

# Disordered Structures in Biology Can Provide Material Properties not Obtained with Precise Hierarchy

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Biological organisms exist in an astounding array of habitats. The materials produced by these organisms correspondingly exhibit significant diversity in function and form. Highly ordered and structured biological materials have been the subject of intense scientific investigation, especially in the context of exploring possible models for functional materials. Biological materials lacking in organized structure, on the other hand, such as barnacle exoskeletons, have largely been ignored. Here disordered biological materials falling into four different classes are discussed: soft and organic, hard and organic, soft and a composite of organic and inorganic, and hard and a composite of organic and inorganic. Across the examples within each class of materials one can see disorder at different length scales providing increases in fracture resistance or flexibility, suggesting disordered biological materials have much to teach us in terms of materials design.

## 1. Introduction

Materials produced by biological organisms vary widely in function and form. Spider silk combines high strength as well as high energy absorption capabilities, enabling arachnids to catch large insects and sail through the air.<sup>[1]</sup> Abalone produces a shell for protection that has an inner nacreous layer that is 3000 times more resistant to fracture than the materials from which it is made.<sup>[2]</sup> Developing an understanding of the unique structures and compositions of biological materials can provide insights into how these organisms evolved and how new materials may be designed.

At the atomic scale, materials are characterized by short-range to long-range order. Short-range order describes the interaction between an atom and its nearest neighbors while

long-range order describes the atomic arrangement over the entire material. Crystalline materials or proteins with a defined tertiary structure have both long-range and short-range orders, while amorphous materials can have short-range order but lack long-range order. At larger length scales, nanometer to millimeter, the structure of a material is determined by the arrangement of the components. An ordered material consists of components arranged in a defined, periodic, or predictable way, while a disordered material consists of components that are arranged at random in terms of distribution, size, shape, and/or orientation.

The majority of biological materials that tend to capture our attention have

very long-range order. They are often structured in such a way that the critical elements are oriented in regular, quasiperiodic structural units, which can be tens of nanometers to tens of micrometers in size. Giant squid sucker disks, whelk egg capsules, and coconut shells are a few examples of biological materials with such higher-level order. Giant squid sucker discs exhibit microstructural order with repeating 200–300 nm protein tubules aligned parallel to one another.<sup>[3]</sup> On the millimeter-scale, the pores separating adjacent tubules smoothly decrease in size and spacing from the inside of the tooth to the outside.<sup>[3]</sup> The protein fibers making up whelk egg capsules arrange into 10–20  $\mu\text{m}$  thick sheets of aligned fibers with each sheet rotated 90° from the next, resulting in a plywood-like structure with 20–40  $\mu\text{m}$ , the thickness of two sheets, periodicity in the direction of sheet stacking.<sup>[4]</sup>


Other ordered materials have hierarchical structures, in which the ordered, repeating units span several length scales. A few examples of such hierarchical structures are wood, seashell nacre, lobster cuticle, and bone.

In the case of bone, for instance, structure scales from the atomic level with bonds within collagen polypeptides to the micrometer and millimeter levels with fibers, lamella, and osteons. The building blocks of bone are three collagen polypeptides, each a left-handed helix, that twist together into a right-handed triple helical molecule that is stabilized by hydrogen bonds. The triple helix collagen is  $\approx 1$  nm in diameter and several hundred nanometers in length. Triple helix collagen combines with nanocrystalline hydroxyapatite platelets to generate well-ordered fibrils almost a micrometer in diameter. Fibers and lamella (plates of collagen fibers) provide larger scale structures at the micrometer to millimeter levels, finally coming together to form the bone. Features at each of

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these length scales play key roles in obtaining the impressive properties such as high toughness that biological materials are well known for.<sup>[5]</sup> In hierarchical materials, such as bone, the smallest structural levels are highly ordered (tropocollagen is arranged with near perfect periodicity within collagen fibrils), but the order and the periodicity decrease at large length scales (the spacing between osteons in cortical bone is nonuniform).

As with many largely ordered materials, there are also features in bone which have no defined order. For example, the extracollagenous matrix within collagen fibrils or the cement lines at the interface of osteons in cortical bone. These structural elements play a major role in mechanical performance, by mediating the gliding of collagen fibrils among one another<sup>[6]</sup> or by deflecting propagating cracks.<sup>[7]</sup> Similarly, recent work on lamellar bone found three types of sub-lamellae: ordered plywood-like mineralized collagen fibrils, unidirectional mineralized collagen fibrils, or disordered lower density mineralized collagen fibrils with the disordered region having greater compliance.<sup>[8]</sup> Thus, in bone, interplay between highly ordered, hierarchical elements and disordered regions provide the material with its overall mechanical properties.<sup>[9]</sup>

In addition to these hierarchical, largely ordered biological materials are those that are largely disordered, with structures consisting of irregular elements with either no clear repeating elements or no distinct organization of elements therein (in these materials the structural correlation decays over very short distances). Such disordered materials have been examined in a few fields including traditional biomechanics of human tissues<sup>[10]</sup> and the amorphous phases found within many biomaterials.<sup>[11]</sup> However, this aspect of structure has been often ignored in the overall exploration of biological materials.

This article aims to explore several disordered biological materials and provide models for how these systems function. As material functionality depends on all levels of structure from the atomic scale to the millimeter-scale, we explore and describe the level of order across each of those scales. In some of the materials, there is order at a particular length scale, while the majority of the material is disordered. In others, the material is disordered across several length scales. As above, we define disorder at a given length scale as a lack in periodicity or predictability of the structure. Collagen molecules provide a perfect example of a highly ordered material given the 67 nm internal periodicity in structure. The disordered sub-lamellae in bone in which these mineralized collagen fibrils are laid down in a random arrangement provide an example of a disordered microstructure. Our investigation of disordered biological materials will focus on systems that are largely disordered and fall within four different classes: soft and organic (Section 2.1), hard and organic (Section 2.2), soft and a composite of organic and inorganic (Section 2.3), and hard and a composite of organic and inorganic (Section 2.4).

