What a Drag...But We Might Have the Cure

Biofouling (the accumulation of biological matter, such as microorganisms, plants, algae, or animals on seagoing vessels) adds significant drag to ship hulls and it is really a drag that it increases the Navy's fuel and maintenance costs. Modern antifouling paints have helped but environmental and regulatory concerns regarding toxic leaching of chemicals from these paints make finding a better solution for use on Navy ships and underwater structures a major priority.

What's the cure? Well, one solution for hard foulants like barnacles is to figure out how barnacles' insoluble glue cures underwater – and then invent advanced marine coatings that can disrupt those specific chemical and biomechanical processes. To do this, a research team from NRL's Chemistry and Materials Science and Technology Divisions and the Duke University Marine Laboratory was assembled and has been looking at barnacles from the inside out. These researchers have been devising new methodologies and using such technologies as CAT scans (X-ray tomography), atomic force microscopy, and infrared and ultraviolet spectroscopy to find out how barnacles secrete and cure their amazing adhesive glue. And the team's own stick-to-itiveness has paid off: they've found that the curing may be accomplished via a process similar to blood clotting in mammals; that the barnacles' glue differs from clotted blood in that its proteins fold in ladder-like amyloid structures, similar to how Alzheimer's disease affects protein conformation; and that barnacles use many simultaneous methods to make the glue they secrete an insoluble adhesive.

Applying more than an ounce of prevention (which is worth at least a pound of cure) seems to be the key, so the push now is to design new coatings that prevent barnacles from curing their adhesives. That should reduce the barnacle bill for sailors!

Marine Biofouling: Grasping Barnacle Cement Curing from the Inside Out

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Biofouling of Navy vessels substantially increases fuel and maintenance costs: the added drag increases fuel consumption, and the tenacious foulants are difficult to dislodge from hull surfaces. Understanding the biochemical and mechanical properties of biofoulants and their often insoluble adhesives will enable development of hull surface treatments that specifically target biofouling without resorting to materials or coatings that contain toxins. Our team from the Naval Research Laboratory and Duke University Marine Laboratory studied the barnacle *Balanus amphitrite* to learn how it secretes and cures its insoluble adhesive glue. We developed new in situ methods to examine the live barnacle/substrate interface, glue releasing structures, and adhesive curing chemistry. We used the first application of computer-aided X-ray tomography to image the shell structure in 3D where the glue is secreted; and used X-ray, UV, IR, and circular dichroism spectroscopy to elucidate the structure, hydration state, and curing mechanism of the adhesive protein. We discovered that curing proceeds, in part, by a coagulation process similar to blood clotting in mammals. In addition, we found that the adhesive consists of amyloid-like fibrils usually associated with disease in humans. Understanding how barnacles develop, apply, and cure the underwater adhesive protein will guide development of new coating formulations to prevent marine biofouling.

A CLINGY PROBLEM

For centuries, mariners and navies have battled biofoulants. These are the microorganisms, plants, algae, and animals that accumulate on ship hulls and other marine structures, sometimes attaching themselves tenaciously with insoluble proteinaceous glues. Biofouling of ship hulls increases drag, reducing the speed of sailing ships and increasing the fuel consumption of modern vessels (Fig. 1). Removing biofoulants is a huge maintenance expense, and new strategies are always being sought to deter or prevent attachment. In the present study, we used novel techniques to investigate barnacles and the adhesive they secrete, with a view to developing targeted, non-toxic solutions to preventing biofouling of U.S. Navy ships.

KEEPING THEM AT BAY

As early as about 400 B.C. there are reports of mariners applying oil laced with arsenic and sulfur onto wooden vessels to prevent biofouling, while the Greeks are reported to have used lead sheathing. Copper sheathing provided better antifouling properties; the British Navy first explored this on the wooden frigate H.M.S. *Alarm* in 1758. In 1781, the U.S. Navy ship *Alliance* was coppered, as was the *Constitution* in 1785.[1] Serious problems due to the corrosive action of copper with iron prevented its direct application to ironclad hulls, and electrochemical protection of the copper with sacrificial metals like zinc eliminated its antifouling properties.

