Title
Impact resistant and energy absorbent natural keratin materials: horns and hooves

Permalink
https://escholarship.org/uc/item/4kn4z9dp

Author
Huang, Wei

Publication Date
2018

Peer reviewed|Thesis/dissertation
UNIVERSITY OF CALIFORNIA SAN DIEGO

Impact resistant and energy absorbent natural keratin materials: horns and hooves

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

in

Materials Science and Engineering

by

Wei Huang

Committee in charge:

Professor Joanna McKittrick, Chair
Professor Shengqiang Cai
Professor Yu Qiao
Professor Jan Talbot
Professor Michael Tolley

2018
The Dissertation of Wei Huang is approved, and is acceptable in quality and form for publication on microfilm and electronically:

____________________________________________________________

________________________________________________________________

________________________________________________________________

________________________________________________________________

________________________________________________________________

________________________________________________________________

Chair

University of California San Diego

2018
# TABLE OF CONTENTS

TABLE OF CONTENTS ........................................................................................................ iv
LIST OF FIGURES ........................................................................................................... ix
LIST OF TABLES ............................................................................................................ xxi
ACKNOWLEDGEMENTS ................................................................................................. xxii
VITA ................................................................................................................................ xxiv
ABSTRACT OF DISSERTATION .................................................................................... xxvii

## CHAPTER 1: INTRODUCTION ....................................................................................... 1

1.1 Introduction to biological materials science ......................................................... 1

1.2 Impact resistance and energy absorption in biological materials ......................... 3
   1.2.1 Bone and antler ............................................................................................... 6
   1.2.2 Nacre ............................................................................................................. 12
   1.2.3 Mantis shrimp dactyl club............................................................................ 14

1.3 Bioinspired designs ............................................................................................... 16
   1.3.1 Freeze casting ............................................................................................... 16
   1.3.2 Additive Manufacturing .............................................................................. 19
   1.3.3 Bottom-up synthesis of nanocomposites .................................................... 22

## CHAPTER 2: BACKGROUND OF KERATIN ................................................................. 25

2.1 Natural keratin materials .................................................................................... 25

2.2 Classification of keratin materials ........................................................................ 26
   2.2.1 Soft vs. hard .................................................................................................. 26
   2.2.2 Mineralized vs. non-mineralized ................................................................. 27
2.2.3 Offensive vs. defensive......................................................................................28

2.3 Structure and properties of keratin .......................................................................29
   2.3.1 Multi-phase compositions..................................................................................29
   2.3.2 Multi-scale hierarchical structure ....................................................................31
   2.3.3 Multi-mechanical properties ...........................................................................33

CHAPTER 3: STRUCTURE AND PROPERTIES OF BIGHORN SHEEP HORN.............37

3.1 Introduction to bighorn sheep horn .......................................................................37

3.2 Experiments and methods ....................................................................................40
   3.2.1 High resolution X-ray micro-computed tomography (HR μ-CT) ....................41
   3.2.2 Optical and scanning electron microscopy imaging ........................................42
   3.2.3 Transmission electron microscopy imaging ..................................................43
   3.2.4 Compression tests .........................................................................................43
   3.2.5 Hopkinson bar impact recovery tests and failure surface imaging ...............45
   3.2.6 Statistical analysis .........................................................................................46

3.3 Results and discussions .......................................................................................46
   3.3.1 Hierarchical structure of horn ........................................................................46
   3.3.2 Strain rate, anisotropy and water dependency of mechanical properties .....52
   3.3.3 Hopkinson bar impact recovery test results and failure mechanisms .............58

3.4 Conclusions ..........................................................................................................65

CHAPTER 4: STRUCTURE AND PROPERTIES OF DIFFERENT HORMS .............69

4.1 Introduction ...........................................................................................................69

4.2 Experiments and methods ....................................................................................74
4.2.1 Materials preparation ................................................................. 74
4.2.2 Structural characterization .......................................................... 74
4.2.3 Water absorption and FTIR ......................................................... 75
4.2.4 Compression and tensile tests ...................................................... 75
4.2.5 Impact tests .............................................................................. 77
4.2.6 Statistical analysis ...................................................................... 78

4.3 Results and discussions .................................................................. 78
4.3.1 Microstructures of the different horns .......................................... 78
4.3.2 Compression tests ...................................................................... 81
4.3.3 Tensile tests ............................................................................ 88
4.3.4 Drop tower impact tests .............................................................. 92

4.4 Conclusions ................................................................................... 95

4.5 Supplementary information ............................................................ 97

CHAPTER 5: WATER EFFECTS ON HORN KERATIN .................................. 101
5.1 Introduction .................................................................................. 101

5.2 Materials and methods .................................................................. 102
5.2.1 Acquisition and preservation of horn samples............................. 102
5.2.2 Synchrotron wide angle X-ray diffraction (WAXD) ..................... 102
5.2.3 Fourier transform infrared spectroscopy (ATR-FTIR) ................. 103
5.2.4 Tensile tests and fracture surface imaging .................................. 103
5.2.5 Viscoelastic behavior: Creep tests ............................................. 104
5.2.6 Compression recovery tests ....................................................... 105
5.3 Results and discussions ..................................................................................................................... 106
  5.3.1 Water effects on the structure of horn keratin ................................................................. 106
  5.3.2 Water effects on tensile and creep properties ............................................................ 109
  5.3.3 Water assisted recoverable behaviors .............................................................................. 113

5.4 Conclusions ........................................................................................................................................ 117

5.5 Supplementary information .............................................................................................................. 119

CHAPTER 6: STRUCTURE AND PROPERTIES OF EQUINE HOOF ................................................. 122

6.1 Introduction ........................................................................................................................................ 122

6.2 Experiments and methods ................................................................................................................. 123
  6.2.1 Micro- and nanoscale structural characterization .......................................................... 123
  6.2.2 Compression tests and failure surface imaging ............................................................... 126
  6.2.3 Modulus and hardness mapping through nanoindentation ........................................... 126
  6.2.4 In-situ synchrotron X-ray computed tomography compression .................................. 127

6.3 Results and discussions ..................................................................................................................... 127
  6.3.1 Hierarchical structure of equine hoof wall ................................................................. 127
  6.3.2 Multi-scale mechanical behavior of equine hoof wall ................................................. 132
  6.3.3 Failure and energy absorption mechanisms ................................................................. 136

6.4 Conclusions ........................................................................................................................................ 140

CHAPTER 7: BIOINSPIRED DESIGNS BASED ON 3D PRINTING ...................................................... 142

7.1 Introduction ........................................................................................................................................ 142

7.2 Experiments and methods ............................................................................................................... 143
  7.2.1 3D printing of bioinspired horn and hoof structures ..................................................... 143
7.2.2 Synchrotron X-ray micro-computed tomography ........................................ 145

7.2.3 Drop-tower impact tests of 3D printed models ........................................ 146

7.3 Results and discussions .................................................................................. 146

7.3.1 Energy absorption properties of bioinspired designs .................................. 146

7.3.2 Failure mechanisms in horns and bioinspired materials .............................. 149

7.3.3 Impact resistance of bioinspired designs ...................................................... 154

7.4 Conclusions .................................................................................................... 156

CHAPTER 8: SUMMARY AND FUTURE WORK ....................................................... 158

8.1 Summary ......................................................................................................... 158

8.2 Future work ..................................................................................................... 164

REFERENCES ....................................................................................................... 166
LIST OF FIGURES

Figure 1.1 The materials development timeline from ancient to modern times. Images taken from: newsletter.echa.eu; dailymail.co.uk; shutterstock.com. ................................. 1

Figure 1.2 Common characteristics of biological materials [4]. .................................................. 3

Figure 1.3 Young’s modulus as a function of density for biological and synthetic materials. Taken from [4]. ........................................................................................................ 4

Figure 1.4 The Wegst-Ashby map of toughness and Young’s modulus in natural materials. Fracture toughness is also shown in the map. Taken from [6]. ............................................. 6

Figure 1.5 Hierarchical structures of human compact bone. From the left to the right, the collagen fibers comprise several mineralized collagen fibrils, which is formed by collagen molecule chain helices in nanoscale. Osteons have a lamellar structure and individual lamella consists of fibers arranged in geometrical patterns. Finally, the osteons and Haversian canals form compact bone structure. Taken from [24]. ........................................................................................................ 7

Figure 1.6 Crack-resistance curves (R-curves) and fracture behaviors of human cortical bones in different orientations. (a) R-curves for crack length less than 500 µm; (b) R-curves for longer crack length at 7000 µm. (c, d) In-situ SEM images and schematic showing crack propagation along transverse and longitudinal directions respectively [26]. .............................................................. 8

Figure 1.7 Intrinsic and extrinsic toughening mechanisms in human cortical bone. (a) Intrinsic toughening mechanisms exist at the front of the crack tip including microvoid coalescence, promotion of plasticity zone and cleavage fracture, whereas extrinsic toughening mechanisms, act as shielding local stresses or strains from promoting fracture, existing behind the crack tip. (b) Extrinsic mechanisms on the left side at the micro- to macro-level; intrinsic toughening mechanisms including microcracking and fibrillar sliding at sub-micron and nanoscale level [24]. ........................................................................................................ 9

Figure 1.8 Compressive stress strain curves of antler in different loading orientations: (a) longitudinally and (b) transversely in both wet and dry conditions at different strain rates: $10^{-3}$, $10^{0}$ and $10^{3}$ s$^{-1}$. Taken from [32]. ................................................................. 10

Figure 1.9 Failure mechanisms of antlers after compression in different orientations: left: transverse direction, which is perpendicular to the osteons; right: longitudinal direction, which is parallel with the osteons. The central arrow shows the changing of the length scale of the features taken by scanning electron microscopy. Taken from [32]. ............................................ 12

Figure 1.10 The hierarchical structure of nacre. (a) Single mineral platelet as “brick” in nacre. The platelet is composed of millions of ~30 nm nanograins glued together by protein and chitins. (b) The microscale platelets stacked layer by layer. The interface between platelets provides several toughening mechanisms, including breakage of mineral bridges, organic glues in the interface, surface roughness due to the nano-asperities and interlocking sliding due to the uneven surface of platelets. (c) SEM image of the lamellar structure in nacre. Taken from [24]. ......................... 13
Figure 1.11 Morphological features and the cross-section analysis of stomatopod dactyl club. (A), (B) shows a general view of O. scyllarus. (C) Backscattered scanning electron micrograph of the club’s morphology. (D) A microcomputed tomographic longitudinal section of the body. (E), (F) Cross-section analysis of the club. Three different areas are identified. (G, H) The helicoidal chitosan fibers in the periodic region shown in schematic and SEM image, respectively. (I) The herringbone patterns of chitosan fibers in the impact region. The inset indicates the elastic modulus map in this area. Taken from [42, 43].

Figure 1.12 The four processing steps of freeze-casting: slurry preparation, solidification, sublimation and sintering. Taken from [55].

Figure 1.13 Schematic of the setup of freeze casting technique and the results of example final products produced by freeze casting. (A) Freeze casting setup. Application of freeze casting on different material systems: ceramic nanoparticles (B), macromolecules (C), and graphene oxide (GO) nanosheets (D). The final products show bio-inspired nacre-like composites [60].

Figure 1.14 Freeze casting of lamellar and brick and mortar Al₂O₃/PMMA nanocomposites. (A) SEM image showing Al₂O₃/PMMA lamellar nanocomposites. (B) Brick-and-mortar structure with 80 vol.% inorganic phases. (C) Fracture toughness of lamellar and brick and mortar samples as a comparison with single PMMA and Al₂O₃ and homogenously mixed nanocomposites. (D) Strength and fracture toughness comparison with synthetic materials [61].

Figure 1.15 (a) 3D magnetic printing setup. (b) The basic steps of 3D magnetic printing. The orientation of the reinforced mineral plates can be controlled by the field shift. The structure in biological materials and printed architecture: (c, d) abalone shell; (e, f) mantis shrimp dactyl club; (g, h) cortical bone [74].

Figure 1.16 (a) Ink-based 3D printing head. (b) Epoxy ink with silicon carbide and carbon fibers. The reinforced fibers are able to align along the printing direction due to the shear forces of ink flow. (c) Different cellular architectures printed by ink-based 3D printing [75].

Figure 1.17 Scanning electron microscopy images of tooth enamel in different species: (a) human tooth; (b) beak of Octopus vulgaris; (c, d) tooth enamel from an ancient walrus Odobenidae family. (e) Fabrication process of ZnO nanowires nanocomposites through polymer infiltration and layer by layer deposition [77].

Figure 1.18 (a) Self -assembly fabrication process of chitosan/MTM nanocomposites. (b) SEM image showing the nacre-like lamellar structure in the nanocomposite film. (c) Tensile stress strain curves of chitosan/MTM nanocomposite films prepared by vacuum infiltration, evaporation and random mixing [78].

Figure 2.2 Keratinization of (a) human hair and (b) skin. (a) The development of follicle and growth of hair from embryonic stage [90]. (b) Epidermis and dermis layers in skin tissue. Dead keratinocytes in the cornified layer after fully keratinization are found at the outermost layer of skin [89].

Figure 2.3 Hierarchical structure of humpback whale baleen: the baleen plate shows tubular structure embedded in the intertubular matrix at the microscale level. Both tubules and intertubular areas consist of keratinized cell lamellae. Intermediate filaments and calcium salt crystallites at nanoscale compose the cytoskeleton of keratin cells. Intermediate filament fibers have coiled-coil structure at molecular scale [91].

Figure 2.4 Molecular structure and phase transformations of α- and β-keratin. (a) Molecular structure of α-keratin, showing a helical structure with a periodicity 0.51 nm; (b) Molecular structure of sheet like β-keratin. The periodicity is 0.7 nm. (c) and (d): Wide angle X-ray diffraction pattern of α- and β-keratin; (e) Tension stress strain curves showing the α to β phase transformations [82].

Figure 2.5 Hierarchical structure of human hair. Coiled-coils keratin protein with a periodicity of 0.5 nm at molecular scale; Intermediate filaments (IFs) composed by the keratin tetramers at the nanoscale; IFs further form macrofibrils at microscale; the macrofibrils finally form hair fibers [115].

Figure 2.6 Hierarchical structure comparison of horn and hoof: (a) Hierarchical structure of horn from macro- to nanostructure. The tubular and laminated structure is showed in the microscale; (b) Tubules and intertubular materials in the hoof wall. Keratinized cell arrangement changing from tubular to the intertubular matrix. Intermediate filaments found in the keratinized cells. Figures adapted from [8, 96].

Figure 2.7 The predicted stress strain curves of individual phases in keratin fibers and crack-stopping mechanisms at the nanoscale. (a) The two-phase model proposed by Feughelman [103]; (b) Series-zone model of tensile behaviors of IFs and matrix [99]; (c) Predicted stress strain curves of IFs and amorphous matrix by Chapman [125]. (d) Schematic of Cook-Gordon crack stopping mechanism in IF/matrix composite in keratin materials at the nanoscale [112].

Figure 2.8 Porosity and mechanical properties gradient at micro scale in bighorn sheep horn. (a) Porosity gradient in bighorn sheep horn from the outer surface to the inner keratin-bone interface (unpublished results); (b) and (c) Hardness and elastic modulus from the outer surface to interior [109].

Figure 3.1 Bighorn sheep horn specimen and the tubular structure: (a) Photograph of the bighorn sheep horn used for further analysis; (b) Outer keratin sheath with hollow interior. Schematic of the tubules and orientations are shown: Longitudinal direction parallel with the tubules, radial direction along the minor axis of the ellipse shape of tubule cross section, and transverse direction along the major axis of the cross section; (c) High resolution X-ray micro-computed tomography (HR m-CT) image of horn sample; (d) Longitudinal section from the HR m-CT image, showing the continuous tubules along the longitudinal direction.
Figure 3.2 Tubular and lamellar structure in the bighorn sheep horn: (a) Differential interference contrast (DIC) optical microscopy image of the cross section. Elliptical tubule cross section and curved cell lamellae are observed; (b) Schematic diagram of the tubular and cell lamellar structure; (c) DIC image of the longitudinal section, showing the cell lamellae. The angle between the cell lamella and tubule is ~30°; (d) Scanning electron microscopy image of the cross section. Cell lamellae stacking layer by layer was noticed; (e) Cell lamellae in the longitudinal section shows ~1–2 mm thickness of the lamellae; (f) Keratin cells connect with each other forming the tubules.

Figure 3.3 3D optical microscopy image of an inclined surface which has ~30° angle with the tubule direction and the keratinous cells arrangement in different surfaces from toluidine blue stained optical microscopy images: (a) The inclined surface in both schematic and 3D optical microscopy image; (b) Toluidine blue stained cross section slice optical microscopy image show the keratin cells along the major axis direction of the ellipse; (c) Needle like keratin cells with thickness ~1–2 mm in the longitudinal section, showing ~30° angle with the tubule edge; (d) Keratin cells in the inclined surface indicating the irregular shapes of keratin cells connected with each other, and having a diameter 20–30 mm.

Figure 3.4 Transmission electron microscopy images of keratin cell lamellae for different surfaces: (a) Parallel cell boundaries (dark lines, indicated with red arrow) are shown, indicating the cells are stacked layer by layer along the thickness direction; (b) Curved characteristic of cell boundary (dark line) in a higher magnification. Cross section of keratin macrofibrils (yellow arrow and circle) exist in the cells, and the diameter of the macrofibril is around 200 nm; (c) Curved cell boundaries are also found in longitudinal section, which is similar as the cross section. Keratin macrofibrils (yellow arrow and circle) cross sections are also indicated here; (d) keratin macrofibrils (yellow arrow and circle) are found parallel with the longitudinal imaging surface; (e) Intermediate filaments in the macrofibrils in a higher magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Figure 3.5 Hierarchical structure of the bighorn sheep horn: From left to right, curved horn with growth lines from base to the tip at the macro level; Elliptical tubular structures along the growth direction; At the micro level, lamellae of keratinized cells are stacked layer by layer around the tubules, and cell lamellae are oriented ~30° to the tubule direction. Keratinized cells are flat pancake-shaped, with diameters ~20–30 mm and thicknesses ~1–2 mm; At the nano level, each cell contains macrofibrils with a diameter of ~200 nm. The macrofibrils are randomly orientated inside the cell plane. The macrofibrils are composted of intermediate filaments with diameter ~12 nm; At the molecular level, intermediate filaments are formed by alpha-helix with hard disulfide bonds and weak hydrogen bonds connected with each other [25].

Figure 3.6 Stress strain curves of compression tests at different strain rates (from ~10^{-3}s^{-1} to 10^{3}s^{-1}), orientations, and hydration states: The top row shows stress-strain curves obtained under dry condition, (a) Radial direction; (b) Longitudinal direction; (c) Transverse direction; Bottom row shows results for the hydrated state, (d) Radial direction; (e) Longitudinal direction; (f) Transverse direction. The stress strain curves are the average values of at least 3 samples.
Figure 3.7 Bar chart of the Young’s modulus of horn samples in different directions, hydration states, and strain rates: (a) strain rate 0.001 s\(^{-1}\); (b) strain rate 0.1 s\(^{-1}\); (c) strain rate 0.5 s\(^{-1}\); (d) strain rate 4000 s\(^{-1}\). t-tests between all the dry and wet conditions. One-way ANOVA tests were conducted for the different directions for each strain rate. “ns” refers to negligible statistically significant difference between the results with the level of 0.05........................................................................... 55

Figure 3.8 Impact energy absorption (area under stress-strain curves) as a function of compressive strain in different loading directions and hydration states at a high strain rate (~10^3 s\(^{-1}\)). The energy absorption data for each condition were averaged from 3 samples................................................. 57

Figure 3.9 Bar impact recovery compression tests performed on dry samples under compression: (a-c) Stress-strain curves with loading and unloading under quasi-static (solid lines, strain rate 10\(^{-3}\)s\(^{-1}\)) and dynamic loading conditions (dashed lines, strain rate 10^3s\(^{-1}\)) along the radial, longitudinal, and transverse directions; f in the plots correspond to the final states in the dynamic tests, showing significant residual strains after recovery tests; (d-f) Differential interference contrast optical images of pristine surfaces in different directions: radial, longitudinal, and transverse, respectively; (g-i) Surfaces in three directions after 30% quasi-static deformation; buckled and kinked lamellae formed shear bands in h; X-shaped shear bands indicated with red lines in i; (j-l) Scanning electron microscopy images of the sample surfaces in the three directions after ~20% impact deformation (strain rate ~ 10^3s\(^{-1}\))............................................................................................................ 59

Figure 3.10 Bar impact recovery compressive tests of hydrated samples under compression: (a-c) Stress-strain curves with loading and unloading under quasi-static (solid lines) and impact loading conditions, the latter with limited strain (~30% strain, dashed lines) along radial, longitudinal, and transverse directions, respectively; f in the plots correspond to the final states in both quasi-static and dynamic tests, indicating almost no residual strain after recovery tests; (d-f) Differential interference contrast optical images of original surfaces before compression in different directions: radial, longitudinal, and transverse, respectively; (g-i) Surfaces in three directions after 30% quasi-static deformation. Red circles in (g) indicate water drops squeezed out after radial compression; (j-l) Scanning electron microscopy images of the surfaces in three directions after ~30% impact deformation................................................................. 61

Figure 3.11 Detailed failure mechanisms under different loading directions, rates (quasi-static ~10\(^{-3}\)s\(^{-1}\) and impact ~10^3 s\(^{-1}\)), and hydration states (dry and hydrated). Quasi-statically loaded samples were imaged in an optical microscope while dynamically loaded samples were imaged using a scanning electron microscopy. Each row of images correspond to radial (a-d), longitudinal (e-h), and transverse (i-l) directions, respectively: (a) Tubule deformed under quasi-static compression in the radial direction; (b) Tubule collapse under high strain rate impact in the radial direction; (c) Tubule tearing at the corner and slightly deformed in the hydrated condition; (d) no obvious failure under impact for wet samples; (e) Lamella buckling, kinking, and fiber bridging under quasi-static compression in the longitudinal direction; (f) Tubule buckling under impact in longitudinal direction; (g) Lamellae buckling and delamination in hydrated samples under quasi-static compression in longitudinal direction; (h) no obvious damage under impact in longitudinal direction; (i) Tubule distortion and fiber bridging in dry samples under quasi-static compression; (j) Tubule coalescence under impact in transverse direction; (k) Tubule rupture in wet samples under quasi-static compression; (l) no obvious damage in wet samples under impact in transverse direction. ........................................................................................................................................ 65
Figure 4.1 Phylogenetic tree and estimated divergence times of four ruminant species with keratinized horns [192, 193]: Bighorn sheep (*Ovis canadensis*), domestic sheep (*Ovis aries*), mountain goat (*Oreamnos americanus*) and pronghorn (*Antilocapra americana*). The evolution of important traits are mapped onto branches of the tree. ‘Cranial appendages’ refers to the horns, ossicones, and antlers of ruminant mammals; keratinous horn sheaths likely evolved independently in the families Bovidae and Antilocapridae [194]. Paintings are by C. Buell (copyrights to J. Gatesy). ......................................................................................................................... 73

Figure 4.2 Combat styles and horn morphologies for the different species: (a) bighorn sheep, (b) domestic sheep, (c) mountain goat and (d) pronghorn. Growth direction is indicated by a black arrow for each horn. Compression and tension samples taken from the keratin sheath are indicated with yellow blocks and red strips, respectively. Combat photographs of different species are taken from: theholepicture.photoshelter.com; gudmann.photoshelter.com; gettyimages.com; nationalgeographic.com. ........................................................................................................................................ 77

Figure 4.3 Schematic diagrams of the tubule distribution, optical microscopy and scanning electron microscopy (SEM) images of horn cross sections for different species. (a-d) bighorn sheep, (e-h) domestic sheep, (i-l) mountain goat and (m-p) pronghorn. The radial, longitudinal and transverse directions are indicated in the schematic diagrams. Different tubular cross section and pore shapes found in optical microscopy images (b, f, j, n). Lamellae surrounding tubules are identified in the SEM images of the cross sections (c, g, k, o). Higher magnification SEM images reveal the cell lamellae in each species with a thickness ~1-2 um (d, h, l, p). ......................................................................................................................... 79

Figure 4.4 High magnification optical microscopy images of cross sections and transverse sections stained with toluidine blue: (a, e) bighorn sheep horn, (b, f) domestic sheep horn, (c, g) mountain goat horn and (d, h) pronghorn horn. Pores and cell lamellae are identified in the cross sections (a-d). Cell lamellae directions show differences in each species: (e) ~30° in the direction between cell lamellae and tubule direction (longitudinal) (f) No specific arrangement is found in domestic sheep. (g) Cell lamellae are parallel to the longitudinal direction in mountain goat horn; (h) Similar to the mountain goat, the cell lamellae in the pronghorn horn are parallel to the orientation of tubules. ........................................................................................................................................ 80

Figure 4.5 (a) Water content (wt.%) of horns in the ambient dry and fully rehydrated conditions. (b) Fourier-transform infrared spectroscopy (FTIR) spectra of ambient dry horns in the different species. ........................................................................................................................................ 83

Figure 4.6 Comparative compressive stress-strain curves of horns in different directions (longitudinal, radial and transverse) and hydration conditions (ambient dry and fully hydrated). Stress strain curves are averages of five samples for each species, and error bars indicate standard deviations. ........................................................................................................................................ 85

Figure 4.7 Energy absorption as a function of compressive strain of dry and rehydrated horns in the (a) longitudinal, (b) radial and (c) transverse directions. For each species, plots are based on an average of five samples for each condition. ........................................................................................................................................ 86
Figure 4.8 Tensile stress strain curves in the longitudinal direction of horns in the ambient dry condition. Averages based on seven samples from each species; error bars indicate standard deviations. ................................................................. 90

Figure 4.9 Scanning electron microscopy images of tensile fracture surfaces of horns from four species. Images at top (a,c,e,g) are low magnification, and high magnification images (b,d,f,h) are below. (a,b) bighorn sheep, (c,d) domestic sheep (e,f) mountain goat and (g,h) pronghorn. .......................... 91

Figure 4.10 External damage modes of domestic sheep (a,b,c) and bighorn sheep (d,e,f) with associated normalized impact energy ($E_n$). Radial direction is the impact direction, which is perpendicular to the imaged surface. Black lines are sample marks. .................................................. 94

Figure 4.11 Damage types (bottom surface) with different normalized impact energy from the (a) domestic sheep and (b) bighorn sheep horn .................................................. 95

Figure 4.12 Statistical analysis of Young’s modulus of different horns in three different directions in ambient dry state. No significant difference is found in both the longitudinal and transverse directions between four different species. In the radial direction, Young’s modulus of mountain goat is significant higher than that in pronghorn................................. 97

Figure 4.13 Statistical analysis of yield strength of different horns in three different directions in ambient dry state. No significant difference is found in the longitudinal direction between four different species. The yield strength of mountain goat is significant higher than that in the other three species in both radial and transverse direction. ......................... 98

Figure 4.14 Statistical analysis of Young’s modulus of different horns in three different directions in fully hydrated state. The Young’s modulus of pronghorn is significantly lower than that in the other three species in all directions. In longitudinal direction, the Young’s modulus of mountain goat is significantly higher than that in bighorn sheep and pronghorn................................. 98

Figure 4.15 Statistical analysis of yield strength of different horns in three different directions in fully hydrated state. The yield strength of mountain goat is significantly higher than that in the other three species in all directions. In radial and transverse directions, the yield strength of pronghorn is significantly lower than that in bighorn sheep and domestic sheep. .................... 99

Figure 4.16 Statistical analysis of Young’s modulus, yield strength, ultimate tensile strength and toughness (loading longitudinally) of different horns in ambient dry state: (a) Young’s modulus; (b) Yield strength; (c) Ultimate tensile strength; (d) Toughness. The Young’s modulus of mountain goat is significantly higher than that in bighorn sheep and pronghorn. The yield strength, ultimate tensile strength and toughness in mountain goat and pronghorn are significantly higher than that in bighorn sheep and domestic sheep. No significant difference is found between all the tensile properties of domestic sheep and bighorn sheep horns. ........................................ 100

Figure 5.1 (a) Hierarchical structure of the bighorn sheep horn (adapted from [24]); (b) Schematic diagram of samples used for the synchrotron wide angle X-ray diffraction (WAXD) experiments. The X-rays pass through transverse direction, parallel with the cell planes. IFs orientation is also shown in the accompanying schematic diagram. (c) Diffraction pattern for WAXD characterization. The 10 Å and 5 Å arcs are indicated in the pattern. ............................... 106
Figure 5.2 Water effects on the nanostructure of horn keratin. (a) Water absorption as a function of time. (b) Diffraction peak integrate in longitudinal and radial direction. The 1D periodicity spacing is shown in the plot. Red curves are dry samples, while blue are fully hydrated samples. The density decreases in both longitudinal and radial directions after hydration. The d-spacings (peak positions indicated with red dashed lines) remains the same after hydration. (c) Schematic diagram showing water in the amorphous matrix during hydration. (d) FTIR spectra of samples with different hydration states. Yellow, blue and black dashed lines show the wavenumber shift of certain characteristic peaks. Green dashed line indicates the relative intensity ratio of C=O stretch and N-H bending peak changes in different hydration states.

Figure 5.3 Water effects on the tensile behavior of horn samples. (a) Schematic diagram the test samples that were loaded in the longitudinal direction. (b) Plot of tensile stress and intermediate filament (IF) strain as a function of sample tensile strain in both dry and fully hydrated conditions measured by in-situ tensile tests. (c) Stress strain curves under ex-situ stress and intermediate filament strain in different hydration states. Dry samples show the highest tensile strength, while fresh sample has the highest tensile strain. (d) Fracture surface of the dry samples showing brittle failure. Cell lamellae are shown in a higher magnification SEM image (yellow box) at the bottom. (e) Fracture surface of fresh samples. Macrofibrils pulled out and breakages are found at a higher magnification image. (f) Fracture surface of sample in fully hydrated condition. Macrofibrils are pulled out from the matrix. (g) Schematic diagrams showing the fracture modes in samples with different hydration states. Macrofibrils and water molecules are indicated.

Figure 5.4 Creep compliance of horn samples (longitudinal, radial and transverse directions in ambient dried and fully hydrated states), as a function of time, measured through flat punch nanoindentation.

Figure 5.5 Recovery tests of dry horn samples in different loading orientations: (a) Stress strain curves in the longitudinal directions. Samples failed after test 3; (b) Stress strain curves in the transverse direction. Samples failed after test 3; (c) Stress strain curves of horn loading in longitudinal direction at different loading cycles. Stiffness and yield strength slightly decrease after the third cycle, then are consistent from test 4 to 6; (d) Photographs and microscopy images of horn sample after 50% compression in the longitudinal direction. Shear bands and microfibrils breakage are observed; (e) Microscopy images of horn after 50% compression in the transverse direction. Shear bands and cracks are observed. Yellow dashed lines show the axis of the major axis of ellipse changed direction after compression; (f) Microscopy images of horn after 50% compression in radial direction. Collapse of tubules are found in this direction; (g) Horn samples in (d) after hydration in water for 24 hrs. Shear bands disappear but the delamination and microfiber breakage still exist; (h) Horn samples in (e) after hydration in water for 24 hrs. Shear bands and cracks are not recovered; (i) Horn samples in (f) after hydration in water for 24 hrs. Tubules were recovered back after hydration.

Figure 5.6 Quasi-static compression behavior of horn samples with and without tubules at ambient dry condition. (a) Compressive stress strain curves of horn samples with tubules loaded in different directions; (b) Compressive stress strain curves of horn samples without tubules loaded in different directions; (c) Young’s modulus and yield strength comparison between samples with and without tubules; (d) Optical microscopy images of samples without tubules before compression in radial direction; (e) Samples without tubules after 50% compression in the radial direction. X-shape shear
bands are shown on the surface; (f) Samples without tubules recovered by hydration showing cracks and delamination; (g) Samples without tubules prior to transverse compression; (h) Samples in (g) after 50% compression. Large shear bands and cracks are observed on the surface; (i) Samples in (h) after recovery by hydration. Large cracks and shear bands are found on the surface.

Figure 5.7 Bighorn sheep horn samples without tubules. (a) Bighorn sheep horn sample, indicating the longitudinal, radial and transverse directions; (b) Scanning electron microscopy image of the fracture surface in the longitudinal direction (green box in (a)), showing the lamellar structure of the keratin cells; (c) Optical microscopy showing the areas (yellow box in (a)) without tubules; (d) Lamellae structure is also found in the transverse direction, indicating that the lamellar structure in longitudinal and transverse direction are identical.

Figure 5.8 Images of dry and wet samples under in-situ tensile tests before and after fracture. Dry sample shows a brittle fracture along 45º to the tensile direction, while cracks in the fully hydrated sample is along the tensile direction.

Figure 5.9 (a) Two-dimensional diffraction pattern showing the qy intensity was integrated azimuthally for an angle of 30 degree over the meridian (yellow dashed lines). (b) Two-dimensional diffraction pattern showing the qx intensity was integrated azimuthally for an angle of 30 degree over the equator (yellow dashed lines). (c) d spacing in both longitudinal and radial direction from the WAXD pattern of dry samples during the in-situ tests. No obvious change was found in dry samples. (d) d spacings changes during the tensile tests of fully hydrated samples. Sample tensile strains increase from red to green, indicating d spacing increases during tensile tests.