## 2. Biomaterial Structures and Properties

### 2.1. Soft and Organic

Organisms make a large number of soft materials to fulfill various functions such as compartmentalization (e.g., phospholipid



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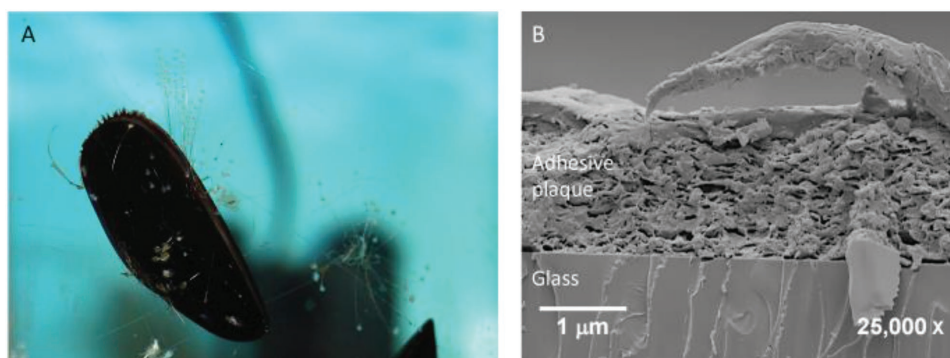
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**Figure 1.** A) The byssal assembly mussels use to attach to a substrate can be seen here emanating from this particular mussel's body. The dark threads have several tan adhesive plaques that are used to adhere the mussel to the glass. B) A scanning electron microscope (SEM) image of a freeze fractured cross-section of the adhesive plaque (top) on glass (bottom) highlights the irregular structure of the adhesive.

bilayers), motion (e.g., actin fibers), transportation (e.g., spider silk), and protection (e.g., skin). Disordered organic, soft materials have several general characteristics, described in greater detail below for two examples: mussel adhesive and skin. Although these two examples have no periodicity regarding how proteins are arranged within the material, the proteins from which they are made do show varying levels of order. Collagen, found within skin, is a highly ordered protein. Skin elastin proteins have no tertiary structure. Mussel byssus proteins show some regions of crystallinity. Thus, both materials lack microstructural order, though have a level of nanometer and atomic scale order. These two examples help to illustrate the importance of ideal chain statistics, cross-links, rubber-like networks, entropic elasticity, and poroelasticity in determining materials properties.

### 2.1.1. Mussel Adhesive

Mussels cling to rocks while often being pounded by waves. When attaching to substrates they are able to cluster together into communities, thereby mitigating disturbances from the waves, preventing predators such as sea gulls from carrying them away, and residing close together for efficient reproduction. These shellfish attach with a byssal adhesive assembly, seen in **Figure 1B**, which contains several fascinating materials aspects. From the substrate up to the organism there is the adhesive plaque, the threads, and then attachment to soft tissue within the shell.

Both the plaques and threads are protein-based materials. As far as we know at this point, the near dozens of plaque proteins do not exhibit any particular structural features with three identified as being intrinsically disordered or having disordered regions.<sup>[12]</sup> The lack of structure within the proteins indicates the plaque has at least some, if not a considerable amount, of long-range atomic disorder. Iron induces oxidative cross-linking of 3,4-dihydroxyphenylalanine (DOPA) residues within these proteins.<sup>[13,14]</sup> However, the level of iron is low, in the parts per thousand range, thus the material is nearly all protein. Several proteins are cross-linked to form the plaque. This reactive chemistry is somewhat indiscriminate, with organic radicals seen to be part of the cross-linking process.<sup>[13]</sup>

Consequently, the ability to generate regular microstructures appears unlikely, with such ordered structures unobserved. In vitro cross-linking of extracted plaque proteins with iron transformed an open hydrogel to a more dense system, with heterogeneity persisting both prior to and after such a reaction. No clearly defined periodic structural features were observed.<sup>[15]</sup> Microscopy images of plaque interiors have shown foam-like structures on the  $\approx 100$  nm and  $\approx 1$  mm scales.<sup>[16]</sup> Large pores are embedded in a mesh network consisting of thin struts  $\approx 100$  nm in diameter.<sup>[16]</sup> This system is covered by a more dense outer coating made from separate DOPA-containing proteins. Although foamy, the plaque interiors do not exhibit organization of pore size or shape or mesh network at the micrometer to millimeter scale.<sup>[16]</sup> **Figure 1C** shows a cross-section obtained after freeze fracturing the adhesive and glass substrate. Note the lumpy, irregular appearance. Based on experiments in which plaques were formed at colder temperatures, resulting in thinner network struts and a weaker plaque, the struts are thought to distribute loads throughout the plaque, making it more robust.<sup>[16]</sup> The plaque is also found to be highly deformable, exhibiting plastic yielding at 20% strain.<sup>[17]</sup> The plaque, thus, dissipates the strain energy through deformation, resulting in stronger surface–plaque adhesion.<sup>[17]</sup> While the exact mechanics behind this plasticity is unknown, protein structure, molecular bonding, and even microstructure, all of which exhibit disorder within the plaque, likely play a role.<sup>[17]</sup>

In potential contrast to adhesive plaques, the mussel byssal threads do exhibit order. The proteins here are collagen-like and arranged into semi-crystalline structures. This material architecture is even more complex in that there are two distinct regions of the threads. The distal portion of the thread, closest to the rock, is more rigid.<sup>[18]</sup> The proximal thread is flexible, being comprised more of proteins resembling those of elastin.<sup>[18]</sup> Much like the plaques, the threads do contain notable levels of metal ions such as iron, although the loadings are low and nearly all of the material is made from proteins.<sup>[19]</sup> Whereas metal ions may be distributed homogeneously to create cross-linking in helping the plaque form, here in the threads, metals are clustered into granules to provide a degree of self-healing capability noted by the simultaneous ability of being both hard and flexible.<sup>[19]</sup>

The byssus is constructed with a modulus gradient to handle the inevitable pulling and tugging from the mussels' high wave intensity environment as well as potential predators.<sup>[20]</sup> If the mussel were to stick its very low modulus internal tissue right onto an extremely high modulus rock there would be concentrations of mechanical stresses at such a hard–soft interface. Each time the animal is pushed around, the stress buildup might cause the adhesive joint to fail. To avoid this problem, these shellfish distribute mechanical stresses by judicious arrangement of the aforementioned materials into gradated moduli.<sup>[18]</sup> The rock is hardest, the adhesive plaque has a lower modulus than the rock but higher than the internal tissues, the thread modulus is lower still than the plaque, and then the inside tissues are the softest.

