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Following the thread: *Mytilus* mussel byssus as an inspired multi-functional biomaterialJ. Herbert Waite^{1*} and Matthew J. Harrington^{2*}¹ Marine Sciences Institute, Lagoon Road, University of California, Santa Barbara, CA, 93106, USA² Dept. of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal, Quebec H3A 0B8, Canada

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Abstract

Over the last 15 years, the byssus of marine mussels (*Mytilus spp.*) has emerged as an important model system for the bio-inspired development and synthesis of advanced polymers and adhesives. But how did these seemingly inconsequential fibers that are routinely discarded in mussel hors d'oeuvres become the focus of intense international research. In the present review, we take a historical perspective to understand this phenomenon. Our purpose is not to review the sizeable literature of mussel-inspired materials as there are numerous excellent reviews that cover this topic in great depth. Instead, we explore how the byssus became a magnet for bio-inspired materials science, with a focus on the specific breakthroughs in the understanding of composition, structure, function and formation of the byssus achieved through fundamental scientific investigation. Extracted principles have led to bio-inspired design of novel materials with both biomedical and technical applications, including surgical adhesives, self-healing polymers, tunable hydrogels and even actuated composites. Continued study into the byssus of Mytilid mussels and other species will provide a rich source of inspiration for years to come.

Keywords: bio-inspired materials, mussel byssus, DOPA-catechol, metal coordination, adhesion

The muddled history of mussel byssus

Mussels and mankind would seem to have nothing in common, but actually they do. Both live in tight, perpetually anxious communities, both are obsessively interconnected, and both are guilty of prolific consumerism. Perhaps these are just the ravings of researchers who have spent too much time investigating the byssus, a multipurpose holdfast made by mussels (Mollusca, Mytilidae). Indeed, byssus is the commodity that manifestly enables mussel community, networking and consumerism. Of course, mussels acquired their behaviors long before the hominids. Humans consumed mussels since prehistoric times, but with the progression of civilization came appetites for more than mussel food. Some mussel species were prized for their byssal threads, which in the eastern Mediterranean region were spun into silken threads and woven into luxurious berets, purses, gloves and stockings beginning perhaps in Hellenistic times (350 BC) and continuing to nearly the 20th century¹. In recent years, scholarly interest in mussel byssus has mushroomed into a sprawling series of scientific and technological initiatives vying to capture or harness the secrets of mussel tenacity and wet adhesion. We aspire in this review to delineate scientific interest in the byssus and to describe where it stands today.

Recorded scholarly interest in mussel byssus started with Aristotle's *Historium Animalium* in which he pedantically noted that *Pinna* mussels were "affixed to the bottom (βυσσός)" of the sea. In a popular translation by Theodorus Gaza (1400-1475), this description was corrupted and embellished to "affixed by a byssus (βύσσοις)", a fine plant-derived linen of the ancient world². The corrupted version percolated among scholarly circles for about 300 years. Réaumur³ and de Heide⁴ did not mention byssus in their detailed anatomical drawings of mussel holdfasts although de Heide considered the holdfast a plant (Fig. 1). Mueller⁵, however, adopted byssus for the mussel holdfast and, after him, so did everyone else. Was Gaza's corruption suggesting a plant-fiber intended to stoke more interest in mussel byssus than Aristotle's original meaning could? Perhaps he had an investment in the byssal silk trade? The questions remain moot.

In 1872, Anton Dohrn's endowment to establish the Zoological Laboratory, now the Stazione Zoologica di Napoli, energized marine biology by introducing sea creatures including mussels to

visiting scientists as well as providing the bench space to study them. Many researchers with interests in byssus anatomy, physiology, biochemistry and biomechanics such as Tullberg⁶, Seydel⁷, Stary & Andraschke⁸ and others too numerous to mention availed themselves of this heaven-sent facility. Within a score of years, similar laboratories were built in the US, Britain, France, and were crucial in enabling the transfer of the latest histochemical and fiber X-ray advances in the medical sciences to marine biology generally and mussels particularly⁹⁻¹⁰. With regard to adventitious histochemistry, there was an amusingly erroneous though prescient report by Tullberg in 1877 in which he marveled at finding a *green* gland in a mussel foot following fixation in cognac and sectioning⁶. Although the green color was an artifact of the acidity and iron impurities in the cognac (during distillation), his result anticipated the popular green ferric chloride stains used later for detecting polyphenols in both foot and byssus material¹¹.

Figure 1. Mussel in a beaker drawing by Antonio de Heide, 1684⁴. The foot (b) is just disengaging from a new plaque (eye-like ellipse, *bottom center*). Translation of title: “Anatomy of the Belgian Mussel that explains its elegant structure and amazing movement”.

Biomimetics

The biomimetic phase of mussel byssus started much earlier – in the 1940s – than most are aware. It was not an institutional program but rather a coalescence of the multidisciplinary

interests of Mark G. M. Pryor, Laurence E. R. Picken, Norman de Bruyne and others spurred by the war effort. Pryor was a senior tutor and Fellow of Trinity College and in the Department of Zoology, Cambridge University and had considerable though divergent expertise in zoology and aircraft construction, the latter wittily described in *The New Science of Strong Materials*¹², so it was natural for him to look for common themes in the two. Pryor's focus on multi-ply fiber-filled insect cuticles undoubtedly led to inclusion of these in discussions about composite lightweight aeronautical material designs. Picken, a Fellow of Jesus College and a graduate research advisor in Zoology at Cambridge, was a pioneer of investigating the multiple length scales of structure in biopolymers¹³. Norman de Bruyne of the Royal Aircraft Establishment (RAE) Farnborough was a founding father of modern adhesion science. He started with Ernest Rutherford at the Cavendish Laboratory in Cambridge before spinning off several aircraft companies and personally building two high-performance airplanes using layers of balsa wood or linen impregnated with glue. In 1940, Pryor's discovery of a pathway for quinone-tanning (QT) in insects that led to sclerotization of the exoskeleton put him on the map¹⁴. It identified the cross-linker needed to harden the protein-chitin mixtures (Fig. 2A). Alexander Todd and others helped Pryor characterize the tanning agent as protocatechuic acid and showed that oxidation to o-quinone made it a very versatile cross-linking agent¹⁵⁻¹⁶. De Bruyne's switch from casein to phenol- or resorcinol-formaldehyde glues in aircraft construction coincided with these discoveries and was informed by them. Multi-talented and decorated research clusters were not uncommon in Cambridge at the time. Baron Lord Todd, Nobel Laureate (for synthesizing ATP and flavin), was Master of Christ College, Cambridge. Pryor was himself a grandson of a baronet and married to Sophie Raverat, a talented artist and a great granddaughter of Charles Darwin, and, finally, Picken was a world traveler, ethnomusicologist of traditional Chinese and Turkish music as well as a polymer physicist. The Cambridge teams' rigor and panache in describing catechols as the keystone of insect sclerotization made QT an instant shibboleth for the greater zoology and biomaterials research community, though not always with adequate justification. Indeed, Julian Vincent, to his credit, has often questioned the universality of the QT pathway proposed by Pryor thereby stoking curiosities about other catechol-mediated pathways¹⁷.

C. H. Brown: *Byssus in the crosshairs*

While at RAE Farnborough during the Second World War, Pryor met a student intern C. H. Brown from Girton College, Cambridge on a National Service assignment to help improve the design of RAF pilot helmets (correspondence, Girton College Archives). When Brown expressed interest in his work on natural load-bearing composites of fiber-reinforced phenolic resins, he apparently suggested *Mytilus* byssus to her as a promising project for studying QT in marine organisms. Brown was smart, scholarly and independent, but also intensely reserved, rarely revealing more about herself than her initials C.H. Between ~1944 to 1949, with Pryor and Picken as mentors and *Mytilus* as her model organism, she succeeded in showing or adding substance to the following: 1) byssal threads contain collagen but extend much more than tendon, 2) byssal threads and foot both contain high-molecular weight catecholic compounds, 3) byssal catechols are covalently tethered to proteins and readily bind to Fe^{III} ¹¹. These were important deviations from Pryor's model of QT in insects and gave mussel byssus its own stamp (Fig. 2B). Brown gained her PhD from Cambridge in 1950 and between then and 1955, published 4 noteworthy single-author papers^{11, 18-20}. In 1950, she also married William Ranulf Brock, a lecturer of US History at Cambridge; then in 1955, distracted by domestic demands and 2 children, she exited from the world of laboratory research.

