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REVIEW

Changing environments and structure–property relationships in marine biomaterials

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Summary

Most marine organisms make functional biomolecular materials that extend to varying degrees 'beyond their skins'. These materials are very diverse and include shells, spines, frustules, tubes, mucus trails, egg capsules and byssal threads, to mention a few. Because they are devoid of cells, these materials lack the dynamic maintenance afforded intra-organismic tissues and thus are usually assumed to be inherently more durable than their internalized counterparts. Recent advances in nanomechanics and submicron spectroscopic imaging have enabled the characterization of structure–property relationships in a variety of extra-organismic materials and provided important new insights about their adaptive functions and stability. Some structure–property relationships in byssal threads are described to show how available analytical methods can reveal hitherto unappreciated interdependences between these materials and their prevailing chemical, physical and ecological environments.

Key words: mechanical properties, molecular structures, length scales, perturbation, mussel byssus.

Introduction

Investigating the relationships between organisms and their environments at many length and time scales is necessary for a mechanistic understanding of the limits of adaptation and the survival strategies of individuals and communities. With dimensions that range from molecular to global, the problem is a staggering one and will require scientific and technical expertise and creative thinking from many different disciplines. At the cellular level, the role of the environment in perturbing the metabolic activity of an organism is no longer hypothetical. Organisms ranging from microbes to metazoans are perpetually making measurable adjustments to maintain their cells and tissues within a narrowly defined standard state in response to internal and environmental changes. The cellular heat shock physiology and redox regulation are two classic examples (Lindquist and Craig, 1988; Jensen et al., 2009).

Living organisms, however, are much more than a collection of cells and organs covered by a membrane or skin. Highly elaborate adaptive materials are constructed 'beyond the skin' of the organism. Turner refers to these materials as 'externalized organs' even though they are usually devoid of cells (Turner, 2000). The bell of diving spiders, midge-induced plant galls, polychaete burrows and termite mounds are a few of the many extended structures examined. How the environment influences the performance of these biomaterials is also crucially important (Byrne et al., 2011). The extended material is, after all, often the first line of defense between an environmental shock and the organism. Bivalve mollusks, for example, can tightly close their shells thereby insulating their living tissues from transient environmental stresses. If biomaterials were also adapted for optimal function within some limited range of environmental conditions, what effect would a shock beyond this range have on their performance? A compromised biomaterial performance could arise from three different scenarios: (1) during a sustained environmental stress, cells and tissues working under austerity survival measures might produce inferior biomaterials; (2) during an abrupt and extreme environmental stress, one or more factors (e.g. waves and acidic run-off) might combine to briefly undermine the performance of materials in service, or (3) during a sustained change in the environment that is not physiologically stressful, the quality of normal biomaterials might deteriorate (Fig. 1). To date, it remains unknown whether the first, second, third, or some combination of the three represents the most realistic perturbation of biomaterials serving an individual or community.

Of the many different properties exhibited by extra-organismic biomaterials in the extended organism, many research groups including ours have focused on those with mechanical or load bearing properties. These are necessary in biomaterials that function in, for example, support, shelter, feeding, prey capture and adhesion. This review is about structure–function relationships in the load-bearing biomaterials of extended organisms and the hypothesis that the functional performance of biomaterials is linked to structures that are perturbed by a changing environment. The proposition is somewhat rhetorical because the toolkits to properly test it are incomplete and few are suited to field ecology. Notwithstanding this, the overview will briefly survey old and new techniques for studying structures and mechanical properties in marine biomaterials, survey how different test conditions affect structure and function in byssal threads, and speculate on what these studies might contribute to ecomechanics.

Approaches to structure–function analysis

A major goal in experimental biology is discovering relationships between structure and function. This goal is equally relevant to studying biomaterials, except that mechanical properties are used as proxies for function. It is understood that the measured mechanical properties may only be one of many in a multifunctional material. The conventional wisdom in materials science is that structure determines properties; thus, altering structure is likely to alter

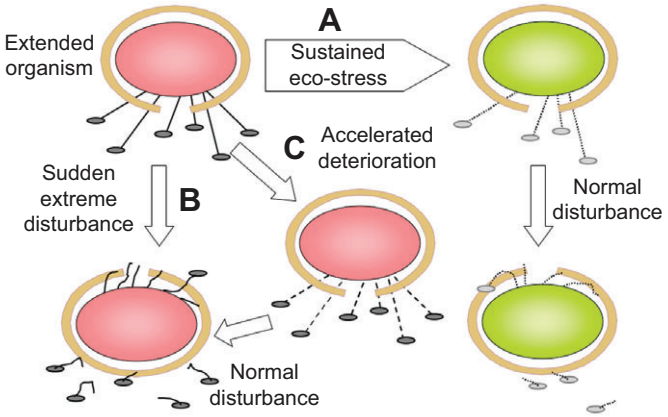

Fig. 1. Three types of compromised quality in the biomaterial of an extended organism. (A) An organism during a sustained environmental stress makes a defective material (dotted tethers). (B) An organism in a normal environment makes normal biomaterial that is suddenly perturbed (perhaps involving a combination of environmental factors) that exceeds its performance capacity. (C) An unstressed organism with a normal biomaterial that undergoes enhanced deterioration (dashed tethers). Green denotes a physiologically stressed organism and red a normal one.

properties. Structure in any given material is defined as the chemical and physical arrangement of constituent molecules. A property is a measurable attribute of function, and performance the degree to which anything functions as intended or adapted. Any given structure and property must be investigated at many length and time scales in order to understand their relationships to one another.

Traditional approaches

Covalent structure

The presence and arrangement of minerals, metal ions, halogens, organics such as proteins, carbohydrates, fatty acids, and even water, in biomaterials all contribute to their structure. However, they must usually be mapped and, in the case of biomolecules, characterized before their contribution can be assessed. Structure analysis of constituent biomacromolecules has long posed difficult obstacles to progress: native biomaterials require prior removal of minerals, degradation with denaturants and painstaking isolation and chemical characterization of constituents. Primary structure determination of proteins still entails the time-consuming and costly sequencing of purified proteins, although this has shifted from Edman chemistry to fragmentation using tandem mass spectrometry (Mann et al., 2001; Papov et al., 1995). The high degree of protein purity for sequencing is less crucial today because the molecular filters on the mass analyzers of mass spectrometers can be tuned to specifically select a single parent mass from a complex mixture for sequencing by fragmentation.

In any case, complete end-to-end sequencing of proteins is only rarely necessary, such as when the protein is highly post-translationally modified. Instead, a few partial sequences are usually adequate to design synthetic degenerate oligonucleotides that can be used to amplify a partial sequence from a cDNA library that, with additional nondegenerate primers, can be extended to its 3′ and 5′ ends and its corresponding mature protein sequence deduced (Frohman et al., 1988). After the purification of proteins and the preparation of specific antibodies, location of proteins in the original biomaterial is possible by immunohistochemical techniques. In our experience, however, more often than not, proteins destined for extra-organismic biomaterials completely lose their antigenicity because of chemical changes that occur during maturation (Anderson and Waite, 2000).

