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Synergistic Roles for Lipids and Proteins in the Permanent Adhesive of Barnacle Larvae

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Abstract

Thoracian barnacles rely heavily upon adhesion to surfaces and are important biofouling pests. Their adhesives also have unique attributes that define them as targets for bio-inspired adhesive development. With the aid of multi-photon and broadband coherent anti-Stokes Raman scatterings, we report that the larval adhesive of barnacles is a bi-phasic system containing lipids and phosphoproteins, working synergistically to maximize adhesion to diverse surfaces under hostile conditions. A lipid-containing material is secreted first, into which the phosphoproteinaceous component is delivered. The former may serve to displace water from the adhesive interface and protect the nascent adhesive plaque from bacterial biodegradation. The bi-phasic material implies an adhesion system with greater complexity than was previously recognised, and that an exclusive focus on proteins in bioadhesives may be an oversimplification. Knowledge and further characterisation of the lipidic contribution will hopefully inspire the development of novel synthetic bioadhesives and environmentally benign antifouling coatings.
Introduction

The ocean is a complex mixture of dissolved ions and organic macromolecules with a pH and ionic strength that vary significantly with geographical location. Over the past 500 million years or so, sessile marine invertebrates have evolved diverse adhesion mechanisms to suit the varied assortment of materials that constitute their settlement substrata, including rocks of differing chemistry, plants and the surfaces of other animals. Performing the complex chemistry of adhesion in such a variable environment is not a trivial matter, yet adhesion to surfaces is routine for most sessile marine invertebrates and it is unusual to find a naturally occurring surface to which barnacles, for example, cannot attach. Indeed, both cosmopolitan barnacle species and those that are highly surface-specific have mechanisms that facilitate tenacious adhesion under these hostile conditions to surfaces that differ in terms of roughness, chemistry, elastic modulus, surface energy and charge\(^1\). In addition, organic materials released into the ocean, particularly to a surface, quickly become a target for bacteria and may be damaged by digestive enzymes and other extracellular polymeric substances. Since many barnacles settle readily onto biogenic surfaces and persist there for years, their adhesives must have been rigorously optimised in a long-running 'arms race', both against bacteria and against organisms that have good reason to remain clear of fouling\(^2\). The bioadhesive systems of barnacles and other sessile marine invertebrates have in fact become so effective that most living surfaces that resist colonisation do so via toxic means\(^3\), grooming\(^4\) or by sloughing\(^5\).

It is imperative that the colonising forms, often larvae, remain securely attached to a chosen substrate during metamorphosis and growth; dislodgement usually equates to death. To be effective, their adhesives must overcome the adverse physical conditions at hydrated interfaces as well as the presence of biofilms. In light of these challenges, the adhesive used by a
barnacle larva during permanent attachment to a surface presents an interesting model for the
development of synthetic adhesives with wide-ranging applications. The adhesives of marine
organisms, and of barnacles in particular, are therefore of significant commercial interest for
novel biomedical adhesives and implants, being already optimised for conditions akin to those in
the human body. However, improved knowledge of their mechanisms of action will also inform
the design of novel coating technologies to combat marine biofouling. Ship hull fouling comes
at a cost of between $180 and $260 million for the US Navy alone and contributes to a loss in
cruising efficiency of up to 86% for a typical Navy ship. The economic and environmental
consequences of fouling, including higher fuel bills, trans-location of invasive species and
greenhouse gas emissions, are also becoming increasingly high-profile as environmental
restrictions on the use of antifouling biocides are tightened.

Barnacle cypris larvae (cyprids) adhere permanently to surfaces using an adhesive
exuded from large glandular structures located posterior to their compound eyes (Fig. 1a-b). This material is apparently unrelated to the cyprid temporary adhesive (used during surface
exploration prior to settlement) and the adult barnacle cement. The permanent adhesive
(cement) glands were described originally by Darwin as, “two long, rather thick, gut-formed
masses...formed of irregular orange balls, about .001 of an inch in diameter, made up of rather
large cells, so to have a grape-like appearance...apparently not enclosed in a membrane.”
Although a rather vague initial description, it is nevertheless the case that further details of the
morphology, biochemistry and mechanism of action of the cyprid adhesive system have
advanced relatively slowly since. The adhesion systems of barnacles have long been known to
differ from those of other sessile marine invertebrates. Whereas mussels, tubeworms and
echinoderms, for example, rely on DOPA-rich or poly-phosphorylated peptides that are stored
separately in glandular structures, crosslinking covalently upon release for adhesion, in contrast, adult barnacle cement components are released from single cells and the sequence of release and the curing mechanism(s) remain subjects of debate\(^1\). Although the permanent adhesive of the cyprid is the most poorly understood of the barnacle adhesives, it is also the system that resembles most closely the seemingly convergent adhesion mechanisms represented by the aforementioned groups of organisms. The presently accepted model for cyprid permanent adhesion, presented by Walker\(^{16,17}\), is that proteinaceous components of the adhesive are stored separately in the large (~120 µm longest length) kidney-shaped cement glands. Two cell types within the glands contain secretory granules with different morphologies (α and β granules) and differing compositions. Walker described release, that we now know to be exocytosis, of the granules into a median collecting duct followed by mixing and expulsion of the adhesive under pressure. The material travelled down the antennules used for surface exploration, culminating in discharge through pores on adhesive disc of the 3\(^{rd}\) antennular segment (Fig. 1a-b). The expressed adhesive then embedded the antennules and, depending on the surface, the two deposits of adhesive (one from each antennule) often merged into one continuous plaque\(^{18}\) (Fig. 1c-e, Supplementary Movie 1). Having identified phenolic peptides and the enzyme polyphenoloxidase in the α-granules, Walker\(^{16}\) surmised that separate adhesive components likely mix during release and cure via quinone tanning, forming a hard adhesive plaque.