FIGURE 1
Fouling of ship hulls results in damage to surface treatments and increased drag. This ship is encrusted with bryozoans (pink patches), an invertebrate animal.

For over a century, the U.S. Navy has explored and used paints to protect ship hulls from fouling and corrosion. Modern examples include tributyltin and copper ablative paints, the latter widely implemented on naval vessels. Environmental and regulatory concerns about toxic leachates have accelerated Navy science and technology exploration of both non-leaching "antifouling" and non-toxic "foul release" strategies as future surface treatments for naval ships and underwater structures, to avoid releasing toxins into oceans and harbors. Strategies currently being researched include coatings with novel surface chemistries or textures, coatings with tethered biocides, and elastomeric release coatings. Each of these approaches provides a different surface functionality to challenge foulants like barnacles, tubeworms, and algae.

STUCK ON YOU!

Barnacles are among the more pervasive marine foulants, with over 1000 species known. Despite studies by preeminent biologists such as Charles Darwin — whose 19th-century taxonomy remains a standard work — much is still unknown about barnacle biology. These crustaceans typically attach to rocks in the water and in intertidal zones, but also to piers, buoys, and ships (Fig. 2). Barnacles settle as larval cyprids, and adhere by exuding a permanent, protein-based adhesive. Once settled, the cyprid metamorphoses into the sessile adult. Barnacles are gregarious foulants — chemical signals from curing adhesives are believed to guide other cyprids to settle close to existing barnacles in order to promote reproduction.[2]

But how does the barnacle make its adhesive? How and where is it stored? If the barnacle is stuck on a rock substrate, how does it expand its space between base and side plates in order to grow? How does it release its glue to tack down the new growth? Understanding the biology and chemistry of barnacle adhesion is of great interest in the areas of marine biofouling prevention and materials science of adhesives: if we understand how the glue cures, we can more effectively develop strategies directly targeted at defeating these pervasive biofoulants.

The authors have developed a multidisciplinary approach to learn how barnacles secrete and cure their adhesive glue. Our team, with scientists from the Naval Research Laboratory and Duke University Marine Laboratory, includes specialists in biology, chemistry, and materials science. Studying *Balanus amphitrite,* or the little striped barnacle, we are developing new tools to examine the barnacle/substrate interface, glue releasing structures, and adhesive curing chemistry. Our experimental approach includes the first application of computer-aided X-ray tomography — CAT scans — to visualize the shell structure in 3D where the glue is secreted; and development of new biochemistry and surface chemistry approaches to elucidate the structure and hydration state of the adhesive protein, as well as its cross-linking, or curing, mechanism. Significantly, we discovered that the mechanism used by barnacles to cure their adhesive proceeds, in part, by a coagulation process similar to blood clotting in mammals.[2]

FIGURE 2
Barnacles are sessile crustaceans that are common in fouling communities (left). They adhere and grow on all kinds of organic and inorganic marine surfaces ranging from stationary rocks and piers to moving objects including lobsters, whales, and ships. The image in the center is a photograph of a hard-shelled barnacle (about 1 cm in diameter) from the top, revealing the interlocking side plates and movable top plates. The top plates protect the barnacle from predators when it is not extending its feeding appendages and seal seawater inside during low tide conditions. On the right is an X-ray tomogram rendering of a barnacle shell (without the top plates) showing the side and base plate structures. The cutaway reveals hollow, fluid-filled openings in the shell carrying ions important for shell mineralization and proteins found in the adhesive.