Structure – higher order

Those fortunate enough to work on a biomaterial containing crystalline or paracrystalline structures are blessed by the rich structural insights revealed by deconvolution of the Bragg and diffuse scattering intensities obtained by X-ray irradiation. These were first applied to biomaterials by Astbury (Astbury, 1933) and are known today as wide angle and small angle X-ray scattering (WAXS and SAXS; Table 1). A substantial database exists on the powder X-ray diffraction of minerals relevant to hard tissues (The International Centre for Diffraction Data; http://www.icdd.com/). The database of distinctive secondary and tertiary structures in natural fibers is somewhat less extensive but every bit as useful (Warwicker, 1960). Indeed, deconvolution of the reflections in the reciprocal space of diffractograms for cocoon silk, wool and tendon collagen often serve as paradigms for different secondary structures such as beta sheet (silk), alpha helix (α-keratin) and polyproline type II helix (collagen), respectively. Insights about the secondary structure of a biomaterial such as spider silk obtained from fiber X-ray diffraction often preceded the publication of protein sequence by several decades (Warwicker, 1960; Guerette et al., 1996). X-ray diffraction of biomaterials has sometimes biased the study of structure. Many biomaterials with complex or unrevealing structures were dismissed as unworthy of further study, whereas others with only a partial fit to prototypes were inaccurately classified. Byssal collagens, for example, were passed off as collagens, when in reality their domain structure combines elastin, silk and collagen motifs (Waite et al., 1998).

Properties

Mechanical properties define how a material deforms in response to an applied force, and biomechanics goes a step further to examine how the response is related to function. In a typical macroscopic mechanical analysis, a biomaterial is subjected to uniaxial tensile and/or compressive testing (e.g. Instron testing with static or quasi-dynamic loading) under dry or hydrated conditions and at several different strain rates. There are widely accepted formulations of critical properties in biomaterials subjected to macroscopic tension, compression or shear (Vogel, 2003). Some of these are initial stiffness, hardness, strength, yield stress, extensibility and fracture toughness. In materials science, the utility of critical properties depends on the validity of certain assumptions pertaining to the tested specimen including: (1) homogeneity of composition and anisotropy, (2) uniform dimensions and (3) the absence of flaws. These assumptions are rarely if ever fully satisfied in biomaterials (De Langre, 2012). Uniform dimensions, for example, are found in simple solid geometries such as beams, sheets, cylinders and spheres. Byssal threads, spider and silkworm silks and macroalgal stipes are presumed to approximate these shapes [although the fit is often less than perfect (Brazee and Carrington, 2006)] (Fig. 2). In most load-bearing biomaterials, however, deviations from ideality are usually too great to allow meaningful macromechanical analysis. A polychaete jaw, for example, does not have simple geometry (Fig. 2E), nor does it have a uniform composition or architecture (Broomell et al., 2006; Lichtenegger et al., 2003).

New approaches

Structure

Although many new techniques have evolved for the determination of chemical structure there is no comprehensive *in situ* method for

Table 1. Comparative survey of techniques for *in situ* structure analysis of biomolecular materials

Structural information	Level of structure	Technique	Probe diameter	Specimen condition	Reference*
Composition	Elemental	XAS	1 μm	Direct; wet or dry	1
		EDX-SEM	<1 μm	Dry; Ag coating	
		PIXE	1 μm	Dry; on capton foil	2
	Molecular	MALDI MS	50 μm	Dry w/ matrix	3
Interactions	Ionic	XANES	0.5 mm	Direct; wet or dry	4
	Coordination	EXAFS	0.5 mm	Direct; wet or dry	4
		Raman microscopy	1 μm	Thin section	5
	Covalent	Solid state NMR	Variable	Wet/dry	6
Conformation	Crystal	WAXS	0.8 mm	Direct; wet or dry	7
	Secondary or higher	WAXS	0.8 mm	Direct; wet or dry	8
		BESSY	30 μm	Direct; wet or dry	9
Orientation	Anisotropy	SAXS	0.8 mm	Direct; wet or dry	4, 9, 10
	Deformation	SAXS BESSY	30 μm	Direct; wet or dry	9

BESSY, Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung; EDX-SEM, energy dispersive X-ray spectroscopy-scanning electron microscopy; EXAFS, extended X-ray absorption fine structure spectroscopy; PIXE, proton induced X-ray emission spectroscopy; SAXS, small angle X-ray scattering; ssNMR, solid-state nuclear magnetic resonance; WAXS, wide angle X-ray scattering; XANES, X-ray absorption for near edge structure; XAS, X-ray absorption spectroscopy; MALDI MS, matrix-assisted laser desorption-ionization mass spectroscopy.

*References are selected from investigations of extra-organismic materials in marine invertebrates. 1, Broomell et al., 2006; 2, Schofield et al., 1992; 3, Zhao et al., 2006; 4, Lichtenegger et al., 2003; 5, Harrington et al., 2010; 6, McDowell et al., 1999; 7, Lichtenegger et al., 2002; 8, Miserez et al., 2007; 9, Harrington et al., 2009; 10, Miserez et al., 2012.

determining structure at the nanoscale in a noncrystalline macromolecular material (Billinge and Levin, 2007). Instead, various methodologies with a wide range of sensitivities, robustness and resolution are available. Essentially all perform a bulk analysis on patches of sample surface and the detected analytes represent the averages over those patches. Testing under physiologically and/or ecologically relevant conditions is desirable but rarely possible.

For characterizing structure *in situ* at many length scales, several options are listed in Table 1. These are not exhaustive but rather represent techniques with which the authors are familiar. Most types of *in situ* elemental analysis of biomaterials are based

