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Structural and Compositional Characterization of the Adhesive Produced by Reef Building Oysters

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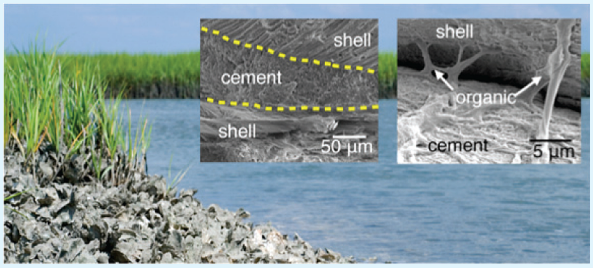
Supporting Information

ABSTRACT: Oysters have an impressive ability to overcome difficulties of life within the stressful intertidal zone. These shellfish produce an adhesive for attaching to each other and building protective reef communities. With their reefs often exceeding kilometers in length, oysters play a major role in balancing the health of coastal marine ecosystems. Few details are available to describe oyster adhesive composition or structure. Here several characterization methods were applied to describe the nature of this material. Microscopy studies indicated that the glue is comprised of organic fiber-like and sheet-like structures surrounded by an inorganic matrix. Phospholipids, cross-linking chemistry, and conjugated organics were found to differentiate this adhesive from the shell. Symbiosis in material synthesis could also be present, with oysters incorporating bacterial polysaccharides into their adhesive. Oyster glue shows that an organic−inorganic composite material can provide adhesion, a property especially important when constructing a marine ecosystem.



KEYWORDS: *adhesive, biomineralization, interface, oyster, reef*

INTRODUCTION

Oysters provide one of the most dominant influences upon healthy coastal ecosystems.[1,2] Their reefs (Figure 1a) give rise to environmental benefits including water filtration, prevention of coastal erosion, absorption of storm surge energy, carbon sequestration, and construction of habitat for other species. By clustering into reef communities, mollusks deter predation, decrease hydrodynamic forces upon individuals, signal to larvae the presence of food, and increase reproductive efficiency. Oysters rely upon an intriguing material for remaining in place. This adhesive must function while subjected to constant waves, turbulence, and temperature swings. Further challenges for this glue include changing salinity and daily transitions from wet to dry. There is no analogous man-made material able to set underwater, bond strongly, and remain affixed for years under such demanding conditions.

Fishermen, marine biologists, and beachgoers have referred to oyster "cement" for decades.[3] However, it was only quite recently that we obtained any direct evidence showing that these shellfish actually produce a chemically distinct adhesive material for bonding together into reefs.[4] The term "cement" usually refers to a material binding particles within. For example, concrete aggregate (e.g., stones) is bound together with cement. The most important characteristic for a true cement is cohesion, or the ability to remain together. A bulk adhesive, by contrast, must join two substrates by balancing cohesion with surface adhesive bonding. With this current study and our prior report,[4] we have seen that oyster "cement" is more properly called an "adhesive" or a "glue," hence the terminology used herein.

Given how much more adept nature can be at materials design than people, we have set out to understand the composition, structure, and formation of oyster glue. A small number of studies have examined the microstructure of adhesives from various adult oyster species.[5−8] These materials have been described to be calcified and crystalline, resembling shell,[5−7] as well as a layer of organics below crystallized cement.[8] Our first examination of oysters showed that attachment is achieved with a chemically distinct, biomineralized material composed of predominantly inorganics (∼86%) with significant levels of organics (∼11%).[4] Infrared spectroscopy demonstrated the presence of both calcite and aragonite in the adhesive but only calcite in shell.[4] Relative to the surrounding shell (∼98% inorganic, ∼2% organic),[9,10] the

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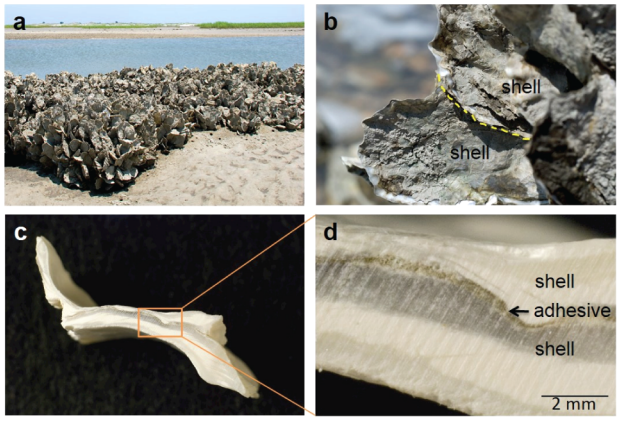

