle is $\sim E_p = 1.29 \pm 0.28$ J.

Figure 4.14 Macroscopic compressive testing of whole saddles. (A) Macro-photograph of a saddle in the testing machine in the unloaded (top) and loaded (bottom) states. The load was applied along the longitudinal C1-C2 direction. (B) Schematic representation of a saddle during testing. During compression in the longitudinal direction (C1-C2), the saddle expands in the transverse (F1-F2) direction, leading to macroscopic flattening of the structure. (C) Compression test results of four saddle samples until failure, associated with deviation from linearity. Lateral expansion during testing is *ca.* 2.5-fold larger (smaller slope) than the longitudinal contraction. (D) Average spring constant of saddles, with the shaded area representing the range of values measured for all samples ($n = 25$). (E) Volume element from the central region of the saddle, with corresponding stress distribution during saddle bending. Note that this stress distribution will be different near the transverse edges, where expansion will be associated with tensile stresses of the outer surfaces. (F) Two-dimensional projection (top view) of a saddle showing the contraction/expansion of the main axes during compressive testing. The compressive stiffness in the longitudinal direction is larger than the tensile stiffness in the transverse direction.

Figure 4.15 Spring constant of the saddle as a function of size. (A) Spring constant versus thickness and **(B)** Spring constant versus length. The data show they are significantly independent (p -value > 0.1). The dash line and red region represent the average spring constant and the standard error, respectively (spring constant = 143.6 ± 31.8 KN/m).

4.3.4 Discussion

Mantis shrimps utilize a well-developed mechanical amplification system for their raptorial appendage, which is employed to magnify the relatively small mechanical power generated by their muscle in order to generate their powerful, ultra-fast strikes. This amplification system contains muscles, tendons, linkages, the saddle, the meral-V and ventral bars, which all synergistically contribute to amplify the extensor muscle power to accelerate the raptorial appendage^[34]. The saddle is among the stiffest part of this complex amplification system and is designed to store, and later release, the elastic energy by flexing in the longitudinal direction, (Figure 4.14). Virtually all biological materials are composite structures^[7, 35], with hierarchical organization, relative content of organic/inorganic components, and molecular-scale interfaces precisely tuned to meet their load-bearing functionality^[36]. The saddle material is no exception; however its specific biomechanical function cannot be achieved alone with a homogeneous distribution of building blocks, even if these are sophisticated composite elements. If the saddle was solely made of the biomineralized composite from the outer layer, it is very likely that the inner layers would prematurely fail due to the relative brittleness of

mineralized phase under tensile load regimes. On the other hand, a predominantly organic structure such as that comprising the inner layer would not be able to store a sufficient amount of elastic energy since its elastic modulus is about 6-times lower than the outer mineralized composite.

Therefore to meet these seemingly exclusive requirements (high stiffness and extensibility), the results show that the distribution of saddle building blocks is precisely tuned. The microCT scans of the whole saddle combined with FESEM observations and confocal Raman mapping show that the outer layer is more mineralized (84 wt. %), with the mineral phases consisting of both ACP and ACC (Figure 4.6 and 4.7), whereas the inner layer is predominantly organic and made of α -chitin and proteins enriched in hydrophobic residues (Figures 4.9, 4.10 and 4.11 and Tables 4.1 and 4.2). Micro-mechanical mapping confirm that the outer layer is stiffer than the inner layer, with larger differences in mechanical properties observed under hydrated conditions (Figure 4.14 and 4.13). This spatial organization has been optimized to take advantage of the intrinsic mechanical characteristics of the structure building blocks. Owing to their much lower susceptibility to micro-flaws (Griffith fracture) under compressive stresses, minerals are much stronger when subjected to compressive than to tensile loading ^[37]. Hence depositing a larger mineral content in the outer layers that are under compressive stresses allows stomatopods to maximize the spring stiffness of their saddle, while at the same time minimizing risks of failure. The inner layers, on the other hand, sustain tensile stresses, a loading regime under which biopolymeric materials are more suitable than highly mineralized structures. This requirement is met by the prevalence of an α -chitin/protein complex in this layer. Saddle compression also results in flattening of the structure due to expansion in the lateral direction (Figure 4.14), leading the lateral extremities of the saddle to also sustain tensile stresses during bending. Just like along the central cross-sections, the organic/inorganic ratio has been spatially tuned to ensure that the lateral extremities are also predominantly biopolymeric, as observed on both microCT (Figure 4.4C) and large view FESEM micrographs (Figure 4.5B). This overall multi-scale design allows the saddle to maximize its elastic storage capability, while at the same time minimizing the risks of macroscopic failure.

The identification of the structural proteins involved in the chitin/protein complex will be critical to better understand the mechanisms of tensile elasticity in the inner layers. Although the full-length sequences of these proteins remain currently unknown, some clues can be gathered from the global amino acid composition. A high content of Gly is a common hallmark of extra-cellular elastic proteins such as elastin, abductin, or resilin^[38]. Enrichment in both Ala and Gly residues is also a notable feature of load-bearing proteins such as silks^[39] or more recently discovered suckerins from the squid sucker ring teeth^[40, 41], with Ala-rich domains favoring the formation of stiff β -sheet domains. Resilin is a classical elastic protein that has notably been identified in the hinges of insect wings^[42], however its relatively low Ala and Val content (< 7 mol. pct and < 4 mol. pct, respectively)^[43], makes it an unlikely candidate given the higher abundance of these residues in the stomatopod's saddle (Table 4.1). Likewise abductin that provides the elasticity of bivalve mollusks hinge ligaments contains an unusually high amount of Methionine (Met) (~ 15 mol. pct)^[44], which is incompatible with the trace amount of Met in the saddle proteins. More recently, it has been established that chitin associates with chitin-binding proteins in softer regions of the robust squid beak^[45] to form a relatively hydrophilic biopolymeric complex in these regions, and the sequences of these chitin-binding proteins has been elucidated^[46]. It is anticipated that a similar complex exists in the saddle inner layers, but this will require further investigations. While such studies have historically proved challenging because of the difficulty in extracting proteins from mineralized structures in high yield, recent advances in combining high-throughput sequencing with proteomics^[41] will greatly facilitate such endeavor.