A highly ordered or crystalline material would likely have a single modulus. Such a design might resist waves up to a point, but then break. Sharp differences in moduli would lead to interfacial stresses and potential mechanical failure. Furthermore, if an adhesive were to be well ordered it would not be able to flow across substrates to maximize interfacial contacts and strong attachment. The entire mussel byssus appears to combine ordered threads with seemingly disordered plaques for enabling surface attachment robust enough to withstand life within the challenging intertidal zone.

### 2.1.2. Human Skin

Skin is a relatively thin and highly deformable organ that covers the body of vertebrates. Skin provides protection against mechanical, chemical, and pathogenic threats from the outside environment. It also fulfills a myriad of other functions including protection against water loss, temperature regulation (sweat), sensing, storage for lipids and water, and oxygen absorption.

The two primary layers of mammalian skin are the outer epidermis (50–150 micrometer thick) and the dermis (1–4 mm thick), which largely governs the mechanical response of skin.<sup>[21]</sup> The dermis is composed of a hydrated random network of collagen fibers (70–80% dry weight) and elastin fibers (4% dry weight) embedded in a proteoglycan matrix. Collagen fibers in skin have the same general structure as collagen in bone, with the polypeptides arranged in a triple helical structure. Elastin fibers are more complex with an amorphous inner core and an outer mantle of microfibrils. On the microscale, the proteinaceous network is largely random, although there is some orientation and prestresses at the local level (the “Langer lines”). Skin and other “natural elastomers” are among the most deformable and weakest biological materials. Elastic moduli fall in the 1–30 MPa range, and strengths are at 0.5–10 MPa.<sup>[22]</sup>

Under uniaxial tension, the initial mechanical response of skin is largely governed by progressive alignment of the fibrillar network,<sup>[23]</sup> which occurs with little viscous dissipation.<sup>[24]</sup> The mechanical resistance is predominantly provided by the progressive rearrangement of collagen fibers with the elastin fibers providing little contribution.<sup>[25]</sup> Instead, elastin provides “recoil” to the network, so that skin can regain its initial state once the forces are removed. The first stage of deformation, which corresponds to normal physiological strains (0–30% strain), can therefore be captured with elastic models based on changes of entropy.<sup>[23]</sup> At larger strains, the alignment of collagen fibers

is high, so that further stretch pulls on the fiber backbone producing a much stiffer and quasilinear response.<sup>[23]</sup> This progressive stiffening of skin is critical to its extraordinary resistance to tear.<sup>[26]</sup> The large deformations near a tear turn a sharp geometrical tip, which can concentrate stresses, into a rounded edge that entails much reduced stresses. In addition, the collagen fibers align in the direction of loading at the tip of the tear thereby reinforcing the material in regions that are most likely to tear further. Skin is therefore a seemingly disordered material in which the disordered microstructure of the fibers, combined with the atomic scale disorder of the nonstructured protein domains, enables them to rearrange into a highly ordered, robust structure on the millimeter scale under stress along any direction. Overall, the resistance to tear is extraordinary.<sup>[26,27]</sup>

## 2.2. Hard and Organic

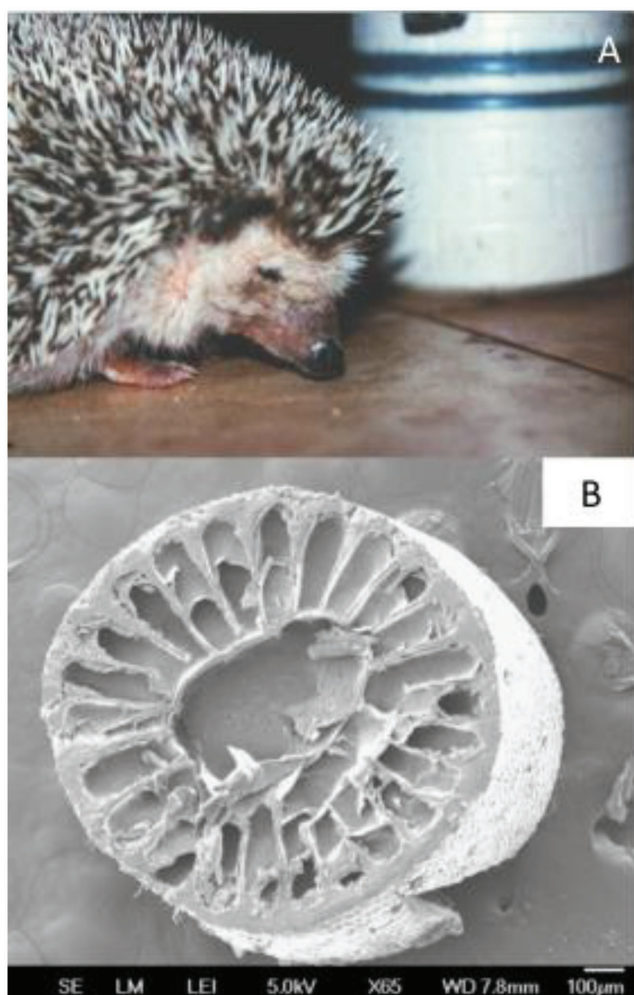
Hard materials are those that are inflexible and resistant to deformation under applied forces. Organisms make hard materials for functions ranging from protection (e.g., exoskeletons) and scaffolding (e.g., bones) to eating (e.g., teeth). The vast majority of hard biomaterials consist of a combination of organic and inorganic components (Section 2.4) or are highly ordered and hierarchical (e.g., coconut shells); there are few disordered, hard, organic materials. Hedgehog quills (Section 2.2.1) are one example of a material with a disordered microstructure that is completely organic, yet hard.

### 2.2.1. Hedgehog Quills

Hedgehogs make up a family of small mammals that are known for their spiny quills that cover the bodies (**Figure 2A**). The uniform quills are used to protect the hedgehog from predators and injury associated with falls.<sup>[28,29]</sup> The hedgehog is able to curl its body into a ball such that the quills are facing outward, offering the necessary protection. These quills are entirely made of keratin, the same basic protein from which human hair is made, though the resulting quill structure is robust and resistant to buckling.