The results of studies aiming to elucidate the permanent adhesive of cyprids have been ambiguous and often contradictory. Although evidence for quinone tanning was first presented by Knight-Jones and Crisp\(^ {19}\), Hillman and Nace\(^ {20}\) obtained none. Hillman and Nace, on the other hand, presented the first evidence for a small quantity of lipid in the adhesive, an observation made by no other investigators. All early studies agreed that cyprid permanent adhesive is
predominantly composed of protein, and this is consistent with what is known from other marine bioadhesives.

Walker's original description of the adhesive plaque morphology\textsuperscript{16} described three well-defined strata (layers 1, 2 and 3 from the outside in) using transmission electron microscopy, but under light microscopy only two layers were visible - a low density reticulum making up the majority of the plaque volume and a high density external 'skin', both staining for protein\textsuperscript{17}. Walker attributed the stratification in the original TEM micrographs to graded curing of the adhesive in the presence of an environmental catalyst; more recent observations using confocal laser-scanning microscopy (CLSM) have cast doubt upon that explanation, however\textsuperscript{18}. Using CLSM, only two well-defined layers were visible: a central proteinaceous bulk and an external non-proteinaceous layer. Based upon these observations it is our view that the high-density external 'skin' observed by Walker under light microscopy was, indeed, the intermediate layer 2 in the original TEMs. We propose that layer 1 in Walker's TEMs is the non-proteinaceous layer that was observed by Aldred et al.\textsuperscript{18}. This layer was probably removed during histological specimen processing in the early studies, which involved the use of ethanol, xylene, acetone and propylene oxide\textsuperscript{16}, leaving only layer 2 as the outer skin. Based upon these recent insights and the apparent removal of layer 1 by organic solvents, we hypothesised that the true outer phase of the adhesive plaque is lipidaceous in nature. If so, this would constitute the first description of a functional lipid in a marine bioadhesive.

To investigate this, we employed Two-Photon Excited Fluorescence Microscopy (TPEFM), Broadband Coherent Anti-Stokes Raman Scattering (BCARS) microscopy, and CLSM, combined with a suite of fluorescent probes for lipids, lipopolysaccharides, proteins and phosphoproteins, to image cyprid adhesive plaques and excised cement glands. We further
applied image analysis techniques, such as 3D segmentation, polygonal-mesh and isosurface generation to spatially delineate the two phases of the expressed adhesive.

**RESULTS**

When adhesive plaques were stained for proteins rendered images defined, with great clarity, the high-density protein skin covering a low-density proteinaceous reticulum (Fig. 1c, d, Supplementary Fig. 2 and Supplementary Movie 1). High-magnification imaging of single embedded antennules labelled specifically for primary amines highlighted a void between the bulk protein core and bacterial growth on the true outer layer of the adhesive plaque, indicating a non-proteinaceous phase between the protein bulk and the ambient seawater (Fig. 2a-f, Supplementary Movie 1 and Supplementary Movie 2 and 3). It is important to note that were it not for the visualisation of bacterial growth on the true outer layer, this non-proteinaceous phase of the cyprid permanent adhesive would have been missed entirely.