4.4 Conclusions

The mantis shrimp raptorial appendage has gathered recent interest as a model biological structure in bio-inspired materials engineering. This chapter has focused on the multi-scale design of the saddle structure. The saddle functionality requires properties that are apparently mutually exclusive, namely high stiffness and extensibility. To meet these requirements, the saddle is constructed from building blocks spatially located within the structure in a way that exploit their intrinsic mechanical properties. Regions

loaded in compression are predominantly made of amorphous mineral phases, whereas regions subjected to tensile loads contain a higher biopolymeric content. The chapter provides useful lessons for the design of multi-materials bio-inspired structures that are both stiff, extensible, and with a high fatigue resistance.

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Chapter 5

Saddle; A Robust Bioactive Energy Storage System

Findings in the previous chapter showed that the saddle, the key structure in the power and speed amplification system of the mantis shrimp appendage, possess a bi-layer structure with tuned spatial distribution to optimize the stiffness and flexibility. In this chapter, however, a more in-depth dynamic mechanical analysis reveals the role and contribution of each layer in storing the elastic energy. The 3D finite element analysis shows the stress distribution in each layer, and the blunt contact indentation elastic-plastic show that the stresses in the saddle never go beyond the elastic region. The failure mechanism of the saddle under compression reveals that no catastrophic failure happens in the interface but shear band cracks in the outer layer after excessive loading.

5.1 Introduction

In Chapter 4, structural and chemo-mechanical studies on the stomatopod saddle revealed that the saddle is comprised of two main layers with distinct mechanical properties. It has been elucidated how the saddle takes advantage of these intrinsic mechanical characteristics of the building blocks to sustain the intensive mechanical condition it undergoes during repetitive strikes and consequently promote its functionality as an efficient spring. A highly mineralized (85 wt.%) phase -predominantly Mg-rich ACC and fluorinated ACP- is in the outer layer which undergoes under compressive stresses, and a very low mineralized chitin/protein matrix presents in the inner layer to sustain the tensile stresses. The spatial distribution of each layer was also depicted using FESEM micrographs and microCT scans.

The studies in this chapter aims to better understand the saddle from a continuum mechanics point of view. It was shown that the saddle's functionality relies on repetitive loadings. These loadings result in different stress regimes in different positions of the saddle, namely maximum compression in the outermost layer and maximum tension in the innermost layer and the extremities due to the flattening phenomena. Another goal of this chapter is to quantify the stresses and design strategies the saddle employs to manage these mechanical load-bearing challenges without any catastrophic failure. Meanwhile, considering that the main role of the saddle in storing the elastic potential energy, the mechanical role of each layer (the outer and the inner) was further investigated.

In accordance with the objectives mentioned above, the samples were evaluated under quasi-static and dynamic loading. Finally, FEA was used to evaluate the stress distribution in the 2D and 3D models of the saddle.

5.2 Experimental Methods

5.2.1 Research samples

All samples were prepared as described in Section 3.2.

5.2.2 Field Emission Scanning Electron Microscopy

Field emission scanning electron microscopy (FESEM) was performed on polished and fracture surfaces of the saddle, meral-V, and merus exoskeleton based on the settings and details which are provided in Section 3.3.2.

5.2.3 Nanoindentation Studies

Indentation studies were conducted on embedded and finely polished samples with a 30 mN standard transducer and a cube corner tip (50-70 nm) and a cono-spherical (10 μm radius) diamond fluid cell tip for analysing the energy expenditure index and the indentation elastic-plastic response, respectively. Refer to Sections 3.6.5 and 3.6.6 for the principals and the detailed descriptions.

Dynamic mechanical analysis of the samples were done by NanoDMA, a DMA transducer and a *CMX* control algorithm. The procedure and the theory are more elaborately discussed in Section 3.6.7.

5.2.4 X-ray microtomography

The 0.012 mm/pix voxel size microCT scans were conducted on the saddle to obtain the 3D model and visualize the crack pattern of the saddle. The specifications and the colour filtering diagrams are described in Section 3.3.3.

5.2.5 Finite elements analysis

To simulate the mantis shrimp saddle under compressive loads in the first step, a 2D Planar model of the saddle was considered and evaluated using the commercial finite

element software Abaqus/Explicit 6.15 under plane-strain conditions. 20 stitched FESEM micrographs (Figure 5.7) showing the whole longitudinal cross-section of the saddle were used to sketch a 2D model of the saddle with the same geometrical shape and dimensions. The model was divided into two layers that corresponded to the FESEM micrographs. The materials properties of each layer were assigned using the nanoindentation studies in hydrated condition obtained in Chapter 4.

For 3D modeling of the saddle, microCT cross-sectional scans of the saddle in transversal direction were used to re-construct the structure. Later the longitudinal cross-section (Figure 5.9) was used as a guide to loft the transversal cross-sections to the 3D model using the SolidWorks software (ver. 2014).

For simplified 3D modeling of the saddle, the two FESEM micrographs of the longitudinal and transversal cross sections (Figure 5.10) were used as a background in OriginPro 9.1 software to fit curve them with two parabolic functions. The club saddle model was constructed with two defined regions, the outer and the inner layer. The interface between the two layers was assumed to be perfectly-bonded through tie interface condition. Each layer of the saddle was assigned the mechanical properties obtained from the nanoindentation studies. A uniform load of 30 N was applied in longitudinal direction (x direction) at the both contact regions and the saddle was assumed to be restrained from the same contact regions. After conducting mesh sensitivity analysis, mesh sizes of 75 μm was chosen for both the outer and inner layers. The finite element mesh contained a total of 621637 nodes and 420106 elements.