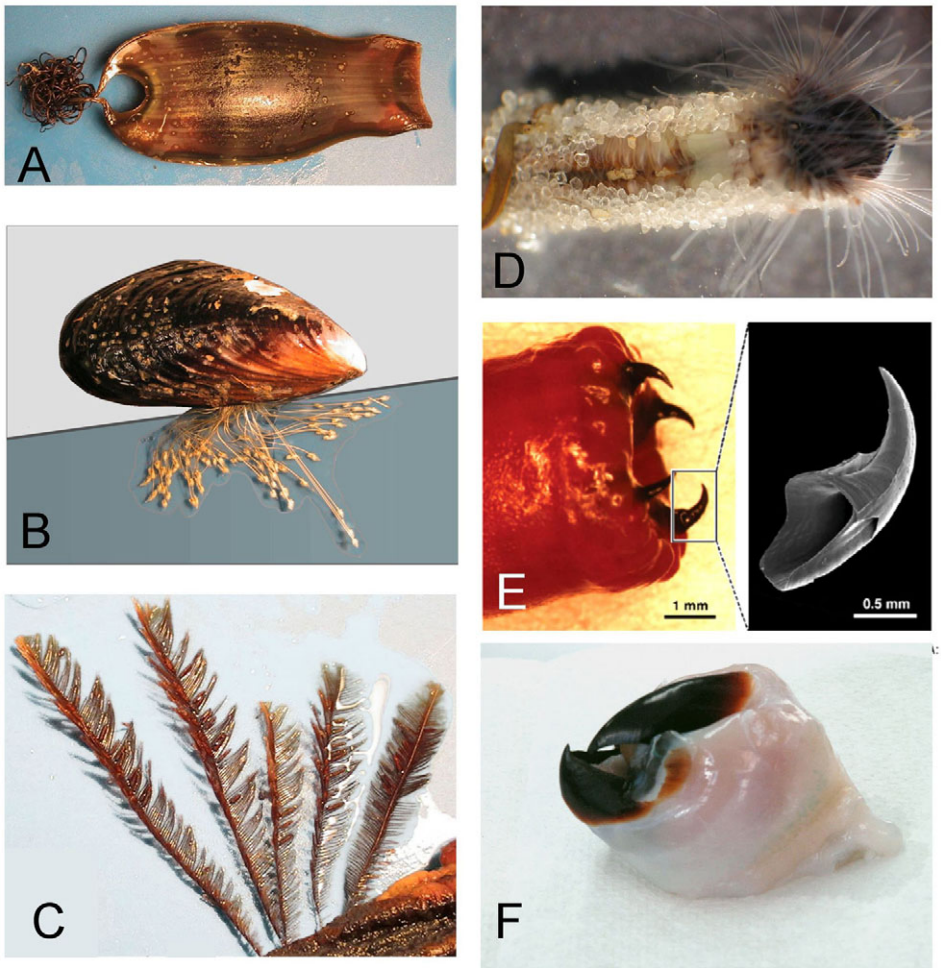

Fig. 2. Biomaterials of extended organisms that highlight some of the geometries and heterogeneous compositions that challenge mechanical characterization. (A) Swell shark (*Cephaloscyllium ventriosum*) egg capsule. (B) Mussel (*Mytilus californianus*) shell and byssus. (C) Perisarc of *Aglaophenia,* a colonial hydroid. (D) Tube of sandcastle worm (*Phragmatopoma californica*). (E) Bloodworm (*Glycera dibranchiata*) jaw. (F) Squid (*Dosidicus gigas*) beak.

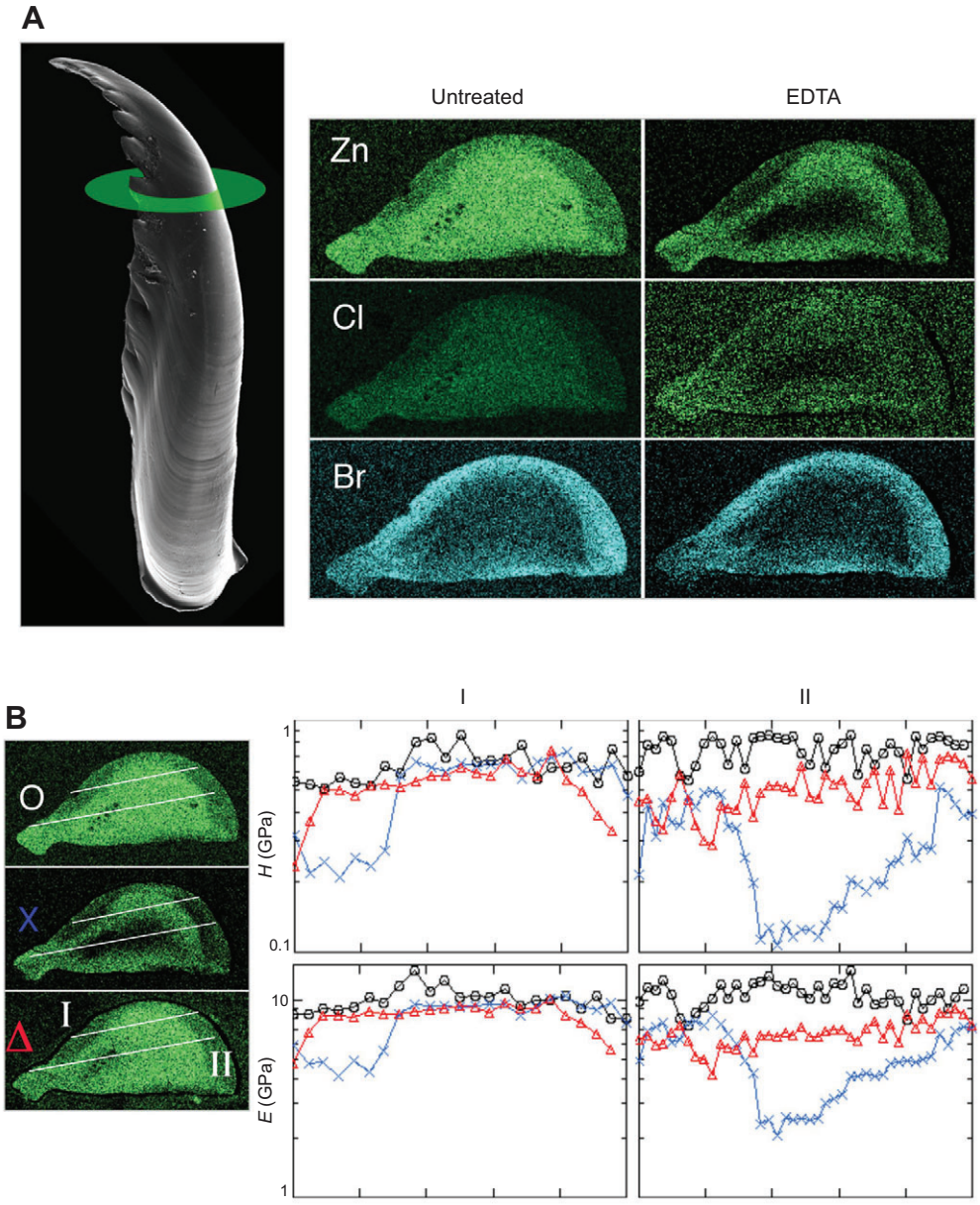

Fig. 3. Mapping chemical structure and mechanical properties in a section of polychaete jaw. (A) Elemental distribution maps of zinc, bromine and chlorine were obtained using EDX in combination with scanning electron microscopy before and after Zn^{2+} removal by EDTA chelation. Note that zinc is only removed from central and peripheral areas, suggesting different affinities in zinc binding by the protein matrix. Chlorine maps as zinc, thus is probably bound to it; bromine in contrast is not affected by EDTA. (B) Nanomechanical maps of the same section of hydrated jaw along trajectories showing different metal depletion patterns. The zinc distribution maps are denoted by O (pristine condition), X (extracted with EDTA) and Δ (restored). I and II indicate the nano-indentation trajectories used. Trajectory II shows particularly well the effect of zinc depletion on stiffness (*E*) and hardness (*H*; blue line). The correlation of stiffness with zinc is confirmed by substantial restoration of stiffness with zinc reconstitution (red line) (Broomell et al., 2006).