Figure 2. Comparing Pryor's and Brown's deductions about the disposition and role of catechols in insects and mussels. Both biomaterials, eggcase and byssus, were observed to consist of catechols, resin proteins and fiber filler. The major difference between the two is that insect

catechols were low molecular weight and needed to be enzymatically deblocked, whereas in mussels, catechols were already fused to the resin proteins. Much more is now known about both pathways.

Pryor, though continuing as Senior Tutor at Trinity, also stopped publishing after 1955 for unknown reasons. In 1967 he suffered a head injury in a car accident that left him in a vegetative coma until he passed away in 1970 at age 51. In the foreword to her extraordinary Lazarus-like treatise "*Structural Materials in Animals*"²¹, Brown wrote of Pryor: "It was he who directed my interest towards this subject and whose suggestions helped shape many of my ideas." Alas, the mussel muse never returned to mussels or catechols and finally passed away in 2000.

Emergence of Byssus Biochemistry

As a graduate student in Zoology at Duke University in the 1970s, Waite was mesmerized by Brown's 1952 paper on structural proteins in *Mytilus*. Everything was so concisely and accurately articulated; so well poised for continuation. If there was any criticism, it was that Brown was parsimonious with illustrations of her analytical data; perhaps photographs were prohibitively costly to make and print at the time. Brown's only research support came through a string of student fellowships. Waite knew of few other biological processes or organisms that involved such a generous stockpile of precursors or speedy assembly than byssal thread formation by mussels (Fig. 3). As there were no reports of efforts after Brown's 1952 paper, Waite accepted her and Pryor's wordless invitation to carry on. In 1976, he got an NSF-Nato Fellowship to study mussel byssus with S. O. Andersen in Copenhagen, and thus, began a long series of peculiar protein characterizations that did not slow down until 2017.

Given a mussel's ability to make a new thread every 1-5 min, a stockpile of byssal precursors had to exist in the mussel foot glands (Fig. 3). Brown¹¹ and later Smyth²² predicted 3 types of stored proteins: collagen from the collagen gland, catecholoxidase from the accessory/enzyme gland, and catecholic proteins from the accessory and phenol gland. Catecholoxidase activity was detected in the foot but the enzyme proved unstable and still awaits proper characterization²³. Brown¹¹ mentioned a sensitive and specific stain for tissues bearing catechols based on exposure to nitrous acid followed by alkaline pH. Unbeknownst to her, this reaction had been adapted by

Arnow²⁴ for analytical solution chemistry and could easily distinguish Dopa from Tyr and Trp. Using 5% acetic acid for extraction, Waite identified 2 catecholic mussel foot proteins (mfp), mfp-1 and mfp-2, simply by applying the Arnow reaction to acid-extracted proteins separated by electrophoresis in polyacrylamide gels. Mfp-1 is from the accessory (or enzyme) gland, consists of short, tandemly repeated sequences, and contains high levels of 3,4-dihydroxyphenyl-L-alanine (Dopa) and modified prolines²⁵⁻²⁷. Mfp-2 is from the phenol gland and has a sequence with longer tandem repeats and Dopa lightly distributed throughout²⁸⁻²⁹. Neither mfp-1 nor mfp-2 proved to be particularly good adhesives, though mfp-1 found a market niche as a cell- and tissue culture attachment factor that persists to this day (BD Cell-Tak™). 5% acetic acid extractions of *M. edulis* mussel feet also liberated collagens or preCols, which will be described below in more detail³⁰. These too contained Dopa, but only at their N- and C-termini.

Figure 3. Relationship between the mussel foot and production of a new byssal thread. Each thread consists of a core shaft, cuticle, and plaque. New threads are injection-molded on demand in the pedal groove and distal depression of the foot from precursors stockpiled in three glands, which were variously known as the phenol (purple), collagen (white) and accessory (enzyme)

glands. Two foot slices - one proximal one distal - stained by Arnow's method, show catechols distributed throughout the foot core, but reaching highest levels in the accessory and phenol glands. The distribution of 8 foot-derived proteins, all Dopa-containing, is shown. Mfp is mussel foot protein, TMP is thread matrix protein; preCol is pre-pepsinized collagen. Adapted with permission from Demartini et al (2017)³¹.

For reasons still not understood today, the really sticky adhesive mfp proteins require more aggressive extraction conditions for liberation from mussel foot tissue. For example, the mfp-3 variant family requires 5% acetic with 8M urea for release³², and mfp-5 is liberated by 5% acetic acid with 6M guanidine-HCl³³. These are highly DOPA-rich precursors at 20 and 30 mol %, respectively. Mfp-3 variants encompass a large family of up to 30 isoforms, and, besides DOPA, contain significant amounts of another unusual modification, 4-hydroxyarginine. Besides Dopa, Mfp-5 contains variable amounts of *O*-phospho-serine. Both proteins are made and stored in the phenol gland^{27, 34} and upon secretion both are confined to byssal plaques near the substrate interface³⁵⁻³⁶. Known Mfp localization in foot and plaque is schematically summarized in Figure 3.

Discovery of mfp-5 with high levels of Dopa, Lys and Gly was a watershed moment because it put the protein on the same plain as many engineered random block copolymers with compositions dominated by a few functionalities e.g. Dopa, Lys and Gly. Then came 2 additional insights: 1) Dopa-bound metal ions such as Fe^{III} serve as cross-linking surrogates³⁷⁻³⁹, and 2), a single cantilever-tethered Dopa exhibited covalent-like but reversible binding to titania surfaces based on atomic force microscopy⁴⁰. In the ensuing pandemonium, research on mussel byssus splintered into groups focusing on collagens, metal ion binding, and individual mfps in adhesion. The golden age of byssus research had begun.

Progress in the last thirty years – the golden age of byssus research

Stimulated by advances in the biochemical understanding of the byssus, a surge in efforts to establish structure-function relationships between the chemical peculiarities of the byssus and their impressive material function followed. These studies have evolved hand-in-hand with the

field of mussel-inspired synthetic materials⁴¹⁻⁴³, and the feedback between biological and biomimetic efforts has been highly conducive to progress on both sides. Below, we will tell the story of how the secrets of the byssus were unlocked with sections focusing on the three most prominent functional parts of the byssus – namely, the core, cuticle and plaque. We conclude with an overview of past and present efforts to understand the fabrication process by which byssal threads are made.

The byssal thread core – a tough and self-healing tether

Functional characterization

Functionally, the byssal thread fiber acts as a tether between the adhesive plaque and the stem, which anchors directly into the soft tissue of the mussel (Fig. 4). As such, the tensile properties of these fibers are extremely relevant for their role in anchoring mussels to hard surfaces where incessant and cyclic application of intense hydrodynamic forces presents a dominant selective pressure. Denny and Carrington investigated the “ecomechanics” of the high intertidal zone utilizing a clever device for mimicking and measuring what organisms experience in the intertidal, showing that water velocities can reach 10 m/s with acceleration approaching 400 m/s² – a formidable mechanical challenge for even the best man-made polymers⁴⁴⁻⁴⁵. Yet, mussel beds often remain firmly intact except during the biggest winter storms⁴⁵.