Figure 1. Photographs showing adhering oysters. (a) An oyster reef in South Carolina, U.S.A. (b) A pair of oysters bound together within a reef. (c) A cross section cut from bonded oysters. The top of the sample is the shell from one animal. The sample bottom is from another animal. These two shells are joined together by an adhesive. (d) Close up image from panel c, showing glue between two animals. Note that the thinner, brown region is the adhesive, whereas the wider, gray area is part of the shell.

adhesive does contain elevated organic content and a lower concentration of inorganic calcium carbonate ($CaCO_3$).[4] Most of the material is made from $CaCO_3$, but we do not know in which form (e.g., aragonite, calcite, or amorphous). Interestingly, $CaCO_3$ of any type is seldom a competent glue. Almost the entire adhesives industry, for example, is dependent upon organic polymers.

With these initial and somewhat puzzling results of high inorganic levels in mind, here, we set out to determine how this biological material can function. Efforts presented below examined adult oyster adhesive from a macroscopic perspective down to the microstructural level. These new findings bring about a proposal for the nature of this unique material. When considered within the broader context of biological materials, oyster glue presents a captivating demonstration of how an organic−inorganic composite material can be used for adhesive bonding.

RESULTS

Experiments began by cutting apart bonding pairs or clusters of Eastern oysters (*Crassostrea virginica*, Figure 1b) to reveal shell−adhesive−shell interfacial regions (Figure 1c). A dark band was observed to reside between each animal (Figure 1d). Although color varied among samples, the adhesive was always distinct and darker than the surrounding shell. Structural features of oyster shells have been well documented, being comprised of myostracal columns, foliated sheets, chalky lenses, and prismatic material.[11−14] No such structures were evident in the glue when examined by eye or optical microscopy.

Narrow shell−adhesive−shell cross sections were fractured to allow examination of interfaces by electron microscopy. The microstructure was consistent with prior reports of crystallinity throughout the shell.[11,12,14] Analogous structural features within the adhesive, however, were not observed. Figure 2 shows typical shell−adhesive interfaces in which the structural differences are stark. This homogeneous appearance of adhesive provided a contrast to the foliated sheets and myostracal columns of oyster shell.[11,12,14] Typical thicknesses for the adhesive region were in the range of ∼10 to ∼100 μm.

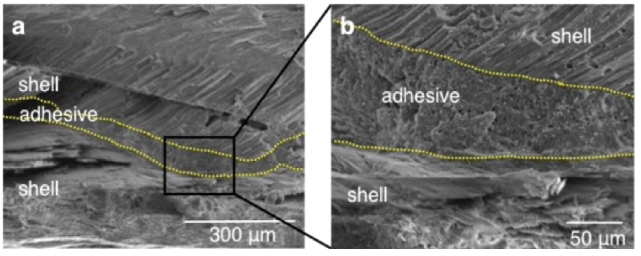

Figure 2. Scanning electron microscopy images of oyster adhesive and shell. Dotted lines have been added to aid viewing of the interfaces between glue and shell. (a) A shell−adhesive−shell interface that was prepared by fracturing the bonded oysters. (b) Close up image from frame a. Note how the glue lacks the microstructural features of the shells. Myostracal columns can be seen in the top shell and foliated sheets are found in the bottom shell.

Mollusks all have an outer, organic coating to protect their shells.[15] For oysters, this periostracum, at <1 μm, is significantly thinner than what we found for the glue.[15] Foreign objects were often trapped within the adhesive such as the embedded diatoms shown in Supporting Information Figure S1.

Samples were subjected to acid and bleach for selective removal of inorganics and organics, respectively. Before (Figures 2 and 3) versus after (Figure 4) acid treatment

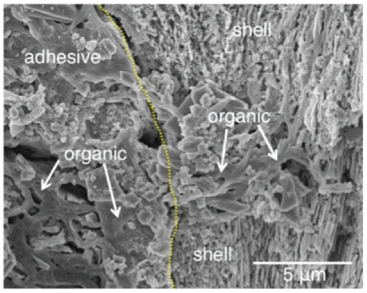

Figure 3. Scanning electron microscopy image of an adhesive-shell interface prior to acid etching. The organic component is seen to be within the glue and making direct contact with shell substrate.