5.3 Results and discussion

Chapter 4 revealed that the saddle is composed of a highly mineralized and compact outer layer. From the structural point of view, this microstructure is very distinct from the other parts of the merus. Figure 5.1 shows the compact structure of the outer layer in comparison with the adjacent parts, including the inner layer, the meral-V and the merus. These distinctions are important to discuss the damping factor presented below.

Figure 5.1 The fractured cross sections of the merus parts. The uniform and compact structure of (A) the outer layer of the saddle differs from (B) the inner layer of the saddle, (C) the meral-V and (D) the merus which all possess a multi layer fibrous structure.

5.3.1 Quasi-static mechanical response

In the first step, in order to investigate the outer layer responses under quasi-static compressive loading, a partial loading-unloading nanoindentation method was used. In this study a blunt contact geometry using a 10 μm cono-spherical tip and Hertzian contact theory (see Chapter 3, Section 3.6, and Chapter 5, Section 5.2) were applied. This technique helps to extract indentation stress-strain curves of the outer and the inner layers. The partial loading-unloading curves indicate that the mechanical response of the outer layer is significantly different from that of the inner layer in both dry and hydrated conditions. Figure 5.2B shows that the outer layer exhibited a stiffer response in compare with the inner layer, and the contrast intensified in hydrated condition (Figure 5.2 C). This behavior is consistent with the structural findings using Raman spectroscopy (see Chapter 4, Section 4.8), which demonstrated that the outer layer had exhibited a higher

degree of mineralization. The extracted indentation stress-strain curves for both dry and hydrated conditions are presented in Figure 5.2 D,E.

Figure 5.2 Partial loading-unloading curves and indentation stress-strain curves of the saddle. (A) The red and the blue colored squares define the zones used to probe the mechanical properties of the outer and the inner layer, respectively. The partial loading-unloading curves in (B) dry and (C) hydrated modes were used to extract the nanoindentation stress-strain curves. The applied load and calculated contact depths were used to calculate the stress and strain values under (D) dry and (E) hydrated conditions. The inset is a zoom-in at low stress highlighting the strain-hardening behavior in the hydrated inner layer.

A linear curve fitting from the elastic region of the stress-strain curves was used to define the yield stress ^[1]. The extracted nanoindentation stress-strain curves revealed that in dry condition the yield stresses were 1.6 ± 0.3 GPa and 0.4 ± 0.1 GPa for the outer and the inner layers, respectively. In the hydrated condition, the yield stress of the outer layer did not significantly change (1.4 ± 0.2 GPa), which is fully consistent with a high degree of mineralization in this layer. However the inner layer stresses decreased more significantly and exhibited a strain-hardening behavior, which parallels the behavior previously reported in the inner layer of the dactyl club ^[1] and lobster endocuticle ^[2]. This mechanical response has been attributed to densification of the micro-channels present around the partially mineralized chitin fibrils.

To find out how the structural properties of the layers affect the energy dissipation of the layers, high-resolution indentation mapping was conducted (see Chapter 3, Section 3.6). In this method, the extracted loading-unloading curves were used to measure the energy expenditure index (Ψ), a normalized term defining the ratio of the dissipated energy to the total input energy spent. It has been shown by Bertassoni *et al* ^[3] that:

$$U_t = U_d + U_e \quad (\text{Eq. 5.1})$$

and

$$\Psi = (U_d/U_t) \times 100 \quad (\text{Eq. 5.2})$$

where U_t corresponds to the total energy spent, U_d is the dissipated energy (colored area in Figure 5.3A), U_e is the recovered energy (grid area in Figure 5.3A) and Ψ is the energy index. Therefore for each material the energy expenditure index, Ψ , can get values ranging between 0 to 100%, *i. e.* from fully elastic to fully plastic material, respectively.

Figure 5.3A shows the indentation curves of the outer and inner layers together with the amount of dissipated energies. The total dissipated energy for each case was calculated by summing the colored areas. The bar chart in Figure 5.3B shows that the total dissipated energy for the outer layer in both dry and hydrated condition is significantly lower than the inner layer. For a constant indentation load of 200 μ N the outer layer dissipates 2.913 ± 0.370 pJ in dry and 1.569 ± 0.024 pJ in hydrated condition,

while the inner layer dissipates 10.644 ± 0.528 pJ and 6.551 ± 0.135 pJ for dry and hydrated condition respectively.

In order to normalize the dissipated energy and to have an independent criterion to compare the amount of recovered energy in the outer and inner layer of the saddle, the energy expenditure index was calculated. It was shown that the energy expenditure index of the outer layer (48.6 ± 1.7) is significantly smaller in comparison to the inner layer (66.7 ± 1.1), indicating that the inner layer dissipates a higher fraction of input energy compared to the outer layer (Figure 5.4). The expenditure indexes varied to 77.7 ± 0.5 and 53.2 ± 0.8 under hydrated condition. The same decrease trend was reported by Bertassoni *et al.* [3] for dentin from dry to hydrated conditions. They suggested that this decrease was related to the ‘bowing out’ behavior followed by a lower residual impression, which was caused a higher recovered energy.

Figure 5.3 (A) Representative loading-unloading indentation curves of the outer and inner layers of the saddle to demonstrate the dissipated energy. The energy expenditure index was calculated by dividing the colored area by the full area under the loading curve (summation of the colored and dashed areas). (B) The bar chart presents the total dissipated energy for the outer and inner layer in both dry and hydrated condition.

Figure 5.4 Energy expenditure index of the saddle in dry and hydrated condition. Energy expenditure index maps (A) and bar plot (B) of the dissipated energy. The index is higher in the inner layer. These indexes decreased in hydrated conditions.