on an energy spectrum following bombardment by X-rays (X-ray absorption spectroscopy or XAS), electrons (energy dispersive X-ray spectroscopy or EDX) or protons (proton induced X-ray emission spectroscopy or PIXE) and are often combined with transmission or scanning electron microscopy. In EDX, the specimen or thin section is dried and sputtered with gold or carbon prior to irradiation, which stimulates X-ray emission from the material in an element-specific manner. Elemental sensitivity of EDX is typically 0.1% dry mass of sample but instruments with higher sensitivity are available. The method is appealing in terms of expense, time and effort required, and revealing about trends in the presence and distribution of metals, minerals and macromolecules (especially using sulfur and phosphorus as markers) that can be pursued in greater depth using other methods. Fig. 3A illustrates the prominence of zinc, iodine and bromine in *Nereis* jaw and reveals preferential localizations and how prone these are to extraction by ethylenediamine tetra-acetic acid (EDTA), a chelating reagent that is widely used to remove metal ions. The abundance of carbon, nitrogen and oxygen was roughly consistent with the presence of protein fibers (not shown).

For chemical information about interactions between different functionalities, there are further analyses that need to be done using X-ray absorption spectroscopy at a synchrotron source. X-ray absorption for near edge structure (XANES) provides information about the oxidation state of metal ions and their counter ions or ligands. In *Nereis* jaw, the XANES spectra suggest that Zn^{2+} is complexed to histidine-like ligands. Extended X-ray absorption fine structure spectroscopy (EXAFS; also synchrotron-dependent) confirms the presence of Zn^{2+} with three imidazoles and one Cl^{-} ligand, which has some similarity to the structure of zinc–insulin complexes (Lichtenegger et al., 2003).

Interestingly, in the jaw of a related polychaete species, *Glycera convoluta*, EDX detected a different metal (copper), and the only detectable halogen was chlorine (Lichtenegger et al., 2002). As in *Nereis* jaw, *Glycera* jaw was predominantly organic with copper

concentrated towards the biting tip. In contrast to *Nereis*, however, most of the copper in *Glycera* jaw was shown by XANES and WAXS to be associated with mineralized fibers of atacamite or $Cu(OH)_3Cl$. A lesser amount occurs as Cu^{2+} associated with the outermost organic layer coating the jaw. Also in contrast to *Nereis*, the organic portion of *Glycera* jaw was not merely protein, but a composite of protein and melanin with the protein fibers perpendicular to the jaw curvature (Moses et al., 2006; Moses et al., 2008).

Even higher order structure can be explored by SAXS, which detects the extent of anisotropy as well as the average diameter of structural elements such as fiber bundles. SAXS requires little in the way of sample preparation and can be done on dry or hydrated specimens. Using this method, *Nereis* jaw has been found to consist of tight bundles of fibers (120±50 nm) that closely follow the contour of jaw curvature (Lichtenegger et al., 2003).

Matrix-assisted laser desorption-ionization (MALDI) is a type of 'soft' mass spectrometry that uses an ultraviolet laser to excite a matrix compound such as α-cyano-3-hydroxycinnamic acid, which, as it ionizes and desorbs from a surface, transfers some of its energy and charge to other analytes in the specimen. These analytes typically include proteins, peptides, lipids etc, from 250 Da to over 50 kDa in mass. Because of an adiabatic energy transfer, larger analytes typically desorb intact, and enter the mass analyzer by which they are detected as ions according to their mass/charge (*m/z*) ratio. *In situ* MALDI analysis of different parts of mussel byssus – plaque, thread and stem – has greatly aided our understanding of protein distribution in that structure. It was MALDI analysis of plaque footprints, for example, that identified three mussel foot proteins (mfp-3, mfp-5 and mfp-6) at the interface between the plaque and substratum (Zhao et al., 2006; Yu et al., 2011). Some currently available MALDI instruments combine programmable specimen rastering with mass analysis and imaging to provide maps of molecular distributions in tissue samples prepared as thin sections (10–20 μm thick) saturated with matrix (Cornett et al., 2007). Creative use of mass spectrometers with imaging capabilities has the potential to significantly advance understanding of the macromolecular organization of biomaterials.

Two final speciality techniques, Raman microscopy and solid-state nuclear magnetic resonance (NMR), are worthy of consideration for the insights they can provide for particular structures. Raman spectroscopy measures mostly ground state molecular vibrations and rotations in a variety of chemical functionalities inducible by visible to near infrared monochromatic light. Most of these vibrations are weak, however, when transitions between ground and excited states occur they are extremely strong. The vibrations of charge-transfer complexes between metals and ligands, for example, are so strong that they overwhelm weaker signals even at excitation wavelengths that are far removed from the maximum wavelength of charge transfer. Charge transfer typically occurs when a nonbonding electron is transferred between a metal ion and a ligand. Just such a transfer occurs between the 3,4-dihydroxyphenylalanine (dopa) residues of mfp-1 and Fe^{3+} in solution (Taylor et al., 1996) and in the cuticle of byssal threads of *Mytilus* species (Harrington et al., 2010). In thread cuticle analysis, Raman microscopy is much more sensitive for dopa–iron detection than EDX; enhanced iron levels were detected in all mytilid thread cuticles analyzed by Raman spectroscopy, whereas EDX detected levels above background in only half of them (Sun and Waite, 2005). Raman microscopy relies on a confocal microscope to image the location of vibrations resulting from dopa–iron complexes in thin sections of byssal threads. Given the excellent resolution (1 μm) of this microscopy, it has been possible to detect a hierarchical pattern in the way that dopa–iron complexes are distributed in byssal cuticles (Harrington et al., 2010).

Solid-state NMR (ssNMR) has potential for measuring concentration- and time-dependent chemical changes in biomaterials. For example, to test whether covalent cross-links form in byssal threads (or portions of byssal threads) during maturation following secretion, ssNMR was used to track changes in chemical shifts of labeled tyrosine incorporated into byssal threads (McDowell et al., 1999). For best results, the method requires doping seawater filtered by living mussels with amino acids thought to be partners in cross-linking. Ordinarily, the dopants are simple amino acids synthesized with nonradioactive isotopes (^2H, ^{13}C and ^{15}N) strategically placed in those molecular positions targeted for the cross-linking bonds. It is the cost of synthesizing these analogs that puts the experiment beyond the reach of most research budgets.

Mechanical properties

The recent introduction of two mechanical techniques – nanoindentation and the surface forces apparatus – has the potential to probe properties of biomaterials at the nano and micrometer length scales in any geometry.

Nanoindentation

Structural details of polychaete jaws highlight many of the challenges facing a meaningful mechanical characterization, i.e. an eccentric shape, small dimensions, inhomogeneous compositions and orientations of the components (Fig. 4). The introduction of force mode atomic force microscopy (AFM), and particularly a dedicated variation of AFM called nano-indentation (Oliver and Pharr, 1992), has made substantial contributions to routine mechanical characterization with a 1–5 μm resolution. The versatility of the method offers specimen testing under a wide range of conditions of hydration, ionic strength, pH, temperature and sample compliance (Moses et al., 2008).