Figure 2 e, f and Supplementary Movie 3, show only the outer phase of the plaque and the bacteria accumulated on it. Simultaneous labelling with various fluorescent probes specific to phosphoproteins and lipids indicated clearly the presence of a lipidaceous outer phase (Fig. 2g-l, Supplementary Fig. 7, 8 and Supplementary Movie 4-7) and allowed detailed analysis of the respective phases of the adhesive plaque. The total volume of the lipid phase was considerably less than that of the protein phase, as evidenced by the difference in fluorescence intensities between lipid and protein-labelled adhesive plaques. The former formed only a thin outer layer around the proteinaceous core (Fig. 2g-n)

Label-free BCARS chemical imaging of the adhesive plaques also showed a marked difference between spectra from these lipidaceous and inner proteinaceous phases. From the BCARS analysis, it was clear that one unique phase encircled the other from the interface
through to the top of the plaque (Fig. 2m-o). The thin outer layer possessed higher lipid content than other regions analysed, retaining a constantly higher ratio of CH$_2$:CH$_3$ at the 2875:2930 cm$^{-1}$ bands, but with a substantial decrease from the phenylalanine and amide I/II protein-specific vibrational modes at 1006, 1668, and 1560 cm$^{-1}$, respectively$^{21,22,23}$ (Fig. 2o). The ratio between the CH$_2$ and protein specific vibrational modes, or lipid:protein ratio, has been shown to be indicative of local concentrations of the two species in biological systems$^{24,25}$. The thin layer surrounding the bulk material was therefore unambiguously lipid-containing. In contrast, the central bulk of the adhesive contained a much higher concentration of protein-specific chemical moieties than that of even the surrounding tissue (e.g. antennules and adhesive disc) of the cyprid.

The presence of a functional lipid component in the adhesive necessitated a re-examination of the adhesive storage apparatus, i.e. the cyprid cement glands and the contents of their two cell types. Previous histological investigations failed to detect a significant lipid component in either the α- or β-granules owing, perhaps, to the use of harsh organic solvents in traditional histological preparations. In fact, a 2006 study$^{26}$ questioned the existence of a second granule type. Not only is this assertion shown here to be unfounded, but the images of toluidine blue-stained sections in the 2006 paper are themselves indicative of two granule types, one of which appeared to be lipid (cyan in colour). In the present study, under physiological conditions and without the use of harsh organic solvents, we present conclusive evidence of two granule types, stored separately in large columnar cells within the cement glands (Fig. 3a and Supplementary Fig. 2d). Our observations, however, depart somewhat from those of Walker$^{16}$ in relation to the biochemistry of the adhesive constituents and the arrangement of the cell types. Whereas α-granules stained positively for protein, as described previously, and constitute the
bulk of the cement gland volume (Fig. 3c, g, h, Supplementary Fig. 6, Supplementary Movie 8),

β-granules contain negligible protein and stained positively for lipids (Fig. 3b, d-f, Supplementary Fig. 5 and Supplementary Movie 9). Walker's arrangement of β-granule-containing cells at the dorsal and ventral extremes of the gland seems to be another artifact of physical sectioning of specimens. Instead, we observed cells containing β-granules along the entire length of the gland, but confined to the lower half closest to the median collecting duct (Fig. 3e, Supplementary Fig. 5 and Supplementary Movie 9). Although cement gland cells only contain one granule type, of either proteinaceous or lipidaceous composition, the close proximity of the β-granules to the median collecting duct would assist in delivery if these are exocytosed first. The natural process of exocytosis has never been observed in cyprids, although artificial stimulation using catecholamines has been achieved\(^9,27\) (Supplementary Movie 10).

**Discussion**

Based upon our observations, we propose a novel mechanism for the permanent adhesion of barnacle larvae in which a lipidaceous primer, stored in the β-granules, is released first. We hypothesize that this material displaces water from the interface creating an environment conducive to the curing of a subsequent proteinaceous component, released by the α-granules (Fig. 4a-b). It is also possible that the two components are released in a gradient, beginning with a high concentration of the lipidaceous phase and ending with the proteinaceous phase, although the clear demarcation between phases observed by Aldred et al.\(^{18}\) suggests that this is unlikely. Mixing of the components, followed by release and subsequent phase-separation *ex vivo*, is considered to be unlikely on the basis of observations early on in cement release, where only the lipid phase was evident on the surface.
As the proteinaceous bulk of the adhesive is released into the lipidaceous phase, the former becomes concentrated at the interface between the two materials, resulting in a protein-dense region. In fact, it is logical to suggest that the role of the lipid layer may, in part, be to control the spreading of the larger volume of bulk protein and this behaviour appears to be surface specific\textsuperscript{18}. From our observations it is also apparent that, being rich in proteins, the proteinaceous phase would serve as a nutrient source for benthic bacteria and that the lipid phase may protect the protein from bacterial biodegradation as it cures. The result is a solid proteinaceous core that embeds the antennules and maintains the cohesive structure of the plaque (Fig 4c). This phase is surrounded and possibly even adhered to the surface by the lipid-based primer; lipid was often observed as faint fluorescence between the adhesive plaque and the surface, but with a signal insufficient to allow isosurface rendering. This is depicted in Fig. 4b.