showed loss of carbonate throughout the sample. The adhesive line took on a porous structure revealing an organic framework, which could then be removed with bleach etching. Closer inspection of the organic fraction in several acid treated samples provided quite interesting structural insights. The organic component of the adhesive appeared to be fashioned into fiber-like (Figure 4c) or sheet-like (Figure 4f) structures. Observation of these fiber- and sheet-like morphologies may be a reflection of how the organics are distributed within the larger matrix or, possibly, a result of agglomeration after sample drying for the microscopy experiment. Perhaps most striking was finding the organic network residing both within the bulk glue and also at the interface between adhesive and shell substrates (Figure 4c).

Energy dispersive X-ray (EDX) spectroscopy yielded elemental composition data further differentiating the adhesive from shell. Line scan analysis was carried out on shell-adhesive-shell cross sections that were polished smooth (Figure 5). When traversing shell-to-adhesive, most discernible was a decrease in calcium. Increases in silicon, aluminum, magnesium, and iron were also observed at the adhesive line. Examination of other elements did not yield sufficient signals or changes. Point analysis of several samples by EDX spectroscopy

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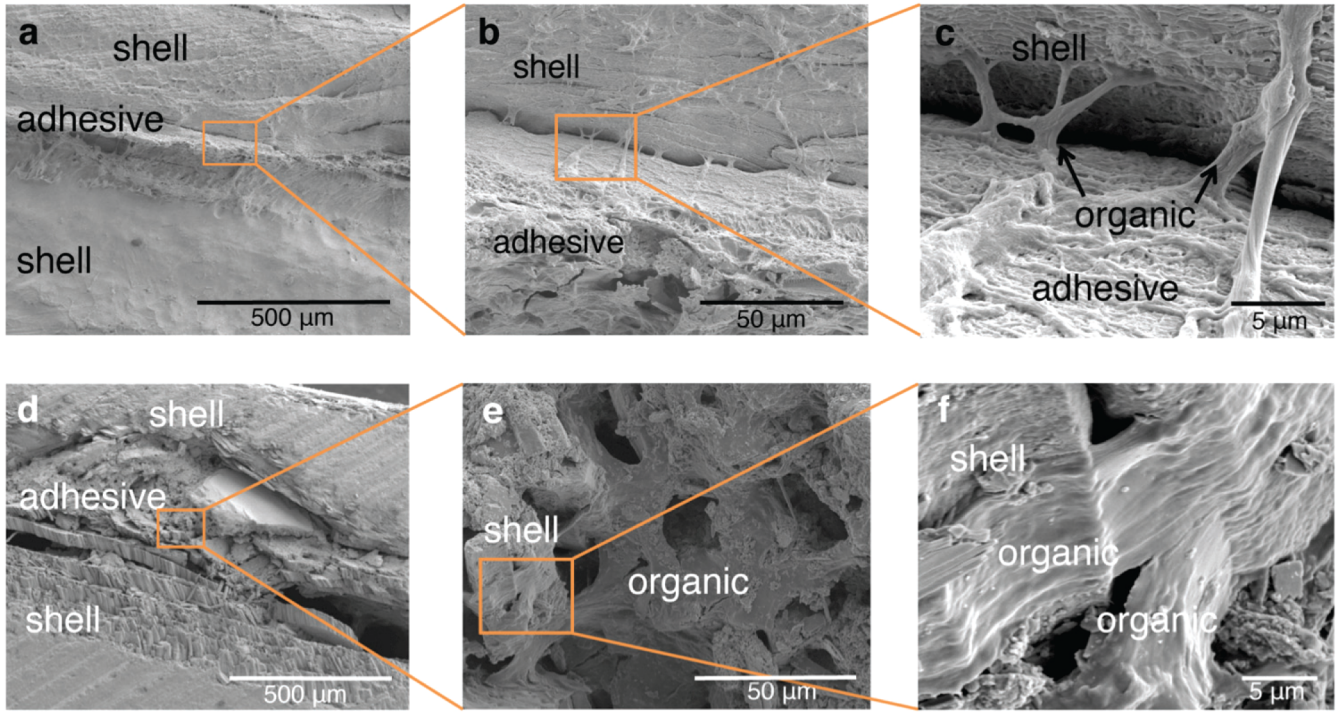

Figure 4. Scanning electron microscopy images showing the organic portion of oyster glue. These two samples (a–c and d–f) are shown after acid etching to remove $CaCO_3$. (a–c) One sample viewed at different magnifications. Note how the persistent organics appear like fibers. This organic glue is both throughout the adhesive and also contacting the shell substrate. (d–f) Another acid treated adhesive region. Here the persisting organic material takes on a sheet-like morphology.