5.3.2 Dynamic mechanical analysis

Viscoelastic materials such as polymers and biomaterials behave differently under dynamic loadings, and consequently quasi-static characterization is not sufficient to study their mechanical response. In order to obtain quantitative data on the dynamical response, the storage modulus, loss modulus and $\tan \delta$ were probed as a function of indentation depth, using the nanoscale dynamic mechanical analysis (nanoDMA) capability of the nanoindenter and the “CMX” algorithm based on Kelvin-Voigt model (see Chapter 3, Section 3.6.7). The calculated storage and loss moduli of the different layers were used to evaluate the energy storage and dissipation in these layers, respectively (Figure 5.5).

Figure 5.5 A CMX depth profile over contact depths in the range of 50 to 300 nm for the two different layers of the saddle. The storage (A) and loss moduli (B) of the outer and the inner layers of the saddle indicate a decrease in both moduli from dry to hydrated modes. In both hydrated and dry conditions, the outer layer storage modulus is higher.

The data show that the storage modulus in the outer layer was *ca.* 13.5 and 11-fold higher than the inner layer in dry and hydrated conditions, respectively, indicating that the outer layer is capable of storing a significantly higher amount of energy in comparison to the inner layer. These differences are depicted more clearly in the bar plots of Figure 5.6.

There is also a clear difference in the damping factor ($\tan \delta$), which quantifies the dissipated energy. In both dry and hydrated conditions, $\tan \delta$ is *ca.* 7 to 8-fold higher in the inner layer than in the outer layer (Figure 5.6 C). Thus the nanoDMA data demonstrate that the inner layer dissipates a higher amount of energy, which corroborates the quasi-static indentation data which also showed a higher dissipation (area under the loading-unloading curves, Figure 5.3). On the other hand, the outer layer has a very small damping factor, again confirming its role in storing energy with minimum loss of energy loss.

Figure 5.6 Storage, loss moduli and $\tan \delta$ of the outer and the inner layers in dry and hydrated conditions. (A) The storage modulus chart bars illustrate the much higher storage modulus of the outer layer in both dry and hydrated condition. (B) The loss modulus is also higher in the outer layer. (C) The damping factor ($\tan \delta$) of the outer and the inner layers of the saddle as more logic way to compare the dynamic response of the two different layers illustrates much higher damping (dissipation) in the inner layers. (D) A schematic representation of the Kelvin-Voigt's model on the FESEM micrograph of the saddle cross-section. It illustrates that the spring-damper couple has a more efficient spring and a bigger damper in the outer layer and inner layer of the saddle, respectively.

5.3.3 Finite elements analysis

Most of the analyses presented so far were focused on the micro-scale properties of each layer. However, the saddle exhibits a complex macroscopic geometrical shape and obtaining the stress distribution in the layers by analytical methods is very challenging. Finite element analysis (FEA) is thus a very useful alternative in order to gain quantitative insights into the stress distribution along the different layers of the saddle. It also gives us the opportunity to explore the role of each layer under different stress regimes. In the context of this project, 2D and 3D finite element analysis (FEA) were conducted on the modeled samples. The dimensional and geometrical aspects of the saddle were extracted from FESEM micrographs and 3D microCT scans of the saddle. Figure 5.7 shows how the 2D model was precisely obtained from FESEM micrographs, by stitching FESEM micrograph to reconstruct the whole 2D structure.

Figure 5.7 Construction of the 2D model of the saddle used for finite elements analysis. (A) 20 FESEM micrographs stitched together of a longitudinal cross-section of the saddle used for the 2D modeling; red: outer layer, grey: inner layer. (B) The 2D model of the saddle “bi-layer” constructed in Abaqus. The mechanical properties of each layer were assigned according to the stress-strain nanoindentation values.

The 2D-planar analysis shows that stresses are minimum in the central region of the saddle, near or at the interface between each layer. As expected from simple beam analysis, the outer layer is under compressive stress, whereas the inner layer is under tensile stress (Figure 5.8). In the outer and inner surfaces, where the compressive and

tensile stresses are maximum, the modeling results indicate a uniform in-plane stress distribution. However, this model could not quantify the overall stress distribution since the geometrical complexity of the hyperbolic-paraboloid structure of the saddle is not considered in this model. This model could only reveal stress distributions in the longitudinal direction.

Figure 5.8 The 2D finite elements analysis of the saddle. The highly mineralized outer layer was under compression stresses while the inner layer was under tension stresses.

For a more comprehensive analysis of the saddle under compressive loading, 3D modeling was necessary. In order to generate the 3D model with the Solidworks software, longitudinal cross-sections of the sagittal plane were used as a guideline. The final model was very similar to the saddle; however due to symmetrical constraints in the real saddle, the model was slightly simplified. In order to simplify the 3D model, the longitudinal and transversal cross-sections of the saddle were curve-fitted with two parabolas (Figure 5.10) and the saddle was then re-constructed in Solidworks. The thickness, width and height of the saddle model were input from their mean values obtained from $n = 26$ saddles. The model was further divided in two layers, namely the inner layer and the outer layer and the width ratios along the longitudinal direction precisely followed those measured by FESEM. For the mechanical response of each layer, the elastic-plastic response extracted from nanoindentation studies was used. The elastic modulus E , density ρ and the Poisson's ratio ν of the outer and the inner layer were as follow:

Outer layer: $E = 6 \text{ GPa}$, $\rho = 2.5 \text{ g/cm}^3$; $\nu = 0.25$

Inner layer: $E = 1 \text{ GPa}$, $\rho = 1.5 \text{ g/cm}^3$; $n = 0.35$

The density and Poisson's ratio assumptions are based on the previous modeling on dactyl club in Ref. [1].

Figure 5.9 Construction of the 3D model of the saddle for finite elements analysis. (A) Contact regions (C1, C2), free edges (F1,F2), and transversal and sagittal planes from microCT images used to reconstruct the saddle in 3D. (B) Stacked microCT images of the cross-sections of the saddle in the transversal direction with fixed intervals. (C) A longitudinal cross-section of a saddle rendered from the microCT, used as a guide to construct the 3D model. (D) Process of the 3D model construction leading to (E) the final model in SolidWorks.

