Nano-Mechanical Investigation of the Byssal Cuticle, a Protective Coating of a Bioelastomer

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ABSTRACT

The mechanical properties of the mussel byssal thread have been investigated via nanoindentation, with the emphasis on the differences between the cuticle and the fibrous interior. The cuticle hardness was found to be 30-40% higher than that of the underlying fibrous interior. In contrast, the Young’s moduli in the two regions were virtually identical to one another. Elemental analysis via energy dispersive spectroscopy indicated surprisingly high levels of Al and Br in the cuticle considering the low amounts found in seawater. A potential role of Al in byssal thread mechanics is discussed in light of the unique capability of the cuticle to accommodate strains of 70% by the underlying fibrils in the core without delamination.

INTRODUCTION

The holdfast structure of mussels, the byssus, has evolved to withstand the challenges of high energy wave action in the intertidal zone [1, 2, 3]. Each byssal thread consists of three functional domains: (i) the glue in the plaques which bonds the thread to a variety of hard surfaces, (ii) the fibrous collagenous core which connects with the glue and undergoes tension due to lift and drag forces, and (iii) the cuticle that covers all parts of the byssus. New byssal threads are formed and cured over a short time-frame (less than 5 min) through a molding process in the groove of the foot. This occurs by secretion of stockpiles of prepolymers of all the molecular constituents from the foot tissue lining the groove [4]. Byssus, as well as its attachment to hard surfaces, is durable for years and hence may serve as an appropriate conceptual paradigm for biomimetic engineering of materials that exhibit excellent extensibility and abrasion resistance.

In the distal portion of the thread the cuticle exhibits a granular structural appearance (see Figure 1). This is the part of the thread outside the mussel shell and hence it presumably endows the threads with robust protection against “sand blasting”: abrasive action of sand dispersed in seawater. While abrasion resistance suggests hardness and stiffness, the distal cuticle must also accommodate strains of up to 70% by the underlying fibrils in the distal core without delamination. Hence the distal cuticle material is from an engineering standpoint truly unique since it serves as an example of a protective coating of a highly extensible elastomeric material.

The major protein of the cuticle is Mussel foot protein-1 (Mfp-1) [5]. The most reactive functionalities in the sequence are the Dopa residues [6-12]. They tend to auto-oxidize at neutral pH resulting in diDOPA or diquinone cross-linking, but, in the presence of metals they exhibit an even greater tendency to form metal-catecholate complexes.

One of the established methods to increase stiffness of a polymeric material without concurrently increasing its brittleness is to incorporate various reinforcements such as
microspheres into the polymer matrix [13]. Based on its granular superficial appearance in the scanning electron microscope (SEM) (see Figure 1) we speculated whether the distal byssal thread cuticle could be a microsphere reinforced composite material. We present here preliminary data from our investigations of the mechanics and the elemental composition of this unique coating material. These efforts are part of an overall strategy for the in vitro formation of Mfp-1 based films and biocompatible coatings.

EXPERIMENTS

Mussels *Mytilus galloprovincialis* were collected from Goleta Pier (Goleta, CA) and transferred to seawater aquaria at 15°C. After removal of the old byssus, whole threads deposited within 5-7 days were rinsed in deionized water, freeze-dried overnight and embedded in Epofix (Electron Microscopy Sciences, Hatfield, PA). Transverse cross-sections of the distal portions of the threads were prepared with a microtome (Leica EM UC6, Leica Mikrosysteme GmbH, Austria, Vienna). Indentation tests were performed on the microtomed surfaces of the specimen in the remaining mold.

Indentation tests were performed using a TriboIndenter® (Hysitron, Minneapolis, MN) with a cube corner diamond tip. The tip area function was established from a PMMA standard. All load-displacement curves were analyzed using the method described by Oliver & Pharr (1992) [14]. Images of the sample surfaces were obtained via Scanning Probe Microscopy (SPM) immediately before and after each indentation to ensure correct placement of indents. All indentations were carried out in the open loop feedback mode under loading rate control. The loading and unloading rates were 100 µN/sec and the peak load was approximately 1000 µN. Once at the peak, the load was held fixed for a period of 30 sec, for the purpose of eliminating creep effects. Following indentation, the test specimens were examined in an environmental scanning electron microscope (XL-30 ESEM-FEG, Phillips, Eindhoven, Netherlands). Specifically, the local compositions at the indent sites were obtained by energy dispersive x-ray analysis (EDX) (PRISM-IG, Princeton Gamma Technology, Princeton, NJ).