Figure 5.10 Force-displacement curves from nanoindentation on horn samples for the dry condition in top panels: (a) radial, (b) longitudinal, and (c) transverse; for hydrated state in bottom panels: (d) radial, (e) longitudinal, and (f) transversal. (For all cases, the creep compliance is calculated at the load 5mN held for 600s)

Figure 6.1 Hierarchical structure of an equine hoof. (a) Frontal and longitudinal section from the distal to proximal photographs of a fresh hoof; (b) Transverse section view from medial to lateral. Three directions are defined: longitudinal direction (distal to proximal); radial direction (dorsal surface to the inside bone tissue); transverse direction (medial to lateral); (c) 3D reconstructed synchrotron X-ray micro-computed tomography (µCT) image of hoof wall and the internal tubules; (d) Optical microscopy (OM) image of cross section of tubules. The red box shows single tubule cross-section at a higher magnification. Tubules and intertubular matrix are pointed out; (e) Toluidine blue stained OM image of the cross section. Keratin cell shapes and sizes in both tubular (yellow) and intertubular areas (red) are shown in the green box at a higher magnification. The white holes inside the tubules are the longitudinal hollow medullary cavities; (f) Optical microscopy image of longitudinal section of tubules; (g) Toluidine blue stained optical microscopy image of longitudinal section of tubules. Cell shapes and dimensions are also shown in the green box at a higher magnification.

Figure 6.2 Micro- and nanostructure of the hoof wall. (a) 3D reconstructed synchrotron X-ray micro-computed tomography (µCT) image of OsO₄ stained hoof sample. Tubule areas show higher
Figure 6.3 Nanoindentation characterization of cross sections. (a, b and c) Reduced modulus ($E_r$) and hardness ($H$) maps of the horse hoof wall in ambient dry, fresh and fully hydrated conditions. (d) Comparison of $E_r$ in tubule and intertubular areas in the dry, fresh and fully hydrated conditions. (e) Comparison of $H$ in tubule and intertubular areas in the dry, fresh and fully hydrated conditions. (f) Plot of $H$ and $E_r$ of dry hoof samples. (g) Plot of $H$ and $E_r$ of fresh hoof samples. (h) Plot of $H$ and $E_r$ of the fully hydrated samples.

Figure 6.4 Compressive stress-strain curves of fresh hoof samples (~ 30% H$_2$O) in different loading orientations and strain rates; (a) strain rate 10$^3$s$^{-1}$; (b) strain rate 10$^2$s$^{-1}$ and (c) strain rate 10$^1$s$^{-1}$. (d) Comparison of Young’s modulus at different strain rates and loading orientations. (e) Comparison of the yield strength at different strain rates and loading orientations. (f) Plots of energy absorption (area under the stress-strain curve) as a function of compressive strain in hoof and horn (~ 30% H$_2$O) [8] in different loading orientations (red: longitudinal, black: radial, blue: transverse).

Figure 6.5 Deformation and failure mechanisms before and after 30% deformation. (a-c) Optical microscopy images of samples surfaces before compression in the different loading orientations: longitudinal section (red color surface in (a) when loading longitudinally; cross sections (green color surface in b and blue color surface in (c) when loading radially and transversely. (d-f) Surface images after 30% compression in longitudinal, radial and transverse direction, respectively. Cracks are observed in the tubule areas in all the three directions. Black dashed lines in (e) and (f) indicate the crack directions.

Figure 6.6 In-situ synchrotron X-ray computed tomography ($\mu$CT) compression of fresh and fully hydrated hoof samples. (a) 3D reconstructed $\mu$CT image of the undeformed sample. (b) Top view of the undeformed sample showing the tubules. (c) Front view of the undeformed tubules. Higher magnification image of tubules shows keratin cells surrounding the chambers of medulla cavities. (d) 3D reconstructed $\mu$CT image of the sample after 30% compression in the longitudinal direction. (e) Top view of after 30% deformation, where buckling of tubules are shown (f) Front view of buckled tubules at 30% deformation. Cracks in the tubules are also observed in a higher magnification image. (g) 3D reconstructed $\mu$CT image after 60% compression in the longitudinal direction. No obvious cracks are observed. (h) Top view after 60% deformation. Tubules start disappearing due to the severe deformation. (i) Front view of severely buckled tubules. Tubules have collapsed and compressed after 60% compression. Red dashed arrow indicates longitudinal deformation. (i) Front view of severely buckled tubules. Tubules have collapsed and compressed after 60% compression. Red dashed arrow indicates longitudinal deformation.
Figure 7.1 (a) Schematic diagrams and scanning electron microscopy images of tubular and lamellar structures in the bighorn sheep horn. (b) Single-phase 3D printed models with and without tubules. (c) Double-phase 3D printed models with and without tubules. (d) 3D printed single-phase VeroClear products with and without tubules. (e) 3D printed double-phase VeroClear and Tangobackplus products with and without tubules.

Figure 7.2 Compressive stress strain curves for bighorn sheep horns and 3D printed samples. (a) Bighorn sheep horn in different loading orientations [121]. (b) Double-phase models with and without tubules in different orientations. (c, d) Compressive stress strain curves of single-phase models with and without tubules in different orientations.

Figure 7.3 In-situ compression of horn samples under synchrotron X-ray computed tomography. Samples are compressed to 30% (a-f) and 60% (g-l) deformation. (a) Compression in the radial direction. No obvious damages were found; (b) Compression in the longitudinal direction. Buckling of tubules was found; (c) Compression in the transverse direction. Shearing of tubules was noticed; (d) Cross-section shows the elliptical tubules. Slightly compressions were observed, some of the tubules are slightly closed; (e) Longitudinal section shows the X-shape shear bands and also buckling of tubules in the shear bands. The orientation of keratin cells surrounding the buckled tubules was changed; (f) Cross section shows tubules orientation changes due to the shear force. Cracks initiate at the corner of the tubule; (g) Compression in the radial direction at 60% deformation; (h) Compression in the longitudinal direction. Sever buckling of tubule were observed; (i) Compression in transverse direction, shear of tubule in this direction; (j) No tubule were found in the cross section due to the complete collapse of tubule after 60% deformation; (k) Buckling of tubules and cracks of tubules were observed; (l) Cross section of sample after 60% compression in transverse direction. Cracks start propagating along 45º (yellow dashed lines).

Figure 7.4 Failure mechanisms of 3D printed samples: (a) Samples without tubules compressed perpendicular to the printing direction (lamellae); (b) Samples without tubules compressed parallel to the printing direction; (c) Samples with tubules compressed perpendicular to the tubules and lamellae (radial direction); (d) Samples with tubules compressed perpendicular to the tubules but parallel with the lamellae (transverse direction); (e) Samples with tubules compressed parallel with the tubules and lamellae (longitudinal direction).

Figure 7.5 Compressive stress strain curves of samples after different cycles of loading and recovery in the radial (a), longitudinal and transverse (b) directions. The stress strain curves almost keep the same shape after 4 cycles of compression in the radial direction, while samples were failure after the 1st recovery in both longitudinal and transverse directions.

Figure 7.6 3D printed samples for drop tower impact tests at an impact energy $E_n = 100 \text{ kJ/m}^2$. (a) Four kinds of sample designs, from left to the right: single-phase VeroClear solid model; double-phase VeroClear and Tangobackplus lamellar model; double-phase VeroClear and
Tangobackplus tubular model; double-phase VeroClear and Tangobackplus tubule reinforced model. (b, c) Top and bottom surfaces of samples after impact tests. (d) Optical microscopy images showing damages and cracks.
LIST OF TABLES

Table 3.1 Comparison of Young’s modulus at different compressive strain rates, loading directions and hydration states.................................................................................................................................55

Table 4.1 Compressive Young’s modulus, yield strength, and toughness of the horns under different loading orientations in the ambient dry condition.................................................................87

Table 4.2 Compressive Young’s modulus, yield strength and toughness of the horns under different loading orientations in the fully hydrated condition .................................................................87

Table 4.3 Tensile properties of different horns in the ambient dry condition ..............................................90

Table 7.1 Manufacturer provided properties of VeroClear® and Tangoblackplus® .........................144
ACKNOWLEDGEMENTS

I would like to first gratefully acknowledge my advisor, Dr. Joanna McKittrick, for her enthusiastic and intellectual guidance and financial support for my whole PhD study. After accepted me to join her group, she showed me not only the research field of biological materials, but also the fascinating and fundamental science behind the world of animals and plants. She taught me how to do research, how to teach, how to write scientific articles, how to present at conferences, how to communicate with people and how to live a passionate life. All of these are and will always be the most precious knowledge I have learned in my whole life.

I would also like to thank my committee members, Dr. Jan Talbot, Dr. Michael Tolley, Dr. Shengqiang Cai, and Dr. Yu Qiao for their kind suggestions and support. I am grateful to all of my coauthors, collaborators, and colleagues who have contributed to the success of my work, especially Alireza Zaheri, Nicolaus Yaraghi, Dr. Wen Yang, Dr. Horacio Espinosa, Dr. David Kisailus, Dr. Robert Ritchie, Dr. Cheryl Hayashi, Dr. John Gatesy and Dr. Marc Meyers.

I would like to thank all of my colleagues at Dr. McKittrick’s group, Dr. Steven Naleway, Dr. Michael Frank, Jae-Young Jung, Frances Su, Jungmin Ha, Keisuke Matsushita, Sean Garner, Isaac Cabrera, Yuchen Zhang and Alexis Velazquez-olivera. Without the kind and inspiring discussions and suggestions from them, I would never make these progresses efficiently and smoothly.

Finally, I would like to thank my parents for their endless support during my whole PhD study. I would also like to give special thanks to my wife, Lijuan Huang, without her selfless accompany and love, this work would be impossible.
This work presented in this dissertation was supported by a Multi-University Research Initiative through the Air Force Office of Scientific Research of the United States (AFOSR-FA9550-15-1-0009) and a National Science Foundation Biomaterials Grant (1507978).

Chapter 2, in part, is a currently being prepared for submission for publication. This work is coauthored by Jae-Young Jung, Keisuke Matsushita, Joanna McKittrick. The dissertation author is the second author of this work.

Chapter 3, in full, is published as “Hierarchical structure and compressive deformation mechanisms of bighorn sheep (Ovis canadensis) horn” Acta Biomaterialia, 64, 1-14 (2017). This work was coauthored by A. Zaheri, J. Y. Jung, H. D. Espinosa, and J. McKittrick. The dissertation author is the first author of this work.

Chapter 4, in full, is published as “Microstructure and mechanical properties of different keratinous horns” Journal of the Royal Society Interface, in press. This work was coauthored by Y. Zhang, J. Gatesy, C. Hayashi, and J. McKittrick. The dissertation author is the co-first author of this work.

Chapter 5, in full, is in preparation “Water effect on keratin: Hydration-driven recovery of bighorn sheep (Ovis canadensis) horns”. This work was coauthored by A. Zaheri, W. Yang, R. Ritchie, H. D. Espinosa and J. McKittrick. The dissertation author is the first author of this work.

Chapter 6, in full, is currently in preparation for publication as “A natural energy absorbent polymer composite: The equine hoof wall”. This work was coauthored by N. Yaraghi, W. Yang, A. Velazquez, R. Ritchie, D. Kisailus, S. Stover and J. McKittrick. The dissertation author is the first author of this work.

Chapter 7, in full, is currently in preparation for publication. This work was coauthored by F. Su, J. McKittrick. The dissertation author is the first author of this work.
VITA

2018  Ph.D. Materials Science and Engineering
University of California, San Diego, La Jolla, CA
Dissertation: Impact resistant and energy absorbent natural keratin materials: horns and hooves
Advisor: Professor Joanna McKittrick

2013  M.E. Materials Engineering
Huazhong University of Science and Technology, Wuhan, China

2011  B.S. Materials Science and Engineering
Huazhong University of Science and Technology, Wuhan, China

2011  B.B.A. Business Administration
Huazhong University of Science and Technology, Wuhan, China

PUBLICATIONS


Wei Huang, Nicholas Yaraghi, Wen Yang, Alexis Velazquez-Olivera, Robert O. Ritchie, David Kisailus, Susan Stover, Joanna McKittrick, A natural energy absorbent polymer composite: The equine hoof wall, in preparation.

Jae-Young Jung, Wei Huang, Keisuke Matsushita, Joanna McKittrick, Impact resistant biological materials, in preparation.

Wei Huang, Frances Su, Alexis Velazquez-Olivera, Joanna McKittrick, 3D printed bioinspired energy absorbent and impact resistant materials, in preparation.

PRESENTATIONS


ABSTRACT OF DISSERTATION

Impact resistant and energy absorbent natural keratin materials: horns and hooves

by

Wei Huang

Doctor of Philosophy in Materials Science and Engineering

University of California San Diego, 2018

Professor Joanna McKittrick, Chair

Keratin is one of the most common structural biopolymers with very high strength and toughness but relatively low density, found in various tissues such as hairs, feathers, horns and hooves. The current study focuses on the impact resistant and energy absorbent properties of two representative keratin tissues: horns and hooves. Bighorn sheep (Ovis canadensis) rams hurl themselves at each other at speeds of ~9 m/s (20 mph) to fight for dominance and mating rights. The equine hoof is also considered as an efficient energy absorption layer that protects the bony skeleton from impacts when the horse is galloping. Keratinized tissues are permanent tissues that
are not able to remodel or regrow once broken or damaged. This is a severe challenge to horns and hooves that are without self-healing mechanisms but are under high risk from various loadings. It is hypothesized that both horn and hoof are impact resistant and energy absorbent, however, due to the different loading directions, the mechanical behaviors and energy absorption capabilities should be different and anisotropic in these two kinds of keratin tissues. This impact resistance and energy absorption abilities is hypothesized to stem from material-structure components in horns and hooves. By understanding the structure designs and energy absorption mechanisms, it is hypothesized that bioinspired impact resistant and energy absorbent synthetic materials can be fabricated.

In this thesis work, the hierarchical structure and correlations between the structure and mechanical properties in both horns and hooves were investigated. Structural characterization techniques such as optical and electron microscopy, synchrotron X-ray computed tomography, wide angle X-ray diffraction (WAXD) were used to reveal the structural features from the molecular to macro scale. Compressive properties at quasi- to high- strain rates were characterized to identify the energy absorption performance under different loading orientations and hydration states. In-situ compression tests under synchrotron WAXD were performed to further analyze the energy dissipation mechanisms. Tensile tests were conducted to determine the behavior of the crystalline intermediate filaments and amorphous keratin phases. It was found that although both horn and hoof had tubular structures, significant differences were observed in terms of tubular compositions, shapes, sizes and keratin cell arrangements. The mechanical properties and energy absorption mechanisms were also different due to the structural differences. The microstructure and mechanical properties of horns from four representative ruminant species: the bighorn sheep (Ovis canadensis), domestic sheep (Ovis aries), mountain goat (Oreamnos americanus) and
pronghorn (*Antilocapra americana*), were studied, aiming to understand the relation between evolved microstructures and mechanical properties. Differences were found in the mechanical properties of these four species, which was partially contributed by the structural differences. The differences in mechanical properties among species may relate to their different fighting behaviors. Water-assisted recovery mechanism in bighorn sheep horn keratin was also investigated. It was found that recovery can occur when the amorphous matrix absorbs water and reorganize the amorphous phases. Damages to the cells and fiber breakages cannot be recovered. This recovery and remodeling mechanism is anisotropic and totally different with living tissue such as bones, which could give inspiration for recoverable energy absorbent materials designs. Finally, 3D printing was applied to mimic the tubular and lamellar structure. The lamellar and tubular structures printed out showed promising impact resistant and energy absorbent properties.
CHAPTER 1: INTRODUCTION

1.1 Introduction to biological materials science

In human history, civilization development and advancement can be directly seen from the level of material design and utilization at different ages. In the Stone Age, human used natural materials such as rocks and wood as tools. During thousands of years development, human has gone through various material ages from Bronze to Iron Ages, finally to polymers, composites and nanomaterials in recent ~100 years (Figure 1.1). Materials science has been evolved to an important field and is directly related to our everyday life. Materials science is an interdisciplinary subject that at the confluence of physics, chemistry, and engineering, focusing on the structure and property relationships of materials [1, 2].

![Materials Development Timeline](image)

Figure 1.1 The materials development timeline from ancient to modern times. Images taken from: newsletter.echa.eu; dailymail.co.uk; shutterstock.com.

During the development of materials science and technologies, nature has been playing important roles by showing materials designs and systems evolved in millions of years. Albert Einstein once said, “Look deep into nature, and then you will understand everything better.” Numerous well-known bioinspiration examples surrounding our daily life such as radar inspired by the sonar systems in bats; swimsuits inspired by shark skin; adhesives inspired by gecko feet;
Shinkansen bullet train inspired by kingfisher’s beak; drug delivery vesicles inspired by cells [1]. The field of biological materials science can be divided into three sub-areas: biological materials, which are the materials and systems evolved by nature; bioinspired materials, which are synthetic materials inspired from the materials and designs in nature; biomaterials, these are materials designed for biomedical applications, such as implants [1, 3].

Unlike synthetic materials, biological materials evolved in nature have unique structures and characteristics due to the limited elements and specific environment. These special characteristics were summarized in previous work (Figure 1.2) [4]:

- Self-assembly: materials are synthesized bottom-up controlled by genes, cells and enzymatic activities;
- Multi-functionality: biological materials usually serve as more than one role;
- Structure hierarchy: different but organized structures, and materials exist from nano- to macro-scale level, conferring properties from one level to another;
- Hydration effects: the biological materials properties are dependent on the hydration level;
- Mild synthesis conditions: most of the biological syntheses occur at 1 atm, low temperatures and in an aqueous environment;
- Evolution and environment constrains: there is a limit on the available local elements and environmental resources.

Due to these special characteristics, biological materials usually show remarkable physical and mechanical properties, which has been attracting significant attention [5, 6]. The study of structural biological materials and bioinspired designs thus become one of the biggest fields in biological material science [1].
1.2 Impact resistance and energy absorption in biological materials

Nature has evolved tremendous materials and structures to realize various functions such as structural support, mobility, and protection [4, 6, 7]. Many of the functions have mechanical requirements:

- Support static and dynamic loads in moving and fighting created by the mass and speeds in large animals, such as bighorn sheep horns, elk antlers and equine hooves [8-10];
- Resistance to buckling, fracture, and penetration in the protection from attack of predators, such as porcupine quills, fish scales, and nacre [11-13];
- Storage and release large amounts of elastic and viscoelastic energy, such as skins, muscles and spider silk [14-16].
- Provide lightweight but stiff, strong and flexible structures and materials for animal movements on land, in water and air, such as bones, animal feathers, and fish fins [17-19].
By using as little material as possible to reduce weight, natural materials show remarkable efficiency to fulfill these functions [6]. Most of them are formed by biopolymers and biominerals, one soft and one hard phase, respectively, arranged in complex hierarchical architectures from the nanoscale to the macroscale [20]. Figure 1.3 shows a map of Young’s modulus and density for natural materials [4]. Nature creates materials with density as low as possible (< 3 g/cm³) with Young’s moduli varying from 0.1 GPa to 100 GPa [3]. From a classical perspective, soft, tough materials are usually weak, while, hard, strong materials are extremely brittle, leading to a conflict between strength and toughness [4].

![Figure 1.3 Young’s modulus as a function of density for biological and synthetic materials. Taken from [4].](image)

Toughness is an important mechanical property, which is the ability of a material to absorb energy during elastic and plastic deformation without fracturing [2]. It can be depicted as the area under the stress-strain curve up to fracture [2]. In the requirement of supporting the static and
dynamic load, hard materials such as minerals undergo small deformations thus limit the local dissipation of high stresses resulting in the fracture [21]. At the same time, soft materials such as collagen can sustain large amounts of plastic deformation thus lose the basic function of support [22]. However, nature helps to defeat the conflict between strength and toughness by designing complex structures that integrate respective material classes from soft to hard [4, 5]. Figure 1.4 shows the Ashby plot for natural materials, which maps toughness and Young’s modulus [6]. In terms of the absorption of a given impact energy, the best material will have the largest value $J_c$, in which $J_c$ is the toughness (kJ/m$^2$). The $K_{IC}$ value is the fracture toughness of a material, indicating the ability of a material to resist crack propagation [5]. It can be concluded that natural materials such as keratin, bone (antler), wood, bamboo and mollusk shell are among the materials with the largest toughness in terms of both energy absorption and resistance to fracture propagation [1, 4, 6]. Examples of strong and tough natural materials will be discussed in details in the following sections.
1.2.1 Bone and antler

Bone is considered to be one of the most important structural materials in nature serving as roles supporting the body and protecting the inner organs [17]. The main compositions of bone are type-I collagen and hydroxyapatite [23]. The hierarchical structure of bone is shown in Figure 1.5 [24]. At the nanoscale, the collagen molecular chains form tropocollagen triple helix. Hydroxyapatite crystals and tropocollagen further form collagen fibrils. These collagen fibrils orient helically around the Haversian canals, which are the main structure composition of osteons at the microscale [24].
Figure 1.5 Hierarchical structures of human compact bone. From the left to the right, the collagen fibers comprise several mineralized collagen fibrils, which is formed by collagen molecule chain helices in nanoscale. Osteons have a lamellar structure and individual lamella consists of fibers. Finally, the osteons and Haversian canals form compact bone structure. Taken from [24].

Because of these hierarchical structures, bone is both a strong and tough material. The fracture behavior of bone has been studied intensively [23, 25-28]. The single value fracture toughness of bone was measured in the range $K_c = 2 - 8 \text{MPa} \sqrt{m}$, and higher toughness was found in the transverse direction, perpendicular to the osteon direction [25]. However, the single value fracture toughness cannot fully represent the fracture behavior of biological materials, since the fracture toughness of most biological materials will increase as the crack length increases [24, 29]. The resistance-curve (R-curve) is thus applied to show the fracture mechanics of bone, which is the resistance to crack propagation as a function of crack length [26]. Figure 1.6a,b show the R-curves of human cortical bone in both transverse and longitudinal directions [26]. It was found that the fracture toughness increased as the crack length grew. However, the resistance to crack propagation increased much rapidly in the transverse direction, which could be explained by the in-situ scanning electron microscopy images that show crack propagation in different orientations (Figure 1.6c,d). It can be found that the osteons will deflect the cracks when they are propagating through the osteons thus increase the fracture toughness in the transverse direction (Figure 1.6c) [26].
Figure 1.6 Crack-resistance curves (R-curves) and fracture behaviors of human cortical bones in different orientations. (a) R-curves for crack length less than 500 µm; (b) R-curves for longer crack length at 7000 µm. (c, d) In-situ SEM images and schematic showing crack propagation along transverse and longitudinal directions respectively [26].

The crack toughening mechanisms in bones has been summarized into two main categories: the intrinsic and extrinsic toughening mechanisms [21]. There is a competition between the intrinsic damage process (operating ahead of a crack tip to promote its propagation) and the extrinsic crack-tip shielding process (acting behind the crack tip to inhibit its propagation). Intrinsic toughening tends to inhibit crack propagation associated with enlarged plasticity, including mechanisms such as microvoid coalescence, promotion plasticity zone, and cleavage fractures. While extrinsic toughening focuses on crack shielding by reducing the local stress on the crack, including fiber and grain bridging [21, 29]. Figure 1.7 shows an intrinsic and extrinsic toughening mechanism schematic diagram, as well as an example of the toughening mechanisms in human cortical bone at different length scales (Figure 1.7b) [24]. In the intrinsic view, collagen fiber sliding is the main toughening mechanism in bone. Other mechanisms such as molecular
uncoiling, microcracking and sacrificial bonding occur at sub-micrometer length scale. In the extrinsic mechanism, the uncracked-ligament bridging and crack deflection serve as the main features that shield the crack tip at the micro- and macro-scales [17, 24].

Figure 1.7 Intrinsic and extrinsic toughening mechanisms in human cortical bone. (a) Intrinsic toughening mechanisms exist at the front of the crack tip including microvoid coalescence, promotion of plasticity zone and cleavage fracture, whereas extrinsic toughening mechanisms, act as shielding local stresses or strains from promoting fracture, existing behind the crack tip. (b) Extrinsic mechanisms on the left side at the micro- to macro-level; intrinsic toughening mechanisms including microcracking and fibrillar sliding at sub-micron and nanoscale level [24].
Another example of impact resistant bone material is antler. Elk can fight with each other at a speed of 11m/s, which will cause a huge impact on the antlers [9, 30]. During impact, a high force or shock is applied over a very short period of time, which causes a large strain rate [9]. Materials may face a catastrophic failure, which may not occur at the same stress level under a static load [31]. Compared with skeletal bones, although antlers and bovine femur bones have similar tensile strength (~100 - 200 MPa), the maximum tensile strain (8% -10%) and work of fracture (~6.2 KJ/m²) for antler is 4 - 5 times greater than bovine femur bones, which means a much higher toughness in antlers [9].

To evaluate the impact resistant mechanism in antler, quasi-static and dynamic mechanical tests were carried out by researchers [9, 32]. Figure 1.8 shows the stress strain curve of antler under different strain rates in both transverse and longitudinal directions [32]. Investigations into these two directions indicated that transverse samples were able to sustain higher maximum compressive stresses than the longitudinal ones, while longitudinal one was stiffer and had higher yield strength. This was explained by the radial compression of osteons, sustaining substantial deformation before

![Figure 1.8 Compressive stress strain curves of antler in different loading orientations: (a) longitudinally and (b) transversely in both wet and dry conditions at different strain rates: $10^{-3}$, $10^{0}$ and $10^{3}$ s$^{-1}$. Taken from [32].](image-url)
the final shear failure occurs, which can be observed on the left side of Figure 1.9 [32]. Compressive strengths and elastic moduli decrease as the hydration of antler specimens increase. However, the anisotropic effects and trend of strain rate sensitivity in longitudinal and transverse directions are maintained in the dry antlers. Hydration of collagen is attributed to an effect on mechanical properties. Collagen becomes more compliant with increasing water content, resulting in a decrease in stiffness and strength [32]. Water acts as a plasticizer in collagen fibers by altering the ionic strength of hydrogen bonds which play a significant role in stabling the triple helical collagen structures [33, 34]. Figure 1.9 shows the scanning electron microscopy images of failure mechanisms from nano- to macro-scales [32]. From the microscale, the main failure mode in the transverse direction is the shear band formation between osteons, while in the longitudinal direction, it is the buckling and separation of osteons. Breakages of individual osteon and tissue ligaments can be observed in transverse direction. Fibrils bridging between ligaments and osteons occur in both of the longitudinal and transverse directions [32]. Antlers show an order of magnitude higher compressive strains and more resistance to high-speed impacts than mammalian bones, because of the ~10% lower mineral contents in antlers, which could provide more toughness in antlers [9, 30, 32].
Figure 1.9 Failure mechanisms of antlers after compression in different orientations: left: transverse direction, which is perpendicular to the osteons; right: longitudinal direction, which is parallel with the osteons. The central arrow shows the changing of the length scale of the features taken by scanning electron microscopy. Taken from [32].

1.2.2 Nacre

Similar to the bone, nacre is another mineralized structural material found in nature, which has been considered as one of the best natural armors with extremely high stiffness, mechanical strength and fracture toughness [29, 35-37]. Nacre is a hierarchical composite structure consisting of polygonal aragonite platelets (~95 vol.%) with thickness ~200~500 nm and 5~20 nm thick interlayer of the organic polymer (~5 vol.%) forming a sandwich lamellar structure. The average diameter of the aragonite platelets is 5-8 µm [13, 38]. It was found that these mineral platelets were composed of millions of single crystal nanograins (~30 nm) bound together by proteins and chitin (Figure 1.10 (a)) [36]. The lamellar structure in nature enables a 40-fold increase of fracture
toughness from aragonite (~0.25 MPa m$^{1/2}$) to nacre (~10 MPa m$^{1/2}$) [24]. Rising R-curve was also found in nacre, showing the increase of toughness as cracks propagating, which did not occur in pure aragonite material [35]. The toughening mechanism of nacre is summarized and shown in Figure 1.10 (b) [24]. In the structural design of nacre, the hard minerals provide stiffness and strength, and interlayer shearing of the organic phase is attributed to the inelastic deformation, which results in the dissipation of energy, thus increases the toughness [24, 35, 37]. As the shearing of organic interlayers occurs, the mineral layers begin sliding over each other. As a result, the fracture of mineral bridges between mineral layers, as well as the nano-roughness of mineral plates contribute to the final toughness [24]. Figure 1.10c shows the fracture surface of nacre, indicating the pulling out of the lamellar tablets. The extrinsic and intrinsic toughening mechanisms were also summarized by researchers [39]. The extrinsic toughening mechanisms include micro-voids, tablet bridging, and viscoplasticity of the biopolymer phase, while the intrinsic toughening mechanisms in front of the crack tip include crack deflection by the soft and hard interfaces and crack trapping at the soft layers [39].

Figure 1.10 The hierarchical structure of nacre. (a) Single mineral platelet as “brick” in nacre. The platelet is composed of millions of ~30 nm nanograins glued together by protein and chitins. (b) The microscale platelets stacked layer by layer. The interface between platelets provides several toughening mechanisms, including breakage of mineral bridges, organic glues in the interface, surface roughness due to the nano-asperities and interlocking sliding due to the uneven surface of platelets. (c) SEM image of the lamellar structure in nacre. Taken from [24].
1.2.3 Mantis shrimp dactyl club

Another extremely damage tolerance example in structure design is the helical structure in the hammer-like dactyl clubs of stomatopods. Stomatopod, also named mantis shrimp, is a marine crustacean. Researchers found the dactyl clubs from one species, *Odontodactylus scyllarus*, exhibit an incredibly high-velocity impact on its prey, sea shells [40]. The dactyl clubs can accelerate at 10,000g to a speed of 23 m/s from a stationary position. This rapid strike can generate a very large instantaneous force to break the shell. However, the impact resistant and damage tolerance keep the clubs survive from the thousands of highly energetic blows [41, 42].

![Morphological features and the cross-section analysis of stomatopod dactyl club](image)

Figure 1.11 Morphological features and the cross-section analysis of stomatopod dactyl club. (A), (B) shows a general view of *O. scyllarus*. (C) Backscattered scanning electron micrograph of the club’s morphology. (D) A microcomputed tomographic longitudinal section of the body. (E), (F) Cross-section analysis of the club. Three different areas are identified. (G, H) The helicoidal chitosan fibers in the periodic region shown in schematic and SEM image, respectively. (I) The herringbone patterns of chitosan fibers in the impact region. The inset indicates the elastic modulus map in this area. Taken from [42, 43].
By investigating the microstructures of the club, three specific regions were identified: the impact region, the periodic region and the striated region [42]. The impact region is the outermost region of the club with the thickness ~50 – 70 μm. Results from nanoindentation and energy-dispersive spectroscopy (EDS) reveal a higher calcium and phosphorus concentration and higher hardness and modulus in impact region. The X-ray diffraction pattern shows a higher crystallinity of hydroxyapatite in the impact region than the other regions [42]. Beneath the impact region, an abrupt decrease in modulus occurs in the following transitional region. Then a periodic region with oscillating modulus values ranging from 10 to 25 GPa occurs. In this region, amorphous mineral phase, as well as an elevated magnesium concentration, occurs. Magnesium has been verified to improve the stabilization of amorphous minerals [44]. A chitinous organic matrix serves as a support of these minerals, exhibiting a helicoidal organization. The striated region occupies the side of the club, which is composed of the thickened circumferential band with parallel chitin fibers. Figure 1.11 shows the characteristic features of the dactyl club, in which (A), (B) shows a general view of *O. scyllarus*, (C) shows a scanning electron microscope image of the club, and a micro-computed tomography image of the longitudinal section of the body is shown in (D) (E), (F) gives an elaborate analysis of the cross-section of the club. It can be seen from the figure there are three specific areas indicated in both (E) (blue, red, green) and (F) [42]. Specific structures in different areas can be observed: a buckled rotated plywood structure in impact region, a pseudo-laminated area in the periodic region, and parallel chitin fibers in the striated region. The Bouligand structure of the chitin fibers in the periodic region (G and H) can twist the cracks propagation thus increase the fracture toughness [45]. A herringbone structure was identified in the impact region (I), which is different from the helicoidal structure found in the periodic region. The inset shows a sinusoidal trend of elastic modulus in the herringbone region due to the orientation of apatite
crystals. This herringbone structure design can have a better stress redistribution than regular Bouligand structure, thus increasing the compression behavior [43].

Apart from the well-documented examples mentioned above, nature evolves other materials with high impact resistance and energy absorption properties, such as tooth enamel and dentin [46, 47], wood and bamboo [48, 49], horn and hoof [8, 50, 51], wood pecker tongue and skull [52, 53], fish scales [12, 33] and crab exoskeletons [54]. The design strategies and energy dissipation mechanisms are very meaningful to be investigated deeply, which could be applied in future bioinspired designs.

1.3 Bioinspired designs

Due to the remarkable structures and designs created by nature, more and more bioinspired designs have been proposed to fulfill numerous functions in our life by learning from nature [4]. Freeze casting, 3D printing, and some self-assembly chemical synthesis methods for bioinspiration are introduced in the following sections as prominent examples of bioinspired designs.

1.3.1 Freeze casting

Freeze casting, also termed as ice templating, has been considered as a powerful technique mimicking the lamellar structures found in nacre and bone [55, 56]. In recent years, freeze casting attracts much more interest because of the flexibility of different organic and inorganic combination as well as the easy process [57, 58]. Freeze casting starts with freezing a liquid suspension of ceramic or metal powders, following by sublimating the solidified solvent phase directly into gas and subsequent sintering to densify the resultant aligned, porous scaffold (Figure 1.12) [55]. The microstructures such as lamellae thickness and porosity can be easily controlled by the freezing speed and slurry composition [58, 59].
Figure 1.12 The four processing steps of freeze-casting: slurry preparation, solidification, sublimation and sintering. Taken from [55].

Figure 1.13 Schematic of the setup of freeze casting technique and the results of example final products produced by freeze casting. (A) Freeze casting setup. Application of freeze casting on different material systems: ceramic nanoparticles (B), macromolecules (C), and graphene oxide (GO) nanosheets (D). The final products show bio-inspired nacre-like composites [60].