In nanoindentation, a hard tapered tip of precise geometric shape and dimensions mounted on the end of a rod with two calibrated linear variable differential transformers for force and depth is pressed into a flat surface at a predetermined rate and load (Fischer-Cripps, 2002). The displacement or depth of penetration (h) is plotted against the applied load (p) to reveal two important materials properties: stiffness and hardness. Indentation stiffness (E_r) is derived from the tangent to unloading at maximum load (P):

$$E_r = 3P / 4R^{1/2} h_e^{3/2}, \quad (1)$$

where R is the indenter radius and h_e is the load point displacement (Fig. 4).

Hardness (H) is determined from the depth of the stab indent (h_p) and for a triangular pyramidal (Berkovich) tip is related to the equation:

$$H = P / 24.5\, h_p^2. \quad (2)$$

It is quite common to program nanoindenters to automatically indent a defined surface at 5 μm steps and then to map the changes in E and H over hundreds, or even thousands, of square micrometers. Smaller steps are programmable but this risks measuring damage due to strain fields of previous indents.

With the help of a nanoindenter, E and H of peculiar biomaterials such as jaws, stylets, stingers, beaks, claws and spicules can be tested, provided that smooth sections or surfaces are available. From a functional perspective, E can be viewed as how well the

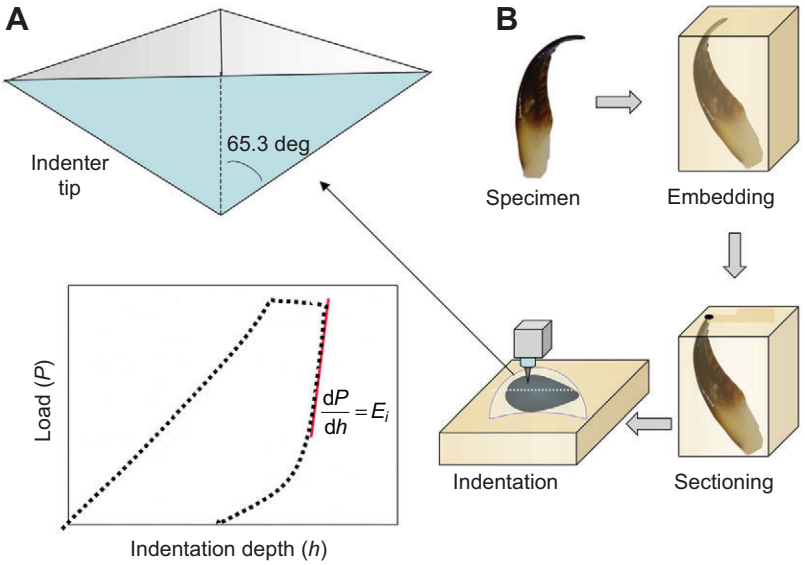

Fig. 4. Nanoindentation of a polychaete jaw. (A) Pyramidal indenter tip, i.e. Berkovich. (B) Steps in processing an embedded specimen for sectioning and indentation; sectioning is done on a microtome to a desired depth with respect to the jaw tip; programmed nanoindentation of hydrated sectioned surface along a trajectory as in Fig. 3B. (C) A load *versus* displacement curve, which, from the unloading portion and its initial slope, allows the determination of a Young's modulus (E_i).

biomaterial resists deformation by tension, compression or bending, whereas H denotes resistance to permanent deformation usually by a sharp object. High H in a jaw is essential for penetrating food or an enemy. High E ensures that jaw architecture will withstand cyclic deformation. Combining E and H, as $H^{3/2}/E$, provides a measure of the resistance to wear or abrasion. These are important parameters in materials such as polychaete jaws adapted for impact and penetration.

Nanoindentation of a microtomed surface of *Nereis* jaw is shown in Fig. 3B highlighting the differences between pristine, chelation-depleted, and zinc-restored jaw sections along defined trajectories. Metal depletion by chelation in metal-stabilized biomaterials becomes probable during two types of environmental stress: high ambient microbially produced siderophore levels, or run-off with high humic acid content (Raymond and Dertz, 2004; Stumm and Morgan, 1996). Note that the material becomes soft and compliant in areas of zinc depletion and that hardness and stiffness are restored by re-exposure to Zn^{2+}. In practice, a biomaterial is carefully dissected from the organism, dehydrated and embedded in resin, and sectioned in a preferred orientation on a microtome to prepare a smooth surface. The plug end of the sectioned sample is then mounted directly in the indenter, which is programmed to map a portion or the entire exposed dry or hydrated surface. Before undertaking such a study there are several parameters that must be carefully examined. The first concerns the chemical and physical effects of embedding on the integrity of native structure. Does resin chemistry modify sample properties? Does the resin completely surround and adhere to the specimen? Does the swelling of rehydration distort the structure? These are valid concerns that often require the testing of many variations. Some preferred general procedures for sclerotized materials have recently been described (Broomell et al., 2006).

Surface forces apparatus details

Surface forces are important for numerous functions in living organisms including their extended nonliving structures. The survival of many marine organisms depends on their ability to attach to solid surfaces. Force gauges have been used in the field to measure the tenacity of individuals, but progress in marine adhesion has been much hampered by the absence of instrumentation to measure adhesion at the micro and nanoscales. The most sensitive and versatile instrument for measuring surface forces is the surface forces apparatus (SFA) (Israelachvili et al., 2010). SFA has nanoNewton force and ångström (Å) distance precision and has been used to measure attractive or repulsive forces between surfaces. Its design is a simple one consisting of two crossed (at 90 deg) half cylinders with the rounded surfaces covered by a thin layer (1 µm) of atomically smooth mica (Fig. 5A). The contact zone has a diameter of 50 µm. Compressive forces are imposed by a calibrated spring attached to the lower hemi-cylinder, while the approach and separation distances are measured by videotaping fringes of equal chromatic order (FECO) produced by a beam of visible light that is directed to the contact zone. Owing to silvering of the mica back surfaces, the light is reflected between the confined mica sheets allowing a single wavelength to pass through the contact zone to the collimator. The FECO video images can be deconvoluted to reveal distance, rate of separation, sample homogeneity, hydrodynamic diameter and extensibility. In adhesion measurements, the coordinates plotted are force/radius *versus* distance. Distance decreases as the upper surface is lowered using a piezoelectrically controlled stage until repulsion between the two surfaces increases sharply to an asymptotic or predetermined level (Fig. 5B). The asymptotic level is referred to as the 'hardwall' beyond which the intervening material cannot be compressed. When a protein film is deposited as a monomolecular layer, the hardwall approximates the protein's hydrodynamic diameter. The stage is then reversed for separation. When there is no adhesion, the separation essentially retraces the approach curve; with adhesion, however, there is a sharp jump-out in force (spike in the negative force direction) as the interface resists separation. The peak force (F) is referred to as the adhesion strength with units of $mN\,nm^{-1}$. It is customary to convert the peak force of the jump out to units of adhesive energy (E_A) using the Derjaguin approximation (E_A=–$F/2\pi R$) where R is the radius of curvature.