Figure 5.10 Simplified 3D saddle model construction. Extracted parabola fitted curves for the (A) longitudinal and (B) transversal cross-sections used for constructing the 3D model of the saddle. (C) 3D model of the saddle constructed in Solidworks, made of two layers and the fine meshed, which was used for running the modelings. (D) Load versus normalized displacement of the saddle under compression (refer to Section 4.3.3), highlighting the compressive load (30 N) used the finite element modeling. The load was selected such as to remain within the elastic region of the macroscopic tests.

After fine meshing, a uniform 30 N force was applied longitudinally (x direction) to the saddle. The results, shown in Figure 5.11, demonstrate that relatively high compressive stresses (*ca.* 430 MPa) were reached in the outermost layer of the saddle, whereas the maximum tensile stresses in the innermost layer is lower in absolute value at *ca.* 230 MPa.

The modeling results also reveal that the saddle's deflection is about 230 μm (2.3%) in longitudinal direction (x direction), which is well consistent with the normalized displacements, recorded in experimental data (Figure 5.10D).

It was noted that the maximum Von Mises stress in the outermost layer (430 MPa) is well below the yield stress measured in the outer layer by nanoindentation (1.4-1.6 GPa) (Figure 5.2), indicating that even at relatively high external loads (30 N), the stresses generated in the saddle are well within the elastic regime. The simulations also show that the surface stresses are uniformly distributed.

Figure 5.11 3D finite elements analysis results of a compressed saddle (external load of 30 N). **(A)** Side view of the saddle. The maximum Von Mises stress (430 MPa) on the saddle outer surface (center of the saddle) is well below the yield stress of the outer layer material. **(B)** Bottom view of the saddle showing the tensile stress distribution, with a maximum Von Mises stress of 230 MPa in the center of the saddle. **(C,D)** Cross-sections of the saddle illustrating that from the outer layer to the inner layer, the stress is maximum, reaches a minimum at the bi-layer interface, and then increases again in the innermost layer.

5.3.4 Failure mechanisms analysis

In addition to finite element modeling, the saddle cracking and failure mechanisms were also investigated after compressive load testing using X-ray micro-computed tomography. The tested samples were loaded past the yield point in Figure 5.10D. Tiled diagonal cracking patterns of the saddle after compression tests were observed, as shown in Figure 5.12. The cracking pattern indicates that uniform cracking occurred on the surface of the outer layer (Figure 5.12, A), with cracks observed at 45° from the applied loading axis, suggesting that cracks are nucleated along shear bands ^[4]. The uniform cracking pattern throughout the outer surface is also consistent with the uniform stress distribution obtained by FEM. Finally, the microCT scans of the inner layers, shown in Figure 5.12b, indicate that no cracking occurred in these layers.

Figure 5.12 MicroCT tomography scan of a damaged saddle after a compression test. **(A)** The outer layer (green color-filtered) indicates the presence of a uniform cracked pattern consisting of two series of cracks oriented at 45° from the loading axis. **(B)** In the inner layer, (blue color-filtered), no macro-cracks are observed. **(C)** Diagram showing the relative densities used for color filtration of the microCT scan of the saddle.

5.3.5 Discussion

The saddle, as a bi-layer geometrical complex spring, is the stiffest element in the stomatopods power amplification system used to store the elastic potential energy prior to striking. The stiffness arises from the geometrical shape, and more significantly from the microstructure and the chemical composition of the outer layer, which consists of a

compact and highly mineralized mixture of Mg-enriched ACC and fluorinated ACP (Chapter 4). Importantly, this compact mineralized structure is exclusively found in the saddle outer layer only. Indeed, other parts of the merus and the adjacent cuticles, including the meral-V, vental bars, and the merus exoskeleton are all microstructurally very similar to the inner layer of the saddle and are made of a fibrous chitin/protein complex partially mineralized with ACC (Figure 5.1). In this chapter, the role of the individual layers of the saddle on the overall energy storage is investigated. The quasi-static and dynamic nano-mechanical analyses (DMA) of each layer have revealed that the outer layer plays a substantial role in storing the elastic energy. The quasi-static nanoindentation stress-strain curves of the layers of the saddle shows that the elastic modulus and the yield strength of the outer layer are much higher than those of the inner layer. This high elastic modulus leads to a higher energy storage capability of the outer layer, while the high yield strength allows a fully elastic recovery. On the other hand, the energy expenditure index, a normalized criterion to quantify the energy absorption, demonstrates that the outer layer dissipates significantly less energy than the inner layers (Figure 5.3 and 5.4).

The lower energy expenditure index in the outer layer of the saddle gives rise to a higher amount of elastic energy storage in this layer. The nanoDMA experiments (Figure 5.5 and 5.6) on the individual layer corroborate these findings. These results reveal that the storage modulus (E') of the outer layer is 11 to 13.5-fold higher than the inner layer in both dry and fully hydrated conditions. This can be directly attributed to the higher amount of mineral phase in the outer layer ^[5]. According to previous dynamic nanomechanical analysis studies on nacre, the presence of mineral phase enhances the storage modulus (E') while the presence of the organic phase leads to a higher loss modulus (E'') and consequently a higher the damping factor of the material ^[5]. This means that the inner layer dissipates more energy, as also evidenced by its higher the damping factor ($\tan \delta = E''/E'$). The higher energy loss and damping factor observed for organic phases are a direct consequence of their viscoelastic properties ^[6, 7]. Collectively, these results imply that the outer layer of the saddle is the main contributor for elastic energy storage and plays a key role in the energy storage of the saddle structure, while the main purpose of the inner layer is to sustain the tensile stresses during saddle loading.

This latter aspect was elaborately in the previous chapter. As a result, it is suggested that the highly mineralized content in the outer layer of the saddle (compared to the other parts of mantis shrimp merus exoskeleton) is present to take advantage of the intrinsic mechanical (dynamic and quasi-static) properties of the mineral phases, which is suitable for potential elastic energy storage.