A schematic of freeze casting setup is shown in Figure 1.13(A), in which the prepared slurry is gradually frozen by the liquid nitrogen underneath [60]. The temperature is controlled by thermocouple and heating system. Three different materials systems: ceramic nanoparticles, macromolecules, and graphene oxide nanosheets are applied in freeze casting as examples shown in Figure 1.13(B), (C) and (D). After infiltration with a second phase and post-processing such as
sintering, pressing and pyrolysis, the final nacre-like nanocomposites with promising mechanical properties are achieved [60].

As an example, aluminum oxide combined with polymethyl methacrylate using the ice-templated method to form a laminated structure, which had a toughness over 300 times that of its constituents [61]. Figure 1.14 shows the structure and mechanical performance of this ice-templated material [61]. From (A), a lamellar structure formed by freeze casting can be easily observed. In order to make a brick and mortar model, after polymer infiltration between the lamella, the bulk material is pressed perpendicular to the lamella and then subsequently sintered. The structure is shown in (B). Ceramic bridges are formed, which has been shown to be an effective toughening mechanism by impeding sliding between lamellae [24]. The differences between lamellar and “brick and mortar” structure are the controls of lamellae thickness and surface features, as well as the organic-inorganic interfaces. Methacrylate groups were grafted to the surface of inorganic lamellae and bricks before the infiltration of PMMA, which could increase the PMMA adhesion by covalent bonding between organic and inorganic phases [61]. The fracture toughness of both grafted and non-grafted lamellar structure were lower than the “brick and mortar” models (Figure 1.13 C). In terms of the toughening mechanisms, delamination and break of ceramic lamellae were observed in the lamellar structure, while no bricks breakage but sliding and pulling out of bricks existed, which could contribute to the fracture toughness because of the strong grafted covalent-bonded interfaces. The strength and fracture toughness of the “brick and mortar” product as a comparison with other synthetic materials are shown in Figure 1.13D. The freeze casting Al₂O₃/PMMA nanocomposites are comparable with the strength and toughness with metallic aluminum alloy [61].
1.3.2 Additive Manufacturing

Additive manufacturing (3D printing is an example) is another promising and popular method to realize natural designs. 3D printing has been developed and used as a method in tissue engineering to build human bone scaffolds and organ printing, which can mimic the structure and function of human tissues and organs [62-64]. It has very broad adaptations of hard and soft materials including ceramics, metals, and polymers, as well as their composites [65-68]. The basic idea of 3D printing method is bottom-up layer by layer fabrication of 3D objects; complicated models thus could be printed out directly. Seven categories of additive manufacturing methods have been summarized: binder jetting; direct energy deposition; material extrusion; material jetting; powder bed fusion; sheet lamination; vat photopolymerization [69]. Polymeric cellular
materials with triply periodic minimal surfaces, which are surfaces without joints and discontinuities that can minimize stress concentration, were fabricated by applying selective laser sintering [70]. The strength and stiffness of the printed cellular designs were comparable with other cellular structures such as the honeycombs. Apart from polymers, metals such as cellular Ti–6Al–4V structures were printed out by applying selective electron beam melting. The metallic cellular structure with interconnected macropores had similar stiffness and strength as bones, which was good for bone implants [71]. Unlike metals and polymers, most of the ceramic 3D printing studies focus on the application in bone implants and tissue engineering because of the mechanical properties and biocompatibilities of ceramics and the interconnected porous structure 3D printing could achieve [64, 72, 73]. Selective laser sintering and direct ink writing are the main 3D printing methods for ceramics [62].

As an example mimicking the nature designs using additive manufacturing, 3D magnetic printing was developed to mimic the ceramic/polymer nanocomposite materials found in nacre, bone and the stomatopod dactyl club [74]. Stereolithography (SLA) based 3D magnetic printer was able to control the reinforced direction of ceramic platelets using a magnetic field. The setup and printing process, as well as the final printed products, are shown in Figure 1.15 [74]. Figure 1.15a is the basic setup of the magnetic 3D printer. The orientation of the mineral plates in different areas can be controlled by the magnetic field and the UV exposure areas (Figure 1.15b). Figure 1.15c-h are natural and printed structures in different organisms: nacre, mantis shrimp dactyl club, and cortical bone, respectively [74]. It was shown that the mechanical properties (stiffness, strength and hardness) were larger in the reinforced direction (along ceramic platelets). The design of the orientation changes in different architectures can steer the crack propagation thus increase the fracture toughness [74].
Figure 1.15 (a) 3D magnetic printing setup. (b) The basic steps of 3D magnetic printing. The orientation of the reinforced mineral plates can be controlled by the field shift. The structure in biological materials and printed architecture: (c, d) abalone shell; (e, f) mantis shrimp dactyl club; (g, h) cortical bone [74].

Fiber reinforced cellular composites were fabricated with an ink-based 3D printing technique [75]. Natural lightweight cellular composites such as wood and bamboo show remarkable mechanical properties and energy absorption performance [5, 76]. Silicon carbide whiskers and carbon fibers were added to the epoxy ink to fabricate the fiber reinforced composites. The 3D printer head and schematic of fiber reinforced ink are shown in Figure 1.16a,b [75]. During the printing process, the high aspect ratio fibers and whiskers were able to align along the printing direction under the shear forces of ink extrusion (Figure 1.16b). Figure 1.16c shows the different cellular architectures printed out by the ink-based 3D printer. It is demonstrated that the customized ink-based 3D printing technique can fabricate lightweight cellular architectures with preferable mechanical properties [75].
1.3.3 **Bottom-up synthesis of nanocomposites**

Bioinspired nanocomposites were also fabricated by using bottom-up self-assembly by previous researchers [77-79]. Tooth enamel is a tough and strong natural material, consist of parallel ceramic nanoscale columns embedded in soft biopolymer matrix. Interestingly, this column motif of biocomposites in tooth enamel has been found in various species during different evolutionary epochs [80, 81]. Figure 1.17 shows the column structure of tooth enamel in different species: (a) enamel of human tooth; (b) beak of *Octopus vulgaris*; (c, d) tooth enamel from an ancient walrus *Odobenidae* family [77]. It is not accident that these column biocomposites evolved in these species. It is also considered as one of the hardest and durable biocomposites in nature.

Bottom-up hydrothermal growth of ZnO nanowires and layer-by-layer deposition of polyallylamine (PAAm) and polyacrylic acid (PAA) was used to create the nanocolumn structure in synthetic materials. The fabrication process is shown in Figure 1.17e. ZnO nanowires were first grown on a Si substrate. PAAm and PAA polymers were then deposited on the top of ZnO nanowires and infiltrated between the gaps of the nanowires. The process was then repeated until a desired thickness of the final product was reached. By investigating the viscoelasticity and dynamic mechanical properties of ZnO nanocomposites, it was concluded that the nanoscale columns embedded in polymer matrix provided efficient energy dissipation [77].
Figure 1.17 Scanning electron microscopy images of tooth enamel in different species: (a) human tooth; (b) beak of *Octopus vulgaris*; (c, d) tooth enamel from an ancient walrus *Odobenidae* family. (e) Fabrication process of ZnO nanowires nanocomposites through polymer infiltration and layer by layer deposition [77].

Bottom-up synthesis has also been applied to mimic nacre structure [78]. Chitosan and montmorillonite (MTM), a natural clay was used as to fabricate strong and tough bionanocomposite films. The self-assembly synthesis of chitosan/MTM nanocomposite film is shown in Figure 1.18(a) [78]. After dispersed in distilled water, the chitosan macromolecules and MTM nanosheets were able to align to form a lamellar structure by vacuum infiltration or water evaporation induced self-assembly. The connection between the chitosan macromolecules and MTM nanosheets were electrostatic forces and hydrogen bonds. Scanning electron microscopy image of the lamellar structure is shown in Figure 1.18(b). The stiffness and final tensile strength were much higher in the lamellar structure prepared by self-assembly than randomly mixed samples (Figure 1.18 (c)) [78].
Figure 1.18 (a) Self-assembly fabrication process of chitosan/MTM nanocomposites. (b) SEM image showing the nacre-like lamellar structure in the nanocomposite film. (c) Tensile stress strain curves of chitosan/MTM nanocomposite films prepared by vacuum infiltration, evaporation and random mixing [78].
CHAPTER 2: BACKGROUND OF KERATIN

2.1 Natural keratin materials

Keratin is a key structural protein produced in epithelial cells that is found in, for example in hair, horns, hooves, feathers and skins [82]. It has been considered as one of the strongest and toughest biological materials in nature [5, 82]. Keratin is a dead tissue that is not vascularized, which means it cannot be remolded or regrow once damaged [83]. Most keratinized materials are made of polygonal cell tiles (tens of microns in diameter, several microns thick) that overlap laterally and are stacked on top of each other to form a relatively dense layer [82]. Keratinized materials have a variety of morphologies that depend on their function. These range from a simple waterproof layer (skin and feathers) to a structurally robust, impact-resistant material (horns and hooves). Keratin is both mechanically efficient in tension (wool and hair) and compression (horns and hooves) [82]. Figure 2.1 shows typical keratinized materials in nature.

2.2 Classification of keratin materials

2.2.1 Soft vs. hard

In terms of the process of biosynthesis and amount of disulfide bonds formed from the presence of cystine, keratinous materials can be classified into soft or hard keratin [82]. Soft keratin such as stratum corneum contains a lower amount of sulfur while hard keratins such as wool and hair show more sulfur crosslinks leading to higher hardness and durability [87, 88]. During the keratinization process, filamentous proteins gradually replace the cytoplasmic contents, turning the living keratinocytes into cornified, stiff and rigid dead keratin cells [82, 89]. Schematic diagrams of the keratinization process and keratin tissue development in hair and skin are shown in Figure 2.2 [89, 90]. The hair development in embryonic stage starts from the neurogenic placode, which will give rise to hair follicles. The hair is then growing from the root of hair follicles using massive stem cells in the bulge, which is located at the outer root sheath (Figure 2.2a). The growth of keratin cells is controlled by the signals from dermal papilla located at the bottom of each hair follicle [90]. In skins, the same keratinization process exists in the keratinocytes. The outer three layers of epidermis make up the skin, in which the outermost stratum corneum layer consists of dead cells because of fully keratinization (Figure 2.2c) [89].
2.2.2 Mineralized vs. non-mineralized

Although almost all the keratin materials are non-mineralized biopolymers, mineralized keratin is found in whale baleen [91, 92]. During the feeding process, whale pushes water through the baleen, small animals such as krill will remain in the mouth as food sources. It was reported that the sei whale has ~14.5 wt.% hydroxyapatite, indicating highly mineralization in the keratin [91]. The percentage of minerals vary from as low as 1 wt.% to 14.5 wt.% in different whale species. The hierarchical structure of humpback whale baleen is shown in Figure 2.3 [91]. Baleen plates have tubular structures embedded in the intertubular matrix at the microscale. Keratinized cells form the tubules and intertubular areas. Inside the keratinized cells, keratin intermediate filaments (IFs) and hydroxyapatite crystals are mixing together as the cytoskeleton of keratinized
cells. The tensile tests of baleen samples from different species showed the Young’s modulus increased as the mineralization degree increased, indicating the minerals acting as reinforced elements in whale baleens [91].

Figure 2.3 Hierarchical structure of humpback whale baleen: the baleen plate shows tubular structure embedded in the intertubular matrix at the microscale level. Both tubules and intertubular areas consist of keratinized cell lamellae. Intermediate filaments and calcium salt crystallites at nanoscale compose the cytoskeleton of keratin cells. Intermediate filament fibers have coiled-coil structure at molecular scale [91].

2.2.3 Offensive vs. defensive

Keratin materials can be found for both offensive and defensive purposes in animals. The most common offensive weapons are horns and claws. Horns in Bovidae family are considered as weapons in intraspecific combats [93, 94]. Bighorn sheep hurl themselves with a speed of 20 miles per hour fighting with each other for dominance or mating rights by clashing their large curved horns. The maximum force during combat can reach 3400 N, with an impact energy near 3500 J.
The deceleration can be as high as 450g [95]. Most of the impact energy is absorbed by plastic deformation, which provides protection to the skull [8]. The equine hoof wall has been found that could stop cracks propagating through it thus protecting the internal bony skeleton [96]. Pangolin scales are also keratin armors that can protect the body from a predator’s teeth penetration [97].

2.3 Structure and properties of keratin

2.3.1 Multi-phase compositions

Although solely composed of keratin, there are two phases in typical keratinized materials: crystalline α- or β-keratin embedded in an amorphous keratin matrix [82, 98, 99]. Thus, keratin can be considered as fiber reinforced polymer composite. Figure 2.6 shows the typical molecular structures of α- and β-keratins [82]. α-keratin has a helical structure with a crystal periodicity of 0.51 nm (Figure 2.6a), while β-keratin shows a sheet-like structure with a crystal periodicity of 0.7 nm (Figure 2.6b). The wide-angle diffraction patterns of these two crystal structures are shown in Figure 2.6c,d [87, 100, 101]. Phase transformations from α to β phase occurs when keratin fibers such as hair and wool are under large tensile deformations (Figure 2.6e) [99, 102]. This phase transformation is a main energy absorption mechanism for keratin materials under tension [102]. Similar to the differences in molecular structures, the mechanical behaviors of the crystalline keratin and amorphous matrix are very different [103, 104]. The IF can be stretched 2.5-fold, indicating a highly extensible property because of its coiled-coil network nanostructure [105]. The amorphous keratin matrix is rich in proteins with a high sulfur that provides stiffness to the structure due to the strong disulfide bonds [87, 106, 107]. As a result, this nanostructure can provide both the stiffness as well as ductility by changing the number ratio of IFs in the amorphous matrix [87, 108].
Figure 2.4 Molecular structure and phase transformations of α- and β-keratin. (a) Molecular structure of α-keratin, showing a helical structure with a periodicity 0.51 nm; (b) Molecular structure of sheet like β-keratin. The periodicity is 0.7 nm. (c) and (d): Wide angle X-ray diffraction pattern of α- and β-keratin; (e) Tension stress strain curves showing the α to β phase transformations [82].

Water exists in almost all biological system, not only serving biological functions, but also affects the mechanical properties [4, 109, 110]. The mechanical properties of keratin materials can vary in a large range with the change of their water contents [83, 86, 111]. Kitchener investigated the water effects on the fracture toughness and notch-sensitivity of horn keratin [104, 112]. Several toughening mechanisms were found in hydrated horns which made the horn as a notch-insensitive material, thus prevents the scratches and cracks propagation rapidly during repeat fighting. The plastic yield of the matrix contributes 50-70% of the total work of fracture in wet horns, the rest is due to the IFs and matrix interface [112]. It was found that water affected the amorphous matrix by breaking down the structural hydrogen bonds, and reduced the stiffness of an oryx horn from
6.1 GPa to 1.8 GPa [113]. Tombolato et al. [8] showed the compressive yield strength and elastic modulus decreased significantly after full hydration. Tensile tests of wet horns showed 60% tensile strain before failure, which is far higher than 5% in dry horns [109]. Under a tensile strain rate as high as 1000 s$^{-1}$, the strain can reach 70% in wet horns compared with less than 5% strain in dry horns [114]. Thus, water as a second phase in keratin can increase the plasticity of the amorphous matrix, thus making the whole structure more energy absorbent.

### 2.3.2 Multi-scale hierarchical structure

Figure 2.4 shows the hierarchical structure of human hair, which is a typical and well-studied keratin material [115]. At the molecular level, the amino acids form the coiled-coil protein structure. The periodicities in the crystal structure were verified by wide angle X-ray diffraction studies [115, 116]. The coiled-coils further forms tetramers, which are the main components of the IFs with diameter ~7-10 nm. The crystalline IFs are embedded in an amorphous keratin matrix forming macrofibrils at the microscale level. The macrofibrils are IF bundles with diameter ~100 – 400 nm. Finally, these macrofibrils assemble to form the hair fibers [115].

![Hierarchical structure of human hair](image)

Figure 2.5 Hierarchical structure of human hair. Coiled-coils keratin protein with a periodicity of 0.5 nm at molecular scale; Intermediate filaments (IFs) composed by the keratin tetramers at the nanoscale; IFs further form macrofibrils at microscale; the macrofibrils finally form hair fibers [115].
Horns and hooves show similar hierarchical structures, which has been considered to play an important role in both energy absorption and impact resistant (Figure 2.5) [8, 117]. At the microscale, tubules and the intertubular matrix consisting of lamellar stacked keratin cells, which have been noticed in both horns and hooves by various researchers [8, 10, 118, 119]. Elliptical tubules with average major axis 100 um and minor axis 40 um were identified in bighorn sheep horn, playing anisotropic mechanical behavior in different directions [8, 109]. Tubule buckling and delamination occurred when the compressive load was applied parallel with the tubules, while tubules collapsed when compressed in the perpendicular direction. Tubules served as a reinforced structure that enhance the stiffness as well as the strength in the longitudinal direction, which is parallel to the tubules in both tensile and compressive tests, while the collapse of tubules in radial direction helps absorbing more energy [8]. Very high strain rate dependency of the strength and stiffness was found in both longitudinal and radial directions [120, 121]. The yield strength and elastic modulus increases as the strain rate increases, thus increasing the energy absorption in a high-speed impact [114]. The lamellar feature of the flat keratin cells in the tubules and the intertubular matrix was found as a factor that contributed to the fracture toughness of cattle horn materials. The wavy like interface between adjacent keratin cells will deviate crack propagation, while the rough surface can also increase friction thus restrain the relative movement [122].

Kasapi and Gosline [10] showed a more complicated tubular and lamellar structure design in equine hoof than that in bighorn sheep horns (Figure 2.5b). Keratin cells that form the tubular and intertubular materials change shape and orientation from the inner tubular to the matrix, which means the orientation of the lamellae change from inner tubular to the matrix materials [10]. Cracks deviated when propagating through the tubules because of the directions of the cell lamellae and interfaces changing from inside to outside tubules [10, 51, 96].
2.3.3 Multi-mechanical properties

The mechanical properties of the crystalline IFs and amorphous matrix were measured in previous studies [103, 123, 124]. Feughelman proposed a two-phase model in which IFs embedded in an amorphous matrix (Figure 2.7a) [98, 103]. A schematic diagram of stress strain curves of wool fiber and the individual phases is shown in Figure 2.7b [99]. The matrix yields at ~2% strain and then maintains a consistent stress level, in which a time-dependent shear thinning behavior occurs [99]. In the IFs, after the elastic stretching of α-helix, the α to β phase transformation occurred [99]. Chapman also developed similar models for the IFs and matrix [125]. The IFs reach an equilibrium between α- and β-keratin phase which is thermal dynamically reversible after the initial elastic region. While the amorphous matrix shows an elastomeric behavior, in which the slope of stress strain curve increases with the extension increasing [125]. These predicted shapes of stress strain curves fit very well with the experimental results of the tensile behaviors of keratin fibers such as wool and hair [125]. The stiffness differences between IFs and amorphous matrix in horns were reported by Kitchner [104]. In fresh horns (20 wt.% water content), Young’s modulus of IFs is ~6.1 GPa, while ~3.1 GPa in matrix [113]. This mechanical property mismatch provides an effective Cook-Gordon crack stopping mechanism at the nanoscale [113]. Cracks are deflected
and blunted when propagating through the IFs due to the weak amorphous matrix between IFs and the interfaces of IFs and matrix (Figure 2.7d) [112]. This crack-stopping mechanism can provide energy dissipation thus increase fracture toughness [112].

![Figure 2.7](image)

**Figure 2.7** The predicted stress strain curves of individual phases in keratin fibers and crack-stopping mechanisms at the nanoscale. (a) The two-phase model proposed by Feughelman [103]; (b) Series-zone model of tensile behaviors of IFs and matrix [99]; (c) Predicted stress strain curves of IFs and amorphous matrix by Chapman [125]. (d) Schematic of Cook-Gordon crack stopping mechanism in IF/matrix composite in keratin materials at the nanoscale [112].

The mismatch of mechanical properties also exists at micro scale in bighorn sheep horns. A porosity gradient from the outer surface to the interior has been noticed in previous studies [8, 109]. The porosity at the outer surface of the horn is ~8%, while decrease to almost ~0% in the interior bone keratin interface. The porosity as a function of distance from the outer surface is shown in Figure 2.8a. As a result of the porosity gradient, both the hardness and elastic modulus show lower values on the outside surfaces (Figure 2.8b and 2.8c) [109]. Since in bighorn sheep horn, the most important requirements are impact resistance, energy absorption, and lightweightness, the porosity gradient helps to meet these requirements [93, 126]. A high porosity increases the energy absorption during impacts by increasing the plastic deformation and maintains...
the stress level at a lower value to protect the skull [121, 126]. The porosity can also decrease the total weight of horn. The higher stiffness and hardness at interior areas can increase the bending stiffness for the whole structure, which could protect horns from bending during high-speed impacts [94, 109].

![Figure 2.8](image)

Figure 2.8 Porosity and mechanical properties gradient at micro scale in bighorn sheep horn. (a) Porosity gradient in bighorn sheep horn from the outer surface to the inner keratin-bone interface (unpublished results); (b) and (c) Hardness and elastic modulus from the outer surface to interior [109].

At the macroscale level, horns are composed of a bony core structure surrounding by a keratin sheath [127]. There is a huge mechanical properties mismatch between the keratin sheath and mineralized bony core [120]. The density of keratin is \(\sim 1.3 \text{ g/cm}^3\), while the bony core density is \(\sim 1.725 \text{ g/cm}^3\). The elastic modulus in keratin horn and the bony core is 2 GPa and 15 GPa, respectively [120]. It has been verified that the outside surface keratin layers are tough and impact resistant which effectively withstand high impacts and localized initial loads [50, 120, 121]. The sponge-like trabecular bone inside the bony core provides significant strain energy absorption and
reduces rotational accelerations of brain cavities at the same time [50]. Due to the higher stiffness of the bone materials, a higher bending resistance was also found in this bony area [120]. As a result, this material and mechanical properties mismatch at macroscale in bighorn sheep horn provides an efficient design in terms of energy absorption and impact resistance [120].

Chapter 2, in part, is a currently being prepared materials for submission for publication. This work is coauthored by Jae-Young Jung, Keisuke Matsushita, Joanna McKittrick. The dissertation author is the second author of this work.
CHAPTER 3: STRUCTURE AND PROPERTIES OF BIGHORN SHEEP HORN

3.1 Introduction to bighorn sheep horn

Natural structural materials possess variety of unique self-assembled hierarchical structures which result in remarkable mechanical efficiency, such as resistance to different loading modes and ability to sustain extreme deformations with limited selection of chemical constitutes along with optimized weight [128]. One example is the bighorn sheep (*Ovis canadensis*) horn, which can support an impact force as large as \( \sim 3400 \) N [94]. The velocity of the intraspecific combat between two males can reach \( \sim 9 \) m/s, decelerating in \( \sim 2 \) ms with a deceleration estimated as \( \sim 450g \) [95]. Hence, sheep horns experience high impact loads during combat with others animals and protection from predators [129]. At the same time, they need to absorb the impact energy to minimize its transmission to the skeletal frame of the animal. Based on the study of sheep collisions, an estimate of the strain rate experience by the horn material is of the order of \( 10^2 \sim 10^3 \) s\(^{-1}\) because of the extremely short impact time (\( \sim 2 \) ms) [95], which is much higher than previous reported result (\( \sim 38 \) s\(^{-1}\) ). The reason of the differences is in latter work, the initial impact speed was set as 4.7 m/s, and the horn shape effects were considered as well. The higher strain rates estimated in the present work are based on the condition of \( \sim 9 \) m/s for the initial impact velocity and \( \sim 450g \) for the acceleration reported by Courtney *et.al* [95]. This reveals the importance of high impact resistance in these materials. Horns are permanent structures which are made of an external keratin sheath covering a spongy bone core, and will not regenerate or recover once fractured [130, 131]. For the efficiency of horn in fighting, they are expected to be: stiff and strong enough to resist the impact force; tough enough to dissipate impact energy without fracture; light enough to be functional [93, 94, 132]. The overall shape of the horn, as well as the bone tissue inside the horn sheath, plays an important role in protection of the sheep brain from impacts. In
this regard, understating the role of structural-material components on mechanical properties of horn keratin sheath provides insight into utilization of these tissues during the lifetime of the animal.

Keratin is selected through the evolutionary process for a plethora of animal tissues, such as hooves, claws, nails, hairs, wools and scales [83, 86]. Horn keratin is composed of α-helical crystalline intermediate filaments (IFs, 7–10 nm in diameter), embedded in an amorphous non-helical keratin matrix [87, 133, 134]. Keratin in horns contains disulfide bonds as well as secondary bonds, such as hydrogen bonds, to stabilize the amorphous matrix, by holding together the non-crystalline polypeptides [107]. The hydrogen bonds are thought to be sensitive to hydration, which result in mechanical properties dependent on water content [106, 111]. Indeed, studies on the effect of hydration revealed a reduction on the stiffness and strength of horn keratin [8, 109, 113, 114, 135]. The Young’s modulus of bighorn sheep horn has been reported to increase from 0.63 to 2.2 GPa as the water content decreased from 34.5 to 10.6 wt.% water content [8]. However, the fracture toughness of oryx horn was found to increase from 2.2 MPa/m$^{1/2}$ in dry condition (0 wt.% water content) to 4.5 MPa/m$^{1/2}$ in fresh condition (20 wt.% water content) [113]. This was argued as the result of limited matrix yielding and plastic deformation in the fully dry state. The hydration sensitivity for many keratinous materials such as human hair and nails [136, 137], feathers [138] and hooves [139] has been reported in the past. It is believed that the sensitivity in general is a result of absorption of water [140] and disruption of hydrogen bonds with the subsequent decrease in the number of effective bonding in the matrix of keratin, which generally decreases the overall strength and stiffness [113]. In conjunction with this, it was reported that the water molecules tend to bind on the hydrophilic sites of protein in both the IFs and amorphous matrix. However, the
matrix showed higher capability of binding water molecules than the IFs, thus absorbing more water [141].

Besides hydration sensitivity, anisotropy plays an influential role on the mechanical properties of keratinized materials. McKittrick et al. [8] conducted quasi-static compression tests on different orientations of sheep horn specimens. Three main loading directions in horns were introduced: Longitudinal, which is along the growth direction of the horn; radial, which is the main impact direction in the bighorn sheep horn; transverse, which is another orthogonal direction perpendicular to the growth direction. Smaller elastic modulus and yield strength were found in the radial direction, compared to the longitudinal and transverse direction. They also observed deformation mechanisms such as microbuckling and delamination of lamellae during specimen compression. In another work, Trim et al. [109] investigated the stress-state dependent structure-property relationship under quasi-static compression and tension tests. They found insignificant variation of properties for specimens obtained from the base to the tip of the horn. They also observed buckling of lamella and shear-type failure mode in compression for longitudinal and transverse orientations, respectively. Recently, Horstemeyer et al. [114] found that as the compressive strain rate increased, the Young’s modulus and yield strength were increased for samples in only two orientations (longitudinal and radial). For the case of tensile behavior, Zhang et al. [142] showed higher tensile strength and elastic modulus in the longitudinal direction than the transverse direction of the horns from domestic bovines.

Understanding the aforementioned anisotropic behavior requires the description of the hierarchical structure at different length scales. Microscopically, most keratinous materials have heterogeneous structures. For the case of horns and hooves, micro-tubules (40 ~ 100 µm in diameter) orientated in the longitudinal directions are embedded in the intratubular matrix [8, 10].
Keratin cells (2 – 5 µm thick) are stacked layer-by-layer forming a lamellar structure around the tubules as well as in the intertubular area. The arrangements of these lamellar cells vary in different tissues and species. Previously, Kasapi and Gosline [10] showed the complex orientation of the IFs around the tubular cortex as well as in the intertubular area of equine hooves. These along with the cell lamellae arrangements were presented as the influential factors on mechanical properties of hooves. However, to the best of our knowledge the keratin cell lamellae arrangements and IFs orientations in the bighorn sheep horn have not previously been identified. Moreover, previous studies were mostly limited to low strain rates (~10^{-3} s^{-1}) [8, 109]. Even though some high-strain-rate mechanical properties were recently reported, deformation mechanisms as a function of strain rates were not sufficiently characterized. We believe this to be due to impedance mismatch between the employed bars used in the Kolsky bar experimental setup and the horn samples. This led to a limited understanding of the energy absorption mechanisms [114]. Moreover, understanding of the mechanical behavior was limited by the lack of knowledge of the keratin microstructure. Thus, the present work aims to understand the following points: 1) The hierarchical structure of bighorn sheep horn, including tubules, cell arrangement, intermediate filaments orientations; 2) The strain rate dependency, anisotropicity, and hydration effects on compressive properties; and 3) The compressive deformation mechanisms and their correlation with the hierarchical structure.

3.2 Experiments and methods

Two horns of different bighorn sheep (*Ovis canadensis*) from Rocky Mountain area were purchased from the Wilderness Trading Company (Pinedale, WY), and were kept at room temperature in a dry environment, as shown in Figure 3.1a. The total lengths of horns are ~60 cm, with diameters ~9 cm at the base part. Based on the length of the horns, they are estimated to be 6
~ 8 years old. However, the time between the harvesting of horns and our tests remains unknown. A section near the base of the original horn was cut, showing a hollow interior (Figure 3.1b). The thickness of the horn sheath in Figure 3.1b changes with the position through the cross section. The outer impact surface has a thickness ~2-3 cm, while the inner is around 1 cm thick. Thus, samples were collected from the thicker area near the proximal region, which is the most probable impact part, to conduct structural characterization and mechanical testing. These samples are assumed to be representative of the whole horn according to previous structural characterization and mechanical results, which indicated no significant difference in density, tensile, and compressive stress-strain behaviors between different locations along the length of the horn [109]. Since the internal regions of the horn are less compromised during impact, they were not investigated in detail in this study. Irregular shapes and closed tubules in the cross sections of horn samples were reported in previous studies [8, 109]. We hypothesize this to be the result of accumulated damage from ramming during the bighorn sheep’s life. To avoid using damaged regions, in this study specimens were carefully inspected and prepared from pristine regions of the horn to avoid history effects because of apparent ramming damage. Likewise, sample preservation and preparation were optimized to avoid generating imperfections that could impact the characterization and mechanical properties here reported.

3.2.1 High resolution X-ray micro-computed tomography (HR µ-CT)

Three 2 x 2 x 4 mm³ horn samples were cut from the middle part of impact area (Figure 2b). A 2.5 vol.% glutaraldehyde solution was applied to fix the samples for 24 hrs. After washing for 3 times, 2% osmium tetroxide (OsO₄) was used to stain the specimen for 3 days to increase the contrast. The samples were then washed with deionized (DI) water 5 times. The microstructure of the outer impact area was evaluated by high resolution X-ray microscopy (XRM, Xradia 510
Versa, Carl Zeiss Microscopy, Pleasanton, CA, USA). The scan parameters employed in the experiment are a tilt increment of 0.15° (for a 360° of rotation angle, a 2401 of tilts) and an isotropic voxel size of 1.95 µm at a 80kV acceleration voltage. The series of tiff images were reconstructed by Amira software (FEI Visualization Sciences Group, Burlington, MA, USA) with a module of volume rendering. The reconstructed three-dimensional rendering model was cropped and visualized to show both transverse and longitudinal cross-sections. A colormap of “physics” (from a minimum intensity with a dark blue to a maximum intensity with a red) was applied to distinguish the different keratin densities based on the acquired X-ray intensities.

3.2.2 Optical and scanning electron microscopy imaging

Samples (six samples in total, three from one horn and three from a second one) were cut into cubes with dimension 4 x 4 x 4 mm³ and were located ~5 mm from the impact surface as showed in Figure 3.1b. An ultramicrotome was used to make flat surfaces of the cross and longitudinal sections in each block. Differential interference contrast (DIC) optical microscopy images were taken on the flat surface by a Keyence VHX 1000 (Keyence, Palatine, IL, USA). Thin slices (~1 µm thick, 6 ~ 9 slices for each cube) were cut by ultramicrotome (Leica EM UC6, Leica Microsystems Inc., Buffalo Grove, IL, USA) and stained with Toluidine blue, which increases the contrast of the keratin cells under the optical microscope. Optical microscopy images with different magnification (5×, 10×, 20×, 40×) were acquired. Porosities/pore sizes, cell lamellae angle and cell sizes were quantified from the optical images. Half of the 6 cubes were immersed in a 2.5 vol.% glutaraldehyde solution overnight to fix the structure. A graded series of ethanol solutions (20%, 40%, 60%, 80%, 95%, and 100% vol.% ethanol) were applied to further dehydrate the samples. Then the samples were freeze-fractured after immersion in liquid nitrogen in both cross and longitudinal directions. Finally, a critical point dryer (Autosamdry-851, Tousimis,
Rockville, MD, USA) was used to further remove the excess ethanol. Samples were sputter coated with iridium (Quorum Technologies Ltd., West Sussex, UK) to enhance the sample electron conductivity before performing scanning electron microscopy (SEM) imaging. An ultra-high resolution microscope (FEI, Hillsboro, OR, USA) was applied to conduct the SEM imaging.

3.2.3 Transmission electron microscopy imaging

Samples (four in total) were cut into small blocks with dimension 2 x 1 x 1 mm\(^3\) from the similar impact areas as the SEM samples. Then the samples were immersed in a 2.5 vol.% glutaraldehyde solution overnight. 2% osmium tetroxide (OsO\(_4\)) was applied to stain the horn specimens. After 24 h of staining at room temperature, the samples were washed with DI water 5 times. Then the samples were further stained with uranyl acetate for 1 day to obtain better contrast. The samples were washed with DI water for two times and then dehydrated with graded series of ethanol solutions (20%, 50%, 70%, 90% and 100% vol.% ethanol). After dehydration, samples were embedded in Spurr’s low viscosity resin (Electron Microscopy Sciences, Hatfield, PA, USA). Ultramicrotome (Leica EM UC6, Leica Microsystems Inc., Buffalo Grove, IL, USA) was applied to prepare \(~80\) nm thin sections to perform further imaging. Sections on copper grids were post-stained by lead citrate solutions to enhance contrast. A FEI Tecnai 12 (Spirit) (80 KV) electron microscope (FEI, Hillsboro, Oregon, USA) was used to image the stained sections with magnification from 1000\(\times\) to 10,000\(\times\). The diameters of macrofibrils and orientations are estimated from TEM images.