Mica, the preferred surface for SFA, is a naturally occurring clay (muscovite) convenient for its smoothness, but mica surfaces can be modified to many other chemistries and topographies by standard nanodeposition techniques and used in the SFA providing that light remains transmissible through the contact area. Nanolayers of silica, alumina, titania, polymethylmethacrylate and polystyrene, among others, have been successfully deposited on mica as have films of biosurfactants, membranes and proteins

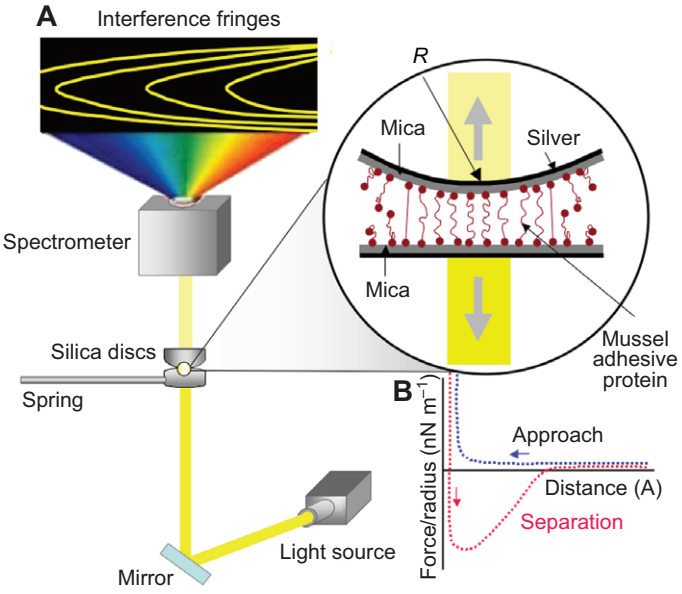

Fig. 5. The surface forces apparatus for measuring adhesion and cohesion in biomaterials. (A) A surface-active substance, such as a mussel byssal plaque adhesive protein, is applied as a thin film to one (asymmetric) or both (symmetric) mica surfaces, then the surfaces are brought into contact (approach) and after a brief contact, are separated. (B) Adhesion is measured by the maximum force resisting separation. *R* is the cylinder radius. In asymmetric tests, the measured adhesion force often reflects the strength of adsorption, whereas in symmetric configurations, it reflects the cohesive strength.

(Anderson et al., 2010; Lin et al., 2007). The SFA has the potential to measure how changing seawater chemistry affects the surface energy of mica, and how interfacial energy affects the performance of adhesive molecules (Yu et al., 2011; Hwang et al., 2010).

Structure–property databases

Any study of structure–property relationships in a biomolecular material necessarily depends on a reliable database of coupled physical or chemical and mechanical data. The ideal database of information for understanding and predicting the effect of environment on the structure and properties of biomaterials is an extensive set of structure–property maps at different length and time scales and under different conditions. The physical–chemical database provides fundamental insights into how a material is put together, whereas the mechanical database reflects critical load-bearing properties such as strength, extensibility, stiffness, hardness, wear and toughness. Typically, at any given set of experimental conditions, the accumulated structural and mechanical datasets are mapped to highlight correlated trends. Given the natural variability of biomaterials, the best correlations are likely to emerge when the same material or specimen is subjected to a simultaneous chemical and mechanical analysis under natural or near-natural conditions. At present this is only possible at facilities such as the BESSY synchrotron (Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung, Berlin, Germany) (Harrington et al., 2009).

Current constraints

Current methodologies for the coupled mapping of structure and mechanical properties in biomolecular materials have many constraints even before considering the special needs of ecological fieldwork. In some cases, specimen preparation, such as embedding and sectioning, is extensive. Whether crucial specimen structures or properties survive preparative treatments should always be explored. Time to analysis can be a factor because many instruments have waiting lists or take many hours to completely map a large specimen. Analytical instruments are cumbersome, delicate and expensive; several require operation under clean room, vibration-free environments, and many require special recalibration for use at different conditions of temperature, pH, ionic strength and hydration. Many chemical analyses can only be done on dry specimens under high vacuum. Synchrotron-based resources, such as those associated with X-ray absorption spectroscopy, are not portable and require advanced authorization for a beam line.

A special focus: mussels, byssus and the environment

Marine mussels, especially those of the genus *Mytilus*, are promising model organisms for investigating ecomechanical properties. Mussels are abundant, robust, easily accessible and influence the spatial distributions of many organisms in the intertidal zone (Van de Koppel et al., 2012). Mytilid physiology, biochemistry, natural history and genetics are well established. An extended biomaterial of mussels, the byssus is a holdfast that mediates the sessile mode of life in individual and clustered mussels and is beautifully adapted to resist forces associated with lift and drag in rocky wave-swept seashores (Yonge, 1962; Bell and Gosline, 1996). Together, clustering tendency and byssus formation provide the vehicle for the dynamics and prominence of mussels as an intertidal foundation species (Denny and Gaylord, 2010).

The byssus is a nonliving biomaterial that consists of a bundle of collagenous threads attached distally to hard surfaces and fused proximally through a stem to 12 byssal retractor muscles at the base of the foot (Fig. 2B and Fig. 6). A byssus, having on average 50–100 byssal threads, is made one thread at a time in the ventral groove of the foot by a 5 min process that resembles the reaction injection molding of polymeric materials (Waite, 1992). Each byssal thread is composed of an interactive consortium of proteins specialized for four major functions: (1) load bearing in tension [prepolymerized collagens (preCOLs) and matrix proteins in the thread core]; (2) a protective outer cuticle (mfp-1 protein); (3) opportunistic adhesion to wet surfaces (mfp-3 and mfp-5); and (4) molecular adaptors that link cuticle and core structures with the adhesives in the plaque. The byssal proteome and mechanical properties of byssal threads have been extensively investigated since 1970, and this growing database has stoked much recent interest in bio-inspired wet adhesive and coating technology (Lee et al., 2011).