LOOKING UNDER THE HOOD

Buried adhesive interfaces provide a challenge to the researcher — how can we access and evaluate the adhesive curing process occurring underwater, underneath the hard calcified shell of an organism settled on a rock or ship hull (Fig. 3)? We have tackled this problem by two approaches. First, we have grown barnacles on silicone foulant-release panels, enabling us to remove them at will and turn them over to examine the adhesive directly. We have also grown barnacles on, or transferred them to, laboratory substrates that allow various spectroscopies to access the adhesive interface.

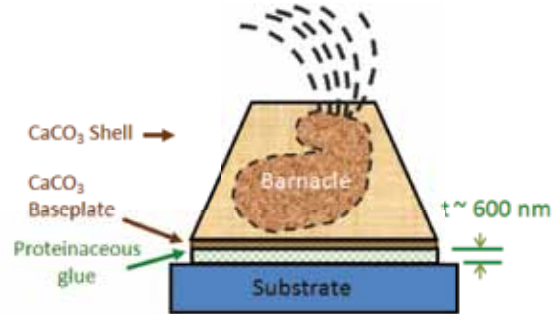

FIGURE 3
Cross-section diagram of a barnacle. The barnacle cures its glue not only underwater but underneath a protective shell. The animal is attached to the shell and feeds by sweeping the water with fringed structures called cirri – essentially it is stuck down on its head and eats with its feet.

We have employed a wide range of spectroscopies (on different substrates) at wavelengths spanning X-ray (polystyrene and polymethylmethacrylate [PMMA]), UV (CaF_2), visible (glass, CaF_2, and polystyrene), and IR (germanium). The substrates supporting the barnacles are selected for transparency at the specified spectral wavelength. These methods have enabled us to evaluate the structure and chemistry of live barnacles (in vivo) by direct, in place (in situ) spectroscopy of the adhesive interfaces, and after removal from surfaces (ex situ) (Fig. 4). This provides a window into the live barnacle adhesive interface with a wide variety of materials science and chemistry tools. We have also found that it is possible to make three-dimensional microtomograms or "digital dissections" of living barnacles in air or submerged in water (Fig. 5). These tomography images are revealing much about the nature of the barnacle shell that was never before attainable (Fig. 6).

THE BARNACLE'S CURE-ALL

Barnacles adhere to surfaces by secreting and curing a liquid, protein-containing adhesive. The barnacle secretes its liquid glue from under the periphery of the shell in growth cycles that result in rings of cured glue resembling the growth bands in a tree stump (see the photomicrograph in the upper center panel of Fig. 4). Liquid glue is released at the junction of the calcified base and lateral plates, and cures rapidly within minutes to hours, although the exact curing rate is not yet known. The lateral plates mate at the periphery and are interlocked to the base plate by mace-like structures (Fig. 6). The adhesive curing occurs under a protective calcium carbonate–rich shell that is simultaneously growing upward and outward to present a fresh surface for the liquid adhesive. The cured protein adhesive layer is less than a micron thick (typically ~600 nanometers [nm]), and the animal is further protected by calcifying a base plate shell on top of this adhesive (Fig. 3).

We have found that barnacle glue secretion and curing occur by a mechanism similar to that of blood clotting, suggesting that it is a specialized form of wound healing.[2] As the barnacle grows, it cracks open its shell at the periphery (see Fig. 6), allowing the adhesive to leak out and cure. At least two enzymes are involved in the adhesive curing process. These enzymes are molecules that activate protein chemistry, allowing proteins to unfold, rearrange, assemble, and lock in place after assembly. Like coagulated blood, cured barnacle glue is composed of nanofibrils intertwined in an insoluble mesh.[3] Topographic imaging by atomic force microscopy (AFM) revealed that the fibrils are very thin (2 to 25 nm in diameter) and unbranched (Fig. 7). The fibrillar mesh differs from coagulated blood, however, in the way the protein folds; this folding is referred to as secondary structure. The proteins comprising the fibrils are linear chains of amino acids that can fold into coiled, globular, or hairpin structures. The sequence of amino acids results in specific, reproducible folding under normal biological conditions. Our IR, UV, and circular dichroism (CD) spectroscopy analyses indicate that the protein fibrils are most likely folding into repetitive, alternating hairpin folds that form ladder-like structures. These ladder-like folding motifs are known as amyloid structure and are most often associated with disease states, like Alzheimer's, in humans. In individuals with diseases like Alzheimer's, proteins do not fold into their normal functional conformation, but instead fold and aggregate into insoluble domains. Barnacle glue appears to be an example of a recently recognized class of proteins comprising naturally occurring amyloid with a functional purpose.[3]