3.2.4 Compression tests

Due to the hollow design of horns, samples used for compression tests were obtained from the proximal and central regions of horn where it is the thickest (Figure 3.1b). Samples were taken at positions \(~10 - 20\) cm from the proximal region of the horns. The dimensions of the samples for
quasi-static and dynamic testing were 4 mm in all directions, which were prepared by use of handsaw and powered-saw with diamond blade. Then, the samples were ground to obtain parallel faces. To examine the anisotropic behavior of horn, samples were cut from three different orientations: longitudinal, transversal and radial as shown in Figure 3.1b. The samples were tested in ambient conditions as well as hydrated, in which they were immersed in deionized (DI) water for 72 hrs. In the dried condition, the moisture content was around 10%, however, it is increased to ~30 ± 0.7% in the hydrated condition (similar to the ~35% reported in a previous study [8]). At least three samples for each direction, both in the dry and hydration conditions, were tested and the final results were averaged. For the quasi-static uniaxial compression test, a universal testing machine with a 30 kN load cell (Instron 3367 Dual Column Testing Systems, Instron, MA, USA) was used. The specimens were tested at strain rates of $1 \times 10^{-3}$ s$^{-1}$, $1 \times 10^{-1}$ s$^{-1}$ and $5 \times 10^{-1}$ s$^{-1}$ (six samples for each condition). In all experiments, the loading process was continued until fracture occurred. Given that the natural striking rates of sheep horns are ~ $10^2$-$10^3$ s$^{-1}$, a split Hopkinson bar system was employed to test the samples dynamically. This system has been extensively used to determine the high strain rate mechanical properties of many materials from monolithic materials such as metals [143, 144], ceramics [145] and polymers [146, 147] to composite materials [148, 149]. However, in the case of low impedance materials such as biological materials proper modifications in the split Hopkinson technique are required in order to obtain reliable and accurate results [150]. These mainly include strain rate constancy and stress equilibrium at the interfaces of the sample [151, 152]. The key to achieve aforementioned criteria is the impedance mismatch ratio between bars and the sample [153]. To this end, woven glass/epoxy composite (G-10) rods with a diameter of 12.7 mm were used for striking, incident, and transmission bars. Lower impedance of these bars in compare to the steel and aluminum with the same size (i.e. one fifth of
steel and one half of aluminum) leads to optimal strain rate constancy and stress uniformity over the specimen length [151]. Moreover, the higher yield strength of G-10 is an advantage compared to polymeric bars, which provides higher load capacity (i.e., compressive stresses) to crush the specimens. To achieve a linear ramp loading, a polycarbonate pulse shaper was used on the impact side of the incident bar. In the present setup, a gas gun was used to fire the striker bar. A compressive pulse is generated on the incident bar, which travels towards the specimen. A portion of the pulse is transmitted through the transmission bar by the specimen sandwiched between two bars and the remaining is reflected at the incident bar-specimen interface. Finally, the stress pulses in the bars were recorded by strain gages, amplified, and recorded in an oscilloscope. The obtained stress-strain curves for all strain rates are given in Section 3.3. Due to the cost of experimentation and analysis, compared to the 6 samples tested in compression at low strain rates, three samples were selected as representative in each testing condition. The average strain rate was \(~4000\) s\(^{-1}\).

### 3.2.5 Hopkinson bar impact recovery tests and failure surface imaging

To further understand the interplay between horns microstructural features such as tubules and their role in determining the overall compressive deformation behavior, bar impact recovery experiments (i.e., experiments in which the sample deformation is limited to a specific strain level) were conducted. For the quasi-static tests, specimens were loaded to a specified strain level and then unloaded with the same rate to the zero load level. Optical images of different surfaces of the deformed specimens were taken before and after the tests to track changes in microstructure. In the bar impact recovery tests, a stopper ring surrounding the sample was employed [154]. The function of the ring is to carry the load after the specimen achieves the desired axial strain. The desired strain was set around 25-30\% since the softening behavior occurs at that level observed from the obtained stress-strain curves in Figure 3.6. The stopper ring was made of G-10 with the
same outer diameter as the bars and an inner diameter large enough to avoid any radial confinement of the sample during axial compression. The faces of the ring were prepared to be as parallel as possible to the bar end faces. Depending upon the desired strain level in the recovered samples, the length of the ring was adjusted. It should be noted that the bar impact recovery results here reported only include the loading part. The recovery configuration here reported does not allow calculation of the unloaded part of the stress-strain curve. For this reason, the recovered part of the strain could only be obtained by comparison between the pristine and the recovered sample lengths, as measured by a caliper. After testing, the specimens were coated with 15nm Au/Pd and imaged in a SEM. It should take into account the fact that the recovery experiments in the present work, especially at high strain rates, were not reported previously. They were performed for understanding energy dissipation mechanisms and revealing the role of microstructure on deformation and failure behavior.

### 3.2.6 Statistical analysis

Detection of statistically significant differences (SSD) of the Young’s modulus among different orientations were performed by a one-way analysis of variance (ANOVA) method. Tukey’s least significant difference procedure was applied to conduct the multiple comparison tests with ANOVA. However, pairwise t-test was employed for SSD of Young’s modulus between dry and wet condition in each direction as well as strain rate. The statistical significance level for both the ANOVA and t-test is assumed 0.05. The mechanical data were collected from multiple specimens in two independent horn samples.

### 3.3 Results and discussions

#### 3.3.1 Hierarchical structure of horn

Figure 3.1 shows the tubular structure of the bighorn sheep horn. Curved growth lines were observed from the base to the tip of the horn (Figure 3.1a). Previous results reported that the tubules (Figure 3.1b) were found to be elliptically-shaped with major axis ~80 µm, and minor axis ~40
µm [8]. HR µ-CT images show the 3D tubular structure (Figure 3.1c). Figure 3.1d is a cross section along the longitudinal direction, showing that the parallel tubules are continuous along this direction. This is the first 3D study that verifies the tubules are hollow and penetrate, in a short distance, through the horn tissue along the longitudinal growth direction. Since the tubules are produced by epidermal cells, their medullary cells develop at the tip of dermal papillae and subsequently extend through the whole horn [155]. Since the 3D reconstruction of tubules over the entire length of the horn is currently impractical, a sample with a 2 mm length in the base part was selected for micro-CT, which showed continuous tubules over that length. Further studies on tubule continuity in the centimeter length scale will become possible as 3D reconstruction capabilities continue to improve.

Figure 3.1 Bighorn sheep horn specimen and the tubular structure: (a) Photograph of the bighorn sheep horn used for further analysis; (b) Outer keratin sheath with hollow interior. Schematic of the tubules and orientations are shown: Longitudinal direction parallel with the tubules, radial direction along the minor axis of the ellipse shape of tubule cross section, and transverse direction along the major axis of the cross section; (c) High resolution X-ray micro-computed tomography (HR m-CT) image of horn sample; (d) Longitudinal section from the HR m-CT image, showing the continuous tubules along the longitudinal direction.

Flat, (keratin) cells were identified forming the lamellar structure in the horn. DIC optical microscopy images of the cross section (Figure 3.2a) show the cell lamellae surrounding the
tubules. Based on the optical microscopy images, the average sizes of the elliptically-shaped cross section of the tubules were calculated. The size of the major axis ranges between 40 – 80 µm with an average of 59 ± 13.8 µm. For the minor axis, the size is in the range of 10 – 40 µm with an average of 24.6 ± 8.9 µm. Both major and minor axes dimensions are similar to what was previously reported [8]. The thickness of each keratin cell is ~ 1-2 µm. Figure 3.2b is a schematic of cell arrangements in a 3D horn model. From the DIC image of the longitudinal section (Figure 3.2c), it can be observed that there is an angle (~30˚) between the cell lamellae and tubules, which implies that the lamellar cells are not exactly parallel to the tubules. SEM images of the cross section (Figure 3.2d) and the longitudinal section (Figure 3.2e) further verify the laminated structure around the tubules, also confirming the thickness observed from optical microscopy. Keratin cell surfaces in Figure 3.2f show that the cell diameters are ~20-30 µm.

Figure 3.2 Tubular and lamellar structure in the bighorn sheep horn: (a) Differential interference contrast (DIC) optical microscopy image of the cross section. Elliptical tubule cross section and curved cell lamellae are observed; (b) Schematic diagram of the tubular and cell lamellar structure; (c) DIC image of the longitudinal section, showing the cell lamellae. The angle between the cell lamella and tubule is ~30˚; (d) Scanning electron microscopy image of the cross section. Cell lamellae stacking layer by layer was noticed; (e) Cell lamellae in the longitudinal section shows ~1–2 mm thickness of the lamellae; (f) Keratin cells connect with each other forming the tubules.
To get further understanding of the lamellar cell size and shape, optical microscopy images of toluidine blue stained thin sections were obtained. The 3D schematic and the optical microscopy images are shown in Figure 3.3a and 3.3c, respectively, revealed that lamellae have an orientation of $30.16 \pm 5.87^\circ$ (averaged by 18 slices from six different samples) with respect to the tubule direction. Examining the inclined surface in Figure 3.3a reveals the surfaces of the cells. Cross- (Figure 3.3b) and longitudinal sections (Figure 3.3c) show the flat cells connected with each other forming a layered structure with the same thickness as the thickness of cells, $\sim 2 \, \mu m$. Figure 3.3d shows the cells arranged in the inclined surface, revealing an irregular organization of the flat cells. The average length of the cells is $\sim 20$-30 $\mu m$, which is more than ten times larger than the thickness. Thus, it can be concluded that the laminated keratinized cells are stacked layer-by-layer in a direction not parallel with the tubules, but at an angle of $\sim 30^\circ$.

Figure 3.3 3D optical microscopy image of an inclined surface which has $\sim 30^\circ$ angle with the tubule direction and the keratinous cells arrangement in different surfaces from toluidine blue stained optical microscopy images: (a) The inclined surface in both schematic and 3D optical microscopy image; (b) Toluidine blue stained cross section slice optical microscopy image show the keratin cells along the major axis direction of the ellipse; (c) Needle like keratin cells with thickness $\sim 1$–2 mm in the longitudinal section, showing $\sim 30^\circ$ angle with the tubule edge; (d) Keratin cells in the inclined surface indicating the irregular shapes of keratin cells connected with each other, and having a diameter 20–30 mm.
TEM images of the cross section (Figure 3.4a) show parallel cell boundaries, corresponding to the laminated structure observed in both optical and SEM images. Curve-shaped cell membranes are evident (Figure 3.4b) revealing the source of surface roughness highlighted in previous work. These rough cell surfaces increase the contact areas between adjacent cells, and result in creation of interlocking interfaces, which lead to higher resistance to delamination between the cell lamellae. Keratin macrofibrils (bundles of IFs) are found inside the cells, with a diameter of ~ 200 nm. Figure 3.4c and 3.4d are TEM images of the longitudinal section, showing different orientations of macrofibrils. The fibrils inside the cells are randomly arranged in the cell planes (since the thickness is much smaller than the diameter of cells, the flat cells can be assumed as “planar”) (Figure 3.4c, d). No fibrils are found perpendicular to the cell plane. Therefore, the mechanical properties of these laminated structures should be transversely isotropic because of the in-plane arrangement of the macrofibrils. Further implications on mechanical properties will be discussed in a later section. The parallel cell boundaries also show a lamellar cell structure in this longitudinal section. IFs, with average diameter of ~12 nm, are observed in the macrofibrils (Figure 3.4e).
Figure 3.4 Transmission electron microscopy images of keratin cell lamellae for different surfaces: (a) Parallel cell boundaries (dark lines, indicated with red arrow) are shown, indicating the cells are stacked layer by layer along the thickness direction; (b) Curved characteristic of cell boundary (dark line) in a higher magnification. Cross section of keratin macrofibres (yellow arrow and circle) exist in the cells, and the diameter of the macrofibril is around 200 nm; (c) Curved cell boundaries are also found in longitudinal section, which is similar as the cross section. Keratin macrofibris (yellow arrow and circle) cross sections are also indicated here; (d) keratin macrofibris (yellow arrow and circle) are found parallel with the longitudinal imaging surface; (e) Intermediate filaments in the macrofibris in a higher magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In summary, the hierarchical structure of bighorn sheep horn from the macro- to nano-scale level is illustrated in Figure 3.5. Tubules with elliptical cross section (~59 µm in major axis, ~25 µm in minor axis) are aligned along the horn growth direction. The whole structure is formed by lamellar keratinized cells (20~30 µm in diameter, 1~2 µm in thickness). The laminae are stacked sequentially with a ~30° angle with respect to the tubule direction. Inside the flat keratinized cell, macrofibrils with diameter ~200 nm are distributed in the plane of the cell with a random orientation. At the nano-level, intermediate filaments (IFs, ~12 nm in diameter) embedded in an amorphous keratin matrix are the components of the macrofibrils [86].
Figure 3.5 Hierarchical structure of the bighorn sheep horn: From left to right, curved horn with growth lines from base to the tip at the macro level; Elliptical tubular structures along the growth direction; At the micro level, lamellae of keratinized cells are stacked layer by layer around the tubules, and cell lamellae are oriented ~30° to the tubule direction. Keratinized cells are flat pancake-shaped, with diameters ~20–30 mm and thicknesses ~1–2 mm; At the nano level, each cell contains macrofibrils with a diameter of ~200 nm. The macrofibrils are randomly orientated inside the cell plane. The macrofibrils are composed of intermediate filaments with diameter ~12 nm; At the molecular level, intermediate filaments are formed by alpha-helix with hard disulfide bonds and weak hydrogen bonds connected with each other [25].

3.3.2 Strain rate, anisotropy and water dependency of mechanical properties

Stress-strain curves obtained for range of quasi-static to dynamic strain rates (1×10^3 s⁻¹ to 4×10^3 s⁻¹) under uniaxial compression are summarized in Figure 3.6. The results for the dried and hydrated specimens compressed along different orientations are plotted in Figure 3.6a-c and 3.6d-f, respectively. Similar to the mechanical response of polymeric materials, which is significantly influenced by strain rate [156], the stress-strain curves, for all three tested orientations, exhibit a strong rate-dependent behavior. The stiffness, based on the initial slope and the yield stress increase with increasing strain rate. The latter observation is a common phenomenon for polymers and is related to secondary molecular processes [157, 158]. However, it has been hypothesized that lack of sufficient time for rearrangement of keratin network, into lower energy configurations, might be another reason of the higher yield stress with increasing rate [8, 159]. Table 3.1 shows
the Young’s modulus comparison for different strain rate, loading directions, and hydration states. The Young’s modulus in the dry state increases 2~3 times when the strain rate changes from $1 \times 10^{-3} \text{s}^{-1}$ to $4 \times 10^3 \text{s}^{-1}$, while it is almost a ten times higher in the hydrated condition. This reveals the importance role of hydration on viscosity characteristics besides the heterogeneous structure in damping the travelling stress waves, which requires further investigations. The data in Table 1 were illustrated in a bar chart (Figure 3.7) with further statistical analyses. Significant differences were found between dry and wet samples in all directions and strain rates based on t-tests. At lower strain rates 0.001 s$^{-1}$ and 0.1 s$^{-1}$ (Figure 8a and b), compressive Young’s modulus of dry samples reveals negligible difference between longitudinal and transverse directions, while they both are significantly higher than the one in the radial direction. However, at higher strain rates (~ 0.5 - 4000 s$^{-1}$, Figure 8c and d), there is no significant difference among the Young’s modulus of all the three directions of dry samples. The comparison reveals transverse isotropicity in lower strain rates, which is believed coming from the in-plane arrangement of keratin macrofibrils. Thus, it can be concluded that the keratin macrofibrils can increase the compression stiffness along the fibril direction.
Figure 3.6 Stress strain curves of compression tests at different strain rates (from $\sim 10^{-3}\text{s}^{-1}$ to $10^3\text{s}^{-1}$), orientations, and hydration states: The top row shows stress-strain curves obtained under dry condition, (a) Radial direction; (b) Longitudinal direction; (c) Transverse direction; Bottom row shows results for the hydrated state, (d) Radial direction; (e) Longitudinal direction; (f) Transverse direction. The stress strain curves are the average values of at least 3 samples.
Figure 3.7 Bar chart of the Young’s modulus of horn samples in different directions, hydration states, and strain rates: (a) strain rate 0.001 s$^{-1}$; (b) strain rate 0.1 s$^{-1}$; (c) strain rate 0.5 s$^{-1}$; (d) strain rate 4000 s$^{-1}$. t-tests between all the dry and wet conditions. One-way ANOVA tests were conducted for the different directions for each strain rate. “ns” refers to negligible statistically significant difference between the results with the level of 0.05.

Table 3.1 Comparison of Young’s modulus at different compressive strain rates, loading directions and hydration states.

<table>
<thead>
<tr>
<th>Strain rate (s$^{-1}$)</th>
<th>Radial (GPa)</th>
<th>Longitudinal (GPa)</th>
<th>Transverse (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry</td>
<td>Wet</td>
<td>Dry</td>
</tr>
<tr>
<td>0.001</td>
<td>$0.96 \pm 0.16$</td>
<td>$0.13 \pm 0.04$</td>
<td>$1.875 \pm 0.17$</td>
</tr>
<tr>
<td>0.1</td>
<td>$1.1 \pm 0.2$</td>
<td>$0.192 \pm 0.07$</td>
<td>$2 \pm 0.2$</td>
</tr>
<tr>
<td>0.5</td>
<td>$1.55 \pm 0.3$</td>
<td>$0.207 \pm 0.08$</td>
<td>$2.1 \pm 0.22$</td>
</tr>
<tr>
<td>4000</td>
<td>$3.486 \pm 0.23$</td>
<td>$1.433 \pm 0.26$</td>
<td>$3.66 \pm 0.25$</td>
</tr>
</tbody>
</table>
Furthermore, it can be observed from Figure 3.6, that a higher strain rate results in greater increase of anisotropic behavior of the dried specimens. The curves for dynamic tests exhibit a linear elastic region up to the yield point. The post-yield behavior is strongly anisotropic with the degree of softening a function of loading direction. The plot for the radial specimens shows a saddle point with a rising flow stress before ultimate failure. For the longitudinal orientation, a very small softening is observed post-yielding. Conversely, the transverse specimens show a significant strain softening up to a local minimum and then a hardening behavior before a catastrophic failure. These kinds of compressive responses were reported for polymeric foams with various densities under dynamic loading [160, 161]. Based on the horn microstructure, the quasi-static and impact forces along the radial direction are almost perpendicular (larger than 60˚) to the cell surface, which will only densify the keratin cell layers. However, forces in both longitudinal and transverse directions, are nearly perpendicular to the cell thickness direction; thus, leading to buckling and collapse of the laminated structure. To better understand the anisotropic behavior in horn, which is rooted in its structural morphology, bar impact recovery test results are described in the next section.

Similar to the dried tested specimens, there is an increasing trend for stiffness and yield strength from quasi-static loading to high strain rate tests under the hydrated condition. However, the stress-strain curves demonstrate the same overall shape for all tested directions, which implies a much milder anisotropic response in the hydrated state. Thus, the influence of water content is dominant over other factors, such as the microstructural elements. Another important feature that is originated from the dominancy of hydration, is the resiliency compared to the dried specimens. The reduction of stiffness and strength due to the hydration reveals how keratin materials are susceptible to water content and become more compliant. Increasing water content also contributes
to changes in viscoelasticity, and subsequent changes in anisotropicity. In this regard, Kitchener [111] studied the effect of water on linear viscoelasticity of horn sheath keratin, which showed more non-linearity of the viscoelastic behavior with increasing hydration.

Figure 3.8 Impact energy absorption (area under stress-strain curves) as a function of compressive strain in different loading directions and hydration states at a high strain rate (~10³ s⁻¹). The energy absorption data for each condition were averaged from 3 samples.

Although the water content in fresh bighorn sheep horn remains unknown, Kitchener and Vincent [113] reported fresh oryx horn had a water content of 20 wt.%. Since both horns are made from crystalline α-keratin embedded in an amorphous keratin matrix, it can be assumed that they have similar range of water content in the fresh status. The in vivo condition of 20% hydration is hard to attain in the laboratory, since partially hydration leads to a nonuniform distribution of water (e.g. a portion of the water may stay inside the tubules), which will cause the inaccurate measurements of the water contents and mechanical properties. Therefore, we aimed to test the dry (~10%) and fully hydrated (~30%) samples, which would be the lower and upper limit of the live horn materials. It is worth noting that the energy absorption per unit volume, which can be
defined as the area under the stress-strain curves, is higher for the radial direction than the other orientations. Figure 3.8 shows the impact energy absorption versus compressive strain in different loading directions and hydration states. This shows that the dry samples absorb more energy than the hydrated ones due to the higher initial stiffness and yield strength under dry conditions. Moreover, it is noticeable that the energy absorption has the highest value for the radial direction. Thus, it can be concluded that the radial direction is the main direction of impact resistance in horns. Further understanding of this observation requires a detailed investigation of the tubule distribution along with a correlation of mechanical properties in the three orthogonal directions. It was previously suggested that increasing the tubule density can lead to higher energy absorption capability [126]. Analysis of these features will be conducted in future studies.

### 3.3.3 Hopkinson bar impact recovery test results and failure mechanisms

Figure 3.9 corresponds to the Hopkinson bar impact recovery tests along with images of sample surfaces before and after testing for the dried specimens. The loading-unloading curves for quasi-static tests show a similar linear elastic region behavior with a plateau for longitudinal and transversal directions, and an increasing stress flow for the radial direction (Figure 3.9a-c). In all directions, the specimens maintained a residual deformation after unloading, even though the elastic portion of the strain was recovered. In the case of dynamic loading, the elastic recovery was obtained by comparing the pristine length of specimen with the deformed length after dynamic loading (labeled as ‘f’ on Figure 3.9a,b,c).
Figure 3.9 Bar impact recovery compression tests performed on dry samples under compression: (a-c) Stress-strain curves with loading and unloading under quasi-static (solid lines, strain rate $10^{-3}$ s$^{-1}$) and dynamic loading conditions (dashed lines, strain rate $10^{3}$ s$^{-1}$) along the radial, longitudinal, and transverse directions; f in the plots correspond to the final states in the dynamic tests, showing significant residual strains after recovery tests; (d-f) Differential interference contrast optical images of pristine surfaces in different directions: radial, longitudinal, and transverse, respectively; (g-i) Surfaces in three directions after 30% quasi-static deformation; buckled and kinked lamellae formed shear bands in h; X-shaped shear bands indicated with red lines in i; (j-l) Scanning electron microscopy images of the sample surfaces in the three directions after ~20% impact deformation (strain rate ~ $10^{3}$ s$^{-1}$).

Comparing the pristine surfaces (Figure 3.9d-f) with the deformed ones (Figure 3.9g-l) reveals the role of tubules in microstructural damage mechanisms. In this regard, the macroscopic observations are discussed first, and then more detailed deformation mechanisms are reported. Optical microscopy images of deformed surfaces were acquired after quasi-static compression
(Figure 3.9g-i), while scanning electron microscopy images (Figure 3.9j-l) were taken from the dynamic compression because of the severely deformed and uneven surface, which made it hard to visualize using optical microscope. For the radial direction, the load was applied along the minor axis of the elliptical tubules. Under quasi-static loading, tubule distortion with increases in major and decreases in minor axis dimensions are observed (Figure 3.9g) when compared with the pristine sample (Figure 3.9d); under dynamic loading more pronounced changes are observed with most of the tubules collapsed (Figure 3.9j). In the longitudinal direction when the load is applied parallel to tubules, buckling of lamellae is noticeable under both quasi-static (Figure 3.9h) and dynamic loading (Figure 3.9k) conditions. In this case, layers of buckled or kinked lamellae form a shear band. Under quasi-static loading along the major axis of the tubules (transverse direction), most of the tubules collapse and cause localized inelastic deformation with X-shaped shear bands (Figure 3.9i). This deformation mode mostly controls plasticity and failure, and is very common in heterogeneous and amorphous material systems [162]. More severe deformation occurs for the dynamic loading in the transverse direction. Figure 3.9l shows that several microcracks formed with a macrocrack propagating diagonally. The specimen exhibited a shear-type failure along the direction of maximum shear stress followed by delamination. This could explain the strongest strain softening measured in this direction compared to the other directions.
Figure 3.10 Bar impact recovery compressive tests of hydrated samples under compression: (a-c) Stress-strain curves with loading and unloading under quasi-static (solid lines) and impact loading conditions, the latter with limited strain (~30% strain, dashed lines) along radial, longitudinal, and transverse directions, respectively; f in the plots correspond to the final states in both quasi-static and dynamic tests, indicating almost no residual strain after recovery tests; (d-f) Differential interference contrast optical images of original surfaces before compression in different directions: radial, longitudinal and transverse, respectively; (g-i) Surfaces in three directions after 30% quasi-static deformation. Red circles in (g) indicate water drops squeezed out after radial compression; (j-l) Scanning electron microscopy images of the surfaces in three directions after ~30% impact deformation.

Images of hydrated samples, recovered after ~30% strain are given in Figure 3.10. In contrast to the dried specimens, most of the strain is recovered under both quasi-static and dynamic loading (Figure 3.10a-c). The residual quasi-static strain is obtained from the unloading data,
while the residual dynamic strain is extracted by the comparison between the initial length (i.e. before tests) of the specimen and the final one (i.e. after tests). In this regard, there is ~5% remaining strain under quasi-static compression, but negligible residual strain under high strain rate impact in all directions based on the measured lengths under loads before and after the tests. Figure 3.10d-f show the optical microscopy image of the original surface in radial, longitudinal, and transverse direction respectively. Water drops were squeezed out under quasi-static compression in radial direction (Figure 3.10g). Slight lamella buckling was noticed after quasi-static compression in longitudinal direction (Figure 3.10h). SEM images of surfaces in different directions when subjected to high strain rates are shown in Figure 3.10j-l. In contrast to the dried tested specimens, no distinguishable damage in the hydrated tests can be observed. This can be explained by the hydration dependence of the glass transition temperature of keratin materials. The glass transition temperature of human hair and wool decreases from ~80°C (~10% water content) to ~20°C (~20% water content) because of the plasticizing effects of water on the keratin matrix [163]. It is well established that the keratinous microfibrils in wool, hair, horn, and hoof possess similar dimensions, with low-Sulphur proteins arranged in the same manner [164]. However, horn keratin contains a smaller proportion of matrix than hair and wool [164-166]. Therefore, horn keratin is expected to experience a reduction in glass transition temperature after hydration but of lower degree compared with the ones observed for hair and wool. The wet horn samples have ~30% water content, indicating both the quasi-static and dynamic compression tests occur around the glass transition temperature, leading to elastomer-like stress strain curves. This is why no residual strains and distinguishable damage occur in totally hydrated samples, while the dry samples show brittle failure. Deformation recovery in animal hairs, in the hydrated state, was also identified in previous work. The advanced explanation was that presence of water molecules in the
keratin matrix allows breakage and reformation of hydrogen bonds, which along with the higher flexibility of macromolecular chains made deformation recovery possible [167]. Since the horn material here studied possesses a similar keratin matrix composition and structure, we infer that the recovery behaviors found in fully hydrated horn samples can be explained by the effect of water on the keratin matrix. In addition to this, we hypothesize that the hollow tubules inside the horns may increase the water absorption, thus further assisting the recovery of horn tissues after impact. This hypothesis will be explored in future studies.

More detailed compressive deformation mechanisms are summarized in Figure 3.11. Deformed tubules in the radial direction are shown in Figure 3.11a-d. In the dry state, tubules are relatively distorted along both major and minor axes under quasi-static load (Figure 3.11a). Under dynamic loading, the tubules compressed significantly and separated into small cavities, and also deform from elliptical shapes to crescent shapes with opposite concavities or in some cases complete flattened shapes (Figure 3.11b). This can stem from the perpendicular deviation of the loading direction respect to the major diameter of tubule due to the non-uniform radially distributed tubule (see Figure 3.11c), which led to the development of homogenous and heterogeneous flattening with different concavities. For the quasi-static hydrated case, absorption of water seems to lead to stress concentration at the vertices of the ellipses. This is noticeable from some damage accumulation at those vertices as observed after the tests (Figure 3.11c). However, no significant deformation is found under impact loading for the hydrated samples (Figure 3.11d). This is consistent with the bar impact recovery test result, where deformation was fully recovered. Longitudinally deformed surfaces in dry condition indicate lamellae buckling and kinking along with fiber bridging (Figure 3.11e-f). Interestingly, buckled lamellae show two opposite curvatures, which surrounded an accumulated damage zone. Slight lamellae buckling and delamination is
noticed in hydrated samples under longitudinal loading (Figure 3.11g). Figure 3.11h is the longitudinal surface of the hydrated samples after dynamic compression. Compared with the dry surface (Figure 3.11f), no damage is observed. Finally, distortion and rupture of the tubules are noticeable for the transversal direction. Figure 3.11i shows tubule distortion and fiber bridging in dry samples under quasi-static compression. However, the image for the dried specimen under dynamic loading demonstrates how a microcrack forms. In this case, similar to the radial direction, tubules adopt crescent shapes and coalescence of them is the source of nucleation sites for cracks (Figure 3.11j). Rupture of tubules also occurs in the hydrated samples in the transverse direction (Figure 3.11k). No evident failure mechanism is observed for the hydrated samples under dynamic loading in all directions (Figure 3.11d, h, l). Figure 3.11 summarizes the failure mechanisms in different loading orientations, loading strain rates, and hydration states, which gives an effective explanation of the stress strain curves shown and described in Section 3.2. Future work will investigate recovery mechanisms of hydrated samples and its relevance to bioinspired designs. The various energy dissipation mechanisms found here could give inspiration on synthesis of multiscale laminated composites with incorporation of tubule for crashworthiness application. In the event of dynamic collision or accident, structures are conventionally made of ductile metals to absorb the crash energy; however, manufacturing viscoelastic composite with the inspired microstructure revealed by the present study may improve energy absorption capacity along with a light-weight design. In this regard, a parametric study and analysis of the interplay between material parameters and microstructural features is essential for design of optimum synthetic material performance.
Figure 3.11 Detailed failure mechanisms under different loading directions, rates (quasi-static ~10<sup>-3</sup>s<sup>-1</sup> and impact ~10<sup>3</sup>s<sup>-1</sup>), and hydration states (dry and hydrated). Quasi-statically loaded samples were imaged in an optical microscope while dynamically loaded samples were imaged using a scanning electron microscopy. Each row of images correspond to radial (a-d), longitudinal (e-h), and transverse (i-l) directions, respectively: (a) Tubule deformed under quasi-static compression in the radial direction; (b) Tubule collapse under high strain rate impact in the radial direction; (c) Tubule tearing at the corner and slightly deformed in the hydrated condition; (d) no obvious failure under impact for wet samples; (e) Lamella buckling, kinking, and fiber bridging under quasi-static compression in the longitudinal direction; (f) Tubule buckling under impact in longitudinal direction; (g) Lamellae buckling and delamination in hydrated samples under quasi-static compression in longitudinal direction; (h) no obvious damage under impact in longitudinal direction; (i) Tubule distortion and fiber bridging in dry samples under quasi-static compression; (j) Tubule coalescence under impact in transverse direction; (k) Tubule rupture in wet samples under quasi-static compression; (l) no obvious damage in wet samples under impact in transverse direction.

3.4 Conclusions

The structure, quasi-static and dynamic mechanical properties and damage mechanisms of a bighorn sheep (*Ovis canadensis*) horn were investigated. The structure was examined by optical, scanning and transmission electron microscopy along with high resolution micro-computed tomography. High strain rate dynamic compression was performed by a Hopkinson bar and results were compared to quasi-static compression experiments. Different compressive deformation mechanisms during testing were observed and summarized in section 3.3. The reason why bighorn sheep horn can withstand blows during ramming are: 1) the radial direction (impact direction) was found to have the highest strength and energy absorption in both dry and hydrated states; 2) The
deformation recovery exhibited by horns in the hydrated states appear to confirm them the ability to withstand multiple blows without fracture during ramming. As a result, the main conclusions in present work are:

- The horn microstructure consists of tubules as well as a laminated structure formed by keratinous cells. The former are located along the longitudinal direction with an elliptically-shaped cross section (major axis ~59 µm, minor axis ~25 µm), confirmed by high resolution X-ray computed tomography. Laminated keratinous cells surround the tubules. The dimension of the keratin cells are 20~30 µm in diameter and 1~2 µm in thickness. There is a ~30.16 ± 5.87° angle between cell lamellae and tubules.

- Keratin macrofibrils with diameter ~200 nm were found randomly oriented in the keratinized cell planes and parallel with the cell surfaces. The fibrils are bundles of intermediate filaments with dimension ~12 nm in diameter. These in-plane arrangements of macrofibrils, reported here for the first time, explain the transverse isotropic behavior identified through compression tests.

- Stress strain curves of quasi-static and dynamic tests indicated higher energy absorption and impact resistance in the radial direction, which is the impact direction of the horn. Initial Young’s modulus of dry samples in longitudinal and transverse directions are significant higher than in the radial direction at lower strain rates (0.001 and 0.1 s⁻¹), showing transverse isotropy due to the laminated structure around the tubules.

- Damage at various strain rates was examined by conducting Hopkinson bar impact recovery tests. More material damage is observed with increasing strain rate in the dry condition. Pre- and post-test microscopy imaging reveals various inelastic deformation mechanisms: kink bands, lamella buckling, tubule collapse, and microcracking, which highlighted the role of
structural elements such as tubules and lamellae in relation to loading. Tubule collapse in the radial direction leads to significant energy absorption, while lamella buckling and shear band formation in the longitudinal and transverse directions cause catastrophic failure of material with less energy absorption.