Although lipid is the majority component of the putative primer, the true composition of the material is likely lipoprotein and/or lipopolysaccharide (Supplementary Fig. 5i-j). As the outer phase is sufficiently robust to survive days to weeks in the marine environment, we do not exclude a degree of crosslinking of functional groups, nor do we discount the possibility of any proteins in the external phase serving in the initiation and maintenance of adhesion to surfaces. AFM was used to demonstrate both hardening of the outer phase over the course of an hour or so post-secretion\textsuperscript{28} and the time-limited susceptibility of the adhesive plaque to attack by protease enzymes\textsuperscript{29}.

The vanishingly small quantity of adhesive available for collection per cyprid, and even smaller amounts of the individual phases, severely limit the use of conventional analytical techniques in elucidating the nature of cyprid adhesive. Surprisingly, while describing larval cement in another genus of barnacles Darwin\textsuperscript{11} noted that “...the cement, or part at least, comes
out of the ultimate segment of the antennae, in the shape of one tube, within another tube of
considerable diameter and length”, and it is our understanding that this refers to the 'fried egg'-
like appearance of the adhesive plaque in low magnification microscopy. This optical effect may
now be assigned to the presence of two concentrically arranged components of differing
composition.

Whether or not this type of bi-phasic mechanism is more widespread in marine
bioadhesives is unclear, as the role of lipids in adhesion has rarely been investigated. ATR-FTIR
peaks corresponding to lipid esters were observed at the adhesive interface of mussel byssal
threads\textsuperscript{28}, but little relevant information is available in the literature regarding putative lipid
fractions of adhesive secretions from other organisms. Having previously received scant
attention in the literature, it is our view that lipids, lipoproteins and/or lipopolysaccharides could
play an important functional role in the adhesive systems of other marine organisms too. Their
role is, in fact, well established in the tarsal adhesive secretions of terrestrial arthropods\textsuperscript{31, 32}.
Importantly, if we accept Darwin’s and, later, Walker's hypotheses that the cyprid cement glands
ultimately constitute the adult cement apparatus in barnacles (recently supported by\textsuperscript{33}), these
observations will also have important repercussions for our understanding of adult barnacle
adhesion and practical applications thereof.

\textbf{Methods}

\textbf{Cyprid Culture}

Barnacle larval culture was carried out at Duke University Marine Laboratory in Beaufort, North
Carolina, USA. \textit{Balanus amphitrite} Darwin (1854) (=\textit{Amphibalanus amphitrite}) nauplii
(planktonic feeding stage) were obtained from field-collected adults and were incubated for 4
days at 27–28 °C. Larvae were raised on a diet of excess *Skeletonema cosatum*. Following 4 days of incubation cyprids (settlement stage larvae) were separated from the culture through a series of fine mesh filters and were isolated, cleaned and shipped to Clemson University via overnight carrier at 6-8 °C. Larvae were maintained at 6-8 °C until use in experiments\textsuperscript{34,35}.

Sample preparation for staining cyprid permanent adhesive plaque

Cyprids maintained 6-8 °C were acclimated to room temperature. Cyprids were then deposited on #1.5 borosilicate coverglass (Thermo-Fisher) in a 1ml drop of artificial seawater (ASW) (33 ‰) and allowed to settle at 23 °C in a humidified incubator for 6-8 hours\textsuperscript{34}. Permanently attached cyprids that had not metamorphosed into juvenile barnacles were subjected to the various staining procedures described below.

Cyprid cement gland dissection

Cyprid cement glands were dissected as described previously\textsuperscript{35,36}. Briefly, the dissections were carried out on #1.5 borosilicate coverglass (Thermo-Fisher) with the aid of a Nikon SMZ 800 stereo-microscope. Cyprids were relaxed by adding a 7.5% (W:V) MgCl\textsubscript{2} drop-wise to Petri dishes containing cyprids in ASW. Once the cyprids were relaxed, the adductor muscle at the junction of the bivalve carapace was severed using acid-etched tungsten micro-needles (Fine Science Tools Inc. No. 10130-10, Foster City, CA, USA). Cement glands were then dissected out, separated and cleaned from associated tissue debris, brain, eyes and antennules. The cement glands were also subjected to staining procedures.