COMING UNGLUED

Why is barnacle glue insoluble? Our results point to the barnacle using multiple, simultaneous methods to cure its glue into an insoluble adhesive. Folding the

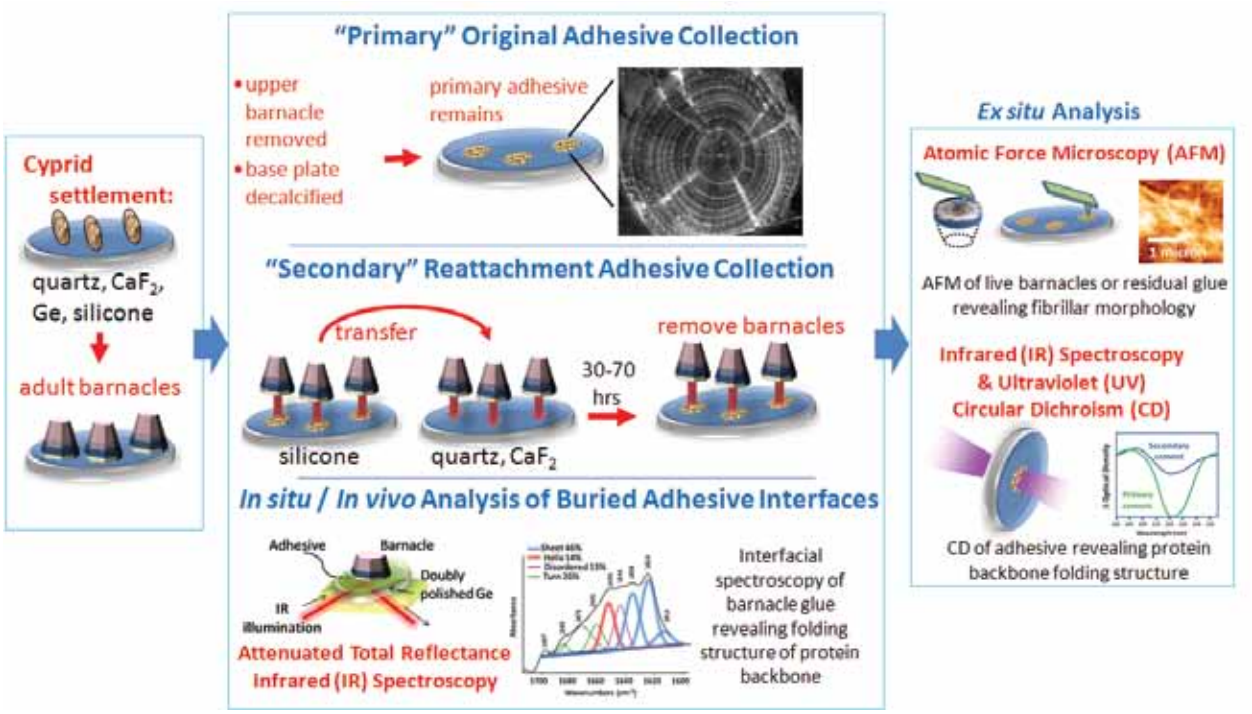

FIGURE 4
Barnacle glue analysis. To facilitate optical spectroscopics, we settle barnacle cyprid larvae on a variety of surfaces including quartz, calcium fluoride, germanium, and silicone rubber (left panel). The latter, silicone rubber, is a model fouling release surface that allows barnacles to be removed intact for examination. We are able to examine the original adhesive applied by the barnacle (upper center panel) after removing the animal and decalcifying the base plate; this glue is called "primary" adhesive. Some experiments require removal and reattachment of barnacles from silicone release coatings to other surfaces (middle center panel). This reattachment glue is called "secondary" adhesive. We have demonstrated that its morphology and chemistry are the same as the primary adhesive. Topographic analysis and transmission spectroscopies can be performed through the substrates or directly on the adhesive (right panel). Finally, spectroscopy of the buried interface of live barnacles (lower center panel) is performed using reflectance IR geometries. These spectroscopies have revealed the folding structure and hydration level (water content) of the protein adhesive.

protein chains into hairpin structures can strengthen the fibrils. Biochemical assays on liquid glue extracted from barnacles[2] point to permanent chemical bonds between protein chains that form in response to enzymes secreted by the barnacle similar to those in blood clotting, to enhance glue curing. The result is an insoluble mesh of tough fibers that resists our attempts to dissolve it, and resists the efforts to remove the protein during hull scraping.