Initial analysis of *Mytilid* byssal threads mechanics by Brown and Mercer focused mainly on the extensibility of the fibers^{11, 46}, while later studies by Vincent, Price and others also reported values for material stiffness and strength using pull-to-break tensile studies⁴⁷⁻⁴⁹. While these investigations mainly focused on measuring whole thread mechanics, even at this stage it was recognized that the proximal (closest to the mussel) region of the thread was considerably more extensible than the distal region (closest to the surface). Later work by Gosline and Carrington more thoroughly examined the mechanical properties of the proximal vs. distal region, demonstrating that the distal portion of threads of *M. californianus* is more than 50-fold stiffer, about two-fold stronger and about half as extensible compared with the proximal region (Fig. 4E)⁵⁰⁻⁵². Importantly, the shape of the stress-strain curves deviates significantly between the distal and the proximal regions. The distal thread is initially stiff, with a clear yield point occurring

at ~12% strain, followed by a post-yield plateau until ~40% strain, after which the stress increases until failure at ~100% strain (Fig. 4E). The proximal thread, on the other hand, does not exhibit a yield point and continues to extend until ~200% strain⁵⁰⁻⁵¹. Waite and co-workers proposed that the divergent mechanical response of the proximal and distal thread reflects a functional gradient that helps to mitigate stress concentrations due to modulus mismatch, considering that the byssus is essentially a direct link between stone ($E \sim 10$ GPa) and the soft tissue of the mussel (~ 200 kPa)⁵³. Thus, by gradually transitioning from stiff to soft, the byssus avoids localized failure at any abrupt transitions including the interface.

Figure 4. Mussel byssus core. A) *Mytilus californianus* mussel attached to a surface with its byssus. B) SEM of byssal thread torn open showing core and cuticle. C) Schematic of hierarchical organization of preCol proteins within the core. D) Schematic of His-Zn metal coordination bond present in His-rich domains of preCols. E) Pull-to-break stress-strain curves for the distal and proximal regions of byssal threads. F) Cyclic stress-strain curve of distal thread, highlighting large hysteresis in the first cycle, damage in the second cycle and self-healing response in the third cycle. Schematics indicate proposed double network model consisting of sacrificial bonds (His-Zn

cross-links) and elastic network (cross-beta sheet flanking domains). Figure adapted from Reinecke et al (2017)⁵⁴ with permission.

In the high intertidal zone, large waves can arrive with a period of just a few seconds⁵⁵, and byssal threads seldom undergo failure upon first impact. Thus, cyclic mechanical testing of byssal threads to strains lower than the breaking strain revealed several other key characteristics relevant for their biological function (Fig. 4F). When cycled below their yield point, distal threads are highly resilient, resembling tendon, with only 9% hysteresis observed^{51, 53, 56}. In contrast, when cycled beyond their yield point, distal threads exhibit a mechanical hysteresis of up to 72% (e.g. when cycled to strains of over 60%)^{51, 53, 56}, enabling significant dissipation of energy from crashing waves. Although yield of synthetic polymeric materials is associated with permanent damage and residual strain, the distal threads, given sufficient time, can recover their initial length when load is removed. Subsequent loading cycles, however, exhibit significantly reduced stiffness and hysteresis (Fig. 4F)^{51, 53, 56}. The softening of the threads that are bearing the brunt of the load was proposed by Carrington and colleagues to functionally recruit other threads in the byssus, by effectively redistributing the load over a greater number of threads⁵⁰. Remarkably, it was observed separately by the groups of Waite and Gosline that threads will recover back toward initial mechanical properties in a time-dependent self-healing process (Fig. 4F)^{51, 56-57}, which later became a main focus on efforts for bio-inspiration⁵⁸. It was determined that threads can recover up to 60% of initial stiffness and hysteresis after only one hour and up to 80% recovery is possible in 1 day⁵⁹. These time scales are relevant to the time spent during low tide, when mussels in the most wave-exposed regions are given a break of several hours, and it was observed that healing rate increased with temperature⁵⁹. Cyclic loading of the proximal region also exhibits a notable hysteresis (47%), but in contrast to the distal region, increases in stiffness with increasing number of loading cycles^{51, 60}.

Compositional characterization

Prior to the advent of specific compositional analyses, early byssus enthusiasts had speculated upon the constitution of the byssal threads with de Heide even considering the byssus to be of plant origin as already mentioned⁴ and Gaza calling it “lana illa pinnali” or Pinna wool². However, initial histological analysis of *Mytilus* threads already hinted at their collagenous composition¹¹,

⁶¹⁻⁶², which was further supported by studies of the precursor proteins within the mussel foot. For example, it was found that collagenase enzymes were capable of digesting the contents of the collagen secretory vesicles used to form the thread⁶³. However, initial efforts to extract proteins directly from the thread were unsuccessful, presumably due to the cross-linking of the proteins¹¹. This challenge was eventually overcome by the group of Waite who acquired the sequence of the byssus fiber proteins by first digesting threads with pepsin, resulting in the purification of three different partial collagen-like sequences which were pepsin-resistant^{30, 64-66}. The full-length sequences of the three proteins were acquired from cDNA libraries, which were given the name pre-pepsinized collagens, shortened to preCols³⁰.

Each of the three preCol variants consists of multiple domains including a large central collagen domain, flanking domains on either side resembling various structural motifs and domains enriched in histidine at both the N- and C-termini (Fig. 4C)⁶¹. The collagen domains were unusual in containing several breaks in the canonical collagen Gly-X-Y triplet motif, which were proposed to result in kinking of the rod-like collagen domains⁶⁷. The flanking domains of the three variants, which are known as preCol-D, preCol-NG and preCol-P, have sequence motifs that resemble dragline silk-like polyalanine runs, Gly-rich flagelliform silk sequence and elastin, respectively³⁰. Finally, the histidine-rich domains contain ~20 mol% His, which given the elevated content of transition metal ions in the threads (Zn and Cu) were proposed to participate in forming metal coordination complexes (Fig. 4D)^{30, 56-57}. Interestingly, it was revealed that the different variants are not uniformly distributed in the different parts of the fiber with preCol-D concentrated in the distal region, preCol-P concentrated in the proximal region and preCol-NG essentially constant along the length, leading to the proposal that this compositional gradient may determine the difference in mechanical properties in proximal vs. distal thread⁵³. Further compositional analysis of the thread by the Waite group revealed that the core also contains at least two different non-collagenous matrix proteins known as thread matrix protein-1 (TMP-1) and proximal thread matrix protein-1 (PTMP-1), which are unevenly distributed along the length of the thread, also possibly contributing to the mechanical gradient⁶⁸⁻⁶⁹.

Structural characterization

In line with the compositional analysis, early X-ray diffraction work by Mercer, Rudall and others provided evidence of a primarily triple helical collagenous structure in the thread^{46, 70}, but also suggested the presence of beta proteins. However, TEM imaging of threads by numerous groups did not show the typical 64-67 nm banding pattern of type I fibrillar collagen, suggesting the higher length-scale organization of the byssal collagen was very different from other collagenous fibers⁷¹. Indeed, TEM of the distal region showed highly aligned fibrils with a lateral spacing of 8-9 nm, while the fibrils in the proximal region exhibited an undulating morphology, with a non-fibrillar material between the fibrils⁷¹. These differences in alignment were clearly observable in polarized light microscopy as well⁷². TEM imaging further suggested that the collagen molecules were organized in 6+1 hexagonal bundles, which was further supported by AFM imaging (Fig. 4C)⁶⁷. Small angle X-ray scattering (SAXS) measurements of the distal thread by the group of Harrington confirmed that the preCols were arranged in 6+1 hexagonal bundles, and that the bundles within the threads were further arranged in a quasi-hexagonal organization⁷³. Axially, the preCol bundles were deduced from SAXS experiments to be arranged end-to-end with adjacent fibrils staggered relative to one another with a periodicity of 13 nm (Fig. 4C)⁷³. Synchrotron wide-angle X-ray diffraction (WAXD) studies confirmed a triple helical conformation resembling that of tendon, with the additional presence of a conspicuous meridional reflection indicating cross beta sheet structure^{54, 59}. The identified beta sheet conformation, which confirmed earlier speculations from the 1950s, was further supported using both FTIR⁷⁴ and solid state NMR⁷⁵ spectroscopy and was proposed to arise from the silk-like flanking domains of preCol-D.