Staining for lipids in cyprid cement glands and cyprid permanent adhesive plaques
i) **Nile Red Staining**: Nile Red is a lipophilic stain that exhibits environment-sensitive fluorescence\(^{37}\). It is not fluorescent in aqueous media but is intensely fluorescent in lipid rich environments\(^{38}\). A 1 mM stock solution of Nile Red (Sigma-Aldrich Cat# N-3013) was prepared in DMSO. From the stock, a working solution at 800 nM was prepared by dilution in phosphate buffered saline (PBS). Cement glands and/or plaques on coverglass were placed in Petri dishes containing 4% formaldehyde solution freshly prepared from paraformaldehyde in PBS and allowed to fix for 30 min at room temperature (RT). Samples were then washed twice with PBS for five minutes each on an orbital shaker to remove the fixative. Glands and plaques on coverglass were immersed in Nile Red staining solution for 15 minutes at RT, followed by two washes for 5 minutes each with PBS on the orbital shaker to remove excess dye. A second #1.5 coverglass was placed gently on top of the coverglass containing specimen and a drop of PBS. The two coverslips were slightly separated and temporarily sealed using silicone grease (General Electric). Two-Photon excitation and emission wavelengths: ex755 nm, band-pass emission filter at 535/50 nm.

ii) **Oil-Red-O staining**\(^{39,40}\): Cement glands or plaques on coverglass were fixed with formaldehyde as described above and immersed in Oil-Red-O (ORO) (Sigma-Aldrich Cat #O0625) staining solution for 20 minutes, followed by washing twice with PBS for five minutes each on an orbital shaker. 100 mL of ORO staining solution was prepared by adding 40 ml of dH\(_2\)O to 60 ml of 12 mM solution of ORO in 100% isopropanol. The ORO staining solution was allowed to stabilize at RT for 20 min and then filtered through a 0.2 \(\mu\)m filter (Nalgene). Coverglass were prepared for
imaging as described above. Two-Photon excitation and emission wavelengths: ex755 nm, band-pass emission filter at 535/50 nm.

iii) Staining for Lipopolysaccharides**: Glands and/or adhesive plaques were subjected to lipopolysaccharide staining using Pro-Q Emerald® stain (Cat# P20495, Life Technologies, Carlsbad, USA). Although the Pro-Q Emerald manufacturer’s protocol is designed for staining polyacrylamide gels, it was modified in this case to stain glands and adhesive plaques. Briefly, formaldehyde fixed and PBS washed glands and/or plaques were treated with the carbohydrate oxidising solution (3% acetic acid and periodic acid solution, provided with the kit) for 15 min, washed twice with PBS for 5 min each on an orbital shaker and then incubated with the Pro-Q Emerald staining solution for 30 minutes. This was followed by two PBS washes for five minutes each on an orbital shaker. Coverglass were prepared for imaging as described above. Two-Photon excitation and emission wavelengths: ex800 nm, band-pass emission filter at 535/50 nm.

iv) Differential staining with lipid and protein dyes: Cement glands and/or adhesive plaques on coverglass were stained with a solution of the lipid membrane dye FM 1-43 FX (Life Technologies) prepared in DMSO in ASW at a final concentration of 5 mM for 10 min. Specimens were then washed twice with ASW for 5 min each on an orbital shaker and fixed with freshly prepared PBS buffered 4% formaldehyde for 30 min. Fixed specimens were washed with PBS twice for 5 min each on an orbital shaker and then incubated with a 1.4 mM solution of fluoresceinamine in PBS for 30 minutes. Fluoresceinamine binds to primary amines in proteins. Specimens were washed twice with PBS and coverglass prepared for imaging as described above.
Staining for proteins in cyprid cement glands and cyprid permanent adhesive plaques

i) **Phosphoprotein staining**: Although the manufacturer’s protocol for Pro-Q Diamond® phosphoprotein stain (Cat# MPP33300, Life Technologies) is optimized for staining SDS-polyacrylamide gels, it was modified to stain cement glands and adhesive plaques. Briefly, cement glands and adhesive plaques were fixed with formaldehyde as described previously. PBS-washed specimens were incubated with the Pro-Q Diamond staining solution for 60 min at RT while gently shaking on an orbital shaker. Following staining, the specimens were washed with PBS three times for 10 min each while on the orbital shaker. Coverglass were prepared for imaging as described above. Two-Photon excitation and emission wavelengths: ex800 nm, band-pass emission filter at 610/75 nm.

ii) **Coomassie Blue staining of adhesive plaques**: Formaldehyde fixed and PBS washed plaques were stained with 0.01% Coomassie Blue R-250 (Sigma) in 50% methanol and 10% acetic acid for 1 hr at RT. Following staining, plaques were de-stained with 40% methanol and 7% acetic acid for 10 minutes on an orbital shaker followed by a gentle rinse with PBS and 10 minute PBS wash on an orbital shaker. Two-Photon excitation and emission wavelengths: ex700 nm, band-pass emission filter at 535/50 nm.

