Protective coatings on extensible biofibres

NIELS HOLTEN-ANDERSEN[1], GEORG E. FANTNER[2], SOPHIA HOHLBAUCH[3], J. HERBERT WAITE[1]* AND FRANK W. ZOK[4]*

[1]Biomolecular Science and Engineering Program, University of California, Santa Barbara, California 93106, USA
[2]Department of Physics, University of California, Santa Barbara, California 93106, USA
[3]Asylum Research, Santa Barbara, California 93117, USA
[4]Materials Department, University of California, Santa Barbara, California 93106, USA
*e-mail: waite@lifesci.ucsb.edu; zok@engineering.ucsb.edu

Published online: 8 July 2007; doi:10.1038/nmat1956

Formulating effective coatings for use in nano- and biotechnology poses considerable technical challenges[1]. If they are to provide abrasion resistance, coatings must be hard and adhere well to the underlying substrate[2]. High hardness, however, comes at the expense of extensibility[3,4]. This property trade-off makes the design of coatings for even moderately compliant substrates problematic, because substrate deformation easily exceeds the strain limit of the coating[5]. Although the highest strain capacity of synthetic fibre coatings is less than 10%, deformable coatings are ubiquitous in biological systems[3,6]. With an eye to heeding the lessons of nature, the cuticular coatings of byssal threads from two species of marine mussels, *Mytilus galloprovincialis* and *Perna canaliculus*, have been investigated. Consistent with their function to protect collagenous fibres in the byssal-thread core, these coatings show hardness and stiffness comparable to those of engineering plastics and yet are surprisingly extensible; the tensile failure strain of *P. canaliculus* cuticle is about 30% and that of *M. galloprovincialis* is a remarkable 70%. The difference in extensibility is attributable to the presence of deformable microphase-separated granules within the cuticle of *M. galloprovincialis*. The results have important implications in the design of bio-inspired extensible coatings.

Mussels are aquatic sessile organisms, whose survival in exposed habitats depends in large part on the byssus: an adaptive holdfast that secures the animal to a solid surface and dissipates the shock of wave impact through repeated large strains. A typical byssus consists of hundreds of collagenous threads. Each thread is formed in the groove of the mussel foot by injection moulding of self-assembling precursors secreted from specialized cells in the groove lining. In the finishing touch of this process, a nascent thread is covered by a thin (2–4 μm) cuticle that consists of proteins with highly repetitive sequences, dominated by proline/hydroxyproline, lysine and 3,4-dihydroxyphenyl-L-alanine (dopa) and collectively termed mussel foot protein-1 (mfp-1)[7,8]. The cuticle protects the underlying collagen fibres from abrasion and microbial attack. Some mussel species are found predominantly on turbulent wave-swept seashores (intertidal), whereas others reside in deeper, stiller waters (subtidal)[9,10]. Survival in the former environment requires greater capacity for energy dissipation by the byssal threads. Hence, a comparative study of the thread cuticles from intertidal and subtidal mussel species is likely to provide insights into the adaptive design of protective and extensible coatings.

When viewed in cross-section, the cuticle of the subtidal *P. canaliculus* seems uniformly homogeneous (Fig. 1a), whereas the intertidal *M. galloprovincialis* cuticle consists of distinct biphasic granules in a homogeneous matrix, evident in both transmission electron microscopy and atomic force microscopy (AFM) images (Fig. 1b–d). The granules show an intriguing phase-separated morphology with a domain size of 20–40 nm, similar to that observed in polymeric bicontinuous microemulsions and supramolecular materials[11]. The granules are typically 0.8 μm in diameter and comprise about 50% of the cuticle volume. They seem to be the only structural feature distinguishing the cuticles of the two mussel species.