Structure-function relationships

The ultimate goal of these studies stretching back over 70 years was to establish clear connections between the composition, structure and mechanical function of these fibers – yet, a detailed understanding remained elusive even until more recently. The peculiar byssal collagens with their flanking domains and His-rich domains³⁰, led to many provocative hypotheses as to why the mechanics of the byssus diverge so significantly from that of tendon. Eventually, application of synchrotron X-ray radiation provided a means of testing these hypotheses directly by enabling the combination of *in situ* mechanical and structural analysis,

tying specific features of the byssus stress-strain curves to specific changes in the conformation and cross-linking of the byssus core proteins. Collaboration between the Waite and Fratzl research groups combined synchrotron WAXD measurements with *in situ* cyclic loading of threads, revealing that the collagen domain is only extended by 2% molecular strain even while the thread is extended to strains of 70%⁵⁹. This indicated that the large extensibility of the byssal threads must arise from unfolding of protein structure in the flanking domains and HRDs based on the knowledge that preCols are organized into fibrils packed tightly in the fiber core. Harrington's group later confirmed this hypothesis by focusing on changes in specific WAXD diffraction peaks for the cross-beta sheet structure, which was deduced to unfold during stretching and refold rapidly upon unloading^{54, 74}. SAXS measurements combined with cyclic loading provided a higher order view of this unfolding process showing that the unraveling of the flanking domains occurs in a stepwise process⁷³. In subsequent cycles showing decreased stiffness, this transition was not observed – the transition occurs smoothly, which was attributed to the presence of sacrificial bonds that are broken in the first cycle and not fully recovered in the second cycle, but which recover over time in a self-healing event (Fig. 4F).

The primary candidate for sacrificial bonding in the thread were the metal coordination bonds between histidine residues in the HRDs and transition metal ions known to be present in the byssus, including Zn and Cu^{57, 76}. Support for this hypothesis came initially by studies in which threads were mechanically tested after undergoing various treatments targeting the integrity of the His-metal complexes including metal chelation with EDTA, histidine protonation with pH and histidine modification with diethylpyrocarbonate (DEPC)⁵⁶⁻⁵⁷. Perhaps most convincingly for the role of histidine in mechanics, threads exhibited a sigmoidal decrease in stiffness from 800 MPa to 400 MPa that almost perfectly matched the titration curve of Histidine – notably, histidine does not bind metal ions efficiently when protonated at acidic pH⁵⁶. Furthermore, threads that were stretched and then rested in acidic buffers lost their ability to self-heal in comparison with control fibers in seawater⁵⁹. Strong evidence for the presence and mechanical role of His-Zn coordination bonds was finally acquired by the group of Harrington using X-ray absorption spectroscopy (XAS) with EXAFS analysis combined with mechanical testing⁷⁷. These studies revealed that on average Zn ions in the threads had a coordination number of 5, with 3 His

residues and one Asp residue as direct ligands (Fig. 4D). When stretched to the end of the yield plateau, it was deduced that several amino acid ligands were replaced by water molecules, consistent with bond rupture. When load was removed, the metal coordination sites were again filled with amino acid ligands; however, the bond lengths were considerably strained compared to the native state. Over time, the bond structure recovered toward the native configuration suggesting that healing involves the formation of an optimal network topology⁷⁷. Combined with the hidden length of the cross-beta sheet structure, these reversibly sacrificial bonds provide a means of dissipating significant mechanical work via rupture of metal coordination cross-links, while the refolding of the beta sheet plus the reorganization of the metal bond network provide the means for self-healing (Fig. 4F)^{54, 77}. These concepts provide fertile ground for developing bio-inspired self-healing polymers.

These findings explained to a large degree the behavior of the distal region of the thread, but not the large difference in the mechanics of the less studied proximal region. Along these lines, it was proposed that the mechanical variation between the distal and proximal regions was largely controlled by the gradient of preCol-P and preCol-D^{30, 53}. Specifically, it was hypothesized that the elastin-like sequence of the preCol-P flanking domain results in a more extensible mechanical behavior in comparison to the silk-like domains of preCol-D. Fibers produced *in vitro* from purified preCol with different proportions of preCol-P and preCol-D showed that at least part of the mechanical difference could be explained by this⁷⁸⁻⁷⁹; however, the clear ultrastructural differences between the proximal and distal region and the larger content of PTMP-1 in the proximal region will also play a large role^{69, 71}.

Investigation of the thread cuticle – a hard and extensible abrasion-resistant coating

Functional characterization

Byssal threads from *Mytilus* and related genera possess a thin outer coating known as the cuticle that is compositionally, structurally and mechanically distinct from the inner core of the threads (Fig. 4B, 5A-C)^{37, 80}. The cuticle, initially referred to as the tanned border or cortical layer, was first noted by Bairati and Vitellaro-Zuccarello⁷¹ about 45 years ago, but at that point, no special attention was given to this thin veneer. The first functional studies of the cuticle were initiated

by Holten-Andersen and Waite in which they explored the hypothesis that the byssus cuticle might play a role in abrasion resistance against sandblasting in the marine intertidal⁸⁰⁻⁸². It was determined through nanoindentation that the cuticle of *M. galloprovincialis* and *M. californianus* possessed hardness and stiffness values comparable to common engineering epoxies, while microcracking studies revealed species-dependent extensibilities of up to 100% strain before cracks propagated through the cuticle⁸⁰. It was hypothesized that the hard coating functions to protect the softer core of the thread and that the high extensibility is adapted to match the high ultimate strain of the fiber core. This combination of hardness and stretchability is unmatched in typical engineering materials, and thus presented an exciting role model for bio-inspiration.

Figure 5. Mussel byssus cuticle. A) Light microscopy image of stained byssus showing cuticle (red) and core (blue). B) SEM image of granular byssus cuticle (red) from *M. edulis* covering the fibrous core (blue). C) TEM image of cuticle (red) showing the granules with their brain-like texture and matrix. D-F) TEM-EDS images showing distribution of (D) sulfur, (E) iron and (F) vanadium in the granules vs. matrix. G) Confocal Raman image showing concentration of DOPA-metal cross-links in the granule vs. matrix with schematic of tris-DOPA-V complex. H) Schematic of mfp-1 protein

highlighting consensus decapeptide motif of the repetitive domain. I) 3D reconstruction of several granules from FIB-SEM image stack showing internal brain-like texture. J) Zoom in of one granule from (G) showing bicontinuous network structure inside granules. Figure adapted from Jehle et al (2020)⁸³ under the creative commons license ([CC BY 4.0](https://creativecommons.org/licenses/by/4.0/)).

Compositional characterization

The most prominent protein component of the cuticle is mussel foot protein-1 (mfp-1), and for quite some time, it was the only cuticle protein identified (Fig. 4H). Originally, purified and characterized by Waite and Tanzer in 1981, mfp-1 was initially believed to be an adhesive protein⁸⁴, but was later localized specifically to the cuticle⁸⁵. As mentioned above, the mfp-1 sequence consists of ~80 tandem repeats of AKP*SY*P**P*TY*K, where P*, Y*, and P**, respectively, are 4-trans-hydroxy-L-proline (Hyp), 3,4-dihydroxyphenyl-L-alanine (Dopa), and 2, 3-trans 3, 4-cis dihydroxy-L-proline (DHP)²⁵⁻²⁷. Mfp-1 was considered to be peculiar at the time and the biochemical evidence for protein bound DOPA-catechol moieties caught the attention of many researchers, who would later pave the way for much of the field of mussel-inspired materials. It is appropriate to emphasize that mussels have no monopoly on protein DOPA; it is widely distributed in the structural materials of animals including molluscan periostracum and shell matrix, trematode eggshells, polychaete cement, tunicate test proteins and selachian capsules⁸⁶⁻⁸⁹. Recent transcriptomic analysis of the accessory gland suggests that a number of cysteine-rich proteins may also be present in the cuticle³¹, with one – mfp-17 – confirmed to be in the cuticle secretory vesicles⁹⁰.