Keratin is found in two basic polymorphs,  $\alpha$ -keratin and  $\beta$ -keratin, which are differentiated by the way in which polypeptide chains making up the protein associated with one another. For both forms of the protein, the polypeptide chains form into filaments, which subsequently interact with an amorphous matrix portion of the protein to generate filament–matrix composites. In  $\beta$ -keratin, the filament is formed of  $\beta$ -pleated sheets, resulting in a filament  $\approx 3$  nm in diameter.<sup>[30]</sup> The overall structure of  $\alpha$ -keratin is more hierarchical with each individual polypeptide helical chain associating, through sulfur cross-links, with a second helical polypeptide chain to form a dimer. The dimers then subsequently form protofilaments, which polymerize to form intermediate filaments. The resulting intermediate filaments are slightly larger than the  $\beta$ -keratin filaments with an  $\approx 7$  nm diameter.<sup>[30]</sup> The intermediate filaments are what bond to and are embedded in the amorphous keratin matrix in  $\alpha$ -keratin. Thus, the  $\alpha$ -keratin protein is a composite consisting of regions of atomic disorder and atomic order.





**Figure 2.** A) A picture of an African pygmy hedgehog shows the white quills that cover the body. Reproduced with permission.<sup>[71]</sup> Copyright P. Van Wynsberghe. B) An SEM image of a hedgehog quill in cross-section. Reproduced with permission.<sup>[32]</sup> Copyright 2016, The Author(s), Published by the Royal Society. The scale bar is 100  $\mu\text{m}$ .

Significant work has been done on the mechanical properties of keratin.<sup>[30–34]</sup> In  $\alpha$ -keratin, the number of intermediate filaments per unit of matrix and the orientation of the intermediate filaments has been found to affect the mechanical properties of the keratin based material, as has the level of hydration.<sup>[30–33]</sup> Despite some disagreement over the mechanism by which hydration affects keratin's materials properties, it is agreed that higher hydration results in an overall decrease in the strength and Young's modulus of a keratinous material, whereas an increase in the amount of intermediate filaments relative to matrix or an increase in the alignment of the intermediate filaments results in a higher Young's modulus.<sup>[31,32]</sup>

Hedgehog quills consist entirely of  $\alpha$ -keratin,<sup>[35]</sup> although with an unknown volume fraction of matrix to intermediate filament. The keratin within the quill is arranged into a circular structure with a distinct interior and exterior, as shown in Figure 2B. The outer portion of the quill is a thin ( $\approx 0.05$  mm) wall that varies in form from fibrous, near the very tip of the quill, to no microstructure everywhere else.<sup>[29]</sup> The interior of the quill is

more complex, consisting of both longitudinal and radial elements (Figure 2B,C).<sup>[29,36]</sup> The longitudinal elements, stringers, run the length of the quill, yet do not span the cross-section.<sup>[29]</sup> The radial elements, called septa, form walled chambers of varying size and shape across the interior cross-section. The result of these features is a multichambered, foam-like core.<sup>[29]</sup> The overall structure thus appears to be a slightly curved tube with an unstructured solid protein exterior and a largely hollow interior broken into randomly sized chambers by protein walls.<sup>[28,29]</sup>

This quill structure and composition enables the system to remain flexible without breaking or buckling. A classic experiment elucidating the mechanical properties of the hedgehog quill was conducted in 1986 by Vincent et al. when they compared the hedgehog quill to that of a porcupine quill.<sup>[29]</sup> The porcupine quill has a similar structure and composition to the hedgehog quill, but with a more organized chambered interior and a fibrous exterior in which the keratin intermediate filaments are largely aligned along the long axis of the porcupine quill.<sup>[29,31]</sup> Vincent et al. found the hedgehog quill to be a remarkable  $3\times$  stronger than the porcupine quill.<sup>[28,29]</sup> The hedgehog quill's resistance to failure when under compression was attributed to the combination of little cross-linking in the keratin making up the outer wall, in addition to the foam-like core made up of the longitudinal and radial support structures in the interior of the quill.<sup>[28,29]</sup> Thus, the robustness of the hedgehog quill has two components—the keratin composition and interactions that occur at a molecular level along with the way in which the keratin proteins are arranged into the overall structure of the quill at the micrometer to millimeter level.

Recent experiments have focused on exploring the role that keratin protein structure plays on the material properties of the quill, by examining how hedgehog quills behave under compression and bending as a function of temperature and humidity.<sup>[37,38]</sup> For individual spines, it was found that, at room temperature, flexural strength and modulus decreased as the level of humidity increased.<sup>[37,38]</sup> For groups of spines in a model pelt, it was found that increased humidity resulted in spines that were more durable to compression impacts, but less able to absorb the energy associated with an impact.<sup>[38]</sup> The authors of these two studies hypothesized that a decrease in strength and stiffness with humidity comes from bonds within the amorphous keratin matrix being disrupted, resulting in an overall weaker material that is more flexible.<sup>[37,38]</sup> This hypothesis supports Vincent and Owers' model that decreased cross-linking or increased atomic disorder within the keratin protein resulting in a more flexible material. Further work is needed to elucidate the finer details of the volume fraction of amorphous matrix in the hedgehog quill and how each component of keratin protein contributes to the hedgehog quill robustness and flexibility.

The second component to the hedgehog quill's robustness, and the location of additional disorder, is in the overall structure at the millimeter scale. Whereas the outer circular wall of the quill provides the quill with overall stiffness and resistance to bending that can also be found in synthetic materials, such as metal struts, it is the core of the quill, with its nonuniform foam-like structure that is largely responsible for the quill's elasticity and resistance to buckling.<sup>[28,31,36]</sup> Given that there is no straight path across the quill, due to the nonuniformity

and disorder of the foam core, stress applied to the quill is dissipated, making the quill more robust. The longitudinal supports in the quill provide further strength by transmitting the stress along the length of the quill, preventing local buckling. Thus, the combination of disorder within the structure of the quill, from macroscopic to nanoscopic scales, provides the quill with more elasticity and flexibility than is observed in similar materials that are more ordered, like the porcupine quill.