To analyze the superficial effects of stretching on the structural organization of the distal cuticle, wet threads were stretched to 70% of their initial length at a speed of 5 mm/min with a Bionix 200 tensile tester (MTS Systems, Cary, NC). The threads were dried in the strained state followed by freeze-drying overnight. Stretched threads did not recoil when kept dry. The stretched specimens were then imaged in a scanning electron microscope (Vega Ts 5130mm, Tescan, Czech Republic). For comparison, unstretched control samples taken through the same drying procedure were also examined.

Figure 1. SEM micrograph illustrating the granular appearance of the distal thread cuticle. The cuticle has been removed to expose the fibrous interior in the lower left half of the image (scale bar is 5 µm).
RESULTS

The scan in Figure 2a highlights topographical contrast in the sample surface with taller areas being lighter. The scan reveals the bright outline of individual spheres within the cuticle with the same dimensions as the superficial granules presented by SEM. From this we conclude that the granular structure apparent on the surface of the distal cuticle is present throughout the entire depth of the distal coat material. Furthermore, the strong outline of the individual granules within the cuticle in the SPM scan indicates a significantly higher stiffness and/or hardness of the granules compared to the matrix revealed upon sectioning.

To test whether the distal cuticle does indeed confer mechanical protection to the interior of the thread we analyzed the thread mechanics via nano-indentation. Hardness measurements of the distal cuticle were performed by placing indents within the boundaries of the coat as presented by SPM of a transverse cross-section of the thread (see Figure 2b), followed by analysis of the load-displacement data as described in the experimental section. These were compared to the same measurements in the interior of the thread. Typical load-displacement data for the cuticle and the interior of the thread are shown in Figure 3. It is clear from the data that the resistance to penetration is greater for the cuticle compared to the interior under the given test conditions. From such data the hardness (H) and modulus (E) of the two materials were calculated (see Table I). The greater resistance to penetration of the cuticle is reflected in the approximately 36% higher H of the cuticle compared to the interior. The modulus however, as obtained by the unloading curve according to Oliver and Pharr (1992) [14], is similar between the cuticle and the interior under the given test conditions.

Figure 2. a) SPM topography image of cross-section of the thread, illustrating the granules throughout the cuticle material. b) SPM topography image of cross-section of thread collected immediately following indentation in the cuticle. Scale bar is 1 µm.

Figure 3. Typical load-displacement data for the cuticle and the interior upon indentation with 1000 µN peak load, 100 µN/sec load and unload, and a 30 sec hold period.
Table I. The hardness (H) and modulus (E) as determined via analysis of the load-displacement data obtained upon nano-indentation of the two different materials (see Experimental section for further details). SD is based on 10 indentations in each of the different materials of two independent samples.

<table>
<thead>
<tr>
<th></th>
<th>cuticle</th>
<th>interior</th>
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<tbody>
<tr>
<td>Hardness (GPa)</td>
<td>0.45±0.02</td>
<td>0.33±0.01</td>
</tr>
<tr>
<td>Modulus (GPa)</td>
<td>7.2±0.2</td>
<td>7.0±0.2</td>
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As mentioned in the introduction, the major protein of the cuticle is Mfp-1 [5]. Preliminary elementary analysis by EDX has intriguingly found high levels of at least Al and Br present in the cuticle compared to the interior (see Figure 4), despite these elements' low concentration in seawater. Whether the higher levels of Al and Br present in the cuticle are responsible for the increased hardness is premature to conclude, but it is nonetheless an interesting finding considering the high binding affinities of the prevalent Dopa-residues in Mfp-1 for transition metals such as Al (see Conclusion for further discussion). The relative distribution and specific form of these elements in the coat is currently under investigation.

Finally, despite the apparent hardness of the distal cuticle our studies show that it accommodates strains of up to 70% by the underlying fibrils in the distal core without delamination (see Figure 5). As seen in Figure 5 the distal coat appears to rearrange in accordance with the tension experienced by a thread. The details of how this structural re-arrangement takes place are currently under investigation.