When a byssal thread from *P. canaliculus* was stretched, its cuticle began to crack at a strain of about 30% (Fig. 2d–f). The cracks propagated through the entire cuticle thickness over a lateral distance of about 10 μm and subsequently deflected at the interface with the underlying collagen fibres. Deflection presumably mitigates the high stresses that would otherwise arise in the thread interior and hence prevents catastrophic thread rupture. Nevertheless, exposure of the thread interior to the ambient environment in the vicinity of the crack would be expected to facilitate abrasion and microbial attack, thereby compromising thread performance. As the strain was increased, numerous additional cracks were formed along the thread length. Thread rupture finally occurred at a strain of about 75–80%.

The sequence of events during stretching of byssal threads from *M. galloprovincialis* differed in two important respects. (1) Cracks first formed within the matrix of the composite cuticle but did not propagate through the entire cuticle. Instead, the granules seemed to arrest impinging cracks, which were evident as microtears in sections and on the external surface of stretched threads (Fig. 3a,b). (2) Cuticle rupture did not occur until strains of about 70%: significantly higher than that of *P. canaliculus* and only slightly below the rupture strain of the thread interior (75–80%) (Fig. 2a–c). Evidently rupture occurs through a mechanism of microtear coalescence, suggestive of the ductile fracture process in metallic alloys. The granules thereby endow the cuticle with enhanced damage tolerance and hence provide protection to the underlying thread during large strain deformation.

Additional insights into the damage tolerance of the *M. galloprovincialis* cuticle were obtained from measurements of the aspect ratios of the granules in longitudinal cross-sections of both pristine and strained threads. In essence, the granule shape