### 2.3. Soft and Organic/Inorganic Composite

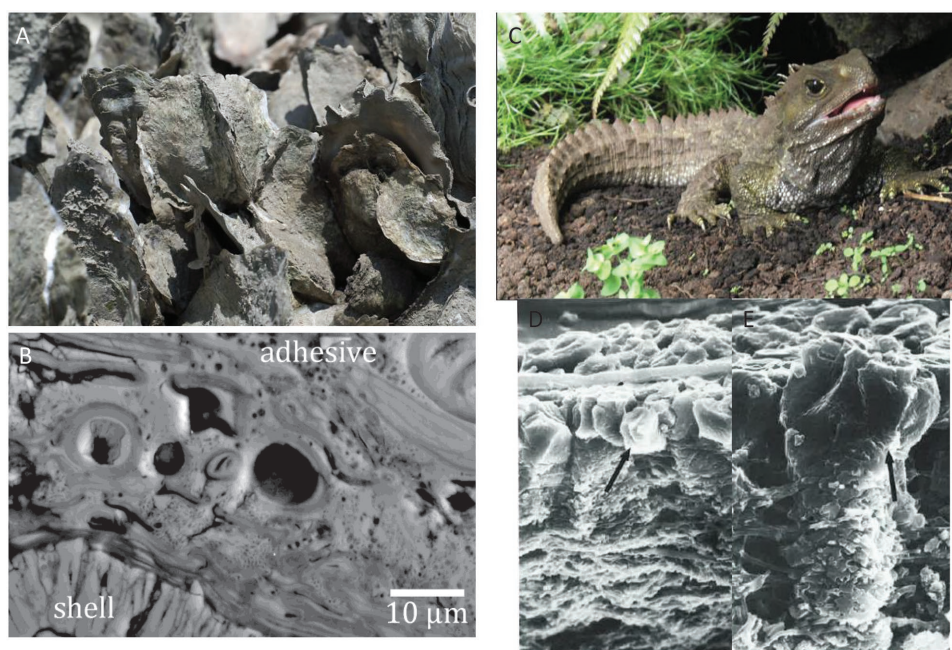
Protein networks are relatively deformable under mechanical stress, making them largely unsuitable for large skeletons or protection. For this reason, organisms very early in the course of evolution developed mineralized soft tissues for structural purposes. Organic–inorganic composites are ubiquitous in nature including, for example, bones, teeth, shells, and whale baleens. The stiffness of these tissues generally scales with mineral content. Bone (30–55 vol% mineral) is stiff,<sup>[39]</sup> whereas highly mineralized tissues such as tooth enamel or mollusk shells (>95 vol% mineral) are much stiffer. Most mineralized tissues display a high degree of organization at the microscopic scale although there are also examples of mineralized tissue with randomly oriented mineral reinforcements, as seen here in Sections 2.3.1–2.3.2. These organic–inorganic disordered materials have been studied even less than the organic disordered materials mentioned above.

#### 2.3.1. Oyster Cement

Much like mussels and other shellfish seen at the beach, oysters live a life dependent upon adhesion. The Eastern oyster, *Crassostrea virginica*, is the dominant species on the US east and gulf coasts. This animal produces a cement that enables the oyster to bond onto substrates, most often other oysters, forming extensive reefs (Figures 3A and 4C). These impressive structures provide the animal with a habitat in which they live for the entirety of their life while also giving a home to other organisms, providing coastal protection from storm surges, and filtering enormous volumes of surrounding water.<sup>[40]</sup>

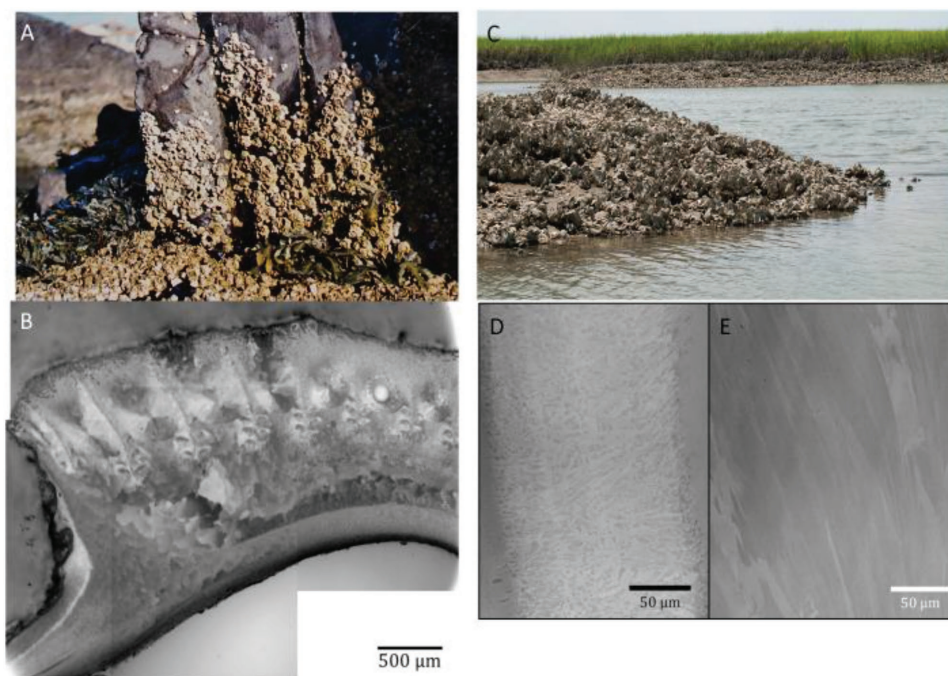
Reef building begins when oyster larvae are dispersed through the water column along the coast and eventually settle onto a substrate.<sup>[40]</sup> This initial settlement becomes irreversible when the larvae eject a premade, organic, hydrated glue.<sup>[41]</sup> Later in life, the animals generate their adult cement, which is a unique combination of organic and inorganic materials.<sup>[42–44]</sup>

The distribution of organic and inorganic components of adult cement is completely random, with no organized structure observed from the sub-micrometer to the millimeter scale.<sup>[42]</sup> The inorganics are largely the calcium carbonate polymorphs aragonite and calcite. The aragonite component is a minority at  $\approx 1:2$  relative to calcite, while in the shell the polymorph is almost entirely calcite. Inclusions within the cement rich in silicon and oxygen are also present, likely sand or dirt. Histology and spectroscopy have been used to provide partial identification of the organic components within cement.



**Figure 3.** A) Eastern oysters adhere to one another, as shown here. B) A back-scatter SEM image of a cross-section taken between two adhered oysters shows the shell of one oyster on the bottom, with the adhesive on top. The heavier elements within the SEM image appear brighter in comparison to the lighter elements. The adhesive is clearly a mixture of heavy and light elements with no apparent order or structure. C) The tuatara looks much like a lizard although has a distinct lineage.<sup>[72]</sup> D,E) Tuatara egg shells are a unique unstructured mixture of calcite crystals and organic membrane. SEM images of the egg shell cross-section show the intimate connection between the calcite and membrane with it being difficult to distinguish between the two components except at the outer shell surface, as indicated by arrows. Reproduced with permission.<sup>[50]</sup> Copyright 1982, Wiley-Liss, Inc.