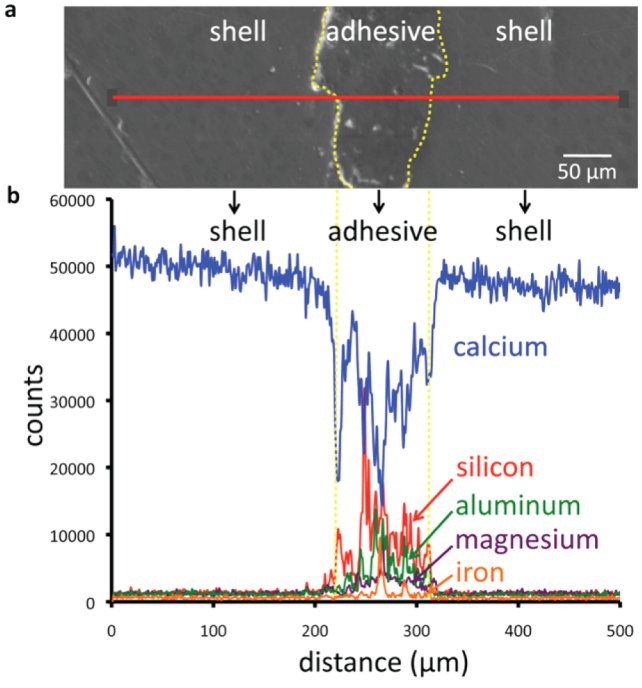

Figure 5. Energy dispersive X-ray spectroscopy line scans of a polished shell-adhesive-shell cross section. Yellow lines have been added to help differentiate regions of adhesive versus shell. A scan shows how the composition differs with regard to calcium, silicon, aluminum, magnesium, and iron. The red line in the top frame (a) correlates to the scans in the bottom graph (b). This sample was made by sawing bound oysters and then polishing the interface to be smooth.

provided a somewhat more quantitative view (Supporting Information Table S1). Although there were small variations from sample to sample, trends did emerge. The adhesive was found to be elevated in carbon (~29% versus ~18%) as well as deficient in oxygen (~40% versus ~46%) and calcium (~19% versus ~35%) when compared to shell.

Several samples were examined by EDX spectroscopy and the standard deviations in Supporting Information Table S1 provide an idea of the reproducibility that was found. For example, carbon in the cement ranged from ~20% to ~40% but was always higher than shell at ~16% to ~20%. Calcium was consistently lower in the adhesive (~10% to ~26%) versus shell (~30% to 40%). Pure $CaCO_3$ (calculated at C = 12%, O = 48%, Ca = 40%) provides a closer standard to shell than the adhesive.

Lower calcium in the glue is likely a result of the elevated organics required for interfacial bonding, present at the expense of $CaCO_3$. Nitrogen, chlorine, magnesium, aluminum, silicon, and iron were all at low levels, but higher in the adhesive than in the shell (Supporting Information Table S1). After having observed diatoms in the glue (Supporting Information Figure S1) the presence of aluminum, iron, and silicon indicated that dirt and silt are plausibly trapped inside.

Several attempts were made to isolate organic components from the adhesive for detailed characterization. Insolubility was a persistent problem. The adhesive was treated with acids, a chelator, a reductant, denaturants, and combinations thereof, followed by extractions. Identifiable organics were not observed by gel electrophoresis or mass spectrometry. Given that radicals[4] and fluorescence (see below) are present in the adhesive, the organic components are likely to be cross-linked extensively, hence the lack of solubility. Consequently, histology studies were used for identifying the organic components present in oyster adhesive. Histology is seldom the optimal characterization tool, given potential problems with

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a lack of staining specificity, dyes not washing away from porous surfaces, and acids dissolving samples. Nonetheless, staining can indicate the broad classes of chemistry present in a system. When faced with the intractable nature of oyster adhesive, histology was well suited to gaining the first insights on composition. The variability of results was diminished, as much as possible, by using consistent lighting and interfacial cross sections to provide shell control samples adjacent to the adhesive.