- Significant differences in behavior were observed as a function of sample hydration. Under the dry condition, the samples exhibited a strong anisotropic behavior as well as strain rate dependency. Specimen hydration leads to a more isotropic behavior, while still rate dependent. The hydrated specimens recover their initial length under dynamic loading at strains as high as 20-30%. This can be explained by the decrease of the glass transition temperature of hydrate samples, thus leading to a strong viscoelastic behavior under compression. This feature is remarkable because it shows that hydrated horn material can absorb significant amounts of energy without damage.

The findings of this study demonstrated how horn dissipates large amount of energy during deformation in different orientations and hydration states. Moreover, the revealed hierarchical organization of horn constituents such as layers of keratin cells along with incorporation of tubules can serve as bio-inspiration for the design of synthetic composites. Compression tests in dry conditions demonstrate the role of tubules in the deformation mechanisms as well as their role in determining the preferable impact orientation. Therefore, the results of this paper hint at a path to tune energy-absorbent engineering materials that incorporate tubular structures as a function of impact direction. Moreover, the water-assisted recoverability of keratin under high-energy impact provides inspiration towards design of recoverable energy-absorbent materials.

Chapter 3, in full, is published as “Hierarchical structure and compressive deformation mechanisms of bighorn sheep (Ovis canadensis) horn” Acta Biomaterilia, 64, 1-14 (2017). This
work was coauthored by Zaheri, A., Jung, J. Y., Espinosa, H. D., and Mckittrick, J.. The dissertation author is the first author of this work.
CHAPTER 4: STRUCTURE AND PROPERTIES OF DIFFERENT HORMS

4.1 Introduction

Animal horns are mainly found in the Bovidae family (e.g. cattle, sheep, goats, buffalo, antelope) and Antilocapridae family (e.g. pronghorn) and serve as attack and defense weapons during intraspecific combat \[127, 168\]. These weapons have diverse forms and shapes arising over millions of years of evolution \[169\], while the fundamental material component remains consistent, which is \(\alpha\)-keratin, a structural protein synthesized by keratinocytes \[4, 86\]. Keratin, typically mineralized to < 1\% (e.g. (0.05 wt.% in ox and rhino horns) \[170\], is one of the most common biopolymers found in nature, showing remarkable mechanical efficiency because of its relatively high fracture toughness and Young’s modulus, but is much lighter than other highly mineralized biological materials, such as bone and teeth \[1, 5, 6\]. Keratinous materials are formed by laminated keratin cell layers, with crystalline keratin intermediate filaments (IF) embedded in an amorphous keratin matrix as the main constituent of the cells \[4, 86\]. The crystalline IFs can be classified into two categories: \(\alpha\)-helix and \(\beta\)-sheet patterns. Wool, hair, hooves and horns of mammals are composed by \(\alpha\)-keratin, while bird feathers, reptilian claws and beaks are all constituted of \(\beta\)-keratin. In terms of the process of biosynthesis and amount of disulfide bonds formed from the presence of cystine, keratinous materials can be classified into soft or hard keratin. Soft keratin such as stratum corneum contains a lower amount of sulfur while hard keratins such as wool and hair show more sulfur cross links leading to higher hardness and durability \[87, 88\]. These keratinous materials serve particular functions such as protection (e.g. waterproof feathers), defense (e.g. horns) and predation (e.g. claws) \[4, 86, 171, 172\].

Mammal horns contain a bony core and an exterior keratin sheath. The sheath develops slowly and attains a definitive size and shape by growing from the epidermal layer surrounding
the bone core from the frontal region of the skull [173, 174]. Given the lifespan of mammals and the inability of most horns to regrow (the pronghorn is an exception), all horns must be resistant to fracture [127]. As the main weapons employed in fights, the horns should meet specific mechanical requirements (impact resistance, energy absorption, high compressive and tensile strength) to accommodate the particular fighting behavior of their species [175, 176]. Thus, the study of hierarchical structure and mechanical properties of keratinous horns can provide principles and design rules for making bioinspired materials with superior mechanical properties and durability.

The hierarchical structure and mechanical properties of bighorn sheep horn have been previously investigated by Horstemeyer et al. [109, 120]. Prior studies showed that tubular and lamellar structures exist in the bighorn sheep horn. Flattened and cornified epithelial cells (keratinized cells) grow from dermal papilla that are arranged in concentric layers around a central medulla, forming the tubular structures [118, 155, 177]. Tubular structures in other keratinized tissues such as equine hooves and rhinoceros horns were identified and characterized in previous studies [155, 178]. The tubular structures in hooves were identified to be continuous hollow medulla cavities, from its origin at the coronet to the ground surface [178, 179]. No clear evidence has shown that the tubular structures could benefit the hydration and thermal insulation properties of hoof [180]. However, it is well established that tubules can redirect crack propagation thus increasing the fracture toughness of biological materials [33, 181]. In bighorn sheep horns, the tubules are hollow and elliptically shaped (in cross-section) with a major axis ~100 μm and minor axis ~40 μm. Surrounding the tubules are concentrically and tightly packed keratin cells (~20 μm in diameter, ~2 μm in thickness) [121]. In hooves, the keratin cells firmly cement together
preventing the passage of water inwards and the loss of body fluids outwards by forming a tough protective barrier [179].

Three orthogonal directions were defined in previous works to show the orientation-dependent mechanical properties: the longitudinal direction is parallel to the tubule direction (growth direction), the radial direction is the impact direction and the transverse direction is perpendicular to both. The compression and tension properties of bighorn sheep horn in different orientations and hydration states were also studied. Anisotropic compression behavior in the different orientations was observed: the Young’s modulus and strength were smaller in radial direction but the energy absorption was the largest. Hydration significantly decreased both the Young’s modulus and compressive strength in different loading states and strain rates [109, 120]. It was reported that water molecules could affect keratin matrix in three ways: water molecules act as a swelling agent and extend the space between molecular chains, thus reducing the interaction between chains; water molecules may replace the secondary hydrogen bonding thereby increasing the chain mobility; water molecules and matrix proteins bind to form a network that acts as a plasticizer, thus the stiffness is reduced and mobility increased [86].

The mechanical properties of other horns have also been examined in previous studies. Experiments revealed that along the length of fresh waterbuck horns, the work of fracture (calculated as the energy differences in loading and unloading curves divided by the area of material fracture in extending the length of the crack [182]) ranged from 10 to 80 kJ/m² [175]. The specific work of fracture (normalized by density) of the horn is 32 kJ/m², which is greater than most other biological and synthetic materials (antler 6.6 kJ/m², bovine femur 1.6 kJ/m², glass 5 kJ/m², mild steel ~ 26 kJ/m²) [183, 184]. Three-point bending tests of cattle horn show that the mechanical properties are position dependent. From the distal to the proximal regions, the stiffness
and strength decreased. This is likely due to the age of the horn. The distal region is the oldest while the proximal region is the youngest. This difference may help during fights, because the distal part needs to be hard enough to stab the opponent, whereas the proximal part should be tough to absorb energy. The gradient of mechanical properties could also be related to water content and the proportions of crystalline and amorphous phases in keratin [122].

The horn shapes of different species have been hypothesized to fit their fighting behavior [127, 169]. It is worth noting that the microstructures and mechanical properties of horns can be related to their fighting behaviors as well. Fighting between bighorn sheep (Ovis canadensis) entails extremely high energy impact on the horns [50, 95], while these collisions in domestic sheep (Ovis aries) are much more moderate with a lower impact energy [185-187]. The mountain goat (Oreamnos americanus) uses a different fighting style, stabbing. The opponents initially whirl in anti-parallel position relative to each other, and then stab using the horn tips. The fights between goats can, in some cases, cause severe injuries [188, 189]. The pronghorn (Antilocapra americana) utilizes two fighting modes. One is the sudden clashing together of two bucks; in the other, two bucks gradually approach each other, lower their heads, and then slowly engage horns. Fights involve thrusts and counterthrusts with the horns. When the horns interlock, pronghorns use their body strength to twist the opponent’s neck and push to make their rival lose balance. Unlike the other species of horned ruminants (family Bovidae), the keratinous horn sheath of the pronghorn (family Antilocapridae) sheds and then regrows every year. Unlike the unbranched horny sheath of bovids, the pronghorn has two branches [190, 191].
Figure 4.1 Phylogenetic tree and estimated divergence times of four ruminant species with keratinized horns [192, 193]: Bighorn sheep (*Ovis canadensis*), domestic sheep (*Ovis aries*), mountain goat (*Oreamnos americanus*) and pronghorn (*Antilocapra americana*). The evolution of important traits are mapped onto branches of the tree. ‘Cranial appendages’ refers to the horns, ossicones, and antlers of ruminant mammals; keratinous horn sheaths likely evolved independently in the families Bovidae and Antilocapridae [194]. Paintings are by C. Buell (copyrights to J. Gatesy).

For the present study, the above four representative species were selected (bighorn sheep, domestic sheep, mountain goat and pronghorn). Both bighorn and domestic sheep are in the same genus (*Ovis*), and the mountain goat is a close relative, but belongs to a different genus (*Oreamnos*) in the family Bovidae. The pronghorn is in the family Antilocapridae that evolved keratinous horns independently of bovids. The phylogenetic tree and divergence times of these four species are shown in Figure 4.1 [192, 193]. The species selected in the present work sample the diversity of microstructures and mechanical behaviors of various horns. The present study aims to understand the following points: The similarities and differences of the microstructures and mechanical properties between horns from different ruminant artiodactyl families; the correlations between
microstructures and mechanical properties in different species; and whether the microstructures that evolved in different species are optimized to accommodate specific fighting behaviors.

4.2 Experiments and methods

4.2.1 Materials preparation

Four pairs of horns (bighorn sheep, domestic sheep, mountain goat, and pronghorn) were purchased from the Wilderness Trading Company (Pinedale, WY). All horns were stored in air at room temperature. We did not have access to fresh horns, but it has been reported that there is a 20 wt.% water content in fresh oryx horns. It is impossible to obtain this value by partial hydration because the water would preferentially be absorbed in the tubules, as well as in the keratin matrix. Thus the controllable conditions of ambient dried and fully hydrated were tested. Figure 4.2 shows the combat styles and horn morphologies of different horns: (a) bighorn sheep; (b) domestic sheep; (c) mountain goat and (d) pronghorn. The keratin sheaths and bony cores are indicated in the cross-section images in the bottom of Figure 4.2. Compression and tension samples were acquired from the midsection of the horns, indicated with yellow and red colors. We choose samples from representative areas that sustain the highest impact force and will most likely be damaged in these extreme conditions. For bighorn and domestic sheep horns, the main forces are impacts and compressions on the middle and outer area as shown in Figure 4.2, thus we took samples for the impact and compression areas. For consistency, the tension samples were also obtained from the same area. For mountain goat, since it is very symmetric and the forces come from the tip, we took samples in the central part. Due to the complicated structure of pronghorn, we took samples under the branch, since samples in this area supported most forces.

4.2.2 Structural characterization
The microstructures were characterized by optical microscopy and scanning electron microscopy (SEM). A Leica Ultracut UCT Ultramicrotome (Leica Microsystems, Wetzlar, Germany) was used to get fine-polished ultra-thin horn slices. Samples were obtained from the central region of the horn and ~5-10 mm away from superficial surfaces. Two samples of each horn were prepared with one cross-section parallel and one cross-section perpendicular to the longitudinal direction. The thickness of horn slices was 1 µm to 1.5 µm. Slices were stained by toluidine blue (a basic thiazine metachromatic dye with high affinity for acidic tissue components). Optical micrographs of stained horn slices were taken from a Zeiss AxioImager M1 optical microscope (Zeiss, Oberkochen, Germany). Scanning electron microscopy (SEM) images of freeze fracture surfaces in liquid nitrogen were acquired using an ultra-high resolution scanning electron microscope (FEI, Hillsboro, OR, USA).

4.2.3 Water absorption and FTIR

Water absorption tests were performed on two samples (6 mm × 6 mm × 6 mm) from each species. Samples were air-dried for 24 hours before weighing. The samples were then soaked in purified water and weighed every four hours. The water was changed every 24 hours. After 96 hours, the samples were taken out and the surface water was removed with a cloth. The samples were then placed into a pre-heated oven (110°C) for five days to fully dehydrate. During dehydration, the samples were weighed every 12 hours. Fourier-transform infrared spectroscopy (FTIR, PerkinElmer, Waltham, MA, USA) was used to characterize the chemical compositions of horns in different species. Three samples with dimension 5 mm × 3 mm × 0.3 mm (length × width × thickness) were acquired from each species and characterized by FTIR.

4.2.4 Compression and tensile tests
Cubic samples with dimensions of 6 mm × 6 mm × 6 mm were prepared for compression tests. A section saw with circular diamond blade was used to cut the samples and obtain parallel surfaces. All samples were cut from the central region of the horn (shown in Figure 4.2). For each species, three sets of samples were prepared with the loading direction along longitudinal, radial and transverse directions. For each species, a total of 30 samples were prepared; 15 were tested in ambient dried condition and 15 in fully rehydrated condition. For each condition, five each were tested in longitudinal, radial and transverse directions. The rehydrated samples were pre-soaked in purified water three days before testing with the water changed daily and kept wet until testing. Compression test experiments were conducted on a universal testing machine equipped with a 50 kN load cell (EM Model 5869, Instron, MA, USA). The testing crosshead speed was controlled at $6 \times 10^{-3}$ mm/s, which corresponds to a $1 \times 10^{-3}$/s strain rate. The machine automatically stopped when strain reached 0.7.

Samples for tensile tests were cut from the central region of the horns. Rectangular samples with dimensions of 15 mm × 3 mm × 0.3 mm (length × width × thickness) were prepared using a water-jet cutting machine. Care was taken to cut the samples such that tubules were aligned parallel to the longitudinal direction. Then the two ends of each sample were attached to 800 grit sandpapers to ensure slippage did not occur. These samples had a gage length of 10 mm. For each species, a total of seven samples were prepared. The samples were kept under ambient conditions for one day until testing. Tests were conducted on a universal testing machine equipped with a 500 N load cell (Instron 3342 Universal Testing Systems, Instron, MA, USA). The crosshead speed was controlled at 0.001 mm/s, which corresponds to a $1 \times 10^{-3}$/s strain rate, same as for the compression tests. Scanning electron microscopy (SEM) images of the fracture surfaces after
tension tests were acquired from ultra-high-resolution scanning electron microscope (FEI, Hillsboro, OR, USA).

Figure 4.2 Combat styles and horn morphologies for the different species: (a) bighorn sheep, (b) domestic sheep, (c) mountain goat and (d) pronghorn. Growth direction is indicated by a black arrow for each horn. Compression and tension samples taken from the keratin sheath are indicated with yellow blocks and red strips, respectively. Combat photographs of different species are taken from: theholepicture.photoshelter.com; gudmann.photoshelter.com; gettyimages.com; nationalgeographic.com.

4.2.5 Impact tests

The ASTM standard D7136/D7136 M-07 [195] is applied for drop weight impact tests of layered multidirectional polymer composite matrix, and the standard requires the test machine to have a rectangular free-standing (unclamped) area with the dimension of 125 mm × 75 mm (length × width). Since it was difficult to get such large testing samples from the horns, a modification of the standard apparatus was used. A lab built drop weight test machine [117] was used with a 1:5 scale of the ASTM standard test machine. With a preset round free-standing area, for this apparatus the optimal test sample dimensions are 20 mm × 20 mm × 5mm (length × width × thickness). The
drop weight is fixed at 1.2 kg and drop height varied from 0 to 0.74 m. The energy of impactor can be expressed by: \( E = mgh \); Where \( E \) is the impact energy imparted to the sample, \( m \) is the mass of the impactor, \( g \) is the gravitational constant and \( h \) is the total drop height. Only bighorn and domestic sheep horn samples were tested because of the limitation of horn size and shape in the other two species.

4.2.6 Statistical analysis

Statistically significant differences of the Young’s modulus and yield strength among the different horns were identified by using one-way ANOVA analysis. The criterion for statistical analyses was \( p < 0.05 \).

4.3 Results and discussions

4.3.1 Microstructures of the different horns

Low magnification optical micrographs of ultra-thin horn slices cut perpendicular to longitudinal direction are shown in Figure 4.3. Figure 4.3a, e, i and m are schematics of the distributions, shapes and sizes of tubules in bighorn sheep horn, domestic sheep horn, mountain goat horn and pronghorn horn, respectively. The transverse section images of each horn are shown in Figure 4.3b, f, j and n. As shown, the shape and size of the pores (cross-section of the tubules) vary among the different species. Elliptically-shaped pores are found in the bighorn sheep and pronghorn. For the bighorn sheep, the pores are more evenly distributed and uniform in size (~70 \( \mu m \) in major axis, ~35 \( \mu m \) in minor axis). For the pronghorn, the pores tend to disperse stochastically between the lamellae and have a wider range of size (from 28 \( \mu m \) x 10 \( \mu m \) to 97 \( \mu m \) x 37 \( \mu m \)). The pronghorn horn has several large pores (only one shown in the Figure 4.3n, with a major and minor axis dimension of 105 \( \mu m \) and 84 \( \mu m \)). These large pores were not observed in the other three horns. Pores from the domestic sheep show a deformed oval shape with a large
length to width ratio of 5.7 (a major axis dimension of 80 μm and a minor axis dimension of 14 μm). For the mountain goat, tubules were not observed (Figure 4.3i,j).

Figure 4.3 Schematic diagrams of the tubule distribution, optical microscopy and scanning electron microscopy (SEM) images of horn cross sections for different species. (a-d) bighorn sheep, (e-h) domestic sheep, (i-l) mountain goat and (m-p) pronghorn. The radial, longitudinal and transverse directions are indicated in the schematic diagrams. Different tubular cross section and pore shapes found in optical microscopy images (b, f, j, n). Lamellae surrounding tubules are identified in the SEM images of the cross sections (c, g, k, o). Higher magnification SEM images reveal the cell lamellae in each species with a thickness ~1-2 μm (d, h, l, p).

SEM micrographs of the fracture surfaces of the four species after fracture in liquid nitrogen are shown in Figure 4.3c, g, k and o. Elliptically shaped tubules were found in the bighorn sheep horn cross section (Figure 4.3c). Oval shaped pores are identified as in the optical microscopy image (Figure 4.3g). No tubules but a wavy lamellar structure is found in the mountain goat horn (Figure 4.3k). Figure 4.3d, h, l and p are SEM images with a higher magnification, showing the cell lamellae in each horn. The thicknesses of the keratinized cells are ~1-2 μm. Tubular density (porosity) in each species was calculated and averaged from five optical microscopy images using ImageJ. The bighorn sheep horn has the highest porosity (11.3%), followed by domestic sheep (7.7%) and pronghorn (5.8%), while the mountain goat has 0%.
porosity. There were some voids identified in the pronghorn cells, while no voids were found in the other three species. Embedded hair fibers in the keratin sheath also were found in pronghorn (Figure 4.3o), which may anchor the sheath in place during the rapid growth of new keratin layers after shedding the old horn sheath [196]. These hair fibers account for the large pores observed in the previous optical microscopy image (Figure 4.3n).

Figure 4.4 High magnification optical microscopy images of cross sections and transverse sections stained with toluidine blue: (a, e) bighorn sheep horn, (b, f) domestic sheep horn, (c, g) mountain goat horn and (d, h) pronghorn horn. Pores and cell lamellae are identified in the cross sections (a-d). Cell lamellae directions show differences in each species: (e) ~30° in the direction between cell lamellae and tubule direction (longitudinal) (f) No specific arrangement is found in domestic sheep. (g) Cell lamellae are parallel to the longitudinal direction in mountain goat horn; (h) Similar to the mountain goat, the cell lamellae in the pronghorn horn are parallel to the orientation of tubules.

Higher magnification optical micrographs of the cross and transverse sections from different horns are shown in Figure 4.4. The lamellar structure can be seen more clearly from these images, showing a high similarity between the different horns. The lamellae are layered in the radial direction as illustrated in Figure 4.4a-d. This result indicates there is a common layering pattern of the lamellae in the different horns. Micrographs of the longitudinal sections reveal the
keratin cell morphology and relationship between keratin cell alignment and tubule growth direction (Figure 4.4e-h). Keratin cells show a disk-shaped morphology, roughly spherical with the radius (~20 µm) much larger than the thickness (~2 µm) [86]. In the longitudinal direction, the cells are attached edge-to-edge, forming the lamella. The tubules extend along the longitudinal direction with no termination observed. However, differences are found between the species. In the bighorn sheep (Figure 4.4e), the parallel lamellae form a ~30° angle to the tubule direction. In the domestic sheep (Figure 4.4f), the lamellae are layered in a more disorderedly manner than in other horns. The lamellae that are close to the tubules tend to be layered parallel to the tubules, whereas away from the tubules, the lamellae form an acute angle to the tubule growth direction. As shown in Figure 4.4g, h, in the mountain goat and pronghorn, the lamellae are layered parallel to each other and to the growth direction. Schematic diagrams in Figure 4.4 shows the tubular and lamellar structures in the horns of different species. Laminated cells are indicated with red lines, showing differences among the species. The inclined lamellae in bighorn sheep and domestic sheep could be a consequence of the strong curvature of the horns in these two species.

4.3.2 Compression tests

Compression specimens of the different horns in both ambient dry and fully hydrated conditions were tested. The water content in each species was determined before the compression tests because of the significant effects of hydration on the mechanical properties, as reported in the previous literature [109, 197]. Figure 4.5a shows the degree of water absorption in each horn. In the ambient dry condition, the water content of different species vary, ranging from mountain goat (~7 wt.% ) to pronghorn (~14 wt.%). After submersion in purified water for 126 hours, the water contents in all four species increased, and pronghorn showed the highest water content (~52 wt.%). Figure 4.3p shows that there are numerous nanopores inside the keratin cells, which are not found
in the other three species. This indicates that the keratinized cells in the pronghorn are not fully cornified and densified, perhaps due to the rapid annual growth and shedding of the horn sheaths, a unique feature of pronghorns. The mountain goat horn has the lowest water content (~22% after hydration) and almost zero porosity. Therefore, both tubules and the nanopores should be considered as reservoirs for water, since the water content of rehydrated samples from mountain goat, domestic and bighorn sheep is positively related to their porosity. In a previous study, hollow tubules in hoof were hypothesized to serve mechanical functions and not transfer water from the surface of the hoof due to the dense layering of keratinized cells [180]. However, based on the present study on the hydration states of horns with different tubule densities, we suggest that tubules can contribute to water absorption by keratin. Thus, it appears that the overall water contents were correlated with increased density of tubules in the different horns.

The chemical compositions of different horns were characterized by FTIR (Figure 4.5b). Bighorn sheep and domestic sheep show very similar spectra, indicating almost the same protein compositions in these two species. This can be explained by the fact they are from the same genus. The mountain goat horn has a higher -S-C- peaks than the other three species, showing a cystine-rich composition [198]. The presence of cystine monoxide (S=O) peak indicates the oxidation of cystine, which could be due to the influence of the mountain goat horn preservation [198-200]. The sharp C=O peak at ~1750 cm⁻¹ in the mountain goat horn indicates a higher fraction of aspartic and glutamic acids [201, 202]. However, the chemical composition analysis by FTIR is a qualitative way to show the differences between various horn keratins. More quantitative amino acid, peptide, and proteomic analyses of different horn keratins are needed to better determine compositional effects on horn properties.
Figure 4.5 (a) Water content (wt.%) of horns in the ambient dry and fully rehydrated conditions. (b) Fourier-transform infrared spectroscopy (FTIR) spectra of ambient dry horns in the different species.

The stress-strain curves of the ambient dry condition and fully hydrated horns are shown in Figure 4.6. The stress-strain curves in all directions exhibited the same trend, which is an initial linear elastic deformation region followed by a long plastic region and ending with a densification region. This type of stress-strain curve is similar to the compression behaviors in typical cellular solids, which have been used for load-bearing and energy absorption purposes [203]. All horns present high strains with a deformation of ~70% without failure. However, anisotropic behavior is evident when comparing the curves loaded in the longitudinal, radial and transverse directions. When compressed along longitudinal direction, as shown in Figure 4.6a, the horns show a long plastic region with nearly constant stress. Based on previous work [121], this may be attributed to delamination and gradual buckling of lamellae, facilitating a large deformation without change of stress level. When compressed along other directions, where tubules extend perpendicular to loading direction, the stress plateau is shorter compared with longitudinal loading conditions. This could result from tubule closure during compression, leading to a fast densification of keratin layers [121]. Therefore, the compressive stress increases more rapidly, as shown in Figure 4.6b, c.

The mechanical properties of ambient dry horns in compression tests are listed in Table 4.1. The
yield strength is taken as the point on the strain-stress curve where the slope deviates from linearity, and the initial slope is the Young’s modulus. The toughness is measured as the area under the strain-stress curve up to a strain of 70%. For all horns, the longitudinal direction has the highest Young’s modulus and yield strength. Since the keratin lamellae are almost parallel with the longitudinal direction, this is suggested to result in a higher stiffness and strength, which has been verified by previous study. When comparing the mechanical properties of different horns loading in different directions, no significant difference is found for the Young’s modulus, which indicates that the stiffness is independent of the tubule density, size and shape (Figure 4.12). The compressive yield strength of mountain goat horn is significantly larger than the other horns when loading in radial and transverse directions, which is likely due to the existence of tubules in the other three horns. The yield strength in longitudinal direction is not affected by the tubules since there is no significant difference between these four species (Figure 4.13). This may correspond to the fighting styles of the different species. The mountain goat fights involve stabbing and thrusting, and the high yield strength and stiffness make the horns strong enough to maintain structural integrity during the stabbing and penetrating into the dermis of their opponents.
In contrast to the ambient dry horn stress-strain curves, Figure 4.6d-f shows the compressive stress-strain curves in the rehydrated state. The maximum compressive stress drops severely (order of magnitude) for all orientations and has a negative relationship to increasing water content. The mechanical properties of the rehydrated horns are shown in Table 4.2. The average Young’s modulus of three directions decreased 97% in pronghorn, 91% in bighorn sheep, 87% in domestic sheep and 84% in mountain goat after being fully hydrated. Given the significant
decrease of all mechanical properties over those of the dry horns, hydration is demonstrated to significantly influence the mechanical response. Comparing the four horns, the strength and stiffness in the pronghorn are significantly lower than that in the other three horns, while the mountain goat has the highest strength and stiffness after hydration (Figures 4.14 and 4.15). These can be related to differences in water content after hydration, since the pronghorn has the highest water absorption while the mountain goat has the lowest (Figure 4.5).

Figure 4.7 Energy absorption as a function of compressive strain of dry and rehydrated horns in the (a) longitudinal, (b) radial and (c) transverse directions. For each species, plots are based on an average of five samples for each condition.
Table 4.1 Compressive Young’s modulus, yield strength, and toughness of the horns under different loading orientations in the ambient dry condition.

<table>
<thead>
<tr>
<th>Species</th>
<th>Orientation</th>
<th>Pronghorn</th>
<th>Bighorn sheep</th>
<th>Domestic sheep</th>
<th>Mountain goat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young’s modulus</td>
<td>Longitudinal</td>
<td>1.8±0.2</td>
<td>1.7±0.5</td>
<td>2.0±0.2</td>
<td>2.1±0.6</td>
</tr>
<tr>
<td>(GPa)</td>
<td>Radial</td>
<td>0.9±0.3</td>
<td>1.2±0.2</td>
<td>1.3±0.5</td>
<td>1.5±0.2</td>
</tr>
<tr>
<td></td>
<td>Transverse</td>
<td>1.2±0.2</td>
<td>1.3±0.6</td>
<td>0.9±0.3</td>
<td>1.4±0.4</td>
</tr>
<tr>
<td>Yield strength</td>
<td>Longitudinal</td>
<td>85.5±6.4</td>
<td>67.8±7.9</td>
<td>64.3±13.5</td>
<td>81.6±22.2</td>
</tr>
<tr>
<td>(MPa)</td>
<td>Radial</td>
<td>67.5±4.2</td>
<td>57.3±5.3</td>
<td>63.1±8.9</td>
<td>82.3±9.8</td>
</tr>
<tr>
<td></td>
<td>Transverse</td>
<td>59.9±7.9</td>
<td>49.3±9.0</td>
<td>59.5±6.1</td>
<td>76.1±2.5</td>
</tr>
<tr>
<td>Toughness (MJ/m³)</td>
<td>Longitudinal</td>
<td>72.5±2.5</td>
<td>92.1±9.5</td>
<td>83.5±5.1</td>
<td>103.4±5.8</td>
</tr>
<tr>
<td></td>
<td>Radial</td>
<td>82.0±5.6</td>
<td>117.0±4.5</td>
<td>98.1±5.6</td>
<td>141.0±16.0</td>
</tr>
<tr>
<td></td>
<td>Transverse</td>
<td>78.1±5.7</td>
<td>98.4±5.4</td>
<td>85.2±3.0</td>
<td>120.1±8.8</td>
</tr>
</tbody>
</table>

As shown in Figure 4.7, for the dry horns (solid lines), the radial direction can absorb more compressive energy than in the longitudinal and transverse directions. This is because the lamellae are layered face-to-face along this direction, which allows them to withstand more compressive stress without overly deforming. The radial direction is also the orientation in which sheep horns clash during combat. In all directions, mountain goat horn exhibits the largest energy absorption compared to the other horns. The nearly zero porosity in the mountain goat horn and parallel alignment of the lamella in the longitudinal direction may be the factors influencing the energy absorption ability. Bighorn sheep also possess high energy absorption capability, especially along...
the radial direction. During combat, the bighorn sheep butt horns at high speeds. The impressive energy absorption prevents horns from cracking and crushing when subjected to high impact loads. When the horns are fully rehydrated, the energy absorbed decreases significantly (Figure 4.7 dashed lines), which is likely due to the softening of the keratin. Also, the difference of energy absorbing ability between different loading directions diminishes, which indicates a matrix dominant property for rehydrated horns.

In summary, there are many common characteristics of the different horns. Anisotropic mechanical properties are found in all the horns in ambient dry conditions. The longitudinal direction has the highest Young’s modulus and yield strength, which indicates the stiffest and strongest direction of the horn. The radial direction (clashing direction) has the largest toughness, which indicates that more energy can be absorbed, compared to the other directions. For all orientations, the mechanical properties decrease with increasing water content. It can be concluded that the change between hard (dry) and ductile (hydrated) horns can be manipulated by changing the water content of the structure. All species present hydration dominated mechanical properties, which indicates a common characteristic for the horns.

4.3.3 Tensile tests

Tensile tests were performed along the longitudinal direction since it is the most likely direction to undergo tensile stress when fights occur. Horns were tested in ambient dry conditions. The stress-strain curves are shown in Figure 4.8. The mountain goat has the largest failure strain (14.7%), while the domestic sheep has the smallest (5.9%). The failure strain in tension is much smaller than in compression, which indicates a different fracture mechanism. The mechanical properties of the horns under tensile stress are listed in Table 4.3. Among the four horns, the mountain goat is the stiffest (1.54 GPa), strongest (yield strength of 88.0 MPa and ultimate strength
107.3 MPa) and toughest (11.9 MJ/m^3) (Figure 4.16). This may be linked to the mountain goat’s stabbing combat style. The horns may be subjected to tensile stresses when the goat tries to pull its horns out from the opponent. A high yield strength and stiffness maintains structural integrity while stabbing and avoids buckling and large plastic deformation. Unlike the other three species, the stabbing behavior requires the mountain goat horns to be thin, sharp and light to penetrate the skin of their opponent. Thus, mountain goat horns are expected to have the highest tensile properties relative to the other species. The pronghorn has the second strongest (yield strength of 69.6 MPa and ultimate strength of 78.3 MPa) and toughest (6.1 MJ/m^3) horn. The pronghorn performs two methods of combat, one involving thrusting and twisting where tensile stress will be generated. To avoid fracture of horns, these would be important properties. In contrast, bighorn and domestic sheep do not engage in stabbing or wrestling behaviors and their horn yield strengths are lower (38.9 GPa and 41.9 GPa for bighorn and domestic sheep, respectively) compared to mountain goat and pronghorn. No significant difference is found between these latter two species.

Mountain goat and pronghorn horns have lamellae that face parallel to the tensile direction (Figure 4.4g, h) while sheep horns have lamellae arranged at angles to tensile direction (Figure 4.4e, f). Therefore, the superior mechanical properties of mountain goat and pronghorn may be a result of the aligned lamellae that are parallel to the tensile direction. The larger amount of disulfide bonds in the mountain goat horn can also be a factor that improves the mechanical behavior.
Figure 4.8 Tensile stress strain curves in the longitudinal direction of horns in the ambient dry condition. Averages based on seven samples from each species; error bars indicate standard deviations.

Table 4.3 Tensile properties of different horns in the ambient dry condition.