**Figure 4.** A) Volcano shaped acorn barnacles attach to a variety of substrates, forming communities consisting of many barnacles, as shown here. B) A polarized light microscopy image of a *Balanus amphitrite* barnacle exoskeleton plate exhibits a variety of grey levels. Each grey level represents a different  $c$ -axis orientation of a calcite crystal, indicating that the calcite within the exoskeleton has no set structure or orientation. C) *Crassostrea virginica* also attaches to a variety of substrates, including other oysters, to make reef communities, as shown here. D,E) Polarized light microscopy images show two of the distinct structures found within an oyster shell. D) The chalky structure is quite porous, while E) the lathed structure exhibits crystals of different orientations, as represented by varied grey levels. The number and variety of these regions vary from one oyster to another.

Proteins, polysaccharides, and lipids have all been seen.<sup>[42,44]</sup> Of particular interest were observations that lipids or phospholipids are present in the cement, but not detected in shell samples. Infrared spectroscopy indicated the presence of phosphoesters, possibly being either phosphorylated proteins or phospholipids. Phosphate compounds can be adhesion promoters, possibly explaining the presence in oyster cement.<sup>[45]</sup> The amount of atomic level order is unknown at this point in time. At the micrometer scale, there is no obvious structure to the distribution of any of the organics within the cement.

The scanning electron microscope (SEM) image in Figure 3B shows a further lack of structure within this adhesive with respect to the distribution of organics and inorganics all the way up to the millimeter scale. Darker regions in Figure 3B represent organic or lower atomic weight elements, whereas the brighter regions are inorganic, heavier atomic weight elements. Phosphate species can prevent biomineralization.<sup>[46]</sup> The unordered appearance here could, thus, be a result of phospholipids and/or phosphoproteins preventing  $\text{CaCO}_3$  crystallization. When coupled with adhesion promotion, these organophosphates may be playing a dual role in biological material function.

Microhardness data acquired on adult oyster cement found a majority of the adhesive to be quite soft (90 hardness values, HV), whereas inclusions within the adhesive were hard (501 HV).<sup>[42]</sup> These results show that the combination of organics and inorganics in the cement provides the oyster with a

material that is largely soft, but with scattered hard inclusions. The current hypothesis, based on other biomineral systems, suggests that the soft portion of the cement provides flexibility and energy dissipation to the material while the hard inclusions and the calcium carbonate crystallites are there for stiffness and strength.<sup>[42,47]</sup> There are not yet models for how the different components interact to strengthen the adhesive, though the random mixture of the two components likely makes it difficult for fractures to propagate through the adhesive as such a fracture would continuously run into high energy boundaries. Thus, one could imagine that interactions between the hard and soft components would be vital to the material performance. Hence, while most of the mechanical properties of oyster cement are still to be explored, the structural details available indicate that micrometer to millimeter scale disorder may be providing flexibility and fracture resistance.

### 2.3.2. Tuatara Egg Shells

Many organisms produce shells that surround the developing embryo of their young. The tuatara (*Sphenodon punctatus*) (Figure 3C) is a lizard-like reptile, the last living species of the Rhynchocephalia order, found in New Zealand.<sup>[48]</sup> The tuatara lay eggs, like their common ancestors, snakes, and lizards of the Squamata order. However, the eggs laid by the tuatara are unique—they are not laid until after 7 months of pregnancy

and the shells surrounding the eggs are flexible, yet are largely inorganic with a small organic component.<sup>[49–51]</sup>

As with other eggshells, the ones produced by the tuatara protect developing embryos from the outside environment. The tuatara are located solely in New Zealand where they are active between temperatures of 7–20 °C.<sup>[52]</sup> While the egg is not laid until the 7th month of pregnancy, it takes an additional 5 months for the young to emerge.<sup>[49–51,53]</sup> From the start of development to hatching, the egg mass increases by 2–3 times.<sup>[54]</sup>

The tuatara eggshell consists of a mixture of calcite crystals and organic molecules.<sup>[49–51,55]</sup> The organic component of the eggshell is of unidentified composition, though consists of a mixture of ribbon-like fibers of varying diameter and an amorphous matrix.<sup>[49–51,55]</sup> The calcite component may have trace amounts of magnesium, as detected by X-ray fluorescence (XRF) and X-ray emission spectroscopy (XES), though trace amounts of phosphorous, silicon, and iron were also detected, with the authors concluding the silicon and iron came from ground contamination.<sup>[50]</sup> Thus, the level of atomic disorder is unknown, though there is likely a level of long-range atomic disorder due to crystal inclusions.

When laid, the fibrous organic membrane and calcite crystals are thoroughly intermixed throughout the ENTIRETY of the shell with variation in density of the two components.<sup>[49–51]</sup> At the exterior of the eggshell, the calcite crystals are more dense and of random shape, size, and orientation.<sup>[49–51]</sup> Moving toward the shell interior, shown in Figure 3D,E, the calcite forms rough columns that gradually become less dense as the organic matrix becomes more dense.<sup>[49–51]</sup> The organic fibers are embedded in the calcite columns of the eggshell while the amorphous matrix surrounds the columns.<sup>[49–51]</sup> At the interior of the shell, the majority of material is organic with only small calcite crystallites randomly distributed on some of the surface of the interior organics.<sup>[49]</sup>

The tuatara eggshell is decidedly distinct from those of chickens or many other organisms that produce rigid calcified shells with distinct layered structures or even snakes and most lizards that produce flexible shells with very little or no mineral component. While no mechanical tests have been done on the tuatara eggshell, functional observations have led to the hypothesis that the unique structure of the tuatara eggshell provides the protection and flexibility needed in the New Zealand environment.<sup>[50,55]</sup> The large amount of calcite found within the eggshell is thought to help prevent water loss during times of low external humidity while also providing a measure of hardness to protect from exterior pressures.<sup>[49]</sup> The varying size, shape, and orientation of the calcite crystals within the eggshell at the micrometer to millimeter scale, in addition to the random network of organic fibers interwoven throughout the calcite, likely provide flexibility while maintaining robustness.<sup>[50]</sup> Given that the calcite crystals are randomly arranged and shaped, they can move among the organic matrix as needed without allowing fractures to develop. The network of organic fibers supports shell expansion in all directions while also binding the calcite columns together to provide toughness.<sup>[50]</sup> Thus, the lack of microstructure of the mineral and organic components throughout the tuatara eggshell appears to provide a higher degree of flexibility than seen in the highly ordered, mineralized eggshells of other organisms.