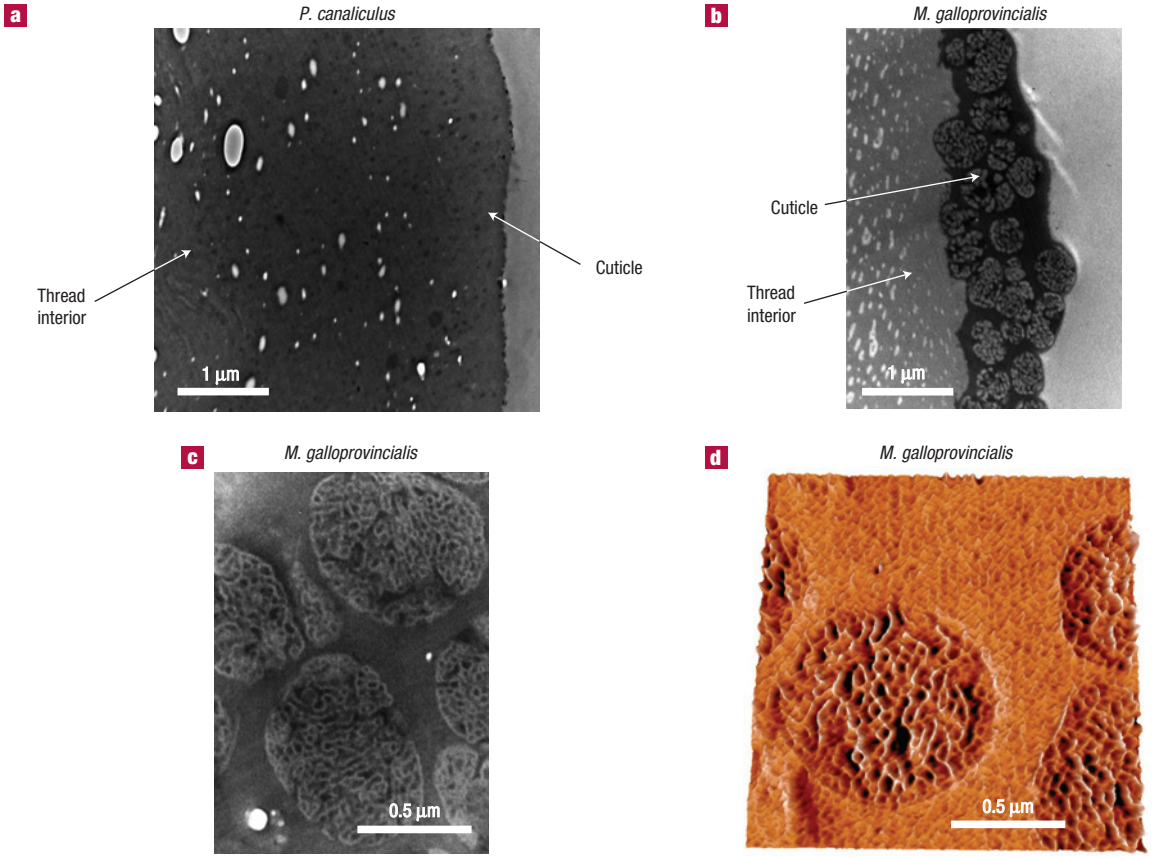

Figure 1 Ultrastructure of mussel thread cuticles. a–c, Transmission electron micrographs of transverse cross-sections through cuticles from both mussel species. **d**, AFM amplitude image (three-dimensional rendering) of longitudinal section of *M. galloprovincialis* cuticle obtained in fluid (Asylum Research).

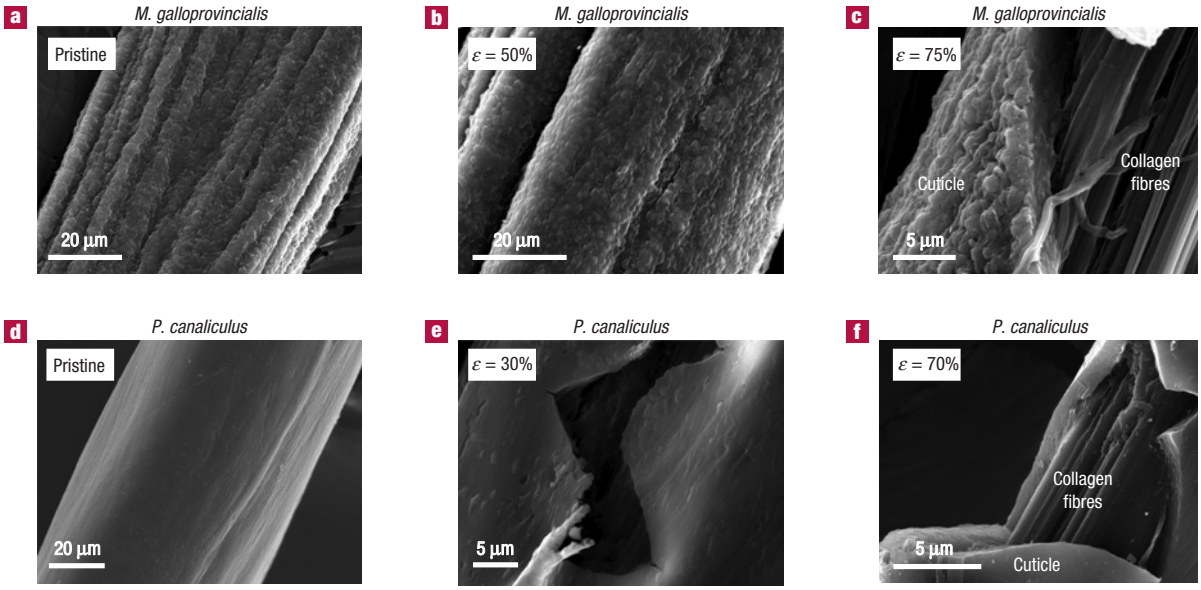

Figure 2 Strain failure of mussel thread cuticles. Scanning electron micrographs of the distal portions of thread surfaces of both mussel species in pristine, strained and fractured states.

provides a measure of local deviatoric strain. Treating the granules as either prolate or oblate spheroids with their principal axes aligned longitudinally, the predicted granule aspect ratio is given by $\bar{S} = \bar{S}_0(1+\varepsilon)^{3/2}$, where $\bar{S}_0$ is the average aspect ratio of the pristine granules and ε is the applied tensile strain (assumed to be purely deviatoric). The measurements and corresponding predictions are