We are just beginning to learn how barnacles cure their adhesive. Our studies of the adhesive from interfaces between live barnacles and model laboratory substrates (as shown in the lowest center panel in Fig. 4) have shown that the native, intact glue is not completely dry. In fact, barnacle glue has a hydration level that we estimate to be between 25% and 50% water by weight.[4] To better address the adhesive curing process, rate, and chemistry, we are developing approaches to access the adhesive curing in real time, rather than after the fact.

CLOSING THE BARN(ACLE) DOOR

How do we stop any or all of the above? Evidence that the glue cures in ways similar to blood clotting provides us with a path forward in devising new means to design marine coatings to resist barnacle fouling. We can now explore development of coatings that contain chemistries that interfere with and counteract the biological processes of adhesive curing. How best to implement these approaches into functional surface treatments that resist these tenacious foulants is the subject of ongoing investigations. Looking at the barnacle from the inside out has provided powerful insight into the chemistry and biomechanics of hard foulants.

As we develop improved real-time and in situ techniques for probing the barnacle and its adhesive, we look forward to grasping more of this amazing creature's secrets. Many of the procedures we are experimenting with and developing are applicable to other marine organisms. Ultimately, they may lead us

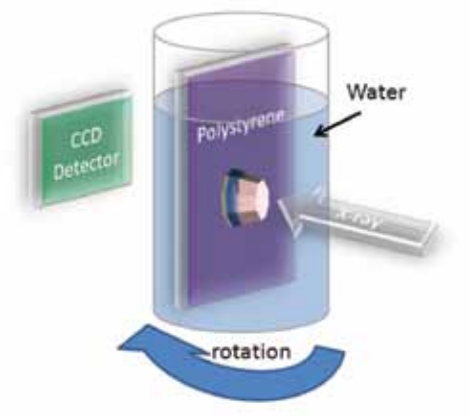

FIGURE 5
We imaged live barnacles using X-ray microtomography. Barnacles were settled on polystyrene or PMMA plastic (transparent to X-rays). Barnacles were imaged in air or submersed in seawater (above) during scans.