<table>
<thead>
<tr>
<th>Species</th>
<th>Pronghorn</th>
<th>Bighorn sheep</th>
<th>Domestic sheep</th>
<th>Mountain goat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young’s modulus (GPa)</td>
<td>1.06±0.2</td>
<td>1.15±0.1</td>
<td>1.33±0.1</td>
<td>1.54±0.2</td>
</tr>
<tr>
<td>Yield strength (MPa)</td>
<td>69.6±12.3</td>
<td>38.9±7.3</td>
<td>41.9±9.9</td>
<td>88.0±12.5</td>
</tr>
<tr>
<td>Ultimate Strength (MPa)</td>
<td>78.3±14.9</td>
<td>45.1±10.5</td>
<td>47.9±11.5</td>
<td>107.3±9.8</td>
</tr>
<tr>
<td>Failure Strain (%)</td>
<td>11.9±1.5</td>
<td>6.5±1.2</td>
<td>5.9±1.4</td>
<td>14.7±1.8</td>
</tr>
<tr>
<td>Toughness (MJ/m³)</td>
<td>6.1±1.6</td>
<td>2.3±0.7</td>
<td>2.1±0.9</td>
<td>11.9±1.7</td>
</tr>
</tbody>
</table>

Compared to compressive tests at the same hydration level (ambient dry) and load direction (longitudinal), the tensile failure strain and stress decrease significantly. Under compressive stress, all horns can sustain a deformation of 70% without failure (Figure 4.6). However, under tensile stress, all horns fracture when the strain is > 15%. Horns can be subjected to a compressive stress of more than 200 MPa without failure while the ultimate tensile strength of horns is four times lower in bighorn sheep and domestic sheep horns (Figure 4.8). Under tensile loading, the toughness
is significantly less (Table 4.3) than under compressive loading (Table 4.1), indicating a more brittle characteristic.

Figure 4.9 Scanning electron microscopy images of tensile fracture surfaces of horns from four species. Images at top (a,c,e,g) are low magnification, and high magnification images (b,d,f,h) are below. (a,b) bighorn sheep, (c,d) domestic sheep (e,f) mountain goat and (g,h) pronghorn.

SEM images of the tensile fracture surfaces are shown in Figure 4.9. For all horns, tubule pull-out is rarely observed, which indicates a high tubule-to-matrix adhesion. However, fiber-to-fiber adhesion may be one factor that influences the tensile properties. For horns having a relatively low yield strength (bighorn and domestic sheep, Figure 4.9a, b, c, d), detachment of the fiber bundles from the matrix is observed. The detached fibers are thin with a small diameter of 1-1.5 μm. High stresses applied on those thin fibers cause fiber fracture at a relatively low tensile load. For horns having a high yield strength (mountain goat, Figure 4.9e, f), detached fibers are not observed, indicating a good fiber-matrix adhesion. For the pronghorn, which also has a high yield strength, there is separation between the fiber bundles and the matrix, but the detached fiber bundles have a larger diameter (> 10 μm). Compared to the thin fibers observed in bighorn and domestic sheep, these thick fibers are presumably stronger under the same load and can withstand more tensile stress.
4.3.4 Drop tower impact tests

According to ASTM standard D7136/D7136 M-07, there are four externally visible damage types and two internal damage types appearing in drop-weight tests. The external damage types include dimple (depression), splits (cracks), combined splits and delamination, and puncture. Internal damage types are sorted into two categories: delamination and splits. Dimpling is the main damage mode on the impact surface for both sheep horns. With an increase in the impact energy, the diameter and depth of dimple increases. On the bottom surface, from low to high impact energy, the domestic sheep horn shows all external damage types. For bighorn sheep, on the bottom surface only split/crack, delamination and puncture were observed.

The normalized impact strength \( (E_n) \) is calculated by: \( E_n = E / d_s t \), where \( E \) is the impact energy derived in section 2.5, \( d_s \) is the cover plate aperture diameter, and \( t \) is the thickness of the specimen. For bighorn and domestic sheep, the typical damage modes under low and high normalized impact energy \( (E_n) \) are shown in Figure 4.10. At low \( E_n \) (30 kJ/m\(^2\)), shown in Figure 4.10a, d, for both horns there is a shallow dimple on the top (impact) surface with an area identical to the size of the hemispherical impactor tip (3.2 mm). On the bottom of the samples, the domestic sheep has a minor crack (length ~ 3 mm), while the bighorn sheep horn exhibits a longer crack (length ~ 10 mm), which indicates that domestic sheep can sustain more stress and exhibit a lighter damage when \( E_n \) is small. At high \( E_n \) (60 kJ/m\(^2\)), for both horns a number of samples fail, and the typical failure modes are exhibited in Figure 4.10c, f. On the bottom surface of the domestic sheep, circular and thin chips fractured away, while for the bighorn sheep thin chips were only punched out but not fractured away. Also, there is delamination combined with the dimple on the impact surface of the domestic sheep, which means that at high \( E_n \) level, bighorn sheep shows a higher capability to withstand the impact load. The number of each damage mode under the same \( E_n \) is
shown in Figure 4.11. Bighorn sheep horn begins to fail when $E_n = 40 \text{ kJ/m}^2$ whereas there is no failure damage for domestic sheep at this energy level, which indicates a higher impact resistance of domestic sheep under low $E_n$. According to Lee, et. al [204], failure impact strength is defined as when 50% of the samples fail (with failure defined when the bottom surface of the sample shows puncture damage). The failure impact strength of domestic sheep is 55 kJ/m$^2$ and for the bighorn sheep is 75 kJ/m$^2$; 36% higher than the domestic sheep. The tubular structures are considered critical for absorbing impact energy in various structural materials [126]. The reason why bighorn sheep horns can withstand greater impact energy than domestic sheep horns could be due to the differences between their tubular structures. Higher tubular density in bighorn sheep horns can absorb more energy by plastic deformation. The failure impact strength reveals that the bighorn sheep horn has better impact resistance when the impact speed is high. Domestic and bighorn sheep have similar fighting styles, but with different fighting speeds. When fighting, domestic sheep stand closer to each other than bighorn sheep, thus generating a lower speed for clashing. The impact test results suggest that horn properties are tailored to fit fighting behavior.
Figure 4.10 External damage modes of domestic sheep (a,b,c) and bighorn sheep (d,e,f) with associated normalized impact energy ($E_n$). Radial direction is the impact direction, which is perpendicular to the imaged surface. Black lines are sample marks.
4.4 Conclusions

The microstructures and mechanical properties of four different species: bighorn sheep, domestic sheep, mountain goat and pronghorn were characterized (optical and scanning electron microscopy, compression and tensile tests, impact tests) and compared. The main conclusions are:

- Keratin cells that form lamellae surrounding elliptically-shaped hollow tubules were identified in the bighorn sheep, domestic sheep and pronghorn, while no tubules were found in the mountain goat. The bighorn sheep horn has the highest porosity (from the presence of the tubules) of 11.3%. The porosity of domestic sheep horn is 7.7% and the tubules have a deformed oval shape. The pronghorn horn has a porosity of 5.8%. The shapes, dimensions and tubule densities were different across the species.

- Laminated keratin cells align parallel to longitudinal direction and show a high tensile strength in the pronghorn and mountain goat. The bighorn sheep horn lamellae arrange \(~30^\circ\) to tubule direction and the domestic sheep horn has a more disordered alignment, resulting in lower tensile strengths for the sheep compared to the pronghorn and mountain goat.

- The pronghorn horn has the largest water content with a maximum of 52% that may be caused by the additional water stored in the nanopores inside of the keratin cells. The water
absorption in bighorn sheep horn is larger than that in domestic sheep, and mountain goat has
the lowest water content. The amount of water absorption is positively related to the tubule
density. In terms of chemical composition, horns from bighorn sheep and domestic sheep have
similar protein compositions, while mountain goat horn has the highest cystine composition.

- All of the horns have similar anisotropic mechanical behaviors related to loading
direction: longitudinal (direction of tubules), radial (direction of impact) and transverse
(perpendicular to both). In the dry condition, the longitudinal direction has the highest Young’s
modulus and yield strength. The radial direction has the largest toughness.

- The mountain goat horn has highest yield strength and energy absorption in all
directions, hydration conditions and loading states, and this likely benefits a stabbing combat
style. Bighorn sheep horns are characterized by high energy absorption capability under
compression in radial direction, which makes them suitable for absorbing energy generated by
high-speed head butting. The pronghorn horn shows relatively high tensile strength that
enables clashing combat and interlocking horn fighting behavior.

- Under low energy impact tests, the domestic sheep has higher impact resistance
than the bighorn sheep. At high normalized impact energy (> 55 kJ/m²), the bighorn sheep has
better impact resistance. The failure impact strength of domestic sheep is 55 kJ/m² and the
impact strength of bighorn sheep is 75 kJ/m². The result indicates that bighorn sheep have
developed horns that can survive large impact forces.

Different structures in horns are found to serve for certain specific mechanical functions.
Cell lamellae in mountain goat and pronghorn are designed for high tension requirements, while
tubular structures in bighorn sheep and domestic sheep enhance energy absorption during impact.
Apart from the hierarchical structures, the chemical compositions in different horns could also
affect the mechanical properties, which need further studies based on quantitative analysis of amino acids, peptide and proteomics. These findings based on the diversity of forms and functions that have evolved in Nature give further inspiration for the design of synthetic materials with a wide range of mechanical applications.

Chapter 4, in full, is published as “Microstructure and mechanical properties of different keratinous horns” Journal of the Royal Society Interface, in press. This work was coauthored by Y. Zhang, J. Gatesy, C. Hayashi, and J. McKittrick. The dissertation author is the co-first author of this work.

4.5 Supplementary information

![Figure 4.12 Statistical analysis of Young’s modulus of different horns in three different directions in ambient dry state. No significant difference is found in both the longitudinal and transverse directions between four different species. In the radial direction, Young’s modulus of mountain goat is significant higher than that in pronghorn.](image)
Figure 4.13 Statistical analysis of yield strength of different horns in three different directions in ambient dry state. No significant difference is found in the longitudinal direction between four different species. The yield strength of mountain goat is significant higher than that in the other three species in both radial and transverse direction.

Figure 4.14 Statistical analysis of Young’s modulus of different horns in three different directions in fully hydrated state. The Young’s modulus of pronghorn is significantly lower than that in the other three species in all directions. In longitudinal direction, the Young’s modulus of mountain goat is significantly higher than that in bighorn sheep and pronghorn.
Figure 4.15 Statistical analysis of yield strength of different horns in three different directions in fully hydrated state. The yield strength of mountain goat is significantly higher than that in the other three species in all directions. In radial and transverse directions, the yield strength of pronghorn is significantly lower than that in bighorn sheep and domestic sheep.
Figure 4.16 Statistical analysis of Young’s modulus, yield strength, ultimate tensile strength and toughness (loading longitudinally) of different horns in ambient dry state: (a) Young’s modulus; (b) Yield strength; (c) Ultimate tensile strength; (d) Toughness. The Young’s modulus of mountain goat is significantly higher than that in bighorn sheep and pronghorn. The yield strength, ultimate tensile strength and toughness in mountain goat and pronghorn are significantly higher than that in bighorn sheep and domestic sheep. No significant difference is found between all the tensile properties of domestic sheep and bighorn sheep horns.
CHAPTER 5: WATER EFFECTS ON HORN KERATIN

5.1 Introduction

An interesting phenomenon stimulated by hydration is the recoverability of shape and properties after deformation. The shape-memory feature of different hair species was conducted to examine the deformation, viscoelasticity, chemical groups, and water assisted recovery [205]. It was found that heat (< 100 °C) could stabilize the deformed hairs, while water stimulated the recovery of hair to its original shape. The molecular recovery mechanism was speculated through the effect that both heat and water molecules could break the original hydrogen bonds (HBs) and reform new HBs to realize shape fixation and recovery [205]. HBs were acting as switches, controlled by the water molecules to turn on the shape memory mechanism in animal hairs [206]. Moreover, microscopic damages caused by indentation in pangolin scales could also be recovered by hydration, which indicated that this dermal armor could be recuperated from penetration injury caused by predators [207]. The shape memory effects of the foam keratin material in peacock tail feathers were also studied [167]. Both of the compressive strength and energy absorption efficiency were able to keep consistent after six cycles under more than 90% compression and recovery tests. The activation energy for relaxation was significantly decreased by hydration, which made this recovery behavior possible. The recovery of horn was introduced in our previous work through hydration under compressive deformation [121]. In this regard, the fully rehydrated horn samples could retain their original length after loading in contrast to the ambient dried specimens for around 30% deformation. It will be of interest to investigate the water assisted shape memory effects in bighorn sheep horn, which could provide further bioinspired designs of recoverable energy absorption structural materials.
Although the hierarchical structure and energy absorption properties in different strain rates of bighorn sheep horn have been investigated in our previous studies [121], the recovery behavior of horns, especially the role of tubules, remains unclear. Thus, the present work focuses on two main points: understanding the water effects on the horn structure at a molecular level and effects on the mechanical behaviors; the hydration-driven recovery properties and mechanisms of horn keratin after severe compression in different directions.

5.2 Materials and methods

5.2.1 Acquisition and preservation of horn samples

Two bighorn sheep (Ovis canadensis) horns were purchased from the Wilderness Trading Company (Pinedale, WY). The horns were then preserved at room temperature in ambient dry condition for future use. Horn samples are estimated to be ~6-8 years old with dimension ~60 cm in length and ~9 cm in diameter at the proximal region (Figure S1(a)). It is unknown how long the horns have been stored in ambient dry condition after harvesting. The testing samples in this study were collected from the impact region shown in Figure 5.7(a).

5.2.2 Synchrotron wide angle X-ray diffraction (WAXD)

Synchrotron wide angle X-ray diffraction (WAXD) were conducted on the beamline 7.3.3 at the Advanced Light Source at Lawrence Berkeley National Laboratory (Berkeley, CA). Samples were cut with a diamond saw into thin films with thickness ~500 µm to improve the penetration of X-rays. The samples were 10 mm in length (longitudinal direction) and 3 mm in width (radial direction). The sample dimensions were based on previous WAXD studies of biological materials [14, 208]. Three samples were scanned before and after hydration. By aligning the thickness direction along the X-rays, the molecular structure in longitudinal and radial direction of horn samples can be determined. A two-dimensional intensity map of total scattering vector could be
acquired ($q_x$ in longitudinal, $q_y$ in radial). The diffraction maps were further analyzed with a data analysis software IGOR Pro (WaveMetrics, Inc., Lake Oswego, Oregon, USA). The corresponding real space length scales periodicity $d$ spacing ($d = 2\pi/q$) in the molecular structures can be further calculated. *In-situ* tensile tests of dry and fully hydrated samples were also conducted with real time simultaneous WAXD measurements. The mechanical tests were performed with a custom-made rig using a 10-mm displacement stage and a 45 N load cell (Omega LC703-10, city, state). The tensile tests were conducted at room temperature with a strain rate $10^{-3}$ s$^{-1}$. The sample was exposed to 10 keV X-rays for 0.5 s for ~5 s intervals during tensile testing.

5.2.3 Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR spectroscopy was conducted using a Nicolet 560 spectrophotometer to characterize the water effects on the chemical bonds of keratin. Nine horn samples with the same dimension for the WAXD tests were prepared. Scans were taken in the thickness direction. Ambient dry samples were first weighed and then put in an oven (~120 °C) for 24 hrs to fully dehydrate; these conditions were based on previous work on horn keratin [8]. After measuring the weight, the samples were immersed in DI water to acquire the water content as a function of immersing time. Samples with three different hydration states (ambient dry, fresh and fully hydrated) were prepared by immersed in water with different time. Due to the unavailability of fresh bighorn sheep horns, samples with partial hydration were prepared to mimic the fresh states. The fresh horn condition has a water content ~15 wt.% - 20 wt.%, which was based on previous studies [113]. Samples with these different hydration states were then characterized by ATR-FTIR. The spectra were compared with previous results on hair [205, 206, 209].

5.2.4 Tensile tests and fracture surface imaging
Samples with dimension 15 mm in length, 3 mm in width and 0.5 mm in thickness for tensile tests in longitudinal direction were prepared. Tensile loads were applied along the length direction. The ex-situ tensile tests were carried out on an Instron 3342 mechanical testing machine (Instron Corp., Norwood, MA) with a load cell of 500 N using the span of 12 mm at strain rates of $10^{-3}$ s$^{-1}$. Samples with three different hydration states (ambient dry, fresh and fully hydrated) were tested. At least five samples were tested in each condition. The fracture surfaces were imaged via an ultra-high-resolution scanning electron microscope (FEI, Hillsboro, OR, USA). Before imaging, samples were fixed in 2.5% glutaraldehyde for 3 hrs and then dehydrated with ethanol series (20%, 40%, 60%, 80%, 90%, 100% volume percent). Samples were then put in critical point dryer (Auto Samdri 815A, Tousimis) after dehydration. The tensile fracture surfaces were sputter coating with Iridium using an Emitech K575X sputter coater (Quorum Technologies Ltd., UK) before the SEM imaging.

5.2.5 Viscoelastic behavior: Creep tests

Using a diamond saw, samples with dimension 4mm × 4 mm × 4 mm for each orientation were cut, and smooth surfaces with roughness of less than 20nm were prepared with utilization of ultramicrotome (Leica UC7/FC7 Cryo-Ultramicrotome). The creep behavior of the ambient dry samples was examined using a nanoindenter (MTS NanoIndenter XP) and a diamond flat-ended tip with 10 µm diameter. At least three different locations on the prepared smooth surfaces were indented with a constant loading rate of 2.5 mN/s and 2 s loading time to avoid any viscous deformation during the loading process. Subsequently, each indentation was followed by 10 min hold time and unloaded with rate as the loading one. To test the creep behavior of hydrated samples, the samples were immersed in deionized water (DI) for three days, and they were kept hydrated throughout indentation by adding droplets of DI water using a pipette. In order to minimize the
effect of thermal drift, the creep tests with the same procedure were conducted on fused silica, and
the subsequent adjustment was conducted on the results for the horn samples. The samples were
placed in the nanoindenter for 48 hrs prior to the tests to adapt the environmental condition.

5.2.6 Compression recovery tests

Compression test cycles were conducted every 24 hr after completion of rehydration process of the specimens. Compression samples were cubes of $5 \times 5 \times 5$ mm$^3$ that were acquired from the region located ~5 mm from the impact surface of the horn (Figure S1). The sample surfaces were polished with ultramicrotome (Leica EM UC6, Leica Microsystems Inc., Buffalo Grove, IL, USA) for optical microscopy (OM) imaging to characterize the microstructure after each cycle. The sample surfaces were imaged by a Keyence VHX 1000 OM (Keyence, Palatine, IL, USA) with differentiate interference contrast (DIC) light to image the surface features. Samples were then compressed with a universal testing machine with a 30 kN load cell (Instron 3367 Dual Column Testing Systems, Instron, MA, USA) to 50% deformation. After compression, samples were immersed in DI water for 24 hrs to obtain fully recovery to the original sample dimensions. The compressive deformation was chosen at 50% so that densification was avoided [121]. Samples were then kept at room temperature for three days to reach a consistent water content (~10 wt.%). Surfaces of the recovered samples were then imaged as reference. Sample dimensions and weight were measured for the consistency with the original dimension and weight before the next cycle of compression. The compression and recovery cycles were repeated until the samples were unable to return to the original dimensions.

Cubic samples ($5 \times 5 \times 5$ mm$^3$) without tubules were found in the regions ~15 mm from the impact surface of the horn (Figure S1). Compression and recovery tests were conducted to examine the role of tubules. Compression and recovery tests were conducted in different
directions. Since there were no tubules, the longitudinal and transverse directions were assumed identical (Figure 5.7). Images of the surfaces were only taken in radial and transverse directions.

5.3 Results and discussions

5.3.1 Water effects on the structure of horn keratin

Synchrotron wide angle X-ray diffraction (WAXD) experiments were conducted to study the water effects at the molecular level. Figure 5.1b shows a schematic diagram of X-rays passing through the transverse direction, in which the X-rays are parallel with the keratinized cells. The diffraction pattern is shown in Figure 5.1c. The periodicities of α-helix crystal structure are indicated in the pattern, including a 0.5 nm arc in the longitudinal direction and a 1 nm arc in the radial direction, which is in agreement with the diffraction patterns of human hair and stratum corneum keratin [115, 116, 210, 211]. This corresponds to the IF arrangements, shown in the schematic diagram in Figure 5.1b. Macrofibrils and crystalline IFs are in the cell planes, and IFs are found perpendicular to the cells.

Figure 5.1 (a) Hierarchical structure of the bighorn sheep horn (adapted from [24]); (b) Schematic diagram of samples used for the synchrotron wide angle X-ray diffraction (WAXD) experiments. The X-rays pass through transverse direction, parallel with the cell planes. IFs orientation is also shown in the accompanying schematic diagram. (c) Diffraction pattern for WAXD characterization. The 10 Å and 5 Å arcs are indicated in the pattern.
Samples after full hydration were also studied by WAXD. By immersing ambient dry horn samples in water, the water absorption as a function of time was plotted in Figure 5.2a, showing an exponential curve. Horn sample in ambient dry condition has a water content ~10 wt.%, ~15 – 20 wt.% in fresh condition and ~30 wt.% in fully hydrated condition. Figure 5.2b shows the 2D diffraction patterns and periodic d-spacing in both longitudinal (x) and radial (y) directions. From the plots of d-spacing, the intensity decreases after full hydration, which could be a result in a decrease of IF density in the matrix because of swelling of the matrix. It has been shown in wool and hair fibers that water acts as a plasticizer in the amorphous matrix and decreased the mechanical strength, while had no effects on the IFs [137, 212]. This corroborates the current results, since the periodicities of the α-helix in the hydrated samples remain the same, indicating that the crystal structure of the IFs was not affected by water molecules. For the first time, this verifies that water molecules are absorbed by the amorphous matrix during hydration by using WAXD. A schematic diagram showing a macrofibril hydration process is presented in Figure 5.2c. Fudge et.al found the IFs could also absorb water by studying the swelling behavior of matrix-free keratin tissue hagfish slime thread [124]. While in IFs and matrix composite α-keratins, the elastomeric matrix will resist IFs from swelling during hydration [124]. This could be a reason why no IFs diameter change in the current study.
Figure 5.2 Water effects on the nanostructure of horn keratin. (a) Water absorption as a function of time. (b) Diffraction peak integrate in longitudinal and radial direction. The 1D periodicity spacing is shown in the plot. Red curves are dry samples, while blue are fully hydrated samples. The density decreases in both longitudinal and radial directions after hydration. The d-spacings (peak positions indicated with red dashed lines) remains the same after hydration. (c) Schematic diagram showing water in the amorphous matrix during hydration. (d) FTIR spectra of samples with different hydration states. Yellow, blue and black dashed lines show the wavenumber shift of certain characteristic peaks. Green dashed line indicates the relative intensity ratio of C=O stretch and N-H bending peak changes in different hydration states.

FTIR spectra on samples with three different hydration states (ambient dry, fresh and fully hydrated) are shown in Figure 5.2d. Amide A&B, I, II and III features are identified, according to previous references [201, 213, 214]. The change of intensities and wavenumbers of the characteristic peaks of amide I, II and III (orange, blue and black dashed lines) indicate that water molecules break the hydrogen bonds [205, 206]. The wavenumbers of C=O stretching (amide I) and N-H bending (amide II) increase during hydration, indicating new hydrogen bonds are forming between the amino acids and water molecules. In terms of the intensity ratios between the two characteristic peaks C=O and N-H, it can be found that the relative intensity of N-H bonds compared to C=O bonds decrease as the water content increase from dry to fully hydrated samples (green dashed lines in Figure 5.2d). This verifies water molecules break the hydrogen bonds
between carbonyl group (C=O) and amino groups (N-H). And then new hydrogen bonds form due to the polar attraction between amino group (N-H) and H-O-H in water molecules, which leads to a decrease of free N-H bonds, and finally a decrease of N-H peak intensity in the hydrated samples. This would help the rationalization of the recovery process in the samples, which will be discussed later.

5.3.2 Water effects on tensile and creep properties

In-situ tensile tests with WAXD in the longitudinal direction of dry and fully hydrated samples were conducted to measure the strains and deformations of the crystalline IFs (Figure 5.8). Figure 5.3a shows a schematic diagram of the X-ray passing through a sample in the transverse direction. The tensile stress and IF strain as a function of sample strain are plotted in Figure 5.3b. The tensile strains of the crystalline IFs were calculated from the periodicity (d-spacing) changes from the WAXD patterns (Figure 5.9). Fully hydrated samples show larger failure strain (~30%) than dry samples (~5%), while dry samples have higher tensile strength. Less than 1% strain was found in the dry samples, which is attributed to brittle fracture. It has been well recognized that the amorphous matrix is stiff and rigid in dry keratin [113, 123, 137], supporting most of the tensile stress, thus leading to a small strain in the IFs. While in the fully hydrated samples, the tensile strain of the IFs can reach ~6%. An interesting aspect is that the shape of the IF strain is similar as the tensile stress, a strong correlation between stress and IF strain. Due to the stiffness and strength of the matrix decreases significantly after full hydration, IFs can be stretched under tensile stress. However, by comparing the IF strain (~6%) to the sample strain (~30%), it appears that the IFs pull out from the matrix, leading to a lesser strain on the IFs. This will be discussed in more detail in the following section.
The stress strain curves of *ex-situ* tensile tests on samples with different hydration states are shown in Figure 5.3c. The dry samples fracture after ~5% strain but have the highest stiffness and tensile strength. The fresh samples (~15 wt.% – 20 wt.% water) fracture at ~40% strain, which is higher than the other two conditions. Figure 5.3d, e and f show SEM images of the fracture surfaces (schematic diagram shown in Figure 5.3g) of ambient dry, fresh and fully hydrated samples, respectively. Brittle fracture of the surface is exhibited in the dry samples. The fractured lamellae are also shown in Figure 5.3b at a higher magnification image, which could explain the small strains under tension. For fresh samples, it can be found that macrofibrils are pulled out and fiber fracture is shown (Figure 5.3c). This tensile behavior shows the macrofibrils are under tension and orienting along the tensile direction, which could increase the final strain considerably due to the ductility of the macrofibrils. It has been shown both hair and wool fibers can sustain a ~50% tensile strains prior to fracture [209, 215]. In Figure 5.3d, the fracture surface of a fully hydrated sample is shown. Although macrofibrils are clearly observed, no fracture is found, indicating the macrofibrils are directly pulled out from the weak matrix. This verifies the previous assumptions made in the *in-situ* test section. Thus, the macrofibrils are not contributing much to the final tensile strain, leading to a smaller strain than in the fresh condition. Figure 5.3g shows the schematic diagrams of tensile fracture behavior of the samples in the three hydration states. Brittle fracture at 45º with respect to the loading direction occurs in the dry condition (Figure 5.8). Macrofibrils are pulled out and fractured in the fresh condition. Due to the high water absorption and significant decrease in the mechanical properties of the matrix in the fully hydrated samples, the macrofibrils are pulled out from the matrix without breakage.

Understanding the effect of hydration on viscoelastic properties such as stress relaxation at the macroscale has been reported on the horn sheath. However, viscoelastic properties of horn
samples were not reported at the small scale. Here, the investigation of hydration effect on viscoelastic behavior of horn samples was performed through nanoindentation. To this end, creep tests at different orientation and hydration levels were conducted. Creep behavior of biomaterials such as bone [216, 217] and synthetic polymeric materials [218, 219] were investigated through nanoindentation in the past. In this regard, indentation creep compliance, $J(t)$ can be measured based on the established method for polymeric materials [219, 220] as: $J(t) = \frac{\varepsilon(t)}{\sigma_0}$. Here, $\sigma_0$ is the contact stress defined as the ratio of the constant indentation load over the contact area at the end of loading segment. $\varepsilon(t)$ is the indentation strain which is measured as the ratio of displacement during the holding constant load over the displacement at which the load becomes constant. The creep compliance can be related to the degree of cross-linking density in molecular structure of material systems. For instance, it was shown higher degree of cross-linking in polymers results in less viscous flow and lower creep compliance [221]. Here, calculation of creep compliance would assist to understand how water influence the molecular structure of keratin cells in horns.
Figure 5.3 Water effects on the tensile behavior of horn samples. (a) Schematic diagram of the test samples that were loaded in the longitudinal direction. (b) Plot of tensile stress and intermediate filament (IF) strain as a function of sample tensile strain in both dry and fully hydrated conditions measured by in-situ tensile tests. (c) Stress strain curves under ex-situ tensile tests in different hydration states. Dry samples show the highest tensile strength, while fresh sample has the highest tensile strain. (d) Fracture surface of the dry samples showing brittle failure. Cell lamellae are shown in a higher magnification SEM image (yellow box) at the bottom. (e) Fracture surface of fresh samples. Macrofibrils pulled out and breakages are found at a higher magnification image. (f) Fracture surface of sample in fully hydrated condition. Macrofibrils are pulled out from the matrix. (g) Schematic diagrams showing the fracture modes in samples with different hydration states. Macrofibrils and water molecules are indicated.

Indentation load-displacement curves for the horn samples are provided in the Supplementary Information (Figure 5.10). The variation of the compliance over time for different orientation and hydration is presented in Figure 5.4. The curves for hydrated samples reveal a much larger increase in creep compliance, which results in higher strain due to the creep or viscous flow. This indicates that water molecules breakdown/replace the extensive secondary bonds such as hydrogen bonds between the protein macromolecules thereby increase the mobility of the matrix molecules rather than increasing the cross-linking [86, 171]. In ambient dry samples, the more differences in creep compliance come from the orientation of cell lamellae as well as IFs arrangements. It is much easier to deform in the direction perpendicular to the cell planes and IFs
(radial direction) than in the directions parallel to the cell planes and IFs (longitudinal and transverse directions). However, for fully hydrated samples, the weakened amorphous matrix increases the creep deformation, leading to more isotropic behavior.

Figure 5.4 Creep compliance of horn samples (longitudinal, radial and transverse odirections in ambient dried and fully hydrated states), as a function of time, measured through flat punch nanoindentation.

### 5.3.3 Water assisted recoverable behaviors

From previous discussions, during the hydration process, water molecules are absorbed into the amorphous matrix and interact with keratin polymer chains by breaking and reforming hydrogen bonds, which makes the shape memory effects on keratin materials possible [206]. It has been verified that impacts in radial direction can absorb more energy than the other two directions due to the collapse of the tubules [121]. Samples were compressed in the dry condition to 50% deformation, and then allowed to recover their original shape in water for 24 hrs. The recovered samples were then dried in air and further compressed to determine if the mechanical properties were recovered. The stress strain curves of different compression cycles in different orientations (longitudinal, transverse and radial) are shown in Figure 5.5a, b and c, respectively. In the longitudinal (Figure 5.5a) and transverse (Figure 5.5b) directions, the samples fractured after three cycles, which indicates the horn samples cannot recover their mechanical properties by hydration in these directions after three cycles. Even within these three cycles, the stiffness and strength
slightly decrease after the first cycle. In Figure 5.5c, the stiffness and yield strength slightly decreased after the third recovery when compressed radially, which might be caused by some unrecoverable tearing and wrinkle of cell membranes [167]. From the fourth to the sixth tests, the samples were able to keep consistent on stiffness and strength when compressed in radial direction. OM images were taken before and after the first recovery. Figure 5.5d, e and f show the sample surfaces after compression to 50% strain after the first cycle in the longitudinal, transverse and radial directions, respectively. Shear bands and microfibrils pull out were found in longitudinal direction (Figure 5.5d). In the transverse direction, more severe shear bands were observed, and cracks propagated from the vertices of adjacent ellipses (Figure 5.5e). The orientation of the elliptical tubule cross section major axis slightly changed (yellow dashed lines) due to the shear force applied along the major axis in transverse direction. In radial direction (Figure 5.5f), no obvious cracks were found but only compression of the tubules occurred after the first cycle. After recovery by hydration, OM images of the damaged surfaces were retaken as comparisons (Figure 5.5g, h and i). The severe shear bands in the longitudinal and transverse directions are recovered after hydration (Figure 5.5g and h). The orientation of major axis changed back to the transverse direction after recovery (Figure 5.5h). However, delamination and pulled out microfibrils were not recovered (Figure 5.5g). In the transverse direction (Figure 5.5h), cracks at the vertices of the ellipses remained. The tubules were reopened by hydration in radial direction (Figure 5.5i). The recovery of the horn samples has limitations: some of the damage modes can be recovered (reopening of the collapsed tubules, buckling of tubules, orientation changes of keratin cells), while the others cannot (delamination, microfibrils pull out, and cracks between laminated cells).
Figure 5.5 Recovery tests of dry horn samples in different loading orientations: (a) Stress strain curves in the longitudinal directions. Samples failed after test 3; (b) Stress strain curves in the transverse direction. Samples failed after test 3; (c) Stress strain curves of horn loading in longitudinal direction at different loading cycles. Stiffness and yield strength slightly decrease after the third cycle, then are consistent from test 4 to 6; (d) Photographs and microscopy images of horn sample after 50% compression in the longitudinal direction. Shear bands and microfibrils breakage are observed; (e) Microscopy images of horn after 50% compression in the transverse direction. Shear bands and cracks are observed. Yellow dashed lines show the axis of the major axis of ellipse changed direction after compression; (f) Microscopy images of horn after 50% compression in radial direction. Collapse of tubules are found in this direction; (g) Horn samples in (d) after hydration in water for 24 hrs. Shear bands disappear but the delamination and microfiber breakage still exist; (h) Horn samples in (e) after hydration in water for 24 hrs. Shear bands and cracks are not recovered; (i) Horn samples in (f) after hydration in water for 24 hrs. Tubules were recovered back after hydration.