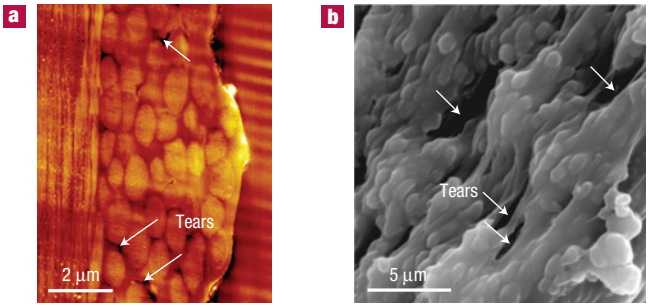

Figure 3 Microtears in granular composite cuticle. a, AFM image of *M. galloprovincialis* thread stretched by 50%, showing early stages of microtearing and deformation of granules. **b**, Corresponding SEM image of cuticle surface at 70% strain, showing extensive microtearing of matrix between granules immediately preceding cuticle rupture.

plotted in Fig. 4. The comparisons indicate that, for a prescribed granule aspect ratio, the predicted strain is considerably lower than the measured value, the difference being attributable to dilation associated with microtearing. Furthermore, the dilational strain component increases rapidly with applied strain and constitutes about 40% of the total for $\varepsilon = 70\%$. The magnitude of the dilation is evidence of the impressive damage tolerance of the cuticle. Finally, on extrapolation of the measurements, the inferred strain at the onset of damage and hence dilation is roughly 30%: consistent with the failure strain of the *P. canaliculus* cuticle. The latter lacks the microstructural elements needed to arrest microtears, and hence fractures catastrophically at the onset of cracking. More importantly, the higher failure strain of the *M. galloprovincialis* cuticle can be attributed to the presence of the granules.

Nanoindentation tests revealed that the Young modulus, E, and hardness, H, of the thread interiors of the two mussel species are similar to each other and comparable to those of soft elastomers: $E = 0.37 \pm 0.04$ GPa and $H = 19.4 \pm 1.9$ MPa for *P. canaliculus* and $E = 0.41 \pm 0.09$ GPa and $H = 23.1 \pm 4.0$ MPa for *M. galloprovincialis*. The cuticles are considerably harder and stiffer, with properties similar to those of structural engineering polymers. Specifically, for the *P. canaliculus* cuticle, $E = 2.3 \pm 0.2$ GPa and $H = 133 \pm 17.4$ MPa, each about six times that of the respective value for the thread interior. Remarkably, in light of the vast differences in extensibility, the indentation properties of *M. galloprovincialis* cuticle are only slightly lower ($E = 1.7 \pm 0.1$ GPa and $H = 100 \pm 6.2$ MPa) and still about five times those of the thread interior. This combination of properties is expected to provide a significant improvement in cuticle durability, necessary in the turbulent environment in which *M. galloprovincialis* resides.

The microstructure of *M. galloprovincialis* cuticle is reminiscent of tough engineering materials such as high-impact polystyrene. High-impact polystyrene typically consists of a polystyrene matrix and two-phase globular particles[12,13]. These so-called salami particles, consisting of polystyrene inclusions in a polybutadiene matrix and ranging in size from 0.5 to 5 μm, enhance fracture resistance by initiating multiple crazes and shear bands in the surrounding matrix during straining[14]. In contrast, in the absence of rubber particles, fracture of polystyrene occurs by catastrophic propagation of a single craze. The deformation induced by the rubber particles provides substantial elevations in both the tensile failure strain and the fracture toughness. It seems that the granules