## 2.4. Hard and Organic/Inorganic Composite

As stated previously, hard biological materials are often a mixture of organic and inorganic materials. Many organic/inorganic hard materials have hierarchical structures that give materials the unique properties required by the organisms. The inner nacreous layer of seashells provides a classic example here. We explore two instances of hard composite materials in which, contrasting with most seashells, the components are not hierarchically structured. Rather, random mineral orientation and size are found. Barnacle exoskeletons and the bulk of oyster shells highlight how, even with a disordered structure, composite materials can be robust and functional.

### 2.4.1. Barnacle Exoskeletons

Barnacles are a group of arthropods originating over 400 million years ago in the early Paleozoic. These crustaceans start life as a free swimming nauplius before undergoing metamorphosis into a cyprid. The focus of the cyprid is to find a substrate for settling onto prior to undergoing a final metamorphosis into a sessile barnacle; while a cyprid, the organism cannot feed but is able to sense the surrounding environment with antennae. As a surface attached barnacle, the animal produces a hard exoskeleton for protection and an adhesive for permanently bonding to the chosen substrate. There are two general types of sessile barnacles.<sup>[56]</sup> The acorn barnacle is perhaps most well-known, with a shape much like a little volcano (Figure 4A). Gooseneck barnacles have an exoskeleton on the animal's top and connect to the rocks with a muscular stalk. Due to their relative prevalence, we focus on acorn barnacles.

The exoskeleton of the majority of acorn barnacles consists of three sets of plates: base plate, parietal plates, and operculum. The base plate is the portion of the exoskeleton between the organism and the thin adhesive that the animal secretes to attach itself onto a substrate. The parietal plates, varying in number between 4 and 8, surround the organism within and make up the majority of the exoskeleton.<sup>[57]</sup> The operculum consists of two different pieces, the scutum and the tergum, that open and close, allowing the feet, cirri, to extend and fan through the water for filtering and catching food.

The exoskeleton consists largely of the calcium carbonate polymorph calcite, with typically less than 3% organics by weight.<sup>[58]</sup> The organic component consists of two fractions: the intercrystalline and the intracrystalline.<sup>[59]</sup> The intercrystalline organics act as a disordered hydrogel, largely consisting of chitin, sulfur rich proteoglycans, and proteins.<sup>[57,59]</sup> The intracrystalline organics are largely highly acidic proteins.<sup>[59]</sup> The structure of these proteins is unknown at this point.

Inorganic calcite forms rhombohedrals that are clear, birefringent, and cleave along the {1011} plane. In the barnacle exoskeleton, as with many other biominerals, magnesium is occasionally substituted for calcium in the calcite crystal lattice, with 1–2 weight percent being reported.<sup>[57,59]</sup> The magnesium, in addition to the intracrystalline organics, disrupts the calcite crystal lattice structure, resulting in a level of atomic disorder.<sup>[59]</sup> The atomic disorder found within the barnacle exoskeleton is greater than that found in geologic calcite, but less than



amorphous calcium carbonate, the completely disordered calcium carbonate.<sup>[59]</sup>

On the micrometer scale, the calcite crystals within the exoskeleton vary greatly in size and orientation depending upon location within the exoskeleton.<sup>[57,59–61]</sup> Figure 4B presents a polarized light microscope image acquired in Metzler's lab of a cross-section of the parietal plates from the barnacle *Balanus amphitrite*. Due to the birefringent nature of calcite, the image indicates *c*-axis orientation of the calcite crystals within the material with grey level; the crystallites with their *c*-axis aligned with the polarized light appear bright while those with their *c*-axis misaligned appear dark. As seen in Figure 4B, the crystallites in the parietal plates are of varied size and largely of random orientation, as shown by different grey levels, with small regions of co-oriented crystals around channels that run vertically from the base of the exoskeleton; the lack of coordinated crystal orientation in the parietal plates has also been observed via X-ray diffraction.<sup>[59,61]</sup> The calcite crystals within the operculum of *Balanus amphitrite* were also found to be poorly ordered with regard to orientation.<sup>[59,62]</sup>

Few mechanical tests have been done on the barnacle exoskeleton. Raman and Kumar used nanoindentation in 2011 to explore the hardness, elasticity, and fracture strength of the *Amphibalanus reticulatus sessile* barnacle.<sup>[57]</sup> The barnacle exoskeleton was found to be harder than geologic calcite ( $3.2 \pm 0.2$  GPa vs  $2.4 \pm 0.1$  GPa) and have a slightly lower elastic modulus ( $60\text{--}70$  GPa vs  $74.4 \pm 2.85$  GPa).<sup>[57]</sup> The remarkable property of the barnacle exoskeleton was a 24 times increase in fracture toughness over geologic calcite.<sup>[57]</sup>

The exact mechanism behind how disorder within the exoskeleton provides the barnacle with a functional advantage is not understood, although several hypotheses have been raised, as discussed below. Namely, the increased atomic disorder within the barnacle exoskeleton is thought to contribute flexibility, hardness, and fracture toughness to the mineral and the disordered hydrogel phase and random orientation and size of the calcite crystals to the fracture resistance and robustness of the exoskeleton.<sup>[57,59]</sup>

Studies on other mineralized tissues have found the presence of organic material, such as the intracrystalline proteins thought to cause the atomic disorder in the barnacle exoskeleton, to decrease the stiffness of the tissue and change the fracture properties.<sup>[63]</sup> The mechanical testing of Raman and Kumar showed a decrease in exoskeleton stiffness with respect to geologic calcite, although in a different species than that for which the atomic disorder studies were conducted.<sup>[57]</sup> The increase in fracture toughness observed in the exoskeleton can also be attributed to the incorporation of intracrystalline organics, as these intracrystalline organics adsorb to and disrupt the natural cleavage planes of calcite.<sup>[59,63,64]</sup>

At a slightly larger scale, the random orientation and size of calcite crystals result in a more robust exoskeleton. The magnesium that is incorporated into the calcite crystals, also contributing to the atomic disorder, results in crystals that are harder than geologic calcite, as seen in many other biominerals.<sup>[65]</sup> Once a crystal is penetrated, the random orientation of the surrounding crystals makes it difficult for cracks to propagate throughout the exoskeleton.<sup>[59]</sup> Forming cracks reach the boundary of a crystal with a different orientation and either

need to go around the crystal or fracture in an energetically unfavorable plane.<sup>[59]</sup> The disordered hydrogel surrounding the calcite crystals is thought to also play a role in dissipating crack energy.<sup>[57,59]</sup> Thus, disorder from the atomic to the micrometer level within the barnacle exoskeleton makes the material more flexible and more resistant to fracture.