To obtain a better understanding the role of tubules played during compression and recovery tests, horn samples without tubules were identified in the region ~15 mm from the impact surface of horn. Quasi-static compression tests in different orientations were performed and compared with the results of samples with tubules. Figure 5.6a and b shows the stress strain curves in different orientations with and without tubules, respectively. The general trend of the stress strain curves was the same in both samples. The Young’s modulus and yield strength are compared in Figure 5.6c. The Young’s modulus in the radial direction in the samples with tubules is less than
samples without tubules. While no significant difference is found in the other two directions between samples with and without tubules. The collapse of tubules in radial direction could decrease the Young’s modulus. The plateau stress level is slightly higher in samples without tubules in all the directions, which could be the result of lack of stress concentration caused by the tubules. The results indicate that tubules could adjust the plateau stress level in all the three directions without changing much of the stiffness. The deformation mechanisms in samples without tubules were also investigated to better identify features that contribute to the energy absorption. Figure 5.6d shows samples in which the cell lamellae are observed. After 50% compression in radial direction (perpendicular to the cell lamellae), X-shaped shear bands were clearly observed (Figure 6e), which was not found in samples with tubules (Figure 5.5f). After recovery by hydration, the cracks caused by shear bands do not recover (Figure 5.6f). The results indicate that the tubules act as a stress redistribution, avoiding shear bands and cracks forming in the real horn samples during impact in radial directions. Samples were also compressed in transverse direction as a comparison. Severe shear bands and cracks formed and could not be recovered after hydration (Figure 5.6g-i). In summary, the recovery of horn samples compressed in the radial can only occur in samples with tubules.
Figure 5.6 Quasi-static compression behavior of horn samples with and without tubules at ambient dry condition. (a) Compressive stress strain curves of horn samples with tubules loaded in different directions; (b) Compressive stress strain curves of horn samples without tubules loaded in different directions; (c) Young’s modulus and yield strength comparison between samples with and without tubules; (d) Optical microscopy images of samples without tubules before compression in radial direction; (e) Samples without tubules after 50% compression in the radial direction. X-shape shear bands are shown on the surface; (f) Samples without tubules recovered by hydration showing cracks and delamination; (g) Samples without tubules prior to transverse compression; (h) Samples in (g) after 50% compression. Large shear bands and cracks are observed on the surface; (i) Samples in (h) after recovery by hydration. Large cracks and shear bands are found on the surface.

5.4 Conclusions

Bighorns sheep horn can be recovered after severe deformation with the assistance of water. Although keratin exists in dead tissues, self-healing mechanisms is found. Water effects on the nanostructure and mechanical properties of keratin were analyzed. Recovery properties after compression in different directions were studied. The main findings are:

- The nanostructure of intermediate filaments (IFs) in horn keratin were investigated by synchrotron wide angle X-ray diffraction (WAXD). Water affects only amorphous keratin matrix but not crystalline IFs. Fourier transform infrared spectroscopy indicate water molecules can break and reform hydrogen bonds in the amorphous matrix phase.
- *In-situ* and *ex-situ* tensile tests of horn samples in different hydration states (ambient dry: ~10 wt.%, fresh: ~15 – 20 wt.%, wet: ~30 wt.%) show different individual IF strain and fracture behaviors under tension. Ambient dry samples had the highest strength but lowest tensile strain due to brittle fracture. Less than 1% strain is observed in the IFs in the dry samples. Fibers pull out is observed in fully hydrated samples due to the weaker matrix after water absorption. ~6% strain in the IFs is found in the fully hydrated samples. The tensile strain on the IFs is directly proportional to the sample tensile stress level. Fiber breakage occur in the fresh samples, leading to the highest tensile strains.

- Creep behavior of horn samples at small scales reveals higher creep strain for all orientations under hydrated state than the dried condition. This was justified as the increase in the mobility of matrix protein molecules coming from interaction with water molecules, which is in agreement with our findings on the effect of water.

- Horn keratin structure can be recovered by hydration after severe compression (50% compressive strain). However, only samples after compression in the radial direction can retain their mechanical properties after recovery, but not in longitudinal and transverse directions. This is due to the damage of the keratin cells during compression in the longitudinal and transverse directions, and these damages cannot be recovered. Recovery occurs after water molecules go into the amorphous matrix and interact with polymer chains by breaking and reforming hydrogen bonds.

- Tubules can redistribute the stress thus protecting the keratin cells from damage under compression in radial direction. X-shape shear bands were found in samples without tubules, which cannot be recovered by hydration.
In summary, the water effects on the nanoscale structure and mechanical properties on the bighorn sheep horn were examined. The hydration-driven recoverable behaviors on the tubular structure of bighorn sheep horn keratin could give further inspirations for shape memory energy absorption materials designs.

Chapter 4, in full, is currently in preparation for publication as “Water effect on keratin: Hydration-driven recovery of bighorn sheep (Ovis canadensis) horns.” This work was coauthored by A. Zaheri, W. Yang, R. Ritchie, H. Espinosa and J. McKittrick. The dissertation author is the first author of this work.

5.5 Supplementary information

Figure 5.7 Bighorn sheep horn samples without tubules. (a) Bighorn sheep horn sample, indicating the longitudinal, radial and transverse directions; (b) Scanning electron microscopy image of the fracture surface in the longitudinal direction (green box in (a)), showing the lamellar structure of the keratin cells; (c) Optical microscopy showing the areas (yellow box in (a)) without tubules; (d) Lamellae structure is also found in the transverse direction, indicating that the lamellar structure in longitudinal and transverse direction are identical.
Figure 5.8 Images of dry and wet samples under in-situ tensile tests before and after fracture. Dry sample shows a brittle fracture along 45° to the tensile direction, while cracks in the fully hydrated sample is along the tensile direction.

Figure 5.9 (a) Two-dimensional diffraction pattern showing the qy intensity was integrated azimuthally for an angle of 30 degree over the meridian (yellow dashed lines). (b) Two-dimensional diffraction pattern showing the qx intensity was integrated azimuthally for an angle of 30 degree over the equator (yellow dashed lines). (c) d spacing in both longitudinal and radial direction from the WAXD pattern of dry samples during the in-situ tests. No obvious change was found in dry samples. (d) d spacings changes during the tensile tests of fully hydrated samples. Sample tensile strains increase from red to green, indicating d spacing increases during tensile tests.
Figure 5.10 Force-displacement curves from nanoindentation on horn samples for the dry condition in top panels: (a) radial, (b) longitudinal, and (c) transverse; for hydrated state in bottom panels: (d) radial, (e) longitudinal, and (f) transversal. (For all cases, the creep compliance is calculated at the load 5mN held for 600s)
CHAPTER 6: STRUCTURE AND PROPERTIES OF EQUINE HOOF

6.1 Introduction

Energy absorption is one of the most important properties of keratin in tissues under extreme loadings [8]. Although solely composed of polymer constitutes, keratin is one of the toughest natural materials. The toughness of keratin is greater than bone and wood, which are considered as structural biological materials with desirable mechanical efficiencies [128, 222]. Bighorn sheep fight with each other at a speed ~9 m/s with deacceleration ~450g, which creates large amounts of impact energy [95, 121]. An unshod horse hoof trotting at ~4 m/s on an asphalt surface had a deceleration ~56g [223]. The vertical landing velocity of the hoof increases slightly with increasing speed, indicating a slightly higher ground reaction force and deceleration at a higher running speed [224]. Although sustaining less impact than horns, the high frequency of contact with the surfaces also requires excellent energy absorption properties [225, 226]. Thus, it is of interest to understand the mechanical properties and fundamental energy absorption mechanisms of these keratinaous materials.

The equine hoof wall structure has been studied previously. Kasapi and Gosline [10] examined the design complexities: tubules sizes and shape, densities, IF arrangements were dependent on the position thorough the thickness direction of the hoof wall. Cells in the tubules were found almost perpendicular to the cell planes in the intertubular matrix in which keratin cell planes are almost parallel with the ground surface. Compact tension and tensile tests were conducted to study the mechanical behavior of the hoof wall [10, 96], and the differences in IF orientation in the tubules and intertubular matrix were proposed to effectively redirect crack propagation, thus increase the fracture toughness. Tensile properties of the tubules and matrix were acquired by micro-tensile testing. The stiffness of the tubular area was found to be ~0.29 GPa and
in the intertubular matrix ~0.14 GPa (center of hoof wall). The yield strength in these two areas 
was similar (~4.8 MPa in tubules, ~4.1 MPa in intertubular matrix). It was concluded that the IFs 
orientations and volume fractions (~23% in tubules, ~30% in intertubular areas) were the main 
factors affecting the mechanical properties [51, 96]. Douglas et al. [227] reported the tensile and 
compressive stiffness were higher at the distal region than that at the proximal region of the hoof 
wall at both medial and lateral sites, which may provide stress protection to the inner living tissues. 
Hydration effects on the mechanical properties were also investigated by previous studies [139], 
that showed a significant decrease of tensile modulus from dry to fully hydrated samples. The 
tensile properties of the hoof wall were also found to be strain rate sensitive [228], both tensile 
stiffness and strength increased as the strain rate increased.

In this study, for the first time a more detailed structural characterization is presented 
through high resolution synchrotron X-ray micro-computed tomography and transmitted electron 
microscopy studies. Compression tests in different orientations and strain rates were conducted to 
evaluate the energy absorption properties. Nanoindentation modulus and hardness mapping were 
performed to acquire mechanical properties of tubule and intertubular matrix areas. In-situ 
synchrotron X-ray computed tomography compression tests were conducted to analyze the energy 
absorption mechanisms of the hoof. The main goals of the current study are: studying the 
hierarchical structure-mechanical property relationship of hoof wall keratin and understanding the 
energy absorption mechanisms during compressive loading. The findings will give inspirations on 
further bioinspired designs of energy absorbent synthetic structures and materials.

6.2 Experiments and methods

6.2.1 Micro- and nanoscale structural characterization
Three fresh horse hooves were acquired from the School of Veterinary Medicine, University of California Davis. The hooves were stored in -20 °C and kept frozen before testing. Hoof samples with three different hydration states were prepared for experiments in this study: ambient dry (~8.8 wt.% H₂O), fresh (~30.2 wt.% H₂O) and fully hydrated (~40 wt.% H₂O). The water contents were measured following methods described previously [121]. In brief, the weight changes of fresh samples were measured before and after drying in an oven at 130 °C for 24 hrs. Fully hydrated samples were acquired by immersing the samples in DI water for three days until there was no change to the final weight. The water contents of fresh and fully hydrated samples in the current study were similar to previous work (~27.9 wt.% - 35.5 wt.% in fresh hooves, ~40.2 wt.% in fully hydrated samples) [227].

Figure 6.1a shows an intact equine hoof and its longitudinal section from distal to proximal; bone tissue and keratinized hoof wall are indicated. A higher magnification of transverse section from the medial to lateral direction is shown in Figure 6.1b, indicating where the samples were extracted. Three directions are defined: the longitudinal direction is distal to proximal; the radial direction is dorsal to plantar; the transverse direction is orthogonal the two other directions. Three samples with dimension 2 × 2 × 2 mm³ were acquired from the central part of the hoof wall as shown in Figure 6.1a. Samples were thawed at room temperature prior to synchrotron X-ray computed tomography (µCT) imaging. Another three samples with the same dimension as above were fixed with 2.5% glutaraldehyde solution for 24 hrs. After washing with deionized (DI) water three times, 2% osmium tetroxide (OsO₄) was used to stain the samples for three days to increase the contrast. The samples were then washed with DI water five times. Both stained and unstained samples were scanned with µCT. The µCT imaging was carried out on the micro-tomography beamline 8.3.2 at the Advanced Light Source (Lawrence Berkeley National Laboratory, Berkeley,
USA). An X-ray accelerating voltage of 25 keV was used for all the scans. The field of view was 1.7 × 1.7 mm at a resolution of 0.65 μm per voxel. During the scanning, 1025 radiographs were collected during a scan over a rotation of 180°. The series of .tiff images were reconstructed by Amira software (FEI Visualization Sciences Group, Burlington, MA, USA) with a module of volume rendering.

Samples (six in total, two samples from each hoof) were cut into 4 × 4× 4 mm³ cubes for further optical and electron microscopy characterization. Flat surfaces of the longitudinal and transverse sections were prepared by an ultramicrotome (Leica EM UC6, Leica Microsystems Inc., Buffalo Grove, IL, USA) for optical microscopy (OM) studies. Differential interference contrast (DIC) OM images were taken on the prepared flat surfaces by a Keyence VHX 1000 microscope (Keyence, Palatine, IL, USA). Toluidine blue stained thin slices (~1 μm thick, 6 slices from each cube) prepared by an ultramicrotome and were imaged to examine cell shapes, sizes and distribution. Three cubes were fixed with a 2.5 vol.% glutaraldehyde solution overnight. Samples were dehydrated in a graded series of ethanol solutions (20%, 40%, 60%, 80%, 95%, and 100% vol.% ethanol). Samples were then freeze-fractured in liquid nitrogen for examination by scanning electron microscopy (SEM) characterization and were sputter coated with iridium (Quorum Technologies Ltd., West Sussex, UK) to enhance the sample electron conductivity. The SEM imaging was conducted with an ultra-high-resolution microscope (FEI, Hillsboro, OR, USA). The three remaining cubes were stained with 2% OsO₄ solution for three days for transmission electron microscopy (TEM) imaging. The stained samples were washed with DI water five times followed by a dehydration process mentioned previously. Samples were then embedded in Spurr’s low viscosity resin (Electron Microscopy Sciences, Hatfield, PA, USA) and cut into ~80 nm thin sections using an ultramicrotome (Leica EM UC6, Leica Microsystems Inc., Buffalo Grove, IL,
USA). The sections were placed on copper grids and post-stained by lead citrate solutions to enhance contrast. TEM imaging was conducted with a FEI Tecnai 12 (Spirit) (80 kV) electron microscope (FEI, Hillsboro, Oregon, USA).

6.2.2 Compression tests and failure surface imaging

Fresh hoof samples with dimension $5 \times 5 \times 5$ mm$^3$ were acquired from the central part of the thickness direction of hoof wall at both dorsal proximal and dorsal distal locations (Figure 6.1b). Compression tests were conducted in three different loading orientations (longitudinal, radial and transverse) and three strain rates ($0.001 \text{ s}^{-1}$, $0.01 \text{ s}^{-1}$, $0.1 \text{ s}^{-1}$). Five samples were tested for each orientation. A universal testing machine with a 30 kN load cell (Instron 3367 Dual Column Testing Systems, Instron, MA, USA) was used to conduct the compression tests. Surfaces of hoof samples before and after 30% compression in different directions were imaged with Keyence VHX 1000 microscope (Keyence, Palatine, IL, USA).

6.2.3 Modulus and hardness mapping through nanoindentation

Nanoindentation has been used as an effective method to characterize the microscale mechanical properties of biological materials [207, 229, 230]. Samples ($\sim 4 \times 4 \times 4$ mm$^3$ cubes) from the same region as SEM and TEM samples were polished with the ultramicrotome to conduct nanoindentation tests. Samples with different water content were tested (ambient dry, fresh and fully hydrated). Fresh samples were kept frozen before testing, while fully hydrated samples were immersed in DI water during testing. Nanoindentation experiments were conducted using a Ti-950 TriboIndenter (Hysitron, USA) with a low-load transducer. Samples in the dried state were tested using a diamond cube corner probe while fresh and fully hydrated samples were tested using a fluid cell Berkovich probe. All maps featured a square array of indents with a spacing of 10 µm in the horizontal and vertical directions. Indents were controlled in displacement to a depth
between 300 nm and 500 nm. Partial unload tests were first conducted in each sample to determine the appropriate minimum depth to overcome surface roughness. A trapezoidal load function consisting of a 5 s load, 5 s hold, and 5 s unload was used for all mapping experiments.

6.2.4 In-situ synchrotron X-ray computed tomography compression

Compressive deformation mechanisms of different materials at the microscale has been investigated by synchrotron µCT characterization, found to be an effective method to study internal failures [231-233]. In-situ quasi-static compression tests were conducted to study the failure and energy absorbent mechanism in the longitudinal direction (impact direction). The in-situ compression tests were carried out on the micro-tomography beamline 8.3.2 at the Advanced Light Source (Lawrence Berkeley National Laboratory, Berkeley, USA). An X-ray accelerating voltage of 25 keV was used for all the scans. The field of view was 3.3 × 3.3 mm at a resolution of 1.3 μm per voxel. Two cubic samples with dimension 2 × 2 × 2 mm³ in the fresh and fully hydrated conditions were compressed longitudinally. Scans were taken after 0%, 30% and 60% deformation. Amira software (FEI Visualization Sciences Group, Burlington, MA, USA) with a module of volume rendering was applied to reconstruct the 3D images.

6.3 Results and discussions

6.3.1 Hierarchical structure of equine hoof wall

Figure 6.1c shows µ-CT images (0.65 μm resolution) of the medial region of the hoof wall. The 3D reconstruction indicates that the tubules are parallel to the longitudinal direction and are continuous from the proximal to distal end. The average porosity of the hollow medullary cavity is ~3% with a diameter 41.2 ± 8.8 μm. OM images in different locations and cross-sections (longitudinal and transverse) are shown in Figure 6.1d-g. From OM with DIC imaging (Figure 6.1d), the tubular areas have elliptically-shaped cross sections with dimension ~206.2 ± 23.8 μm.
(major axis) and 107.1 ± 14.8 µm (minor axis). The tubular areas account for ~30% of the whole hoof wall cross section. To obtain further understanding of the cell shapes and arrangements in different regions, toluidine blue stained thin sections were imaged. Cell boundaries are clearly shown in Figure 6.1e. Comparing the cell sizes and shapes in the tubules and intertubular matrix, significant differences are identified: Irregular polygonal shapes are found in intertubular matrix area (red color), while cells are lens-shaped in the tubular areas (yellow color). The average cell size in the intertubular matrix is ~19.5 ± 4.8 µm, while in the tubular region is ~18.8 ± 2.6 µm in length and ~4.6 ± 1.1 µm in thickness. Figure 6.1f shows the longitudinal cross-section showing the tubules and intertubular matrix. Toluidine blue stained thin slice images are shown in Figure 6.1g. The tubular areas also have lens-shaped cells with dimension ~23.8 ± 5.3 µm in length and ~4.4 ± 1.2 in thickness, while irregular polygonal cell shapes with dimension ~16.9 ± 4.7 µm are found in intertubular areas. By combining cross and longitudinal section images the cell size and shapes in tubular and intertubular areas are: the cells in intertubular areas are ~15-25 µm irregular polyhedrons, while cells in tubular areas are thin lamellae with ~20 µm in diameter, ~5 µm in thickness, with the thickness direction perpendicular to and surrounding the tubules. This is the first report that finds differences between the cell sizes and shapes between the tubular and intertubular areas.
Figure 6.1 Hierarchical structure of an equine hoof. (a) Frontal and longitudinal section from the distal to proximal photographs of a fresh hoof; (b) Transverse section view from medial to lateral. Three directions are defined: longitudinal direction (distal to proximal); radial direction (dorsal surface to the inside bone tissue); transverse direction (medial to lateral); (c) 3D reconstructed synchrotron X-ray micro-computed tomography (µCT) image of hoof wall and the internal tubules; (d) Optical microscopy (OM) image of cross section of tubules. The red box shows single tubule cross-section at a higher magnification. Tubules and intertubular matrix are pointed out; (e) Toluidine blue stained OM image of the cross section. Keratin cell shapes and sizes in both tubular (yellow) and intertubular areas (red) are shown in the green box at a higher magnification. The white holes inside the tubules are the longitudinal hollow medullary cavities; (f) Optical microscopy image of longitudinal section of tubules; (g) Toluidine blue stained optical microscopy image of longitudinal section of tubules. Cell shapes and dimensions are also shown in the green box at a higher magnification.

Further detailed characterization was performed by synchrotron µCT with OsO₄ stained samples, SEM and TEM. Figure 6.2 shows the difference between cells in the intertubular and tubule regions though different imaging methods. Figure 6.2a is a 3D reconstructed image in which the tubular areas show a higher material density. This indicates the tubules absorb more OsO₄ than the intertubular matrix, leading to the higher density. Cross- and longitudinal sections are shown in Figure 6.2b and c, respectively. The cell morphology can be visualized in both images. The cell boundaries, which mainly consist of phospholipid layers, are preferentially stained by OsO₄, leading to the high contrast at the cell boundaries. The cell morphologies and sizes in the tubules
corresponding to the toluidine blue stained images in Figure 6.1e and g. The tubule medullary cavities are not completely hollow. Thin bridges are found inside dividing the medullary cavities into small chambers (Figure 6.2c). Figure 6.2d shows a 3D reconstructed image of a tubule wall, where the lamellar structure of cells (yellow dashed line) is indicated. The irregular green lines indicate cell boundaries that form a wavy (suture-like) structure. SEM images of freeze-fractured cross sections are shown in Figure 6.2e-h. An overview of the cross section shows the tubule distribution in Figure 6.2e. A concentric layered structure is found surrounding tubules in a higher magnification SEM image (Figure 6.2f). Cells are also found with the same shape (Figure 6.2h, yellow dashed lines) corroborating the findings from OM and µCT. At a higher magnification (Figure 6.2g), cell surfaces are found to be rough with ~100 nm thick ridges (yellow dashed arrows), which has been observed in the surfaces of the stratum corneum and pangolin scales [86]. This correspond to the rough surface found in the 3D CT image in Figure 6.2d. More detailed structure and features of the cell boundaries and cytoskeleton were characterized with TEM (Figure 6.2i-l). Figure 6.2i shows TEM images of the cell morphology in the intertubular areas. The irregular polygonal shape of cells is similar to the results from OM. Suture structured cell boundaries are identified as the dark, wavy lines, due to more OsO₄ staining. Macrofibrils with diameter ~710 ± 130 nm are found inside the cells (Figure 6.2k) which is larger than that in hair fibers (~100 – 400 nm) and horns (~200 nm) [121, 234]. This larger diameter of the macrofibrils might have higher stiffness compared to smaller ones based on previous studies on hair [235]. In Figure 6.2j, the cells in the tubular area are elliptically-shaped with dimension ~20 µm in length and ~5 µm in thickness, which verifies the findings in the OM studies. Detailed image of macrofibrils shows the cross sections are not circular, which indicates the macrofibrils are not perfectly aligned along the tubule (longitudinal) direction (Figure 6.2l). Intermediate filaments
(white dots, ~7-10 nm in diameter) embedded in an amorphous matrix in the intertubular and tubular areas are shown in Figure 6.2k and l (yellow box), respectively. The percentage of intermediate filaments are different in these two areas: ~32.2% in the intertubular area and ~21.3% in the tubular area, which is similar as the reported data in previous work [96]. The cells in tubular area (Figure 6.2l) are darker than that in intertubular areas (Figure 6.2k) because more OsO₄ is concentrated in the tubular area, due to the higher fraction of amorphous matrix.

In summary, a tubular structure was identified in the equine hoof wall. The medulla cavity inside the tubules are not completely hollow but have thin bridges forming chambers. The
structural and morphological differences between tubular and intertubular areas were found in both micro- and nanoscale levels. Keratin cells from irregular polyhedrons in intertubular areas and change to ellipsoid-shaped in tubular areas. At the nanoscale, crystalline IFs (~7-10 nm) embedded in an amorphous matrix were found in both tubules and intertubular areas, while the intertubular areas have a higher intermediate IF fraction. These structural differences at different scales may lead to the differences in mechanical behaviors, which will be discussed in the following sections.

6.3.2 Multi-scale mechanical behavior of equine hoof wall

From previous work [109, 120, 121], the mechanical properties of big horn sheep horns have been strongly found to depend on the level of hydration. Nanoindentation tests were conducted on samples with three hydration states: ambient dry, fresh and fully hydrated. The reduced modulus ($E_r$) and hardness ($H$) maps were acquired with the three hydration states to determine the differences between the tubular and intertubular regions. Figure 6.3a, b and c show the reduced modulus and hardness maps. Figure 6.3d summarizes the reduced modulus of both tubular and intertubular areas in the different hydration states. In the dry condition, $E_r$ is ~7.91±0.47 GPa in tubular areas and ~6.96±0.3 GPa in intertubular areas. In the fresh condition $E_r$ is ~6.26±0.44 GPa in tubular areas and ~5.41±0.22 GPa in intertubular areas. This suggests that tubules reinforce the intertubular areas. In the fully hydrated condition, $E_r$ decreased to 0.12±0.02 GPa in tubular areas and 0.19±0.02 GPa in intertubular areas, orders of magnitude smaller than in the dry and fresh conditions. A similar trend is also found in $H$ measurements (Figure 6.3e). $H$ in tubular and intertubular areas are ~0.34±0.03 GPa and ~0.38±0.03 GPa, respectively in the dry condition, which are similar values. In the fresh condition, $H$ in tubular areas (~0.26±0.04 GPa) is higher than that in intertubular areas (0.19±0.02 GPa). In the fully hydrated condition, $H$ values in the tubular and intertubular areas are similar: 0.011±0.002 GPa and 0.013±0.002 GPa,
respectively, again orders of magnitude smaller than in the dry and fresh conditions. The relationship $H$ and $E_r$ was also investigated, combining both tubular and intertubular regions. From the plots of $E_r$ vs $H$ in the dry samples (Figure 6.3f), $H$ remains constant while $E_r$ increases, indicating that local ability for local plastic deformation of different regions is not affected by the reduced modulus. $H$ increases as $E_r$ increases in both the fresh and fully hydrated conditions (Figure 6.3g and h), showing after hydration the resistance to local plastic deformation is more sensitive to modulus changes. Due to the indentation nature that hardness is the hybrid measurement of both reversible and irreversible deformations, changes in indentation modulus could lead to changes in hardness [236]. However, the constant $H$ in dry conditions indicates the irreversible plastic deformation dominants the tests, which is reasonable because keratin materials are brittle without water molecule “plasticizers”, and more irreversible deformation will be produced than elastic reversible deformations [86].

In summary, both $E_r$ and $H$ in the tubular and intertubular areas decrease significantly after full hydration. There is only ~20% decrease in $E_r$ and $H$ from the dry to fresh samples, but almost ~98% decrease from fresh to fully hydrated samples. This indicates water effects dominate the mechanical properties once fully hydrated. It is also found that the tubular area has higher $E_r$ than the intertubular areas in both dry and fresh conditions, while the opposite trend is found in the fully hydrated condition. This can be explained by the basic composition in these two areas that the tubular areas have a greater fraction of the amorphous matrix. Based on previous work [237, 238], the amorphous matrix has a higher amount of cystine, which is a sulfur-rich amino acid. The enriched disulfide bonds between the IF-matrix and matrix-matrix in these areas could lead to higher rigidity and mechanical stiffness in the tubule areas [107, 239]. Thus, the tubules are stiffer than the intertubular areas, serving to form a tubular reinforced component in the structure. These
findings are different from results found in the previous literature \([179, 240]\), where it was concluded that it is the intertubular matrix that accounts for hoof mechanical strength and stiffness, while the tubules are only acting as crack stopping interface. After full hydration, the stiffness of amorphous matrix decreases significantly due to water incorporation, while the crystalline IFs are not affected \([98, 103, 104, 110]\), leading to a greater decrease \(E_r\) in the tubular areas due to the higher fraction of the amorphous matrix.

![Figure 6.3 Nanoindentation characterization of cross sections.](image)

(a, b and c) Reduced modulus \((E_r)\) and hardness \((H)\) maps of the horse hoof wall in ambient dry, fresh and fully hydrated conditions. (d) Comparison of \(E_r\) in tubule and intertubular areas in the dry, fresh and fully hydrated conditions. (e) Comparison of \(H\) in tubule and intertubular areas in the dry, fresh and fully hydrated conditions. (f) Plot of \(H\) and \(E_r\) of dry hoof samples. (g) Plot of \(H\) and \(E_r\) of fresh hoof samples. (h) Plot of \(H\) and \(E_r\) of the fully hydrated samples.

Macroscale compression behavior of fresh hooves was characterized under different loading orientations (longitudinal, radial and transverse) and different strain rates \((10^{-3}\text{s}^{-1}, 10^{-2}\text{s}^{-1}, 10^{-1}\text{s}^{-1})\). Figure 6.4a, b and c show the compressive stress strain curves in different orientations and strain rates. The red color indicates compression in longitudinal direction (parallel to tubules),
black and blue colors show compression in radial and transverse direction (perpendicular to tubules). The stress strain curves indicate that the hoof is an ideal energy absorption material with similar representative compression behavior of traditional cellular and foam structures [49, 76]. Starting with an initial elastic region that is followed by a region with a long plateau (main energy absorption area), then followed by a densification region (stress increases significantly). Interestingly, even though the structure is anisotropic, the stress strain curves appear isotropic at the different strain rates. This isotropic behavior is different from the anisotropic compressive behaviors of bighorn sheep horns, which have a larger amount of porosity and do not have reinforced tubules [121]. The Young’s modulus and yield strength under different loading orientations and strain rates are shown in Figure 6.4d and e. Both increase as the strain rate is increased. This strain rate dependent behavior is due to the viscoelastic property of keratin, which has been studied in human hair and bighorn sheep horn [102, 120]. By integrating the compressive stress strain curves, the amount of energy absorption as a function of compressive strain can be calculated and are plotted in Figure 6.4f. As a comparison, the energy absorption of the bighorn sheep horn with the same amount of water content (~30% wt.%) is also shown [121]. It is found that hoof samples absorb more energy than bighorn sheep horn in all the three loading directions. The average Young’s modulus of the hoof (~0.5 GPa) is also more than twice of the value in horns (~0.2 GPa).
Figure 6.4 Compressive stress-strain curves of fresh hoof samples (~ 30% H$_2$O) in different loading orientations and strain rates; (a) strain rate $10^{-3}$s$^{-1}$, (b) strain rate $10^{-2}$s$^{-1}$ and (c) strain rate $10^{-1}$s$^{-1}$. (d) Comparison of Young’s modulus at different strain rates and loading orientations. (e) Comparison of the yield strength at different strain rates and loading orientations. (f) Plots of energy absorption (area under the stress-strain curve) as a function of compressive strain in hoof and horn (~ 30% H$_2$O) [8] in different loading orientations (red: longitudinal, black: radial, blue: transverse).

6.3.3 Failure and energy absorption mechanisms

Failure mechanisms under compression were studied in detail with both OM and in-situ synchrotron μCT to understand the energy absorption mechanisms (deformation modes) in the three different directions. OM images of the cross-sections in the longitudinal (Figure 6.5a), radial (Figure 6.5b) and transverse (Figure 6.5c) directions were examined before compression and after 30% compression (Figure 6.5d, e and f). No obvious damage to the microstructure is observed but small cracks in the tubular region were found in the sample when compressed in longitudinal direction (Figure 6.5d). Cracks in diagonal directions (black dashed arrows in Figure 6.5e) are found inside each tubule when compressed in radial direction. Cracks are only observed in the tubule areas (Figure 6.5e). In the transverse direction, small cracks (black dashed arrow) start propagating from the vertices of one elliptically-shaped tubule to another, forming long cracks (Figure 6.5f). Compared with the bighorn sheep horn, no shear bands or catastrophic failures were
observed [121]. This may explain the isotropic compression stress strain curves in Figure 6.4a-c, since all the three directions show similar failure mechanisms. From previous work [121], shear bands, together with lamella buckling formed in bighorn sheep horn causing severe failure when compressed longitudinally, leading to lower energy absorption in this direction. In contrast in the hoof, due to the higher stiffness of tubules, more force will be applied on the tubules, thus protects the whole matrix structure from shear-banding failure.

Figure 6.5 Deformation and failure mechanisms before and after 30% deformation. (a-c) Optical microscopy images of samples surfaces before compression in the different loading orientations: longitudinal section (red color surface in (a) when loading longitudinally; cross sections (green color surface in b and blue color surface in (c) when loading radially and transversely. (d-f) Surface images after 30% compression in longitudinal, radial and transverse direction, respectively. Cracks are observed in the tubule areas in all the three directions. Black dashed lines in (e) and (f) indicate the crack directions.
When the horse is galloping, the main loading come from the ground and is applied along the longitudinal direction. The deformation of the tubules in the longitudinal direction was studied with in-situ μCT compression tests. Figure 6.6 shows the 3D reconstructed images of fresh hoof samples before (Figure 6.6a) and after 30% (Figure 6.6d) and 60% (Figure 6.6g) compression. Figure 6.6b and c show the top and front view of the samples before compression. Higher magnification image indicates tubules consist of segments of cells (Figure 6.6c). After 30% compression in the longitudinal direction, the tubules are buckled (Figure 6.6d). The top and front view of the buckled tubules are shown in Figure 6.6e and f. Clear curvature of the tubules are
observed. In Figure 6.6f, cracks on the tubules are found after compression, which can be correlated to previous findings shown in Figure 6.5d. Interestingly, in Figure 6.5d, no buckling of tubules is found in the OM image after 30% compressive strain, only cracks are observed in the tubules. These findings suggest that the buckling of tubules may recover once the load is released, indicating a viscoelastic behavior. Figure 6.6g-k shows images after 60% compression. Few tubules can be found in Figure 6.6g. From the top and front view of the sample (Figure 6.6h, i), it can be found that the buckled tubules become discontinuous and apparently disappear. Figure 6.6i shows severe damage, in which the tubules are found along a diagonal direction (red dashed line) due to buckling. Cracks (red box) and cell shear and shape change (Figure 6.6i, yellow dashed line) on the tubules compared to the undeformed cells (Figure 6.6c, yellow dashed line) are observed due to the shear forces on the tubules. This indicates at 60% compression, the buckled tubules have compressed and densified within the intertubular matrix, leading to the disappearance of the tubules. This densification can be correlated to the stress strain curves in Figure 6.4a, in which a sharp increase of the stress occurs around 60% strain due to this densification. Figure 6.6j shows 3D reconstructed block image and tubules after 30% compression in the fully hydrated condition. No buckling of the tubules is observed; only cracks are found in the tubules. This indicates the tubules are no longer serving as reinforcing structures due to the stiffness decrease after hydration (Figure 6.3). After 60% compression, no tubules are found, which is attributed to densification. Therefore, buckling and cracking of the tubules are the main deformation and energy absorption mechanisms in the fresh equine hoof when compressed longitudinally. Due to the higher stiffness and yield strength of the tubules, more energy will be absorbed because of this tubule-reinforced structural designs. However, hoof samples in the fully hydration state loses this tubule reinforcement.
6.4 Conclusions

The hierarchical structure, multi-scale mechanical behavior and energy absorption mechanisms of equine hoof wall under compression were investigated. The hierarchical structure was examined by optical and scanning and transmission electron microscopy (OM, SEM and TEM) and high-resolution synchrotron X-ray computed tomography (µCT). Multi-scale mechanical analysis was tested by nanoindentation and compression tests at different strain rates. Failure and energy absorption mechanisms of hoof samples under compression were studied via OM and in situ µCT compression tests. Tubules, serving as a reinforced element in hoof structure, were identified and verified. The main conclusions are:

- Tubules embedded in the intertubular matrix were identified in the transverse cross-section. The total porosity is ~3% (medullary cavity), and the tubules consist of ~30% of the whole area. The size and shapes of the keratin cells are different in the tubular and intertubular regions: cells in intertubular areas are irregular polygons ~19.5 ± 4.8 µm, while cells in tubular areas are lamellar ~18.8 ± 2.6 µm in diameter, ~4.8 ± 1.1 µm in thickness with the thickness direction perpendicular to the tubules.