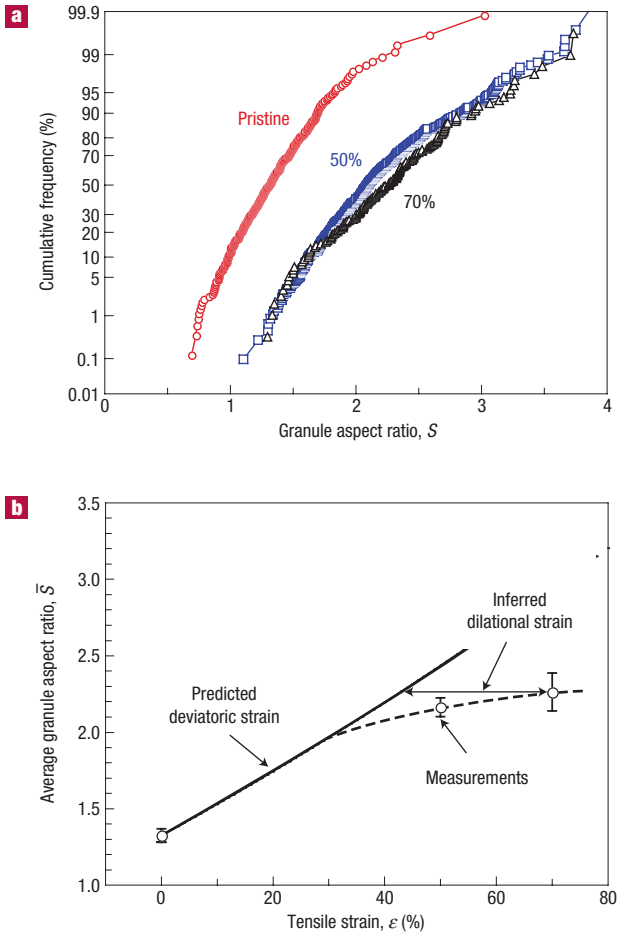

Figure 4 Damage tolerance in granular composite cuticle. a, Distributions in granule aspect ratio in *M. galloprovincialis* cuticle in pristine condition and after straining to 50 or 70%. **b**, Variation in average granule aspect ratio with strain. The solid line is based on a prediction assuming that the granule strain is the same as the applied strain.

in the *M. galloprovincialis* cuticle may serve similar functions, enhancing damage tolerance and extensibility.

The greater extensibility of the matrix in the composite cuticle distinguishes it from the matrices used in synthetic composites. We surmise that this difference is related to mfp-1's high level (~10 mol%) of dopa—a functionality that has been implicated in the adhesion of the mussel holdfast fibres—along with moderate levels of Fe, Al and Si in byssal cuticles[7,8,15–17]. Dopa/metal cross-links are expected to increase the hardness of the proteins (relative to those with only secondary intermolecular bonds), yet be sufficiently weak to enable molecules to undergo large-scale sliding and rearrangement without catastrophic fracture. Furthermore, unlike the covalent cross-links typical of synthetic network polymers, the dopa/metal cross-links can reform after being broken, leading to potential recovery of hardness after unloading. This mechanism might also facilitate healing of the matrix microtears on removal of the stress.

Although the factors leading to the distinctive granular morphology in *M. galloprovincialis* cuticle, on the one hand, and the featureless *P. canaliculus* cuticle, on the other, remain unknown, a critical role for the mfp-1 coating proteins is indicated.

An ultrastructural examination of byssal cuticle formation by the accessory cells in *M. galloprovincialis* revealed that the phase separation of mfp-1 is gradual and occurs within the secretory granules of the cells before thread formation[18]. Mfp-1 variants have little known secondary structure and are highly positively charged ($pI > 10$), both excellent properties for polymers involved in phase-separated systems[19]. The mfp-1 coating proteins of the two species are similar in repetitiveness, fidelity of repeat sequences and amino-acid composition, but differences are also evident[8]. Of the two, the *Perna* mfp-1 is half the mass, has shorter consensus repeats and lacks hydroxyprolines.