#### 2.4.2. Eastern Oyster Shells

Like barnacles, shells of the Eastern oyster protect the animal within from the intertidal environment in which they live (Figure 4C). With the oyster being a bivalve, it has two valves (i.e., shells) that are denoted left and right. These valves are made from  $\approx 98\%$  calcium carbonate, predominantly of calcitic form, with a minority of organics in the 0.3–3% range.<sup>[66]</sup> Phosphorylated proteins are likely to comprise the bulk of these organics, although structural features associated with the proteins are not known.<sup>[67]</sup> Each valve has several microcrystalline components: prismatic, foliated, and chalky (Figure 4D).<sup>[68]</sup> There is no consistent distribution of these components between animals or even within shells of an individual.<sup>[68]</sup> The lack of consistent relation between these microcrystalline regions appears to scale up to the macroscopic: oyster shells are like fingerprints in that they all tend to look different.

Each of the microcrystalline regions consists of the calcium carbonate polymorphs calcite or aragonite. The prismatic region is on the exterior of the shell, facing the outside environment. Here, calcite crystals of various sizes, shapes, and orientations are separated from one another by thick organic matrices. The chalky regions appear porous in nature with pores distributed randomly and varying in size from 1 to 10  $\mu\text{m}$ . The lamellar regions consist of small calcite crystal lathes that are nanometers in size and arranged in varied crystallographic orientations with no set pattern. Thus each region, though having a distinct structure, has a level of structural disorder at the micrometer scale.

The oyster shell is also unique in that, other than the prismatic layer, there is no set number, location, or orientation of the structural elements. Each of these regions appears to have no spatial or orientational relationship among one another with the shell, looking to be a random mixture of these microcrystalline components. The degree to which such components are present does not even seem to be consistent from one valve to the next. To that end, some oysters have no chalky regions, whereas others have multiple. Typically, the left valve is thinner than the right valve with both of these shells consisting of the above structural elements.

The shell, itself, is considerably harder than geologic calcite or aragonite, as shown by microindentation data.<sup>[42]</sup> In addition, preliminary data show that the chalky regions within the oyster shell are more resistant to fracture than the foliated or prismatic regions, likely due to fractures hitting pores (pockets of air) and not being able to propagate further. Thus, the random assortment of foliated and chalky regions at the millimeter scale makes the overall shell more resistant to fracture. Cracks moving across the shell are disrupted when they reach regions with crystals of different orientation or chalky regions. While the exact mechanism by which each structural

component contributes to the shell's overall strength is still not fully clear, it is likely that a lack of organized structure helps with toughness and dissipating energy from applied forces.

### 3. Discussion

The exploration of biological systems presented here helps illuminate materials properties that can be linked to disordered structure, independent of composition or level of disorder. Specifically, disorder in both hard and soft materials provides a degree of flexibility that can be difficult to achieve with highly structured materials. Increased flexibility can correspond to greater elasticity in soft materials and an increase in fracture resistance for hard materials. Indeed, the lives of some animals are completely dependent upon these combinations of properties brought about by disorder. The lessons we can learn from these organisms may prove especially useful in biomimetic material design given that disordered materials are generally easier to produce than precise highly ordered structures.

Each of the soft materials examined above (mussel adhesive, human skin, and tuatara egg shells) is required to change shape, resisting applied stresses. By having a completely disordered network of proteins, as seen in skin, stresses from all different directions can be applied to the material without failure. In creating a structure with both inherently hard minerals and soft organics that are arranged in a disordered manner, the tuatara produces an egg shell that can expand and contract in response to different environmental conditions without breaking easily under applied stresses. In all of these examples, the unstructured components, at different length scales, allow the materials to rearrange and adapt to external pressures, resulting in a more robust system overall.

When nature requires a delicate functional balance between hardness and flexibility, disorder can often be found. The hedgehog quill, barnacle exoskeleton, and oyster shell each use combinations of materials or structures—or a lack thereof—to achieve the necessary properties. The disordered keratin exterior seen in the hedgehog quill prevents splitting fractures and buckling, with there being no single weak direction. A similar mechanism looks to be at play for the interior foam-like structure of the quill, preventing compressional collapse. The quill is additionally made tough through cross-linking bonds between the keratin proteins. The lack of order in crystallographic orientation within the calcite crystals making up the barnacle exoskeleton prevents fractures from propagating throughout the material. Organics within the crystals and surrounding the crystals provide hardness and flexibility. Similar observations are made in the oyster shell. Thus, in the hard disordered materials, irregular structures from the atomic to the millimeter level prevent fracture planes from forming, resulting in materials that are hard and resistant to breaking.

The disordered structures observed in both the hard and soft biological materials explored here result in materials that can resist fracture or deform under stresses applied from multiple directions. Similar findings have been found in synthetic materials exploring the variation in material properties between a crystalline and disordered material with the same chemical make-up.<sup>[69]</sup> Even within largely ordered, hierarchical

biological materials, such as bone, a level of disorder can be found, playing an important role in increasing compliance and flexibility.<sup>[6–9]</sup> Thus, while we are unable, at this point, to quantify disorder in biological materials, correlating the amount of disorder within a material to measureable increases in flexibility or toughness is a worthy goal that will likely aid in future materials design. Here we see in making a disordered structure without a preferred orientation, organisms have found a way to make incredibly robust materials without the use of highly ordered, hierarchical structures.

### 4. Conclusion

Throughout biology one common theme is conservation of energy. Organisms often attempt to expend as little energy as possible when fulfilling a given function. According to the laws of thermodynamics, energy is necessary to achieve and maintain orderliness.<sup>[70]</sup> Thus, the creation of ordered structures, in general, requires more energy than the creation of disordered structures. We see here that impressive functionality and simplicity need not be mutually exclusive. Disorder might not only be from a lack of effort. Rather, nature has discovered that there are many places wherein keeping components from settling to more ordered structures can actually confer significant mechanical advantage. We may want to keep such lessons in mind when designing future generations of biomimetic materials.

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### Conflict of Interest

The authors declare no conflict of interest.

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