To be adaptively beneficial, byssal tenacity ought to be adjustable, and several reports confirm that tenacity is influenced by season (Price, 1980; Carrington, 2002), by spawning stress (Babarro and Fernandez-Reiriz, 2010; Carrington, 2002), as well as by epiphytes (Witman and Suchanek, 1984) and the presence of predators (Caro et al., 2008). One might reasonably expect tenacity to be largely determined by the number of threads in the byssus; however, in the intertidal mussels of Narragansett Bay (Rhode Island), Moeser and Carrington observed that thread quality, more than number, determined tenacity (Moeser and Carrington, 2006). A similar trend was observed in cultured subtidal mussels at Ile de Madeleine lagoon, Quebec, Canada (Lachance et al., 2009). In the Narragansett mussels, the mean breaking strength and extensibility of threads made in late winter to early spring was 50–60% higher than that of threads in other seasons. These are mechanical thread qualities; apparently, changes in structural thread qualities also occur, e.g. a significant (~30%) drop in the thread histidine content

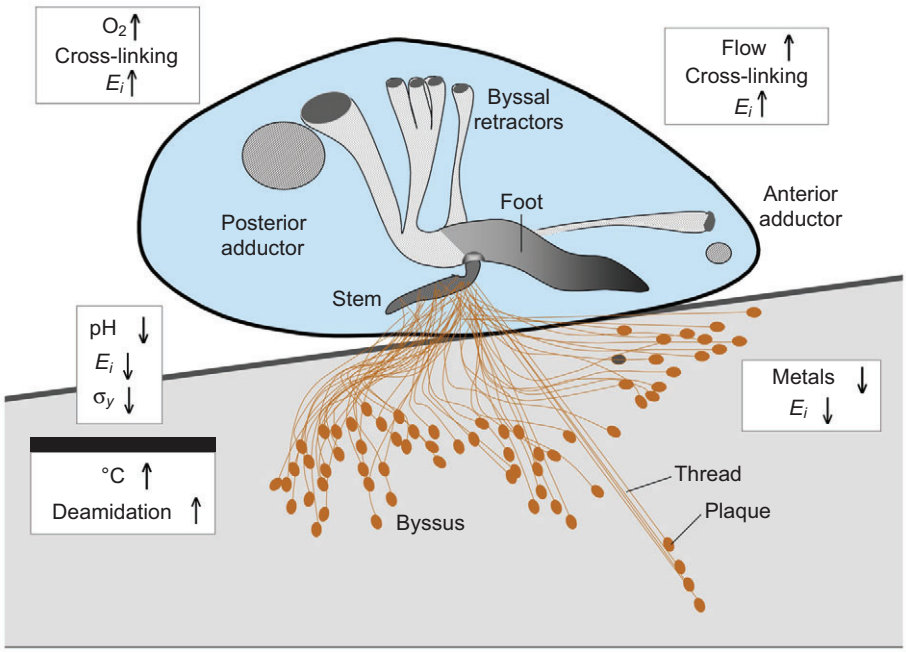

Fig. 6. Mussel byssus and the effect of selected local environmental factors on chemical and mechanical properties of byssal threads. Everything beyond the foot, such as stem, threads and plaques, are nonliving extended biomaterials. Thread tension is controlled by six retractor muscles per valve. E_i and σ_y are stiffness (initial modulus) and yield stress, respectively. See text for discussion.

of spawning mussels (Babarro and Fernandez-Reiriz, 2010). Whether seasonally adjusted thread quality has adaptive value remains unclear, but what is evident is that the lower thread quality in September–October makes entire mussel communities vulnerable to dislodgement and destruction when hurricanes occur (Carrington et al., 2009).

The thread quality issue is an intriguing new aspect in ecomechanics – that individual and community survival can depend on biomaterial quality (which in turn depends of the effect of environment on structure). In the previous mindset that tenacity was determined by thread number, and researchers investigated primarily factors that stimulated or inhibited byssal thread formation, such as water agitation (Young, 1985), aerial exposure (van Winkle, 1970), temperature (Young, 1985), salinity (Pelc and Alexander, 1999) and sediment (Young, 1983a; Meadows and Shand, 1989). Investigations of environmental factors influencing the quality of thread structure and mechanics have not been as extensive but several merit mention here and are illustrated in Fig. 6.

Temperature

In cultured subtidal *Mytilus edulis*, byssal thread breaking strength was strongly and negatively correlated with temperature in mussels (Lachance et al., 2009). At least one byssal protein (thread matrix protein, TMP) is extremely prone to temperature-dependent deamidation, but the effect of deamidation on mechanical properties is not yet known. L-asparagine side chain deamidation in TMP leads to extensive amino acid racemization, i.e. D, L-aspartate. This would substantially increase TMP backbone flexibility and conceivably impact load bearing by byssal threads as well as alter thread disposition to degradation (Sagert and Waite, 2009).

pH

The stiffness and yield strength of byssal threads are reversibly lowered by 20% when pH is lowered from 8.2 to 7.2 (Harrington and Waite, 2007). The titration of thread stiffness with pH coincides closely with the titration of histidines, ~pK_a 6.5 in the byssal preCOL proteins (Harrington and Waite, 2007). Below pH 6.5, histidine is charged as imidazolium and repulsive, whereas at pH>6.5 it is predominantly uncharged (imidazole) and avidly binds metal ions such as Zn^{2+} in the collagenous preCOLs of the thread core. Because Zn^{2+} can coordinate as many as four different imidazoles, it can be viewed as a reversible protein cross-linker. Byssal proteins also contain another amino acid, dopa, capable of high affinity metal binding in a pH-dependent fashion. In the thread cuticle, for example, the dopa side chains of mfp-1 are extensively complexed with iron. The high stiffness and extensibility of byssal cuticle are proposed to be an attribute of this unusual interaction (Harrington et al., 2010). The number of dopa groups bound to Fe^{3+} is concentration and pH dependent, e.g. at pH 5, only one dopa binds Fe^{3+}, whereas at pH 6–7 there are on average two dopas per Fe^{3+}; at pH>8.5, there are three dopas per Fe^{3+} (Zeng et al., 2010). The mussel probably constructs a pH gradient in the foot during the deposition of the byssal cuticle to gradually ramp mussel foot proteins from being soluble monometallocomplexes to insoluble cross-linked bis and tris complexes in the mature thread (Holten-Andersen et al., 2011). The pH of seawater can also be lowered: inundated sulfate-rich coastlands can be as low as pH 4 and run-off from pyrite mines can dip into negative pH (Johnston et al., 2009; Nordstrom et al., 2000).

Oxygen

Byssal threads, though not endowed with living cells, consume oxygen easily measurable with an oxygen electrode (J.H.W., unpublished observations). Perhaps the enzyme catecholoxidase, which is present in byssal threads and catalyzes the oxidative dehydrogenation of catechols (such as dopa) to quinones (Waite, 1985), is responsible. Quinones are capable of covalently cross-linking proteins, and quinone derived cross-links have been detected in byssus (McDowell et al., 1999; Zhao and Waite, 2006). Catecholoxidase exogenously added to the proximal portion of byssal threads in aerated seawater made threads two to four times stiffer than control threads (Sun et al., 2001). Oxygen supply to O_2-consuming processes in byssal threads is dependent on ambient flow regimes.

Water flow

Water movement around clustered mussels is complex and often modeled as a purely physical regime contributing to lift and drag, but flow-dependent mixing and thinning of stationary boundary layers also have profound chemical consequences. With respect to the tensile forces associated with lift and drag, byssal threads stretched beyond their yield stress (at ~10% strain) undergo stress softening distally; in contrast, proximally there is strain stiffening (Bell and Gosline, 1996; Carrington and Gosline, 2004). The former is argued to have adaptive value in that it recruits remaining stiff threads into load bearing, but softened threads take many hours to fully recover their stiffness (Bell and Gosline, 1996).