Our comparative study of the thread cuticles from two mussel species has uncovered a natural coating strategy that provides protection of vulnerable components under very large strain. As we develop a further understanding of the factors responsible for the microstructural features of these cuticles as well as the mechanisms governing their deformation and fracture, strategies such as these are likely to spawn a new generation of bio-inspired protective coatings with novel combinations of mechanical properties.

METHODS

M. galloprovincialis were collected from Goleta Pier (Goleta, California), and *P. canaliculus* from New Zealand were obtained from Harbor Meat and Seafood (Santa Barbara, California). Both species were maintained in open circulating seawater tanks at 15 °C. After removal of the old byssus, whole threads deposited within 5–7 days were rinsed in deionized water, and stored at 4 °C until needed.

To analyse the effects of stretching on the structural organization of the cuticle, threads were stretched under water to 30, 50, 70 and 80% of their initial length at a speed of 5 mm min^{-1} with a Bionix 200 tensile tester (MTS Systems, Cary, North Carolina). 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 5,130 mm, Tescan, Czech Republic). For comparison, unstretched control samples taken through the same drying procedure were also examined. To measure the local effects of strain in the granular coating of *M. galloprovincialis*, fibres stretched to 50 and 70% and unstretched fibres were embedded in Epofix (Electron Microscopy Sciences, Hatfield, Pennsylvania) and longitudinal cross-sections of the distal portions of the threads were prepared with a microtome (Leica EM UC6, Leica Mikrosysteme GmbH, Vienna). The local deformation in the granular coating was then imaged by AFM in air (Dimension 3000/3100, Veeco Metrology, Santa Barbara, California).

Young's moduli and hardness values for the cuticle and the thread interior were obtained for both species by nanoindentation. Tests were carried out on microtomed surfaces of transverse cross-sections of threads embedded in Epofix, with test specimens submerged in milliQ water. Indentation tests were carried out using a TriboIndenter (Hysitron, Minneapolis, Minnesota) with a cube corner diamond tip. The tip area function was established from a PMMA standard. All load–displacement curves were analysed using the method described by Oliver and Pharr[20]. 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 s^{-1} and the peak load was approximately 1,000 μN. Once at the peak, the load was held fixed for a period of 30 s, for the purpose of eliminating creep effects.

For transmission electron microscopy, threads were stained with osmium tetroxide, fixed in glutaraldehyde/paraformaldehyde, embedded in Spurrs resin (Ted Pella, Redding, California) and microtomed to produce thin sections of 80 nm, following standard protocols. Micrographs were obtained using a JEOL 123 transmission electron microscope (Pleasanton, California) operated at 80 kV.

For high-resolution AFM under water, sections of unstrained *M. galloprovincialis* threads were prepared as for AFM in air but imaged under water in soft-tapping mode in a closed fluid cell using a MFP-3D-BIO AFM (Asylum Research, Santa Barbara, California).

Received 28 March 2007; accepted 4 June 2007; published 8 July 2007.