Noradrenaline induced exocytosis of cement granules from explanted cement glands:

Cyprid cement glands were dissected on coverglass as described above, the coverglass were then placed on the microscope stage and a 20 µM solution of noradrenaline prepared in 0.2 µm filtered ASW was added drop-wise to immerse the glands. An image series was recorded at multiple time points in differential interference contrast (DIC) mode.
Prior to carrying out any of the staining methods described above, unstained specimen (adhesive plaques and cement glands) were checked for auto-fluorescence at excitation and emission wavelengths used in the experiments using both single photon confocal laser scanning microscopy and two-photon exited fluorescence microscopy. Control samples which were carried through the staining procedures, but without the addition of stains, were also imaged to check for non-specific fluorescence and auto-fluorescence. Neither the unstained nor the control samples exhibited auto-fluorescence in the emission wavelengths used for the assays.

**Single photon confocal laser scanning microscopy (CLSM)**

Confocal laser scanning microscopy on cement glands and/or plaques was performed as described previously. Briefly, specimens were imaged on a Nikon Eclipse TiE inverted microscope (Nikon, Melville, NY, USA), equipped with a C1Si spectral confocal scan head, following laser lines, 405, 457, 476, 488, 514, 561, 638 nm and appropriate band-pass filter sets for the excitation and emission wavelengths of fluorophores used. Imaging was carried out using Nikon CFI Plan Fluor 20X, 0.45 Numerical Aperture (NA) and Nikon CFI Plan Apo 60X, 1.2 NA objectives. The microscope was also equipped with a CoolSnap-HQ2 monochrome CCD camera (Photometrics, USA) and a Lambda 10-3 Motorized filter wheel (Sutter Instruments, Novato, California, USA). Multi-channel time lapse of noradrenaline induced cement gland exocytosis was imaged by setting up a ‘time lapse’ routine in Nikon NIS Elements software controlling the microscope which acquired images automatically over a defined period of time.

**Two-Photon excited fluorescence microscopy (TPEFM)**
Two-photon excited fluorescence microscopy was performed by coupling a mode locked beam of a Ti:Saphire oscillator at 140 fs pulses at 80 MHz repetition rate (Chameleon Ultra-II, Coherent, Santa Clara, CA, USA) to a Movable Objective Microscope (Sutter Instruments, Novato, CA, USA)\(^{45,46}\). The laser intensity delivered to the microscope was controlled using a pockel cell, model 302RM driver and a model 350-80-LA-02 EO modulator (Conoptics, Danbury, CT). The laser beam was raster scanned in the microscope via a pair of galvanometric scanners (Cambridge Technologies, MA, USA) and projected onto a back aperture of a Nikon CFI Apo LWD 25X, 1.1 NA water immersion objective through a scan and tube lens. The four channel non de-scanned detector setup consisted of four GaAsP photomultiplier tubes H7422P40-MOD (Hamamatsu, Middlesex, NJ, USA) with appropriate band pass filters for excitation and emission wavelengths of the fluorophores used in the experiments. The microscope’s X,Y,Z movements, laser intensity modulation by the pockel cell and data acquisition were carried out using MScan software (Sutter Instruments).

Broadband Coherent Anti-Stokes Raman Scattering (BCARS) Microscopy

Coherent anti-Stokes Raman scattering\(^{47}\) is used as an analytical tool and an imaging contrast mechanism in the realms of materials and biological sciences\(^{47-50}\). Broadband CARS (BCARS) contributes to the suite of traditional CARS capabilities by providing improved chemical selectivity and sensitivity over a 3000 cm\(^{-1}\) spectral range. The ability of BCARS to chemically image complex samples for obtaining a broad spectral signal at very low image acquisition times (< 30 ms / pixel offfor blue-shifted light) and at high spatial resolution (< 1 µm\(^3\) / spectral voxel) arises entirely from CARS being a nonlinear, third-order optical process whose signal goes as I\(^2\) and \(\omega_{\text{anti-Stokes}} = \omega_{\text{Probe}} + \omega_{\text{Pump}} - \omega_{\text{Stokes}}\)\(^{51-54}\). As a result, BCARS has the ability to non-
invaseively measure localized molecular susceptibilities at high speeds without auto-fluorescence of biological samples and in 3D sample space completely free of any sample preparation and labeling.