In addition to cuticle proteins, Holten-Andersen provided spectroscopic evidence for the specific localization of metal ions, including Fe, concentrated in the cuticle⁸¹. Given the well-characterized tendency of catechols like DOPA to form highly stable catecholate coordination complexes with transition metals, it was posited that mfp-1 in the cuticle may be cross-linked via DOPA-metal bonds^{39, 81}. Direct evidence for this hypothesis was obtained using confocal Raman spectroscopic imaging of the native cuticle, revealing characteristic resonance Raman bands of the catechol-metal complex (Fig. 5G)³⁷. Notably these bonds were reversible since the metals could be largely removed with a metal chelator such as EDTA and recaptured by soaking EDTA-treated threads

with metal ion solutions^{37, 91}. While initial studies ascribed these Raman spectra to DOPA-Fe coordination bonds, it was recently revealed that mussels also integrate and coordinate vanadium ions with mfp-1 perhaps preferentially over iron ions⁸³, although mussels do appear to be opportunistic in terms of what metals they integrate into their byssus⁹¹.

Structural characterization

The tanned border originally identified by Bairati and Vitellaro-Zuccarello referred to the outer coating of the whole thread including stem⁷¹, but the specific structure was not examined in detail at this point. A detailed TEM image of the structure of the “cortical layer” was presented by Vitellaro-Zuccarello showing for the first time that the bumpy surface of the cuticle of *Mytilus galloprovincialis* was due to the presence of small round inclusions with a mottled texture within a continuous homogenous phase (Fig. 5C)⁹². Yet, following this basic ultrastructural analysis, very little was done to further investigate the byssus cuticle until over 25 years later when Holten-Andersen and Waite examined the cuticles of several different species, discovering that cuticle micro and nanostructure varied significantly between different species⁸⁰⁻⁸². *Mytilus* species all possess the small spherical inclusions, termed granules, which are embedded in a continuous phase termed the matrix. Comparative interspecies analysis revealed that both the size and submicron texture of the granules varies between different *Mytilus* species, and that certain *Perna* and *Modiolus* mussel byssal threads were found to be missing the granular substructure entirely^{80, 82, 93}. For example, *M. edulis* and *M. galloprovincialis* possess granules of approximately 1 μm in diameter that possess a biphasic brain-like nanoarchitecture, while the granules in *M. californianus* have an average diameter of about 200 nm with no substructure visible in TEM^{80, 82-83}. Harrington’s group studied the micro- and nano-scale structure of the *M. edulis* cuticle in 3D using focused ion beam scanning electron microscopy (FIB-SEM), revealing a bicontinuous network of 20 nm thick layers superficially resembling a block copolymer gyroid phase (Fig. 5I-J)⁸³. These studies were coupled with elemental analysis showing a strong vanadium signal coming from the granules, while the matrix was enriched in sulfur and iron (Fig. 5D-F). This was completely in line with resonance Raman studies that showed the localization of DOPA-V cross-links specifically at higher density in the granules than in the matrix^{37, 91}, while the localization of

sulfur in the matrix suggests that this might be where the Cys-rich cuticle proteins such as mfp-17 are localized^{31, 90}.

Structure-function relationships

Even though the structure and composition of the cuticle are now much better understood, the structure-function relationships defining this material are incomplete. Holten-Andersen and Waite's pioneering studies of this material suggested that the high hardness of the cuticle arises from the granules, which act as hard inclusions, and that the high extensibility comes from the matrix, which exhibits reversible microcracking during tensile deformation that circumvents propagation of larger cracks through to the core⁸⁰. Raman spectral mapping showing the elevated DOPA-metal cross-link density in the granules vis-à-vis the matrix supported this model of cuticle mechanics (Fig. 5G)³⁷. Monnier and Waite later proposed a modified model based on AFM-based nanoindentation of granules under semi-hydrated conditions suggesting that the granules retain a higher degree of water, and actually behave softer than the matrix under these specific conditions⁹³. In either case, studies by Harrington demonstrated that removal of metal ions from the cuticle with EDTA resulted in a decrease in both hardness and stiffness of the cuticle of over 80% based on nanoindentation analysis, which could be fully recovered by soaking the metal-depleted cuticle in metal chloride solutions of Fe, V and Al⁹¹. Notably, EDTA treatment resulted in a loss of the resonance Raman spectral peaks for DOPA-metal coordination, which were recovered after metal replenishment⁶⁵. This strongly suggests that the DOPA-metal coordination bonds, which are presumably localized in the granules, provide much of the cross-linking and that covalent bonds only make up a maximum of ~15% of total cross-links. Cohesion measurements between thin layers of mfp-1 in the presence of metal ions support this model of metal-dependent mechanics⁹⁴⁻⁹⁵. Indeed, very recent studies further indicate that DOPA-V complexation can provide a nearly two-fold increase of cohesive forces between layers of mfp-1, providing some insight into why this metal might be preferentially used to cross-link the hard granules⁹⁵. Nonetheless, one must also consider the possibility that cysteine-metal interactions might be present in the matrix based on the co-localization of sulfur and Fe in elemental analysis studies⁸³, and that metal removal and recovery studies also affect the matrix mechanics. However, this remains speculation for the time being.

Investigation of the byssal plaque – a versatile glue for aquatic environments

Functional characterization

The ability of the byssus to securely adhere to wet marine intertidal surfaces is undeniably extraordinary and has captured the attention of biologists, chemists, materials scientists and engineers motivated by current limitations in adhesion technology. A byssus first appears as a single fiber called a drifting thread in the veliger stage of almost all bivalves where it functions like a kite tail to stabilize larval drift. Later, in settling pediveligers, byssal threads are produced as anchoring tethers to marine surfaces during metamorphosis into the adult form which, depending on the species, can possess or lack a byssus⁹⁶. Indeed, in a smaller subset of mussel genera including *Mytilus spp.*, the ability to produce a byssus is retained into adulthood where it enables colonization of intertidal zones with the highest wave impact.

Mytilus byssus adheres via a disc-like structure known as the plaque, into which the core fiber is rooted⁹⁷⁻⁹⁸. The wet adhesive strength of a single *M. californianus* plaque on glass pulled at 45° and at 30 µm/s ranges from 1-5 MN/m²⁹⁹. Similar adhesion is achieved on shell, wood pilings, various metal/minerals, plastic and even non-polar surfaces such as Teflon¹⁰⁰⁻¹⁰¹. This versatility in a hydrated, salt-encrusted and corrosive environment has been a key factor in the evolutionary success of *Mytilid* mussels and a major driver of research into mussel-inspired glues with both technical and biomedical applications⁴¹. However, a fundamental understanding of the complex chemistry of the byssus plaque is incomplete and still holds many secrets.

Structural characterization

Early investigations of the internal structure of the plaque by Tamarin⁹⁷ and Waite⁹⁸ revealed a porous sponge-like architecture. At the interface with the substrate, the plaque was observed to form a thin dense layer, while the collagenous fibers of the core are anchored like tree roots in the spongy bulk of the plaque. Subsequent work by Waite, Helgeson and Valentine utilizing SEM and SANS revealed characteristic porosities of approximately 2 µm and 100 nm in the plaque interior¹⁰² (Fig. 6A-C). The specific localization of the different protein building blocks within the complex plaque structure has been an area of intense experimental effort with more details emerging over the last decade or so. Biochemical and spectroscopic investigations revealed that

the bulk trabecular scaffold is comprised primarily of mfp-2, which consists of 11 tandem repeats of a ~45-residue long epidermal growth factor (EGF) motif, and that these DOPA-rich proteins (~5 mol%) are cross-linked via DOPA-metal interactions (likely Fe/V and Ca)¹⁰³⁻¹⁰⁵. Conversely, MALDI analysis of plaque footprints remaining after plaque removal showed that mfp-3, mfp-5 and mfp-6 were localized nearest the plaque-substrate interface³⁵. Recent findings by Waite's group suggest that mfp-5 comprises the adhesive layer directly at the substrate interface, while the pores near the interface are filled with a fluid protein condensate containing mfp-3 and mfp-6¹⁰⁶ (Fig. 6D). At the plaque-core interface, mfp-4 was proposed to comprise the biochemical linkage between the preCol fibers and mfp-2, although this was never specifically demonstrated¹⁰⁷.