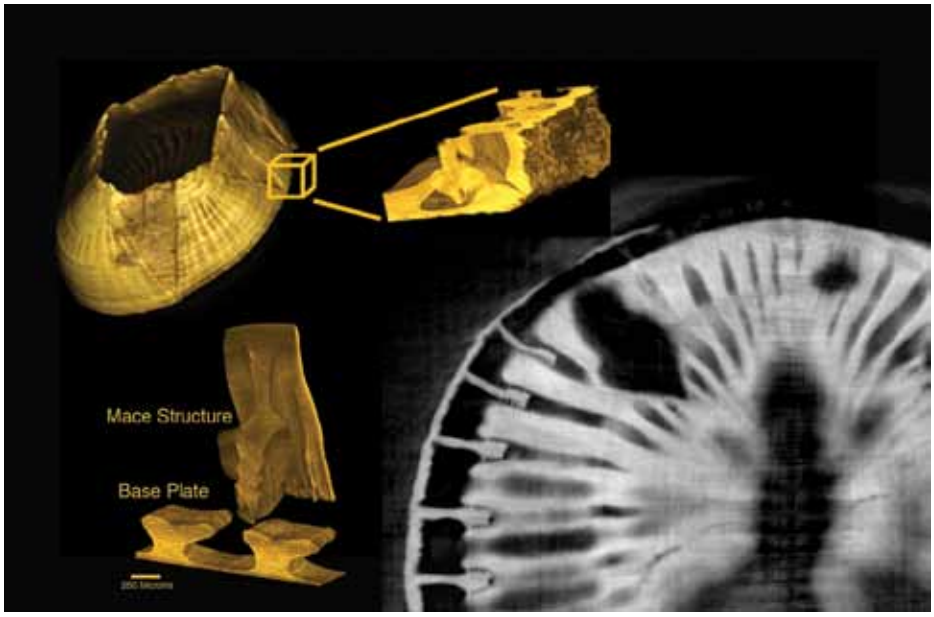

FIGURE 6
X-ray tomography showing 3D rendering of the barnacle shell (upper left) and (in the callout) a section from the growing edge where the side plates join the lower base plate. The grayscale image (lower right) shows a 2D tomograph section of the base plate viewed in plan view (from the bottom of the barnacle). Dark regions are hollow or low mass density regions, and bright regions are calcified shell. The image reveals the hollow structures in the side plates (rectangular holes at left edge), and mace-like structures found in the intersections of the side and base plates. These structures are interleaved with the base plate (lower left 3D rendering). The base plate shell is capable of growing conformally around defects in the substrate, adhesive, or adjoining animals. The dark, upside-down, spade-shaped region (in the lower right image) resulted from calcification above a thickened adhesive region under this particular barnacle.

FIGURE 7
Atomic force microscopy (AFM) images of barnacle glue. Background image shows plan view image where bright and dark scale correspond to height (bright regions are higher) revealing the fibrillar morphology of the cured adhesive. The image on the right is a high-resolution AFM rendered image of the fibrillar topography (1 micron square), showing that the individual fibril crossing from left to right is segmented and is about 25 nm in diameter.

not only to the prevention of biofouling on Navy ships, but also to a broader understanding of marine biomineralization and to development of better underwater adhesives.

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[Sponsored by ONR and NRL]

References

[1] Woods Hole Oceanographic Institute, *Marine Fouling and Its Prevention*, Chapter 11: The History of the Prevention of Fouling (Banta Publishing Co, Menasha, WI, 1952).

[2] G.H. Dickinson, I.E. Vega, K.J. Wahl, B. Orihuela, V. Beyley, E.N. Rodriguez, R.K. Everett, J. Bonaventura, and D. Rittschof, "Barnacle Cement: A Polymerization Model Based on Evolutionary Concepts," *Journal of Experimental Biology* **212**, 3499–3510 (2009).

[3] D.E. Barlow, G.H. Dickinson, B. Orihuela, J.L. Kulp III, D. Rittschof, and K.J. Wahl, "Characterization of the Adhesive Plaque of the Barnacle *Balanus amphitrite*: Amyloid-Like Nanofibrils Are a Major Component," *Langmuir* **26**(9), 6549–6556 (2010). DOI: 10.1021/la9041309.

[4] D.E. Barlow, G.H. Dickinson, B. Orihuela, D. Rittschof, and K.J. Wahl, "*In situ* ATR-FTIR Characterization of Primary Cement Interfaces of the Barnacle *Balanus amphitrite*," *Biofouling* **25**, 359–366 (2009).

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