The coherent Raman (broadband CARS) microscope is detailed in the schematic diagram in Extended Data Fig. 3. In detail, the output (70 fs pulses centered at 830 nm and a 80 MHz repetition rate) from a single Ti:Sapphire oscillator (MaiTai-DeepSee, Spectra Physics, Santa Clara, CA, USA) was split into two separate beams for the pump / probe and Stokes pulses. The bandwidth of one oscillator output (narrowband pulse) was reduced by adjusting the slit width in a 4-f dispersionless filter to 8 cm\(^{-1}\) at full width half-maximum (fwhm) with the center wavelength at 830 nm. The other beam was sent through a photonic crystal fiber (Crystal Fiber-Femtowhite 800, NKT Photonics, Morgnaville, NJ, USA) for supercontinuum generation to produce a spectrally broad (850-1200 nm) pulse and then collimated using a parabolic mirror. The two beams were then collinearly combined before entering into the side port of an inverted microscope (Olympus IX71, Olympus, Allentown, PA, USA). The beam was focused onto the fiber using a 40x, 0.95 NA objective lens (Olympus) for a lateral resolution spot size of ~0.5 µm. The average power at the focal point of the beam was ~14 mW. The scattered light was collected by a 60x, 0.8 NA objective lens (Olympus) and then passed through an 830 nm notch filter and an 810 nm short-pass filter to remove the source and sample emission. After filtering, the blue-shifted BCARS signal was introduced into the spectrograph (SP-2300, Acton with 600 lines / mm grating) and measured with a charged-coupled device (CCD, DU920-BR-DD, Andor, South Windsor, CT, USA). The exposure time on the CCD was 30 ms per sample spatial location over a spectral region of 800-3400 cm\(^{-1}\) across 1024 pixels of the CCD chip. Images were produced by laterally and axially scanning a piezo-electrically controlled stage in 0.5 µm
increments. Prior to sample imaging, the collection efficiency of the light was optimized at the entrance slit to the spectrograph by adjustment of beam overlap, temporal spacing, and collection beam mirrors. Image acquisition and initial analysis were performed using a custom developed LabView (LabView 2011, National Instruments, Austin, TX, USA) program and then exported for further data analysis. BCARS spectral data processing included cosmic ray spike removal, spectral phase retrieval using the time-domain Kramers-Kronig approach to extract the imaginary portion of the resonant Raman signal, and baseline detrending using a cubic-spline algorithm\textsuperscript{55,56}.

**Data analysis and visualization for single photon confocal and two-photon microscopy**

Optical Z-sections (Z-series) collected using TPEFM were first converted to multipage TIFF (tagged image file format) files using Mview image analysis program (Sutter Instruments). Multi-page TIFFs were then converted to Image Cytometry Standard file formats (.ics) using NIS Elements (Nikon). Image files in .ics formats from CLSM and TPEFM were analysed using NIS Elements and Imaris (Bitplane, Zurich, Switzerland) data analysis and visualization software. Two-dimensional images were exported and saved in TIFF format and assembled into a figure panel using PowerPoint (Microsoft). Three-dimensional volume renderings created in NIS Elements and Imaris were exported as .AVI files for supplementary movies.

Three dimensional polygonal mesh surface renderings were created from the confocal and two-photon datasets using Imaris. Briefly, Imaris allows segmentation of the datasets and use of algorithms like Marching Cubes, to extract a polygonal mesh of an isosurface from voxels of the three dimensional dataset. Adobe Photoshop CS5 was only used for creating Extended Data Figure 2. For that figure, images were inverted using the ‘invert’ function in Photoshop and grayscale gradient mapping was assigned to the images using the ‘gradient map’ function.
Brightness, contrast and gamma were then adjusted for the images and the figure panel was assembled using Microsoft PowerPoint.

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12 Naldrett


References for Methods


Figure 1: Morphology of the cyprid adhesive apparatus and the expressed adhesive plaque. a) A schematic re-drawn from Walker (1971) of the anterior section of a barnacle cypris larva, with particular focus on the cementation apparatus: cement gland: c.g., muscular sac: m.s., cement duct: c.d., adhesive disc: a.d., oil bodies: o., compound eye: c.e., fourth antennular segment: IV. b) A confocal volume projection of a cyprid antennule labeled with a membrane dye showing the 2nd, 3rd and 4th segments of the antennule, the adhesive disc (a.d.) and setae of the 4th segment (IV). c) Confocal micrograph of a cyprid adhesive plaque stained for proteins. Embedded within the plaque are the adhesive discs appearing as dark central regions and setae of
the 4th segment. When viewed this way, the plaque appears to approximate the homogeneously proteinaceous, low-density structure originally described by Walker (1981), surrounded by a more protein dense ‘skin’ (white arrow). d) An isosurface rendering of c showing its three dimensional structure. e) A sketch of the cyprid adhesive plaque redrawn from Walker’s 1981 description; adhesive plaque: a.p. Scale bars: b=∼20 µm, c-d= ∼20 µm.