Figure 6: *Mytilus* plaque structure and adhesion of Mfps. A) Light stereomicroscopy image of plaque with attached thread from *M. californianus*; red bar indicates location of section. B) plaque section viewed by SEM showing two length scales of porosity at 2000 and 100 nm. Outer cuticle (OC), spongy core (SC), collagen fibers (CF) and the continuous film at plaque interface (PI) as labelled; C) higher magnification of the TEM plaque section (B, red box) featuring the 100 nm pores and continuous PI film. D) schematic of interfacial plaque protein distribution. The fine sub-100nm mesh is thought to be hydrogel filled with coacervate droplets of mfp-3 and mfp-6, acting as a reducing reservoir against oxidative damage. E) Measuring the adhesion of plaque proteins by SFA. Asymmetric and symmetric modes of mfp-3 application allow separate

determination of adhesive and cohesive contributions from pull-off forces (F/R). F) With asymmetric mode, force versus distance (D) curves show significant pH dependence for adhesion and hardwall (i.e. how much a protein compresses). Adhesion decreases and hardwall increases as pH increases. G) Nearly complete adhesion loss at pH 7.5 is attributed to Dopa oxidation, which cannot be restored by return to pH 5.5. However, addition of thiol-rich mfp-6, provides electrons for reduction of quinones to Dopa and restoration of adhesion. H) Adhesion to mica (aluminosilicate) requires both the catechol moiety as in Dopa and a cationic sidechain like lysine ($\oplus$) in 3-armed-TREN constructs. Having only one of these reduces adhesion to near control levels. Lysyl- ammonium evicts hydrated surface cations opening the door for catechol-binding to oxides and/or metals. Key: stalk (|) to $\oplus$ is long (Lys) or short (Orn); stalk only has no cation; stalk with circle is an acetylated amine. Panels C and D adapted from Valois et al (2020)¹⁰⁶ with permission, Panels F and G adapted from Yu et al (2011)¹⁰⁸ with permission and panel H adapted from Maier et al (2015)¹⁰⁹ with permission.

Compositional characterization

As mentioned above, Waite's group systematically identified and characterized 6 different proteins that were localized specifically to the plaque – mfp-2, mfp-3, mfp-4, mfp-5, mfp-6 and mfp-7^{28, 31-33, 36, 107}, whose defining properties are highlighted along with others in Table 1. More recently, transcriptomic analyses have suggested an additional 10 phenol gland proteins that might be present in the plaque³¹ (Table 1), one of which has been confirmed via proteomics¹⁰⁶. A common feature of most plaque proteins is the post-translational modification of tyrosine into DOPA ranging from 2 mol% for mfp-4 to 30 mol% for mfp-5¹¹⁰, but other modifications include phosphoserine and 4-hydroxyarginine. These proteins all tend to have basic isoelectric points, no doubt due to elevated arginine and lysine contents. Among the mfps confirmed in the plaque, mfp-6 is unusual in its high content of thiol/thiolate cysteines^{36, 108}. Of particular note, Waite's group and the group of Eli Sone also discovered homologous DOPA-rich proteins in the adhesive plaques of freshwater *Dreissenid* mussels (e.g. Zebra and Quagga mussels), suggesting that this biochemical adhesion strategy may be evolutionarily conserved across different orders of mussels¹¹¹⁻¹¹³.

Table 1. **Known** and *putative* mussel foot proteins (MFPs) in *Mytilus* spp. byssus plaques and/or the phenol gland

MFP	Mass, kD ^a	pI ^b	DOPA mol%	Variants	Miscellaneous	Ref
1	110	10.5	10-15	2	[decapeptide] ₇₅₋₈₀	25
2	45	9.5	5	4-5	[EGF repeat] ₁₁	28
3 (S & F)	5-7	10.5	10-25	~30	HO-Arginine	32
4	93	8	2	1	15% His	107
5	9	9	30	1-2	10% pSer	33
6	11.5	9.5	5	5	11% Cys	36
7	4	10.9	? (Y 17) ^c	2	GKY; GGH repeats	31
8	4.8	10	? (Y 24)	1	[GKY] ₁₀ repeat	31
9	11.4	9.8	? (Y 8)	2	H ₆ & H ₃	31
10	35	8.5	? (Y 11)	1	96 AA repeat	31
11	57	10	? (Y 11)	3	[GH] ₃₀ repeat	31
12	80	9	? (Y 12)	1	[RR, KK, KR] ₁₇	31
13	13	10.4	? (Y 16)	1	10% Cys	31
14	10.6	8.5	? (Y 3)	1	10% Cys	31
15	27	9.5	5 (Y 9)	1	6% Cys	31

^a average mass in K Daltons was estimated experimentally or predicted from deduced sequence

^b iso-electric point was experimentally determined or predicted from amino acid pK_ss

^c in the case of transcriptome-deduced sequences Dopa is unknown but Y provides an upper limit.

Structure-function relationships

Considering the large number of proteins in the byssus plaque, delineating clear structure-function relationships for specific proteins has been challenging. A shared trait of the most prominent interfacial proteins is high DOPA content – up to 30 mol%. That the DOPA catechol moiety might be the smoking gun of adhesion was first floated in 1981⁸⁴. In 1987, as a predecessor to the synthetic mussel-inspired adhesives that would emerge decades later, Yamamoto demonstrated for the first time that synthetic DOPA-containing peptides modeled after mussel protein motifs could mediate adhesion on metal oxide surfaces¹¹⁴. In the early 2000s, the Northwestern group led by Messersmith directly tested the hypothesis that single catechol groups could mediate surface adhesion on metal oxide surfaces using AFM based single molecule force spectroscopy, revealing that the interaction was strong (up to 900 pN), yet completely reversible over hundreds of cycles when shielded from oxidation⁴⁰.

While this specific focus on the catechol moiety of DOPA provided crucial new insights, the deeper question of how the specific plaque proteins interact with the surfaces remains debated. Between 2007-2017, the Waite and Israelachvili groups chipped away at this puzzle through an interdisciplinary approach combining classical biochemical analysis with advanced surface science^{103, 115-123}. Methodology critical to this approach was the surface forces apparatus (SFA) – an experimental platform developed by Israelachvili capable of measuring adhesive and cohesive forces of molecules, including proteins, with 1 Å distance and 0.1 nano Newton force accuracy¹²⁴⁻¹²⁵. The different byssus plaque proteins were systematically investigated in both symmetric and asymmetric measurement modes to probe cohesion and adhesion (Fig. 6E), respectively, revealing that certain plaque proteins such as mfp-3 and mfp-5 are superior at bridging between two surfaces (usually mica)^{115, 123}, while others, such as mfp-2 (and the cuticle protein mfp-1) are inferior adhesives, but reveal large cohesive improvements in the presence of metal ions^{94, 103}. These observations are consistent with the localization of mfp-3 and mfp-5 positioned at/near the substrate interface³⁵ and mfp-2 in the trabeculae of the porous plaque structure¹⁰³ – corroborating their proposed function in plaque adhesion and cohesion, respectively. While adhesion was initially thought to be mediated via chelation of metals in the metal oxide surfaces by the DOPA oxygen groups, the fact that strong adhesion is observed at acidic pH, suggests that H-bond and coulombic interactions are also key contributors in the surface chemistry¹²². Moreover, it was observed that the mussel proteins are highly versatile and may interact with non-polar surfaces via hydrophobic forces^{119, 121}.