By thinning boundary layers on surfaces, accelerated flow can replenish nutrient supply to surface reactions. For example, covalent cross-link formation in byssal threads, which follows dopa oxidation, was increased by flow, presumably by providing more O_2 needed to oxidize dopa residues to dopaquinones (McDowell et al., 1999). Perhaps, the up to fourfold stronger byssal threads from mussels maintained under high flow regimes ~20 cm s^{-1} (Dolmer and Svane, 1994) have higher cross-link densities than those maintained in stationary water.

Water chemistry

Seawater chemistry is as complex as that of any biological fluid and varies extensively over temporal and spatial scales (Jackson, 2012). Besides abundant salt, seawater contains soluble trace elements and diverse particulate matter that arise from natural or anthropogenic sources. Particulate abundance in offshore seawater undergoes extreme fluctuation (Wozniak et al., 2010). The functional integrity of mussel byssus depends on efficient collection of certain trace metals such as iron, zinc and copper from particulate or soluble sources in seawater. Nanoindentational analysis of thread cuticles, for example, suggests that iron is necessary for the cohesiveness, stiffness and perhaps self-healing properties (Holten-Andersen et al., 2007; Harrington et al., 2010). Zinc and copper similarly perform as reversible protein cross-linkers in the thread core (Vaccaro and Waite, 2001; Harrington and Waite, 2007). Most transition metal concentrations in seawater are very low, e.g. the concentration of soluble Fe^{3+} is only 10^{-59} mol l^{-1} (Raymond and Dertz, 2004), so passive diffusion into byssal threads is not a plausible pathway. In the case of iron uptake by mussels, iron-containing particulates and colloids are first captured by filtration, ingested, solubilized (usually after transient reduction to Fe^{2+}), reoxidized and distributed to various tissues, notably the byssal glands, and finally co-secreted with proteins during the formation of a new thread (George et al., 1976). Although mussels are able to obtain essential metals from insoluble colloids or particulates (Davies and Simkiss, 1996), it is not clear whether this ability is intrinsic or dependent on symbiotic microbes. To obtain metal ions from insoluble particulates in seawater, microbes release siderophores, molecules with metal binding affinities that resemble or exceed the binding of EDTA mentioned earlier (Raymond and Dertz, 2004; Shi et al., 2010).

Byssus is a key sentinel of anthropogenic changes and heavy metals in seawater (Farrington, 1983; Coombs and Keller, 1982; Koide et al., 1982; Coulon et al., 1987). Apparently, the intrinsic iron-, copper- and zinc-binding ability of pristine *Mytilus* byssus can be considerably expanded to bind other metal ions when ambient levels of the latter are elevated such as by anthropogenic pollution. Does exogenous metal binding by byssal threads result in measurable changes in their mechanical properties? Surprisingly, this important question has never been investigated in byssus. Studies on metal replacement in *Nereis* jaw, however, suggest that specific mechanical properties are bestowed only by certain metals in well-defined locations (Broomell et al., 2008).

Surface energy

The surface energy of available surfaces can influence the number of threads a mussel attaches to any particular surface as well as the spreading and strength of adhesion of the plaques to a surface (Young, 1983; Crisp et al., 1985; Aldred et al., 2006). This has been studied entirely by presenting surfaces with a range of surface energies to mussels. Adsorption of solutes in seawater can also change the interfacial energy of surfaces (Schneider, 1996) but the tensile properties of byssus on such adsorbates have yet to be investigated.

Microbes

The microbial ecology around mussel clusters merits deeper study, particularly how it impacts the byssus. Although generally resistant to degradation, the byssus represents a food source for those microbes that can digest it, and there are some that degrade it with gusto (Venkateswaran and Dohmoto, 2000; Vitellaro-Zuccarello, 1973). The strategies for degradation are of particular interest. It has been proposed that the thin outer cuticle is the primary barrier against microbial attack. Perhaps because of this, fungi are observed to eat threads from the inside out. Microbes are also found encapsulated within the byssus (Kadar and Azevedo, 2006), but may gain access to the collagenous thread core from outside by using their powerful siderophores (Shi et al., 2010) to first remove cuticular metals.

Mussels themselves

Mussels have the peculiar ability to jettison the entire byssus from the point where the stem is rooted to living tissue at the base of the foot (Waite, 1992). This is best illustrated in juveniles scaling a vertical surface – initial threads placed in the scaling direction are jettisoned once new threads reach beyond that position. Byssal jettison is a separate issue from thread quality but should not be overlooked. At this time, there are no known cues for inducing the jettison of byssus.

Underlying factors responsible for the reported inferior thread quality of Rhode Island mussels (Moeser and Carrington, 2006) remain unknown but some conjecture is possible. The tested threads had inferior mechanical properties under normal testing conditions, therefore scenarios A or C seem more likely than B (Fig. 1). Moreover, mussels in these studies were in poor physiological condition, suggesting pathway A (Moeser and Carrington, 2006); added to this, even the quality of normal threads made earlier became compromised, indicating a combination of A and C. In a related study involving subtidal mussels (Lachance et al., 2009), loss in thread quality was shown to happen quite rapidly – in the course of days not months. As proposed earlier (Moeser and Carrington, 2006), enhanced thread deterioration seems the best explanation. Microbial attack, metal depletion by siderophores or humic acids, and temperature- or pH-triggered asparagine–glycine deamidation in byssal proteins are possible candidates.

Clearly, deterioration of biomaterial structure leading to impaired function can result from many different episodic or cyclic changes in the ambient environment. Future research should better determine which changes or combinations of changes affect structure the most.

Conclusions

There is now adequate technology for compiling databases of structure–property relationships in biomaterials of key species at

many length and time scales and under a variety of physiologically relevant conditions, including changes in hydration, pH, ionic strength, aeration, flow, temperature and seawater chemistry. These databases would provide an important experimental basis for theory and predictive modeling in ecomechanics. As ecomechanics develops as a discipline, it must embrace chemistry as fervently as it does physics to understand the interplay between organisms and environment. Mussel byssus is ideal for investigating how structure–property relationships in an extended biomaterial depend on the ambient physical, chemical and biological environments, and how biomaterial quality can affect the survival of individuals as well as communities.

List of abbreviations

AFM	atomic force microscopy
BESSY	Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung
EDX	energy dispersive X-ray spectroscopy
EXAFS	extended X-ray absorption fine structure spectroscopy
FECO	fringes of equal chromatic order
MALDI	matrix-assisted laser desorption-ionization mass spectroscopy
PIXE	proton induced X-ray emission spectroscopy
SAXS	small angle X-ray scattering
SFA	surface forces apparatus
ssNMR	solid-state nuclear magnetic resonance
WAXS	wide angle X-ray scattering
XANES	X-ray absorption for near edge structure
XAS	X-ray absorption spectroscopy

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