Shell−adhesive−shell cross sections were subjected to several stains (Supporting Information Table S2), with one example shown in Supporting Information Figure S2. Basic proteins, mucopolysaccharides, and protein amines were observed in shell, pseudonacre, and the adhesive to varying degrees. Fibrillar morphologies and β-sheet or amyloid structures have been found in the cement of barnacles.[16−20] Much like what was found with barnacle cement, oyster adhesive also stained positive with Congo red and thioflavin T. These results raise the possibility of β-sheet structure in oyster adhesive, but these stains are not specific enough to provide a definitive conclusion in this regard.

Unique to the adhesive was intense staining for lipids and phospholipids, neither of which were indicated strongly in shell or pseudonacre (Supporting Information Table S2 and Figure S2). In an effort to determine if these lipids resided within a specific adhesive location, samples were examined by optical, fluorescence, and scanning electron microscopies before then after exposure to Nile blue (Supporting Information Figures S3 and S4). Interestingly, this stain did not show lipids throughout the entire adhesive. Lipids might be aggregated along one interface between adhesive and mineralized shell and be absent from the opposite interface. This result could arise from how one animal deposits its glue onto another or altered staining of variations in the shell microstructure (e.g., chalky lenses versus foliated sheets).

Examination of unstained adhesive and shell by fluorescence microscopy showed emission from the adhesive line as well as some areas of shell (Figure 6). The glue of all samples fluoresced, although the intensities varied. Differences in fluorescence emission intensity, along with variations in the degree of coloration observed by eye, indicated fluctuations in the levels of conjugated organic compounds present. Such species can be produced as a result of oxidative cross-linking. When coupled with prior observation of an organic radical,[4] these results signal that such reactions and products are contributing to the curing of oyster glue.

With higher magnification imaging, the fluorescence appeared to be concentrated at the immediate interface between adhesive and shell (Figure 6b). Eastern oysters are known to harbor a Gram-negative bacterium on their shell exteriors.[21] These bacteria produce exopolysaccharides and melanins.[21,22] Although such organisms were not seen within the glue by microscopy, histology did indicate the presence of bacteria (Supporting Information Table S2).

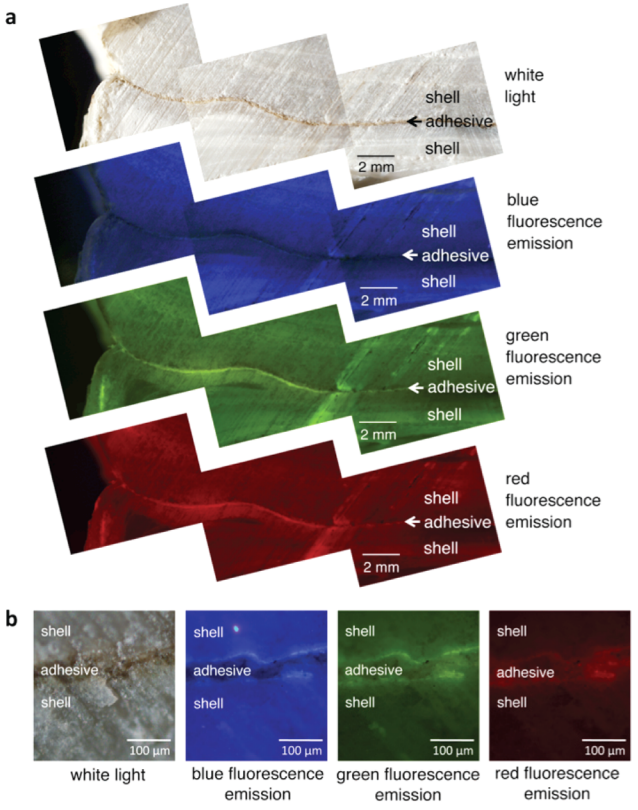

Figure 6. Fluorescence microscopy of shell-adhesive-shell interfaces. (a) White light optical microscope image of a cross section cut from a pair of bound oysters. Note the dark glue region. A 2X objective was used. Fluorescence microscopy images of the sample with blue (excitation ≈ 310−390 nm, emission ≈ 420+ nm), green (excitation ≈ 450−490 nm, emission ≈ 520+ nm), and red (excitation ≈ 510−550 nm, emission ≈ 590+ nm) emission are shown. (b) Close up images of the same sample, using a 10× objective.