References

1. Ghosh, S. K. in *Functional Coatings* (ed. Ghosh, S. K.) (Wiley, Weinheim, 2006).
2. Rossi, S., Deflorian, F. & Risatti, M. Reduction of corrosion protection properties of organic coatings due to abrasive damage produced by natural sands. *Wear* **261**, 922–931 (2006).
3. Fabbri, P. *et al.* Cohesive and adhesive properties of polycaprolactone/silica hybrid coatings on poly(methyl methacrylate) substrates. *Surf. Coat. Technol.* **200**, 6706–6712 (2006).
4. Leyland, A. & Matthews, A. On the significance of the H/E ratio in wear control: A nanocomposite coating approach to optimised tribological behavior. *Wear* **246**, 1–11 (2000).
5. Messori, M. *et al.* Prevention of plasticizer leaching from PVC medical devices by using organic–inorganic hybrid coatings. *Polymer* **45**, 805–813 (2004).
6. Das, D., Datta, M., Chavan, R. B. & Datta, S. K. Coating of jute with natural rubber. *J. Appl. Polym. Sci.* **98**, 484–489 (2005).
7. Sun, C. & Waite, J. H. Mapping chemical gradients within and along a fibrous structural tissue, mussel byssal threads. *J. Biol. Chem.* **280**, 39332–39336 (2005).
8. Zhao, H. & Waite, J. H. Coating proteins: Structure and cross-linking in fp-1 from the green shell mussel *Perna canaliculis*. *Biochemistry* **44**, 15915–15923 (2005).
9. Rius, M. & McQuaid, C. D. Wave action and competitive interaction between the invasive mussel *Mytilus galloprovincialis* and the indigenous *Perna perna* in South Africa. *Mar. Biol.* **150**, 69–78 (2006).
10. Zardi, G. I., Nicastri, K. R., Porri, F. & McQuaid, C. D. Sand stress as a non-determinant of habitat segregation of indigenous (*Perna perna*) and invasive (*Mytilus galloprovincialis*) mussels in South Africa. *Mar. Biol.* **148**, 1031–1038 (2006).
11. Faul, C. F. J. & Antonnietti, M. Ionic self-assembly: Facile synthesis of supramolecular materials. *Adv. Mater.* **15**, 673–683 (2003).
12. Unnikrishnan, K. P. & Thachi, E. T. Toughening of epoxy resins. *Design. Monomers Polym.* **9**, 129–152 (2006).
13. Gao, G., Zhang, J., Yang, H., Zhou, C. & Zhang, H. Deformation mechanisms of polystyrene toughened with sub-micrometer monodisperse rubber particles. *Polym. Int.* **55**, 1215–1221 (2006).
14. Newman, S. & Strella, S. Stress–strain behavior of rubber-reinforced glassy polymers. *J. Appl. Polym. Sci.* **9**, 2297–2310 (1965).
15. Inoue, K., Waite, J. H., Matsuoka, M., Odo, S. & Harayama, S. Interspecific variations in adhesive protein sequences of *Mytilus edulis*, *M. galloprovincialis* and *M. trossulus*. *Biol. Bull.* **189**, 370–375 (1995).
16. Zhao, H. & Waite, J. H. Linking adhesive and structural proteins in the attachment plaque of *Mytilus californianus*. *J. Biol. Chem.* **281**, 26150–26158 (2006).
17. Lee, H., Scherer, N. F. & Messersmith, P. B. Single-molecule mechanics of mussel adhesion. *Proc. Natl Acad. Sci. USA* **103**, 12999–13003 (2006).
18. Zuccarello, L. V. Ultrastructural and cytochemical study on the enzyme gland of the foot of a mollusc. *Tissue Cell* **13**, 701–713 (1981).
19. Williams, T., Marumo, K., Waite, J. H. & Henkens, R. W. Mussel glue protein has an open conformation. *Arch. Biochem. Biophys.* **269**, 415–422 (1989).
20. Oliver, W. C. & Pharr, G. M. An improved technique for determining hardness and elastic-modulus using load and displacement sensing indentation experiments. *J. Mater. Res.* **7**, 1564–1583 (1992).

Acknowledgements

The authors thank J. Weaver for continued discussion of this project. This work was supported by the National Institute of Health under awards No. R01 DE015415 and No. R01-GM65354. G.E.F. thanks the Austrian Academy of Science for a DOC fellowship. This work made use of the MRL central facilities at UCSB supported by the MRSEC Program of the National Science Foundation under award No. DMR00-80034.
Correspondence and requests for materials should be addressed to J.H.W. or F.W.Z.

Author contributions

G.E.F. and S.H. contributed with experimental work and data analysis. J.H.W. contributed with experimental design, data analysis, manuscript writing and project research management. F.W.Z. contributed with experimental design, data analysis and manuscript writing.

Competing financial interests

The authors declare no competing financial interests.