The SFA also revealed the extreme pH-dependence of adhesion in experiments with individual proteins (Fig. 6C) as well as the role of protein mixtures to enable adhesion under natural conditions (e.g. pH 7-8). The conspicuous cysteine residues of mfp-6 were particularly crucial in maintaining adhesion in marine environments¹⁰⁸. It is well documented that DOPA has a strong tendency to oxidize from its catechol to its quinone form under the basic conditions present in seawater. Moreover, Messersmith's single molecule force measurements revealed that the quinone form is much less effective at adhering to anything but amine-modified surfaces⁴⁰, and SFA experiments by Waite and Israelachvili showed a large decrease in adhesive energy for mfp-3 and mfp-5 under basic conditions^{122, 126}. Given the alkaline pH of seawater, one would predict

that DOPA would be ineffective at sticking to surfaces under these conditions due to rampant DOPA oxidation. Indeed, Waite's group demonstrated that mfp-3 and -5 adhesion losses at pH 7.5 were correlated to Dopa oxidation (Fig. 6F); however, the losses were easily recovered, not by returning conditions to acidic pH, but rather by reductive restoration of Dopa-quinone to Dopa^{108, 115}. With its reservoir of cysteine thiols, mfp-6 proved to be an ideal interfacial antioxidant maintaining mfp-3 adhesion at pH 7.5 by reductive electron exchange¹⁰⁸ (Fig. 6G). Based on these observations, interfacial mfp-6 appears to be an adaptation for maintaining catechol-mediated adhesion in an oxidizing environment - a strategy that could be critical for future mussel-inspired adhesives as well.

While DOPA has received the most attention in synthetic biomimetic circles, subsequent scrutiny of plaque Mfp sequences showed other amino acids to be important. For example, DOPA residues in the adhesive proteins mfp-3 and mfp-5 (as well as in the cuticle protein mfp-1) are very often situated beside a positively charged amino acid – lysine or arginine^{33, 84, 110}. Waite and Israelachvili teamed up with Butler to explore the functional implications of this phenomenon using model molecules and found that the presence of a cationic amine group in close proximity with catechol significantly enhances adhesion to aluminosilicates like mica.¹⁰⁹ (Fig. 6H). This 1-2 synergy arises by dint of the charged amine's eviction of hydrated cations in the electric double layer on mica surfaces, clearing up binding sites e.g. by chelation on the surface for the catechol. While molecular studies like these have yielded many compelling insights, collaborative work by Waite and Valentine on the native plaque adhesion provided a sobering epilogue to single Dopa, even single protein adhesion results⁹⁹. When plaque adhesion to mica or glass was measured to break at 45°, the fracture energy (95 J m^{-2}) was shown to be nearly 5000-fold greater than the best performance of mfp-5 adhesion ($\sim 20 \text{ mJ m}^{-2}$). This is an eloquent reminder of the pitfalls of reductionism as well as a hint of how important molecular interactions between the interface and supramolecular architectures may be.

Fabrication of the byssus

Figure 7. Byssus fabrication overview. A) Light microscopy image of a stained histological section of a mussel foot (left) with different glands identified schematically (right). Magnification of cuticle, core and plaque vesicles are shown. B) 3D reconstruction of serial sections similar to (A) along the whole length of the mussel foot showing gland distribution. C-E) TEM images of vesicles used to form (C) core, (D) cuticle and (E) plaque. F) Schematic overview of thread secretion resembling injection molding, highlighting passive and active aspects of the assembly process. Panels A, B and F adapted from Priemel et al (2017)¹²⁷ under the creative commons license ([CC BY 4.0](https://creativecommons.org/licenses/by/4.0/)), panel D adapted from Jehle et al (2020)⁸³ under the creative commons license ([CC BY 4.0](https://creativecommons.org/licenses/by/4.0/)), panels C and E kindly provided by Franziska Jehle.

As seen above, the structure-function relationships defining the remarkable properties of the byssus arise largely from the complex hierarchical organization of around 20 different protein

precursors and a delicate balance of covalent and non-covalent cross-linking. While reductionist approaches to mimic the chemistry of the byssus have been successful by focusing expressly on DOPA and histidine moieties^{41, 43}, most of these mussel-inspired materials achieve neither the structural complexity nor the mechanical prowess of the native byssus. Thus, there has recently been a renewed interest in understanding the byssus fabrication process to elucidate how this complex structure is achieved in the first place. However, this is by no means a new area of research. The ability mussels to rapidly fashion tough anchoring fibers has fascinated researchers for more than three centuries^{3, 6}, leading to focused investigation of the organ responsible for forming the byssus, which is known as the foot. The foot – tongue-like in appearance – ventures outside the shell during the thread-formation process, extending by several times its initial length as it searches for a suitable location to lay down a thread (Fig. 3)¹²⁷⁻¹²⁸. Once the mussel commits, the foot firmly attaches to the surface, undergoing rhythmic muscular contractions for about 3-5 minutes, after which it releases a fully formed and functional thread. Hidden from the view of curious researchers, a complex process not dissimilar from polymer injection molding is taking place at the cellular to organ level.

Early anatomical and histological studies of the foot by Brown, Pujol and Smyth provided evidence that the organ is a fully equipped byssus forming factory^{11, 22, 62}. Running along the ventral surface of the foot is a narrow groove beginning at the base and terminating at the tip of the foot in a cup-like divot known as the distal depression¹²⁹. Histological analysis of dissected foot tissue revealed the presence of several secretory glands surrounding the foot groove and distal depression, which comprises much of the foot tissue volume^{6, 22}. In the 1950s-60s, histochemical studies of the mussel foot^{11, 22} converged on the identification of three glands in the foot: the white, phenol, and enzyme glands. Later, based on more specific cytological criteria these were renamed– the collagen gland, the enzyme (or accessory) gland and the phenol gland as described below (Fig. 3, 7A). In the seventies and early eighties, the groups of Tamarin, Bairati and Vitellaro-Zuccarello explored the ultrastructure of the various byssus glands using TEM^{63, 71, 92, 97, 129}. Of the three glands that had been identified histologically, each was packed with cells containing massive quantities of micron-sized secretory vesicles, and the vesicles in each gland possessed a unique morphology and ultrastructural features (Fig. 7). The foot glands and their

vesicles went relatively unstudied for nearly forty years with few new insights, but recently several studies from the groups of Waite and Harrington have again focused on understanding the byssus assembly process from a more dynamic perspective than these earlier studies and armed with a deeper understanding of the biochemistry than existed at that time^{83, 90, 105-106, 110, 127, 130-133}. Indeed, there is an obvious disparity between the nearly 20 proteins identified through biochemical and “-omic” analysis, and the three secretory vesicle types currently characterized, implying either that there are multiple different proteins in each vesicle type, that there are more than three different types of vesicles or most likely, that there is a combination of both scenarios.

Collagen gland (core)

The largest of the three glands in the foot is responsible for forming the core running from the base of the foot to the distal depression surrounding the groove. This was the white gland initially identified by Tullberg in 1877⁶; however, the revelation that the vesicles within the gland contained collagenous proteins, led to the renaming as the collagen gland by Pujol in 1967⁶² and later as the core gland based on the part of thread, which it forms¹²⁷. The vesicles of the collagen gland possess elliptical shapes with a long axis of 1.5 – 2 μm (Fig. 4C)^{63, 130, 132}. The vesicles stain positively for collagen using Masson’s trichrome and Sirius red staining and exhibit a strong birefringence indicating a high degree of molecular alignment^{127, 132}. Using TEM, both Tamarin and Vitellaro-Zucarello highlighted the characteristic banding pattern of dark and light staining layers with a periodicity of ~ 180 nm, with the dark-staining layers exhibiting a filamentous texture (Fig. 4C)^{63, 129-130}. These studies were followed forty years later by Harrington’s group use of FIB-SEM, revealing the 3-dimensional structure of the layers and confirming a long-standing hypothesis that the preCols are stored as a smectic mesophase within the vesicles, with clear disclinations¹³⁰. It was hypothesized that this smectic LC, which has not been reported in other systems in nature, arises from the pentablock copolymer-like distribution of charge and hydrophathy of the preCols, and that the smectic phase combines the fluidity required to mold the fiber during thread fabrication with the molecular alignment required to achieve the high degree of hierarchical structure observed in the native thread^{67, 130}. While this has proved challenging to confirm *in vivo*, there is substantial evidence *in vitro* and *in situ* that this is the case.