Finite element analysis of the saddle under high external compressive forces (30 N) revealed that the maximum Von Mises stress in the outer layer (430 MPa) remains well below the yield stress (1.4 GPa in the hydrated state) as measured by Hertzian contact mechanics with a partial loading/unloading method (Figure 5.2D). Given the relative brittleness of the outer mineralized layer, such a high “safety factor” ensures that this layer is loaded well within the elastic regime, thereby preventing micro-cracking, which would be highly detrimental for repetitive strikes.

Another key feature of the saddle revealed by FEA is that the neutral axis during compressive bending lies at, or close to, the interface between the outer and the inner layer. This design ensures that each layer is loaded in a way that takes advantage of the intrinsic mechanical properties of their building blocks. Furthermore, this design likely minimizes the risk of failure by delamination of the interface. Indeed, interfacial delamination is often a weak point leading to failure in bi-layer materials ^[8]. Since the saddle bi-layer interface is subjected to minimal stresses, this failure mechanism is likely to be prevented. When the saddle is loaded past the linear regime (i.e. a regime that is not achieved in native conditions), uniform cracking is observed in the outer layer. However, even at such high loading, no sign of internal damage was detected in the inner layer. Furthermore, the inner layer dissipates much higher energy. These data further strengthen the results from Chapter 4, namely that the inner layer mainly acts to enhance the tensile and fracture tolerance of the saddle, but plays a minor role in storing the elastic energy.

Experimental analysis on the failure process of the saddle under compression reveals that the failure starts with the crack formation and propagation in the outer layer while the inner layer is still intact and supporting the whole structure (Figure 5.12). The uniform cracking on the other hand shows the relatively uniform stress distribution in the outer layer. It is considered that although the inner layers have higher damping properties as a

drawback, they are extremely robust in sustaining the tensile stresses. Consequently, it can be suggested that the inner layer principally act as a support to sustain the tensile stresses rather than helping in elastic energy storage. A saddle that were only made of the highly mineralized outer layer material would likely fail because of the intrinsic weakness of mineralized phases when subjected to tensile stresses.

5.4 Conclusions

The mechanical evaluation of the saddle using both quasi-static and dynamic mechanical testing, as well as finite element modeling revealed that the different layers of this biomineralized spring are designed to address specific functional requirements. The outer layer is under compression loading, which is why it is made of a compact mineralized material, designed to resist compression stresses. On the other hand, the inner layer is designed to tolerate tensile stresses. This requirement is met by having a helicoidally fibrous structure providing higher flexibility. Dynamic nano-mechanical investigations of the saddle give insights into the contribution of each layer in storing the elastic energy. The sharp contrast in mechanical properties of the different layers provides the required energy amplification mechanism allowing the saddle to store a significant amount of muscle-generated energy prior to releasing its appendage at high speed.

Finite element analyses elucidated the spatial geometry of the saddle, which is designed to uniformly distribute the applied stresses and to sustain compressive and tensile stresses in the outer and the inner layers, respectively. Overlapping the neutral axis with the inner/outer layer interface provides further protection to the bi-layer saddle against delamination. To the best of the author's knowledge, the presence of a neutral axis/surface that overlaps with a bi-layer interface has not previously been reported in biological materials. Uniform stress distribution deduced from the observation of surface and internal damage by X-ray microCT confirmed the FEA findings, namely that compressive stresses are uniformly distributed on the surface of the outer layer. The results in this chapter further emphasize that the saddle is designed to provide maximum

energy storage through the rigidity of the highly mineralized outer layer, while support and flexibility is supported by the highly organic/fibrous inner layer.

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Chapter 6

Discussions and Proposed Future Work

The two previous chapters investigated the design strategies Nature employs to build an efficient biomineralized spring. In the current chapter, a summary of the whole thesis findings, which rely on the comprehensive chemo-structural and mechanical investigations, is offered. The most significant ones are the optimization of stiffness and flexibility, imposing the neutral surface in the interface of the two layers, and the significance of the biomineralized outer layer in contributing to the energy storage. In the last part of this thesis, the “protein sequencing” is proposed for analyzing the role of the organics and the geometrical aspects effects on the elastic bending energy as future research areas.

6.1 General Discussion

Mantis shrimps take advantage of a well-developed biomineralized hard tissue appendage to amplify the relatively small amount of energy provided by their muscles and generate extremely powerful and ultra-fast strikes. In the context of this project and in reference to the objectives, a comprehensive study was performed on the mantis shrimp “saddle” as the first reported biomineralized spring ^[1] (Figure 6.1). This thesis reveals how the mantis shrimp’s dactyl club has evolved its design to meet its functional requirements. This includes: *(i)* fine tuning of apparently mutually-exclusive properties, stiffness and flexibility, which dictates its efficient spring-like mechanics, *(ii)* enhancing the saddle’s efficiency in storing a high amount of elastic energy, and lastly *(iii)* optimization of its mechanical stability and minimization of the risk of internal failure during repetitive bending. Understanding these underlying design principles can guide the development of a range of industrial, biomedical and architectural applications.

Figure 6.1 MicroCT imaging of the mantis shrimp appendage and the contribution of the saddle as a spring to restore the elastic energy for its fatal strikes.

6.1.1 Chemo-Structural Aspects of the Saddle

Like any other biological materials ^[2], the saddle is a composite structure with a hierarchical organization, a relative content of organic/inorganic components, and molecular-scale interfaces that are precisely tuned to meet their load-bearing function.