DISCUSSION

Results present here show that oysters generate a dark colored and fluorescing adhesive material. Both organic and inorganic components are present, with significantly more of the organics in the adhesive than the shell. Proteins and polysaccharides are within the adhesive as well as shell. Phospholipids, unique to the adhesive, may be providing properties needed in the specific case of a glue. The organic portion is distributed throughout the $CaCO_3$ matrix and positioned to make interfacial contacts. Trapped inside the unstructured adhesive are aluminum, silicon, iron, diatoms, and bacteria. Oysters appear to bond by secreting a flowing adhesive, filling in the space between shell and substrate.

Discovery of such an organic−inorganic composite system is reminiscent of other biological materials including nacre and bone[23−25] but with the added feature of interfacial adhesive contacts. When viewed within the context of other marine biological adhesives, both similarities and unique aspects exist. Sandcastle worms use organic and inorganic components, but in quite a different arrangement.[26] Macroscopic particles such as sand are bonded together with relatively small amounts of glue.[26] Serpulid worm adhesive contains organic and inorganic fractions with defined, crystalline microstructure.[27] Mussel adhesive is almost entirely made from protein.[28] Starfish,[29] limpets,[30] and barnacles[31] each stick with glues containing significant percentages of inorganics. At this time, however, we do not yet have details with regard to the structures or arrangements of these different components. For the organic fraction of oyster adhesive, both polysaccharides and bacteria appear to be present. These observations raise the possibility of symbiosis in biological materials synthesis. Oysters may rely upon bacteria to provide biofilm exopolysaccharides for a portion of their adhesive organics.[32] Tubeworms are known to require a bacteria-laden surface for settlement.[33]

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Phosphorylated proteins are an emerging theme in marine bioadhesion.[34,35] Both lipids and phosphoproteins are key to the adhesion of larval barnacles.[36] Phosphates are also a common class of industrial adhesion promoters.[35] These ions bind to surfaces strongly, even in the presence of water.[35] Metal chelation, hydrogen bonding, and anion−cation interactions can account for such surface adhesion.[35] Beyond this chemical perspective, we must consider the structural nature of oyster adhesive. A crystalline material, such as shell, will not be able to flow onto a substrate and make beneficial bonding contacts in the same way that a liquid or gel might. If the material were to be crystalline, step edges would produce gaps between the glue and substrate. The resulting system would not benefit from the greater degree of surface interaction that could be brought about if the adhesive were first deposited in a liquid form. Phosphates play a role in biomineralization[37,38] but are also known inhibitors of crystallization.[39,40] Perhaps phospholipids are present to both prevent detrimental crystallization of the adhesive inorganics while also promoting bonding.

When considering the origin of oyster adhesive, the well-studied mussel provides a reference point. Mussels have their foot, a specialized organ to synthesize and deposit glue onto substrates.[28] No analogous feature has been described in adult oyster anatomy.[41] Oysters do have a shell synthesis apparatus from which an extrapallial fluid, concentrated in organics and inorganic ions, is secreted out from the mantle.[10,42] Perhaps this extrapallial fluid also gives rise to the adhesive.[5] Both shell and adhesive are comprised of mostly inorganic $CaCO_3$, with protein and polysaccharides also present. However, the organic/inorganic ratio is significantly higher in the adhesive. Also seemingly unique to the glue are organic radicals[4] and phospholipids. Radical−radical, as well as radical−surface, couplings may give rise to the cohesive and surface adhesive bonding required to form a bulk glue. These changes in composition and reactivity may be the oyster's way of differentiating the functions of adhesive versus shell, even if both materials start out from the same place.

Biological materials are adept at capturing our attention, with several aspects being more elegant than synthetic materials.[23,24] Like many other natural systems, oyster glue appears subject to evolutionary design constraints, mild synthesis conditions, hydration, and multifunctionality (e.g., one system to make both shell and adhesive).[25] Self-assembly could be present, with matrix templating of calcium by phospholipids, proteins, or polysaccharides.[25] Bones, shells, and teeth are examples of biological materials in which organic and inorganic components are arranged to bring about strength, elasticity, and toughness. When constructing an oyster ecosystem, the primary design constraints are likely to be making strong adhesive contact with substrates, even in the presence of water. Oyster adhesive presents a fascinating case in which shellfish construct an organic−inorganic composite material for bonding together into reef communities. The results of these efforts benefit both these animals as well as the coastal environment around them.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods, electron microscopy images, energy dispersive X-ray data, and histology results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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