Figure 4. EDX spectra illustrating the high Al and Br content in the cuticle compared to the interior.

Figure 5. Micrographs illustrating the structural reorganization of the cuticle upon 70% strain of thread. Direction of strain indicated by arrow. Scale bar is 1 µm.
CONCLUSION

The nature and properties of the distal byssal thread cuticle from the mussel *Mytilus galloprovincialis* have been the focus of the present study. The cuticle hardness was found to be 30-40% higher than that of the underlying fibrous interior while the Young’s moduli in the two regions were virtually identical to one another (see Table I). Hence, this unique material appears to protect the thread from abrasion while concurrently accommodating strains of up to 70% by the underlying fibrils in the core without delamination.

The origin of the hardness of the cuticle is still unknown. SPM of sectioned threads supports the idea of the distal cuticle as a microsphere reinforced composite material (see Figure 2a). However, the mechanical analysis has so far not allowed us to resolve quantitatively what unique mechanical properties the granules posses compared to the composite cuticle material as a whole. Future work must furthermore characterize the contents of the granules as well as determine the exact localization of Mfp-1 in the granular composite overall. Whether Mfp-1 solely constitutes the polymer matrix between the granules, or if the protein also infiltrates the granules, is therefore still unknown. It is tempting to speculate that the high levels of Al and Br found in the cuticle are concentrated in the granules in some form accounting for their reinforcing mechanical behavior. Considering the high binding affinities for transition metals such as Al of the prevalent Dopa-residues in Mfp-1, this is an interesting possibility. Such metal-catecholate complexes would be an intriguing alternative cross-linking strategy in the cuticle compared to covalent cross-links via diDOPA. It has recently been shown by Kong *et al* (2003) [13] that modifying the properties of the cross-links in a polymeric material, where stress is likely to be localized, had a profound effect on the resulting mechanical properties. Specifically, they showed that the modulus and the toughness of the polymeric materials investigated both increased in ionically cross-linked polymers with increasing cross-linking density, while covalent cross-linking led to increases in the modulus and the brittleness with higher cross-linking densities. The mechanism underlying these observations is still unknown. However, since ionic bonds, in contrast to covalent bonds, are reversible, we speculate that during deformation ionic cross-links could be reversibly broken and reformed thereby maintaining material integrity and resisting catastrophic failure. The result would be a concurrent increase in strength and toughness with ionic cross-linking density compared to strength alone with covalent cross-linking density. This principle would of course apply to all non-covalent bonds exploited as cross-links. Therefore, if the cuticle material is in fact dominated by a metal-ligand based cross-linking chemistry rather than a covalent one, the mechanical consequences could be very similar to the above study. Due to their reversibility, during the extension of the cuticle, metal-catecholate complexes could continuously be broken and reformed as proposed above, thereby upholding the integrity of the cuticle. In fact, the unique strength of metal-catecholate complexes would make them superior candidates among the non-covalent material alternatives. Hence, with strength and toughness as the driving forces, the cuticle could potentially be optimized throughout the hierarchical levels of material design from the spherical reinforced composite structure down to the chemical properties of the individual cross-links.

The metal-ligand cross-linking strategy could also play another important role in thread mechanics apart from increasing toughness. Upon relaxation after tension, byssal threads display the unique behavior of “self-healing” [15]. That is, despite a yield-like behavior observed at about 25% strain of a distal portion of a thread, with time the thread will recover and perform as
new in repeated stress-strain cycles. Hence, upon cessation of loading, the cuticle must reorganize into the native relaxed structure in which the metal-catecholate complexes could reform in their original locations. Covalent cross-links would not allow such “self-healing” properties. On a final note it is very interesting that this “self-healing” capacity observed under macroscopic tension likewise has been suggested by the present microscopic mechanical tests. Hence, upon indentation of the thread cuticle it was observed that the indents “recovered” over time, whereas the indents remained unchanged in the covalently cross-linked epoxy (data not shown) supporting the above speculations that the cuticle material is cross-linked predominantly by reversible non-covalent bonds. Future work will show if and how metal-catecholate cross-links endow the cuticle, and any other material, with a unique combination of mechanical properties such as strength, toughness and a self-healing capacity.

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REFERENCES