Two distinct layers were observed in the saddle by optical and scanning electron microscopies, namely a compact and dense outer layer, and a fibrous inner layer. Chemo-structural analysis has shown that the outer layer is an amorphous mineralized (84 wt.%) biocomposite. Mg-rich ACC and fluorinated ACP are the dominant phases in this region. The inner layer is a fibrous biopolymer, which is only partially mineralized. It consists of an α -chitin/protein complex enriched with hydrophobic amino acids, a composition that is reminiscent of elastic proteins, *i.e.* elastin, abductin, and resilin ^[3]. The high abundance of Ala and Gly residues also parallels load-bearing proteins, such a silk ^[4] or more recently discovered suckerins from the squid sucker ring teeth ^[5, 6], where Ala-rich sequences favor the formation of stiff β -sheet domains. The other structural elements that contribute to the power amplification system, including the ventral bars, meral-V, and the merus exoskeleton ^[7], exhibit a very similar microstructure as the inner layer of the saddle. However, the saddle is unique in containing an outer dense mineralized layer that primarily provides stiffness to the saddle

6.1.2 Tuned Flexibility and Stiffness

To the best of the author's knowledge, the saddle is the first known hyperbolic-paraboloid structure with two opposite curvatures in biological materials. MicroCT scans of the saddle, in combination with FESEM observations, provided a detailed picture of its geometrical characteristics at the meso-scale. These observations have shown that the overall thickness of the saddle is almost uniform, while the thickness of the outer layer decreases from a maximum at its center point and almost vanishes on the free edges.

The other unique feature of the saddle's geometry is observed when it is compressively bent to store the potential elastic energy: when the saddle is compressed in the longitudinal direction, it concomitantly expands in the transverse direction due to the

presence of a flexible membrane flanking the saddle. Experimental data have shown that the reduction of the saddle's curvature in the transverse direction is about 2.5-fold higher than the increase in curvature in the longitudinal direction. The overall geometry of the saddle and the spatial distribution of the outer layer provide high flexibility in the transversal direction together with high stiffness in the longitudinal direction. The stiffness (or spring constant) of the saddle was measured to be 144 ± 32 KN/mm and did not significantly differ across a range of saddle sizes tested ($n = 25$, p -value > 0.1).

Under compression, both the curvature increase of the longitudinal parabola and the curvature decrease of the transversal parabola give rise to compressive stresses in the outer layer and to tensile stresses in the inner layer. The mineralized outer layer is under compression, a design that matches the higher strength of biominerals since these are much stronger in compression than in tension [8]. Hence, depositing a larger mineral content in the outer layers allows stomatopods to maximize the spring stiffness of their saddle, while at the same time minimizing the risks of failure. The inner layers, on the other hand, sustain tensile stresses and this requirement is met by the predominance of biopolymeric phases in this layer, which are stronger in tension. The biopolymeric phase is an α -chitin/protein complex, with the protein component enriched with Ala and Gly amino acid, a composition which is commonly found in elastic and load bearing proteins [3, 4]. The flattening of the saddle leads to its lateral extremities to also sustain tensile stresses during bending. Both microCT and large view FESEM micrographs of the central cross-sections show that the organic/inorganic ratio has been spatially tuned to ensure that the lateral extremities are also predominantly biopolymeric. This overall design allows the saddle to maximize its elastic storage capability, while at the same time minimizing the risks of macroscopic failure.

6.1.3 Significance of Mineralization in Elastic Energy Storage Efficiency

There is no ideal mechanical system with one hundred percent efficiency, and the saddle is no exception. Two complementary methods have been used, the calculation of the energy expenditure index and the loss modulus measurement by nanoDMA, to shed light on the key role played by the outer layer in storing the elastic energy. The

measurement of the energy expenditure was used as a criterion to evaluate the absorption energy^[9], which is ideally zero for fully elastic materials. This study has revealed that the amount of normalized dissipated energy is higher in the inner layer than in outer one.

NanoDMA experiments, a powerful technique to investigate the viscoelastic behavior of materials at the microscopic level, revealed that the damping factor ($\tan d$) of the outer layer is 7 to 8-fold lower than that of the inner layer. This finding suggests that the outer layer plays the central role of storing the elastic energy, while the inner layer mostly acts as a support to prevent catastrophic mechanical failure. Lastly, these data confirm the key contribution of the saddle in the power amplification system of the dactyl clubs in comparison to other building blocks, such as the ventral bars, the merus exoskeleton, and the meral-V.

6.1.4 Presence of Neutral Axis and Uniform Stress Distribution

The stress regimes in the outer layer, inner layer, and at the extremities of the saddle were investigated by analyzing the saddle's deflections under compression. However, the quantification of the stresses was not experimentally possible. Three-dimensional finite element modeling of the saddle gave insights into the overall stress distribution during compressive bending. The modeling results showed *(i)* a relatively uniform stress distribution on the top and bottom surfaces of the saddle, and *(ii)* that the neutral axis coincides with the bi-layer interface. This design likely shields the saddle against interfacial delamination. To the best of the author's knowledge, taking advantage of a neutral axis that matches a bi-layer interface has previously not been reported in biological materials. Comparing the maximum values of stresses obtained by FEA modeling (430 MPa) with the yield stress measured by Hertzian nanoindentation (1300 MPa) revealed that the internal stresses during saddle bending remain well below the yield strength. This safety factor ensures that the saddle can be repetitively loaded without sustaining internal damage, thereby providing the adequate fatigue resistance needed for its dynamic mechanical functionality.

6.2 Lessons and Applications

The “material world” we are living in has many perspectives. Regarding the different applications of the system, different material properties should be targeted. In many cases, the intrinsic limitations of materials lead to the limitations in design and engineering. With better materials, we would have better products and tools. However, a better material in one application might not be suitable for another application. Considering a similar material property, one application may need minimization, while another requires maximization, and another application an optimization. The principal assignment of materials science is to discover ways to tune and control material properties in different applications.

It has always been a great challenge to optimize mutually exclusive properties like hardness and toughness, stiffness and flexibility, in a diversity of mechanical applications.