- The fraction of intermediate filaments (IFs) and amorphous matrix were revealed by synchrotron µCT and TEM images. The intertubular matrix shows a higher amount of IFs (32.2%) while the tubular area has a lower value (21.3%).

- The stiffness and hardness of the tubular areas are higher than that in the intertubular areas in both the dry and fresh hoof samples. This indicates that the tubules serve as reinforced structures to support the load. When the samples are fully hydrated, the stiffness and hardness of tubules are smaller than in the intertubular areas, which is due to the higher water absorption in the tubular areas. The overall compression behavior shows a strain rate
dependency and isotropy. Full hydration leads to a 98% decrease of the modulus and hardness over that of fresh hoof samples.

- The compressive mechanical properties show isotropy when loading at strain rates between $10^{-3}\text{s}^{-1}$ - $10^{-1}\text{s}^{-1}$ is different from the anisotropic behavior found in bighorn sheep horns. Elastic buckling and fracture of the tubules are the main failure and energy absorption mechanisms when compressed longitudinally. The reinforced tubules protect the whole hoof structure from shear-banding or developing cracks in all the three directions, leading to a higher energy absorption compared with bighorn sheep horn at the same hydration level.

The findings in this work presented and identified a natural tubule reinforced polymer composite structure in the equine hoof, which can absorb large amount of energy because of the stiff and strong tubules. Thus, the results here may inspire of designs of light weight energy absorption synthetic structure and materials.

Chapter 6, in full, is currently in preparation for publication as “A natural energy absorbent polymer composite: The equine hoof wall”. This work was coauthored by N. Yaraghi, W. Yang, A. Velazquez, R. Ritchie, D. Kisailus, S. Stover and J. McKittrick. The dissertation author is the first author of this work.
CHAPTER 7: BIOINSPIRED DESIGNS BASED ON 3D PRINTING

7.1 Introduction

Biomimetic impact resistant and energy absorbent materials have been previously studied based on several strong and tough structural biological materials found in nature, such as bone, nacre, and wood [24, 61]. Al₂O₃/PMMA nanocomposites were fabricated by freeze casting, showing > 300× greater toughness than the single components [61]. Layer by layer self-assembly of ZnO nanowires was prepared to mimic the column structure in tooth enamel, showing good viscoelastic behavior and energy absorption [77]. Impact resistant fiber-epoxy composites with helicoidal structures inspired from dactyl club of mantis shrimp was fabricated by laminating, showing the cracks were redirected by the rotating fibers thus the energy dissipation was increased [241]. Apart from these methods, 3D printing has been considered as one of the most useful technique to mimic the complicated hierarchical structures and materials compositions in nature [62, 66, 74, 75].

Buehler et.al fabricated a bioinspired conch shell composites using 3D printing [68]. The printed hierarchical cross-lamellae composites showed 85% more impact resistance than a single stiff material. The crack deviation during the impacts in the cross lamellae structure was identified as the main toughening mechanism. Natural energy absorbent materials with cellular structures such as wood and bamboo were also replicated by 3D printing methods, showing promising energy absorption capabilities [75, 242, 243]. Carbon fiber reinforced epoxy composites with different morphologies of the lightweight cellular structure inspired by balsa wood were fabricated [75]. The aligned fibers along the printing direction showed structure reinforcement and enhanced mechanical properties. Cellular polymeric structures with negative stiffness (stress decreases as strain increases) were fabricated with 3D ink-jetting method [242]. The negative stiffness was
realized by tuning the microarchitectures in the 3D designs, which could be applied in energy absorption cushions by decreasing the stress threshold thus preventing damages. Apart from cellular structures, double-phase co-continuous polymer composites with both soft and stiff phases have also been printed out with multi-materials 3D printer, which mimicked the hydroxyapatite-collagen double-phase combinations in bones [67, 244]. The energy absorption properties could be tuned by changing the fraction of stiff and soft phases. Due to the stiffness difference between the stiff phase (Young’s modulus ~3.3GPa) and soft phase (Young’s modulus ~3.3 MPa), the yield strength of the final products can increase by a factor of 2.7 by increasing the fraction of stiff phase from 50% to 65% [67]. Multi-materials direct ink-writing 3D printing assisted with magnetic fields was developed by researchers to fabricate complicate microstructures and composite materials [245]. By controlling the orientation of aluminum platelets with magnetic fields, complicated structures found in nature such as helicoidal designs in crab exoskeleton were acquired [245].

Although bioinspired impact resistant and energy absorbent materials have been fabricated and studied, bioinspired structures based on the horn and hoof structure have not been developed. Due to the promising mechanical performance of horns and hooves, 3D printing was used to replicate the tubular and lamellar structures and subsequently tested to evaluate the compressive properties and energy absorbing capabilities.

7.2 Experiments and methods

7.2.1 3D printing of bioinspired horn and hoof structures

To mimic the tubular and lamellar structures in both horns and hooves, a multi-material 3D printer (Objet350 Connex3, Stratasys, Poway, CA) was used to fabricate horn and hoof models. The polymers used were VeroClear® for the stiff phase and Tangoblackplus® for the soft phase. Table 7.1 list the properties provided by the manufacturer. For static compression tests, four cubic
(1 cm³) designs were fabricated: single-phase cubes with and without tubules (Figure 7.1b); double-phase materials arranged in a lamellar structure with and without tubules (Figure 7.1c). The single-phase cubes were printed out using VeroClear®, with Young’s modulus ~2-3 GPa (similar to keratin). The samples with tubules (major axis = 1 mm, minor axis = 0.5 mm) were printed with a tubule density of 10%, which is similar to the density in real horn structure. The thickness of the VeroClear® layer was 1 mm, while the Tangoblackplus® layer was 0.2 mm, which mimics the ratio between the cell lamellae and cell-cell interface in real horns. In the real horn, the average thickness of cells are ~2-5 µm, while the interfaces (width of the suture structure) between cells (cell boundaries) are ~200 nm – 500 nm thick, which are ~5-10 times smaller than the thickness of keratin cells [121]. Cell boundaries are lipid membranes and extracellular space connected by a few intermediate filaments that are much weaker than the cell lamellae with keratin intermediate filaments reinforced cytoskeletons [246-248].

Table 7.1 Manufacturer provided properties of VeroClear® and Tangoblackplus®.

<table>
<thead>
<tr>
<th></th>
<th>Young’s modulus (GPa)</th>
<th>Tensile strength (MPa)</th>
<th>Density (g/cm³)</th>
<th>Glass transition temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VeroClear®</td>
<td>2-3</td>
<td>50-65</td>
<td>1.18-1.19</td>
<td>52-54</td>
</tr>
<tr>
<td>Tangoblackplus®</td>
<td>-</td>
<td>0.8-1.5</td>
<td>1.12-1.13</td>
<td>-</td>
</tr>
</tbody>
</table>

Quasi-static compression tests were conducted in different directions (parallel and perpendicular to tubules and printing directions) with a strain rate 10⁻³ s⁻¹ on a universal testing machine with a 30 kN load cell (Instron 3367 Dual Column Testing Systems, Instron, MA, USA). Failure surface images were taken after 30% compression. At least three samples were tested in each condition.

The recovery behavior in the 3D printed samples was also studied to evaluate if the models could replicate the deformation/recovery behavior found in real horns (Chapter 5). Shape memory
effects occur when heating a polymer above a transition temperature, normally the glass transition temperature ($T_g$) or melting temperature ($T_m$) [249, 250]. Based on previous studies, VeroClear® materials can be recovered (return to their original dimensions after deformation) by increasing the temperature slightly above their glass transition temperature (62 °C) [251]. In the present study, single-phase samples with 10% tubules were printed out with VeroClear®, and then compressed in three different directions: perpendicular to both the tubules and printing direction, perpendicular to the tubules but parallel with the printing direction, and parallel with the tubules and printing direction. The ultimate compressive strain was selected to be 50%, which is to keep consistent with previous recovery studies of the real horn [121]. The compressed samples were then heated to 62 °C and held for 15 min to recover. After recovery, samples were compressed to 50% deformation for the next cycle and repeated until until failure.

7.2.2 Synchrotron X-ray micro-computed tomography

To compare the failure mechanisms of real horns and the bioinspired 3D printed samples, in-situ synchrotron X-ray micro-computed tomography was conducted. Real horn cubes with dimensions 2mm³ were cut by diamond saw. The synchrotron experiments were conducted on the micro-tomography beamline 8.3.2 at the Advanced Light Source (Lawrence Berkeley National Laboratory, Berkeley, USA). A 1000 N loading cell was applied for compression tests in the beamline. Compression tests were done in three different loading directions (longitudinal, radial and transverse). The scans during compression had a resolution of 1.3 µm and FOV 3.3 mm × 3.3 mm, thus the whole sample can be monitored during the in-situ compression tests. The loading rate was set at $10^{-2}$ s⁻¹. Scans were acquired at initial stage 0% deformation, at the stress plateau stage (30% deformation), and at the densification stage (60% deformation) according to previous compression stress strain curves [121]. 3D reconstruction of acquired tiff images from scanning
was conducted using Amira software (FEI Visualization Sciences Group, Burlington, MA, USA) with the volume rendering module. Colormap (from a minimum intensity shown as dark blue to a maximum intensity shown as red) was applied to distinguish the different keratin densities based on the acquired X-ray intensities.

**7.2.3 Drop-tower impact tests of 3D printed models**

The four model designs (single-phase solid blocks, double-phase lamellar, tubular and tubule reinforced designs) with dimension (20 mm × 20 mm × 5mm) were printed. The 3D structural designs are shown in Figure 7.6a. Drop tower impact tests were conducted on a lab-built drop tower, which has been used previously for biological materials [117]. The impact tests were performed based on a modified ASTM standard D 7136/D 7136 M-07 [195]. The details of the testing apparatus were illustrated in the previous literature. The drop weight of the impactor was 1.2 kg and the maximum drop height was 0.74 m. The highest impact energy ($E = mgh$) that could be reached is 8.7 J with an impact speed 3.8 m/s [117]. Both of the top and bottom failure surfaces were imaged after the impact tests.

**7.3 Results and discussions**

**7.3.1 Energy absorption properties of bioinspired designs**

Schematic diagrams and scanning electron microscopy (SEM) images of the tubular and lamellar structure are shown in Figure 7.1a. The printed samples and microscale features are shown in Figure 7.1d,e. Due to the layer-by-layer printing nature of 3D printing, the single-phase VeroClear® samples show laminated structures (Figure 7.1d). In the double-phase sample, lamellae of VeroClear® and Tangoblockplus® are shown in Figure 7.1e.
The stress strain curves of the 3D printed samples as well as the original horn samples are shown in Figure 7.2. Figure 7.2a shows the stress strain curves of dry horns in different orientations [121]. The horn samples were able to be compressed up to 70% strain, indicating large energy absorption in all loading orientations. The double-phase models were compressed in directions perpendicular and parallel to the lamellae. The stress strain curves are shown in Figure 7.2b. When compressed perpendicular to the lamellae, samples without tubules (black curve) showed higher stiffness and strength, but lower compressive strain than samples with tubules (red curve). The closure of the tubules results in lower stiffness and strength, while allowing for large plastic deformations, increasing the final compressive strain. Compression parallel with the lamellae in both samples with (purple) and without tubules (blue) showed much lower strength than compression perpendicular to the lamellae, which is attributed to delamination and fracture in the
weak soft phase. For all the designs, the highest compressive yield strength was ~8 MPa, which is an order of magnitude lower than the real horn samples. The weak connection between the soft and stiff lamellae is the possible reason. The stress strain curves of the single-phase model are shown in Figure 7.2c. Both the strength and shape of curves are very similar to the real horn samples, indicating promising energy absorbent performance. The longitudinal direction (red arrow) is the direction parallel with the printing direction, while the radial direction (black arrow) is the direction perpendicular to the printing direction. The yield strengths in samples without tubules (black and red curves) were slightly higher than those of samples with tubules, which is the same trend as seen in real horn samples (Chapter 5, Figure 5.6). No obvious anisotropy was found in these samples, indicating the printing direction does not have obvious effects on the compressive strength. In the samples with tubules, the Young’s modulus and yield strength in radial direction were smaller than those found in the longitudinal and transverse directions. Comparing the plateau strength in samples with and without tubules, the plateau strength was found to decrease because of the tubules. The stress plateau areas are regions that absorb most of the energy by limiting the stress at a constant level, which can further protect the internal structures or materials as an energy absorption cushion [76, 203].
Figure 7.2 Compressive stress strain curves for bighorn sheep horns and 3D printed samples. (a) Bighorn sheep horn in different loading orientations [121]. (b) Double-phase models with and without tubules in different orientations. (c, d) Compressive stress strain curves of single-phase models with and without tubules in different orientations.

7.3.2 Failure mechanisms in horns and bioinspired materials

Microscale deformation of tubules and keratinized cells were studied by in-situ synchrotron X-ray computed tomography (CT) compression tests. Horn samples were compressed to 30% and 60% deformations in different orientations during CT scans. 3D reconstructed images after compression to 30% deformation are shown in Figure 7.3. Figure 7.3(a-c) show the tubules in the horn samples after compression in radial, longitudinal and transverse directions, respectively. No obvious damage was found in the sample after compression in the radial direction (Figure 7.3a). From the top view slice, tubules were observed slightly deformed after compression in the minor axis of the ellipse (Figure 7.3d). Compression in the longitudinal direction led to the buckling of the tubules and X-shape shear bands were observed (Figure 7.3b,e). Figure 7.3c shows the results
of compression in the transverse direction. Tubules tilted (~15°) from the original orientation due to the shear force and cracks were observed (Figure 7.3f).

Figure 7.3 (g-i) shows the more severe deformation of samples after compression to 60% strain. In the radial direction, tubules were squeezed to form dense lines (Figure 7.3g). Almost no tubules were identified in the cross section (Figure 7.3j) due to the compression and closure of the tubules. In the longitudinal direction, more severe buckling of the tubules and cracks were observed (Figure 7.3h,k). Buckling of tubules appear to be the main energy dissipation mechanisms in the longitudinal direction. In the transverse direction (Figure 3i), compression led to closure and orientation changes of tubules. The tubules sheared forming a 45° diagonal crack (Figure 7.3l). The energy dissipation mechanisms can be related to the cracks initiation at the verticies of the elliptical tubules and propagating at a 45° direction.

In summary, after compression to a 60% strain, the tubules were severely damaged in all the three directions. However, unlike the buckling of and damages to tubules and cell lamellae in longitudinal and transverse directions, the bonding between adjacent lamellae remained intact in the radial direction. This could further explain the high impact resistance and energy absorption in the radial direction.
Figure 7.3 In-situ compression of horn samples under synchrotron X-ray computed tomography. Samples are compressed to 30% (a-f) and 60% (g-l) deformation. (a) Compression in the radial direction. No obvious damages were found; (b) Compression in the longitudinal direction. Buckling of tubules was found; (c) Compression in the transverse direction. Shearing of tubules was noticed; (d) Cross-section shows the elliptical tubules. Slightly compressions were observed, some of the tubules are slightly closed; (e) Longitudinal section shows the X-shape shear bands and also buckling of tubules in the shear bands. The orientation of keratin cells surrounding the buckled tubules was changed; (f) Cross section shows tubules orientation changes due to the shear force. Cracks initiate at the corner of the tubule; (g) Compression in the radial direction at 60% deformation; (h) Compression in the longitudinal direction. Sever buckling of tubule were observed; (i) Compression in transverse direction, shear of tubule in this direction; (j) No tubule were found in the cross section due to the complete collapse of tubule after 60% deformation; (k) Buckling of tubules and cracks of tubules were observed; (l) Cross section of sample after 60% compression in transverse direction. Cracks start propagating along 45° (yellow dashed lines).

The failure mechanisms of single-phase 3D printed VeroClear® samples were also investigated. Samples with and without tubules were compressed to 30% strain in different orientations. Samples without tubules were compressed along the directions parallel and
perpendicular to the printing directions. For samples with tubules, compression in three directions were performed: perpendicular to both the tubule and printing direction, perpendicular to the tubules but parallel with the printing direction, and parallel with the tubules and printing direction.

The failure surfaces are shown in Figure 7.4. No shear bands were observed in samples without tubules in directions perpendicular to printing direction (a) and parallel to the printing direction (b). Small cracks were observed when compressed perpendicular to the lamellae (printing direction) and buckling of lamellae was observed parallel to the lamellae (b). Figure 7.4c shows the samples with tubules after compression in the direction perpendicular to the tubules, in which the elliptical tubules closed and cracks formed between the tubules. While tubule closure occurred in the sheep horns, no cracks were, possibly indicating that a more uniform stress distribution exists in the real horns. In the transverse direction (Figure 7.4d), shear bands formed along the diagonal direction, and the shape of the tubules changed under shear forces, which is similar to what occurs in the sheep horn. Figure 7.4e shows the failure surface after 30% compression in the longitudinal direction (parallel with the tubules), no buckling of the tubules was found, but buckling of the lamellae occurred.
Although similar compressive stress strain curves were found between 3D printed samples and real horn samples, the failure mechanisms were different. More stress concentration was found in 3D printed samples leading to the crack formation when compressed radially. Instead of buckling of tubules, buckling of lamellae was found in the 3D printed samples when compressed in the longitudinal direction. In terms of the energy absorption, more designs and simulations are still needed to decrease the stress concentration in the 3D printed samples.

The recovery properties of the single-phase 3D printed samples with tubules were also investigated. In the present study, the 3D printed VeroClear® models were compressed to 50% (strain before densification) at a strain rate $10^{-3} \text{s}^{-1}$, and then recovered at 62°C ($> T_g$) for 15 min. The recovered samples were tested again for the next cycle. Stress strain curves under different test cycles in different directions are shown in Figure 7.5a,b. When compressed in the radial direction (perpendicular to the tubules and print direction) (Figure 7.5a), the shape of the curves remained the same with only a slight decrease in the Young’s modulus and strength after three
cycles. However, in the longitudinal direction (parallel with the tubules and print direction) and transverse direction (perpendicular to the tubules but parallel with print direction), the samples were damaged and could not be recovered even after the first cycle (Figure 7.5b). This was due to the shear band formation and lamellae buckling found in Figure 7.4d,e. These findings correspond to the results found in the real horn samples, in which only samples compressed in radial directions can be recovered (Chapter 5, Figure 5.6). This could further inspire of the designs of recoverable energy absorption materials.

Figure 7.5 Compressive stress strain curves of samples after different cycles of loading and recovery in the radial (a), longitudinal and transverse (b) directions. The stress strain curves almost keep the same shape after 4 cycles of compression in the radial direction, while samples were failure after the 1st recovery in both longitudinal and transverse directions.

7.3.3 Impact resistance of bioinspired designs

Drop-tower impact tests were also conducted to investigate the impact resistant properties of the 3D printed samples. Four bioinspired designs were printed out, shown in Figure 7.6a: from the left to the right: single-phase VeroClear® solid structure, double-phase VeroClear® and Tangoblackplus® lamellar structure, double-phase tubular structure, and double-phase tubule reinforced structure. The double-phase tubular and double-phase tubule reinforced structures were bioinspired designs based on horn and hoof structure, respectively. After 100 kJ/m² impacts in the drop tower impact tests, samples failed differently. The top and bottom failure surfaces are shown
in Figure 7.6b and c. The single-phase solid model fractured into pieces (left side), while the other three double-phase samples were intact, although damage and cracks were found. This indicates the lamellar structure with both soft and stiff layers could dissipate impact energy and protect the whole structure from fracture.

The crack propagation behavior was studied with optical microscopy (Figure 7.6d). Among the three double-phase designs, crack propagation to the corners of the samples were observed in both double-phase lamellae and tubular structure (middle two models), while in the tubule reinforced designs (right one), damages and cracks were limited in between the reinforced tubules, and no cracks were found beyond the tubules. This can be explained by the presence of reinforced tubules, which may redirect crack propagation, thus increasing the toughness. More work is still needed to further quantify the fracture toughness of different designs.
Figure 7.6 3D printed samples for drop tower impact tests at an impact energy $E_n = 100 \text{ kJ/m}^2$. (a) Four kinds of sample designs, from left to the right: single-phase VeroClear solid model; double-phase VeroClear and Tangoblack plus lamellar model; double-phase VeroClear and Tangoblack plus tubular model; double-phase VeroClear and Tangoblack plus tubule reinforced model. (b, c) Top and bottom surfaces of samples after impact tests. (d) Optical microscopy images showing damages and cracks.

### 7.4 Conclusions

3D printed bioinspired designs based on the lamellar and tubular structures found in the horn and hoof were fabricated. Both quasi-static energy absorbent properties and drop tower impact resistances were investigated. The recovery properties of the 3D printed VeroClear® were also studied. The main findings are:
• For quasi-static compression tests, the single-phase VeroClear© designs absorbed more energy than the double-phase models, which have weak interfaces that caused a significant decrease of strength and stiffness. Anisotropic behavior was also found in the designs with tubules, similar to the bighorn sheep horn.

• More severe stress concentrations were identified in the 3D printed samples, leading to lower energy absorption. Closure of tubules, shear banding and lamellae buckling were energy absorption mechanisms found in both horns and bioinspired designs.

• The recovery (by heat) of the 3D printed samples was studied. 3D printed tubular models can recover back to their original shape, stiffness, and strength after compression to 50% in the radial direction (perpendicular to the tubules). Compression in the other two directions cannot be recovered due to the presence of severe defects. This anisotropic recovery was similar to the bighorn sheep horn.

• Drop tower tests showed that the double-phase designs had higher impact resistance than single-phase designs. The tubule reinforced designs show promising crack deflection and redirection.

Chapter 7, in full, is currently under preparation for publication. This work is coauthored by F. Su, J. McKittrick. The dissertation author is the first author of this work.
CHAPTER 8: SUMMARY AND FUTURE WORK

8.1 Summary

Keratinized tissues found in nature were efficient impact resistant and energy absorbent materials. Bighorn sheep (*Ovis canadensis*) horns can absorb most of the impact energy thus protecting the skulls and brains during the intraspecific fights at a speed of ~ 9 m/s (20 mph). Horse (*Equus ferus caballus*) hooves protect the inside bony skeletons by dissipating the energy transmitted from the uneven ground surfaces. The motivation of the present study is trying to understand the impact resistant and energy absorbent mechanisms in both horns and hooves, and further develop bioinspired strategies to mimic the structure and material designs found in nature, thus fabricating synthetic materials with high impact resistance and energy absorption. Our hypotheses are:

- Both horns and hooves are impact resistant and energy absorption materials due to the general loading conditions in these two tissues. The mechanical properties should be related to their characteristic structural and materials designs.

- Anisotropic behaviors exist in both tissues, since the bighorn sheep horns are under impacts perpendicular to the horn surface and the tubules inside, while horse hoof is suffering forces from the ground, which is parallel with the hoof surface and tubules. The preferable impact directions should be same as the real impact direction occurs in nature.

- Synthetic bioinspired materials based on the above findings will have ideal impact resistant and energy absorbent properties.

To validate these hypotheses, several experiments were conducted. The hierarchical structures of bighorn sheep horn and equine hoof were studied from molecular to macroscale using wide-angle X-ray diffraction (WAXD), scanning and transmitting electron microscopy (SEM and
TEM), optical microscopy and micro-computed tomography (µ-CT). Mechanical tests on horn and hoof samples such as compression at different strain rates ($10^{-3}$ s$^{-1}$ to $10^{3}$ s$^{-1}$) and nanoindentation to characterize the local mechanical properties were performed. By investigating the structure-property relationship in these keratin tissues, the fundamental energy absorption mechanisms thus can be understood. Bioinspired designs based on the structural designs found in horns and hooves were further fabricated using 3D printing.

The major microstructural elements of horns were tubules and cell lamellae, which were oriented with (~30°) angle with respect to each other. The cell lamellae contained keratin cells in the shape of discs, having an average thickness of ~2 µm and a diameter of ~20–30 µm. The morphology of keratin cells revealed the presence of keratin fibers and intermediate filaments (IFs) with a diameter of ~200 nm and ~12 nm, respectively, in the cell plane. Tubular structures were also found in equine hooves, however, the structures were different. Tubules were found embedded in the intertubular matrix forming the hoof wall at the microscale. The total porosity was ~3% (medullary cavity), with the tubules comprising ~30% of the whole area, while the porosity was ~7% in bighorn sheep horn. Cell sizes, shapes and IF fractions are different between tubular and intertubular regions: cells in intertubular areas are irregular polygons ~19.5 ± 4.8 µm, while cells in tubular areas are lamellar ~18.8 ± 2.6 µm in diameter, ~4.8 ± 1.1 µm in thickness with the thickness direction perpendicular to the tubules. The intertubular matrix showed a higher amount of IFs (32.2%) than that of the tubular area (21.3%).

The structural features in both horns and hooves were correlated to the mechanical behaviors and energy absorption performance in different orientations and hydration states. Quasi-static and high strain rate impact experiments, in different loading directions and hydration states, revealed a strong strain rate dependence for both dried and hydrated conditions. A strong
anisotropic behavior was observed under impact for the dried state. The results showed that the radial direction was the most preferable impact orientation because of its superior energy absorption. Detailed failure mechanisms under the aforementioned conditions were examined by bar impact recovery experiments. Shear banding, buckling of cell lamellae, and delamination in longitudinal and transverse direction were identified as the cause for strain softening under high strain rate impact. While closure of the tubules occurred in both quasi-static and impact tests (radial and transverse directions), the radial direction led to more energy absorption and impact resistance. Due to the structural differences between horn and hoof, the mechanical properties and energy absorption mechanisms were different. The stiffness and hardness in tubule areas were higher than those in the intertubular areas in the dry and fresh samples. When the samples were fully hydrated, the intertubular areas became stiffer than tubular areas due to the higher water absorption in that region. The compression behavior of samples in different loading directions and strain rates were studied. Isotropy and strain rate dependence of the mechanical properties were documented. The radial direction was no longer the preferable impact directions as it was in the horns, since the loadings in real hoof were from the ground surface, which was along the longitudinal direction. In the samples, the tubules served as a reinforcement, supporting the whole structure under compression. Elastic buckling and cracking of tubules were observed after compression in the direction of the tubules. No shear-banding or severe cracks were found in the intertubular areas even after 60% compression longitudinally, indicating efficient energy absorption properties without failure.

By comparing the energy absorption mechanisms in horns and hooves, one can find that the horn structure was designed for impacts in one direction, while hoof showed similar energy
absorption capabilities in all the three directions, which were also higher than horns at the same hydration level.

Due to the remarkable impact resistant and energy absorbent properties found in bighorn sheep horns, the keratin materials and tubular structure were investigated from an evolutionary perspective. The microstructures as well as the mechanical properties of horns from four different species: the bighorn sheep (*Ovis canadensis*), domestic sheep (*Ovis aries*), mountain goat (*Oreamnos americanus*) and pronghorn (*Antilocapra americana*) were investigated. Microstructural similarity was found where disk-shaped keratin cells attach edge-to-edge along the growth direction of the horn core (longitudinal direction) forming a lamella; multiple lamellae were layered face-to-face along the impact direction (radial direction, perpendicular to horn core growth direction), forming a wavy pattern surrounding a common feature, the tubules. Differences among species included the number and shape of the tubules, the orientation of aligned lamellae, and the shape of keratin cells. The differences in mechanical properties among species might relate to their different fighting behaviors: high stiffness and strength in mountain goat to support the forces during stabbing; high tensile strength in pronghorn for interlocked pulling; impact energy absorption properties in domestic and bighorn sheep to protect the skull during butting.

Water-assisted recovery behavior of horn was investigated. WAXD experiments showed that water went into the amorphous matrix of keratin, but did not have effects on the structure of crystalline IFs. Water affected the structure further, causing differences in tensile behaviors of horn samples in different hydration states. *In-situ* tensile tests showed more IFs deformation in fully hydrated samples due to the weak amorphous matrix. Higher creep strain was also noticed in hydrated conditions. Horn keratin could recover back from severe deformations after hydration,
which was due to the water effects on the amorphous matrix by breaking and reforming hydrogen bonds.

The hierarchical structures as well as impact resistant and energy absorbent properties found in horns and hooves in the present work, were also applied to fabricated bioinspired synthetic materials using 3D printing. Preliminary energy absorbent and impact resistant materials were fabricated using single-phase VeroClear® and double-phase VeroClear® and Tangoblackplus® materials. The 3D printed designs with lamellae and tubules showed promising quasi-static energy absorption and high-speed impact resistance. However, more quantitative analysis and experiments are still needed in the future work.

In summary, the original findings in this thesis work are listed as follows:

- The hierarchical structures in bighorn sheep horns and equine hooves were different:
  - At microscale level, tubular and lamellar structures were found in both horns and hooves, however, the hoof had ~30% tubular and ~70% intertubular areas while no tubular areas were found in the horn.
  - Lamellae were formed by keratin cells in both horns and hooves, however, keratin cell shapes and sizes were different. In the hoof, the cells in intertubular areas were irregular, while cells in tubular areas were formed a lamellar structure. In the horn, all cells had a lamellar structure.
  - At the nanoscale, IFs were identified arranged in the cell planes in both horns and hooves. The IF fraction in horns was uniform, while the IFs fraction was higher in intertubular areas than that in tubular areas in the hoof.
- The anisotropic mechanical behavior in horn and isotropic mechanical behavior in hoof were found, resulting in different energy absorption mechanisms:
➢ The most impact resistant and energy absorbent direction in the horn was the radial direction, which was perpendicular to the horn surface, while the hoof showed isotropic energy absorption.

➢ Closure of the tubules was the main energy absorption mechanism in the radial direction in the horn, while buckling of tubules and shear-banding were failure mechanisms in the other two directions, which led to lower energy absorption.

➢ The tubules in hoof served as a reinforced structure due to the higher stiffness than the intertubular areas. Elastic buckling and cracking of the tubules were the main energy absorption mechanisms in the hoof. The hoof showed higher energy absorption capability than the horn.

• Microstructures evolved in horns from different species that were directly related to their fighting styles. Highest tubular density was identified in bighorn sheep horn and showed better impact resistance than domestic sheep horns. No tubules were found in mountain goat horn, resulting in the highest compressive and tensile strength among the different species, which benefited a stabbing fighting style.

• Self-healing mechanisms with the assistance of water in horns were discovered for the first time. ~70% of the strength and ~64% of the energy absorption amount remained after six cycles of compression and recovery. Water molecules entered the amorphous matrix, broke and reformed hydrogen bonds, which was the main recovery mechanism. The intermediate filament crystal structure was not affected by hydration.

• The tubular and lamellar structures found in horns and hooves were replicated by using multi-materials 3D printing. The 3D printed materials showed promising energy absorption and impact resistance.
8.2 Future work

Based on the results of the present work, some problems still remain unanswered. Although it is known that animal horns and hooves can withstand forceful and dynamic impact loads, the fundamental mechanisms behind this behavior are not known. In terms of the bioinspired designs for impact resistance and energy absorption applications, more work is needed to improve the impact resistant properties and acquire statistical results. Finite element analysis can be applied as a validation method to support the design strategies and mechanical tests results from the experiments. Thus, the future work could focus on the following two points:

- How the two-phase arrangements of crystalline intermediate filaments embedded in an amorphous matrix at nanoscale along with the hierarchical structural features of animal horns assist in energy absorption and resist high-speed impacts.

- Fabrication of synthetic materials based on the hierarchical structures found in horn and hoof. 3D printing can be used to realize the tubular and lamellae as well as the IF/matrix structures at different scales. Mechanical properties as well as energy absorbent mechanisms will be investigated.

In order to answer the first question, experiments at smaller scales (nano- to micro-scale) on the horn and hoof samples are needed. Nanomechanical tests such as in-situ compression and tensile tests in TEM will provide deformation information on the intermediate filaments and amorphous matrix phases. Nano-impacts will be also helpful for understanding the roles of individual IFs and matrix under compression at different strain rates. Thus, energy dissipation mechanisms at small scales can be understood clearly. Small-angle X-ray scattering (SAXS) and wide-angle X-ray diffraction (WAXD) can be applied to visualize and monitor the deformations on the crystal structure of IFs. Orientation changing and phase transformation of IFs under
different loading conditions thus can be determined. Energy absorption mechanisms then can be better understood at a molecular level.

For the development of bioinspired designs of impact resistant and energy absorbent materials based on 3D printing, the hierarchical structure of horn and hoof will be considered and mimicked. Although the tubular and lamellar structures were printed out and tested in the present study, there is a lot of room to improve the impact resistant and energy absorbent behaviors such as changing tubule sizes, porosities and distributions. Finite element analysis (FEA) can be supplemented to support the experiments. Since the IF/matrix fiber reinforced composite structure at the nanoscale is important in all keratin materials, in future 3D printing designs, these nanoscale features can be included in the designs. By changing the IF fractions and orientations, the relationship between the nanostructure and the mechanical properties can be revealed.
REFERENCES


167


[214] Cardamone JM, "Investigating the microstructure of keratin extracted from wool: Peptide sequence (maldi-tof/tof) and protein conformation (ftir)," *Journal of Molecular Structure, 969*, 97-105 (2010).