Tamarin demonstrated that mussels could be induced to secrete byssus vesicles via injection of KCl into the pedal ganglion at the base of the foot¹²⁹. Harrington's group showed thread induction resulted in the coalescence of the contents of the collagen vesicles into an immature fiber showing birefringence and thus, alignment of preCols over hundreds of microns¹²⁷. It was suggested that mechanical forces are required to achieve the high degree of molecular alignment observed in the native threads. Indeed, Harrington and Waite showed that purified preCols can be easily drawn into aligned fibers with native-like properties by simple mechanical drawing from dilute solutions⁷⁸⁻⁷⁹, while purified core vesicles could be formed into fibers through applied shear forces¹³².

Another question that has attracted much recent attention is how the preCol LC phase transitions rapidly from a liquid to a solid. Evidence supports the hypothesis initially proposed by Waite that this may in part be initiated by a pH transition going from the acidic storage conditions of the vesicle to the basic conditions of seawater and that the histidine residues in the termini of the preCols are key players in this process due to their pK_a of ~ 6.5 ^{30, 78}. In support, Harrington's group has showed recently that peptides based on histidine-rich domain (HRD) sequence undergo a transition into an amyloid-like structure when the pH is increased and can be used to initiate self-assembly of materials¹³⁴⁻¹³⁷. While initially it was proposed that metal ions might be critical as cross-linking agents in this process, these studies demonstrated that metal ions are not required at least *in vitro*. However, there is far more to be elucidated concerning this mechanism.

Enzyme or accessory gland (cuticle)

After the collagenous core is secreted and formed during the thread fabrication process, it is surmised that the thin protective cuticle is laid down from vesicles in a separate gland. Smyth's finding that polyphenoloxidase activity and catechols were colocalized in a long slender bi-lobed gland along the ventral groove prompted him to refer to this as the enzyme gland (EG)²². The polyphenoloxidase has been partially characterized²³, but as typical for others of this type¹³⁸, is highly labile even after partial purification. Later work by Allen led to relabeling of the gland as "accessory gland"⁴⁷. Vitellaro-Zuccarello, however, concluded correctly that these vesicles contained structural proteins for building the cuticle due to the morphological similarity in TEM

imaging of the vesicles and cuticle, stating “the EG does not completely deserve its name”⁹². Harrington later rechristened it as the cuticle gland¹²⁷, although all three monikers can still be found throughout the literature.

Early TEM analysis of the vesicles from *M. galloprovincialis* showed a biphasic internal texture resembling a brain-like whorled structure based on differential staining of two different components (Fig. 7D)⁹². Interestingly, analysis of the cuticle forming vesicles from *M. californianus* by Tamarin also showed a biphasic structure, but with lighter staining droplets present in a heavily stained continuous phase¹²⁹. For nearly 40 years, this initial TEM work remained the only focused investigation of the vesicles responsible for forming the cuticles until the topic was revisited independently by the groups of Waite and Harrington. Monnier and Waite revealed that different mussel species have vesicles with different nanoscale textures with two differently staining phases observed, but always showing evidence of phase separation⁹³. Nanoscale compositional analysis of *M. edulis* vesicles by Harrington’s group that the brain-like phase given the name protogranule is enriched in DOPA-rich proteins (i.e. mfp-1) while the outer phase termed the protomatrix is enriched in sulfur⁸³, presumably comprised of putative Cys-rich proteins observed in transcriptomic studies³¹. During induced secretion, it was observed that the protomatrix of the secreted vesicle contents fuse and spread over the surface of the already formed core with the protogranules remaining as separate entities producing a cohesive coating on the core. Notably, the vesicles do not show DOPA-metal coordination, indicating that metal ions are added separately⁸³. The forces driving phase separation are still unclear, but one compelling hypothesis posits that the non-repetitive and slightly less polar tail of mfp-1 (Fig. 5H) might drive phase separation when mixed with the Cys-rich matrix proteins in block-copolymer like behavior⁸³. In addition, cation- π interactions are powerful initiators of mfp-1 phase separation *in vitro*¹³⁹.

Phenol gland (plaque)

The phenol gland, originally part of the purple gland in Tullberg’s analysis (the other part being the accessory gland), is comprised of cells filled with spherical vesicles (1.5 – 2 μm) that appear

homogenous in TEM imaging and are secreted into the distal depression of the foot, forming the adhesive plaque (Fig. 7E)^{61, 97, 129, 139}. For this reason, it has also more recently been referred to as the plaque gland¹²⁷. Early histological analysis of the gland suggested the presence of proteins rich in phenolic amino acids from which the gland's name is derived^{11, 22, 140}, which is consistent with the later biochemical studies of Waite described above. Currently, little is understood about the exact contents of the vesicles in the phenol gland, however, considering the sheer number of different plaque proteins and their precise localization within the plaque structure, it seems plausible that there are multiple variants of the plaque-forming vesicles stored in the phenol gland, each containing different assortments of plaque proteins. However, this conjecture remains to be substantiated. Nonetheless, several studies have shown that the proteins in the plaque vesicles are stored under very acidic pH (pH 2) and under reducing conditions^{127, 131, 133}. As posited initially by Waite, these conditions likely provide a controlled microenvironment conducive to DOPA catechol storage and adhesion¹¹⁰.

The fabrication process by which the plaque proteins transform from vesicle to adhesive has received less attention than the proteins themselves. Tamarin showed in 1976 that there are cilia-lined channels running through the phenol gland termed the longitudinal ducts (LDs), and that during induced thread formation, the contents of the vesicles are secreted through the LDs into the distal depression⁹⁷. For nearly 50 years, there was not much further analysis of the LDs until very recent work by Harrington's group which combined histology, confocal Raman imaging, TEM and FIB-SEM to follow the plaque formation¹⁰⁵. Most interesting was the discovery of the metal storage particles (MSPs) which contain concentrated Fe and V stabilized by catechol coordination. Assembly studies revealed that the MSPs are mixed with the secreted plaque proteins within the lumen of the LDs in which the proteins gradually cross-link with the metal ions via DOPA residues hardening on their way to the distal depression¹⁰⁵. This microfluidic-like process by which the metals are introduced into the plaque holds great potential for inspiring future advances in mussel-inspired materials.

Continuing the thread: Closing remarks and ruminations

The story of human fascination with the mussel byssus is a long one dating back to Aristotle and the ancient Greeks, continuing with stops and starts passing through France,

Germany, Italy and the UK, while currently concentrated at the sunny coast of Santa Barbara and the icy Atlantic waters of Québec. Early scientific interest in mussel byssus was stoked by serendipitous errors, a rich legacy of natural history, the exigencies of the War and insights by Brown, Picken and Pryor who merged curiosity, scholarship and a readiness to cross disciplinary boundaries. The biomimetics of the 1940s are particularly interesting, not only because biology inspired new technologies such as aircraft construction, but also because when those technologies became passé, the biology did not. Indeed, the legacy of these trailblazers continues to rouse and inspire the current and future generation of biological materials scientists to uncover the secrets of materials such as mussel byssus, spider silk¹⁴¹, velvet worm slime¹⁴² or hagfish slime¹⁴³, providing extractable chemical design principles for driving development and sustainable production of next-generation polymers, adhesives, composites and coatings. Along these lines, the dynamic feedback loop between fundamental investigation of biological mechanisms of materials formation/function and the efforts to engineer synthetic bio-inspired materials provides the momentum that drives this field forward. Thus, the story told in this review is only half the story, and we direct readers to several excellent reviews on mussel-inspired materials that tell the rest⁴¹⁻⁴³.

Finally, it behooves us to emphasize that *Mytilus spp.* byssus represents an infinitesimal fraction of the diversity of biological materials in nature. Over seventy years of focused research on this material has yielded a deep, yet still growing understanding of these fibers, their function and their formation. By comparison, much less is known about the byssal threads of other mussel orders such as Ostreida (including *Pinna nobilis*)¹⁴⁴ and freshwater invasive species from *Dreissena spp.*¹¹¹⁻¹¹². Given the variation between fibers from different *Mytilus* species, many exciting insights are yet to be discovered by studying the byssus fibers from other mussel orders. Thus, we end with a call to expand the exploration beyond the *Mytilus* byssus. Who knows where the story will end if we continue to follow the beckoning threads.

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