In this study, stomatopods have been chosen for a number of reasons based on their outstanding capabilities and their long evolutionary history. These living fossils have had plenty of time to refine their materials for their function. They take advantage of a spring-like system in their hunting appendages to provide the huge amount of energy for their fatal ultra-fast strikes. This key component, the saddle, has worked for million years and no failure has been observed in this structure. In this ideal spring, there is a trade-off between stiffness and flexibility and other specific design listed below:

- Balanced stiffness, which is desirable to minimize local buckling.
- Well-tuned flexibility, which is essential as rigid structures are not adequate to store sufficient elastic potential energy.
- Bi-layer structure to withstand the two different forms of stress: compressive stress in the outer layer and tensile stress in the inner layer.
- Taking advantage of the intrinsic properties of the building blocks made from simple elements to sustain applied stresses: a bio-ceramic in the outer layer and a bio-polymer in the inner layer, with their yield stress well above the stresses sustained in the native saddle.

- Spatial distribution of the layers and engineered geometry of the structure (hyperbolic paraboloid), to ensure well-balanced stress distributions as well as adequate combination of stiffness and flexibility.
- Presence of neutral surface at the interface of the two layers, the location where catastrophic mechanical failure may occur due to mechanical properties mismatch at the interface.

These design strategies may be applicable for a wide range of industrial (leaf springs and spring washers), biomedical (wrist saddle joint implants), and architectural (saddle roofs) applications. In all these applications there should be a balance in stiffness and flexibility, and the design strategies unveiled in the mantis shrimp saddle may provide valuable insights to optimize such applications.

6.3 Future Perspectives

6.3.1 Protein Sequencing

The inner layer of the saddle contains a fibrous and predominantly organic structure and is designed to support tensile stresses. A preliminary analysis of the extracted proteins with SDS-PAGE and MALDI-TOF (Figure 6.2 and 6.3) has shown the presence of different proteins in the saddle. The global amino acid composition of the saddle's proteins demonstrated different relative amounts of amino acid residues in the inner and outer layer (Figure 6.4). For instance, in the inner layer the overall amount of Pro and Val amino acids, which are abundant in load-bearing and elastic proteins ^[3, 4], is higher (inner layer: 21 mol. pct; outer layer: 8 mol. pct). On the other hand, the relative amount of negatively-charged amino acids (Glu and Asp) in the outer and inner layers is 17 mol. pct and 11 mol. pct, respectively. Negatively-charged proteins are well-known to be involved in regulating biomineralization, such as in bones ^[10-12]. However, the full-length sequences of these proteins remain to be determined. The identification and sequencing of elastic proteins located in the inner regions of the saddle and their specific interactions with chitin fibrils will shed light on the mechanisms of elastic energy storage at the molecular scale.

Figure 6.2 SDS-PAGE results for different proteins in the saddle indicated by the red boxes with the corresponding molecular weights.

Figure 6.3 MALDI-TOF spectrum for all proteins extracted from the saddle.

It is possible that the chitin/protein complex of the saddle inner layers share similarity with the chitin/protein complexes recently discovered in the squid beak^[13] and this comparison demands further investigations. Although such studies have historically proved challenging because of the difficulties associated with extracting proteins from mineralized structures in high yield and because of the lack of sequenced genomes for most crustaceans^[14], recent advances in combining high-throughput mRNA sequencing with proteomics^[5] will greatly facilitate such endeavor.

Figure 6.4 Amino acid analysis of the proteins extracted from (A) the outer layer and (B) the inner layer of the saddle.

6.3.2 Effect of the Geometry on Elastic Bending Energy

The overall geometrical aspects of the saddle were studied in this work. In Chapter 5, the two opposite curvatures (longitudinal and transversal cross-sections) that define the hyperbolic-paraboloid structure of the saddle were fitted in order to construct a simplified 3D model for finite element modeling. However, the effect of the radius of curvature of these two parabolas on the elastic bending energy has not been examined. For this purpose, another future area of interest will be the application of elastic analytical modeling based on the Helfrich equations^[15], which have previously been established for lipid bilayers. These equations correlate the amount of stored elastic energy during bending deformation to the radii of curvatures and the elastic properties of each layer. Such analytical modeling may give useful insights to mimic such structures with an optimized energy storage capability.

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List of Publications

Journal Papers

1. Alam, P., Amini, S., **Tadayon, M.**, et al., Properties and architecture of the sperm whale skull amphitheatre. **Journal of zoology** 2016, under press.
2. **Tadayon, M.**, et al., The Mantis Shrimp Saddle: A Biological Spring Combining Stiffness and Flexibility, **Advanced Functional Materials** 2015, 25:6437-6447.
3. Amini, S., **Tadayon, M.**, et al., The role of quasi-plasticity in the extreme contact damage tolerance of the stomatopod dactyl club, **Nature Materials** 2015, 14:943-950.
4. Alam, P., et al., **Tadayon, M.**, et al., l-Lysine templated CaCO₃ precipitated to flax develops flowery crystal structures that improve the mechanical properties of natural fibre reinforced composites. **Composites Part A: Applied Science and Manufacturing** 2015, 75:84-88.
5. Shakerzadeh, M., Ma, Y., **Tadayon, M.**, et al., Thickness dependency of the structure and laser irradiation stability of filtered cathodic vacuum arc grown carbon films for heat assisted magnetic recording overcoat, **Surface and Coatings Technology** 2013, 236:207-211.

Oral presentation

1. How Nature Tunes the Hierarchical and Mechanical Features of a Biomineralized Spring, ICMOBT 2015, Waikoloa, Hawaii, USA.
2. Interplay between Chemistry and Micromechanical Properties in a Spring-Like Mineralized Tissue, Euromat 2014, EPFL Lausanne, Switzerland.

Poster presentations

1. Compositional and Micromechanical Features of a Bio-mineralized Spring, ICMAT 2015, Singapore.
2. Indentation Stress-Strain Response of Graded, Ultra Impact-Resistant Biocomposites, ICMAT 2015, Singapore.