Figure 2: Spatial arrangement of lipidaceous and proteinaceous components of the expressed adhesive. a) CLSM volume projection of a single antennular adhesive plaque stained for proteins showing a clear void between the proteinaceous phase (green) and bacterial growth on the true outer phase of the adhesive plaque which is not stained for proteins (bacteria: b., lipid phase: l.p., protein phase: p.p., adhesive disc: a.d.). b-d) Isosurface rendering of a viewed from various angles, it is clear that proteinaceous phase (blue) and the bacterial growth (green) are separated by a material which does not stain for proteins. e-f) Imaged during the adhesive secretion, the cyprid adhesive discs (blue) are embedded in a phase of the cyprid adhesive that did not stain for proteins but which accrued bacterial growth (green). g) XY view from a TPEFM volume projection of the adhesive plaque embedding the paired antennules stained with lysochrome Oil-Red-O. Relative fluorescence intensity units (RFU) plot (inset) shows lower intensity values when compared to the labeling of the proteinaceous phase j. h-i) Isosurface renderings of the lipidaceous phase viewed from above (i) and beneath the plaque (j). The lipidaceous phase appears to entirely cover the bulk phase of the adhesive plaque which is proteinaceous. j) XY view from a TPEFM volume projection of an adhesive plaque stained for phosphoproteins shows that the bulk of cement plaque is phosphoproteinaceous and with much higher RFU as compared to g. k-l) Isosurface renderings of phosphoprotein stained plaque from j.
viewed from above (k) and beneath the plaque (l) shows that the bulk of the plaque is proteinaceous (blue) with bacterial growth (green) separated from the proteinaceous phase. m-o)

Scale bars: a-c= 5 µm, d= ~2 µm, e-f= ~5 µm, g= ~20 µm, h-l= ~10 µm.

Figure 3: Spatial arrangement of lipidaceous and proteinaceous adhesive granules within the cyprid cement gland. Using a combination of lipid specific (FM 1-43 FX) and protein specific (fluoresceinamine) dyes we confirm: a) the presence of two granule types within separate columnar cells of the cement gland; lipidaceous granules (white arrows) and proteinaceous granules (black arrows) in this confocal micrograph. Cell nuclei are blue. b) Lipidaceous granule-containing cells are present along the entire length of the gland, and not restricted to the dorso-ventral extremes, with their contents concentrated closest to the median cement collecting duct. c) Proteinaceous granule-containing cells are also distributed throughout the length of the gland. Lipidaceous granules appear to be larger than the proteinaceous granules and are packed more loosely. d) TPEFM volume projection of cement gland stained with Nile-Red, showing lipidaceous granules (white arrow) in the lipid granule containing cells are present only in the lower ventral half of the gland, closest to the median cement collecting duct (arrow head). e) Isosurface rendered view of the lipidaceous granule containing cells superimposed onto the volume projection of the gland. f) YZ orthogonal view of the Nile-Red labelled cement gland. Since Nile-Red does not label the proteinaceous granules the bulk of the cement gland appears empty (green arrow). g) TPEFM volume projection of cement gland stained specifically for phosphoproteins showing that proteinaceous granules constitute the bulk of the gland, also seen in YZ orthogonal view in h. Lipidaceous granules present around the collecting duct are not
labelled by the phosphoprotein stain and appear as vacuoles (yellow arrow). Also see Supplementary Fig. 5 and 6. Scale bars: a-c= ~20 µm. d, f= ~20 µm. g, h= ~30 µm.

**Figure 4: A new Model for the permanent adhesion of barnacle cyprids.** a) A schematic of the sagittal section of the cement gland. A lipidaceous precursor material (l.p.) is released from the lipid granule-containing cells (orange) closest to the cement collecting duct. Subsequently, the contents of the protein granule-containing cells (blue) are released into the lipidaceous phase, already delivered to the surface. b) A lipidaceous phase (orange) that surrounds the proteinaceous core (blue) of the adhesive plaque is the result, and is even present at the adhesive interface. E.g. c) TPEFM isosurface rendered volume projection showing two individual cyprid cement plaques, each embedding a single antennule, labelled for proteins (blue) and lipids (brown). Lipidaceous phase: l.p., and a proteinaceous phase: p.p. Schematics not drawn to scale. Scale bars: c= ~20 µm.

**Supplementary Figure Legends**

**Supplementary Figure 1: Spatial arrangement of lipidaceous and proteinaceous components of the expressed adhesive.** a-b) CLSM volume projection of two individual cyprid cement plaques embedding the cyprid antennules labelled for proteins (fluoresceinamine, green) and bacterial growth labelled for nucleic acids (Hoechst, pseudocolored red). The bacterial growth is on the true outer phase of the adhesive plaque, which does not label with the protein dye and hence there is an appearance of a gap between the bacterial growth (red) and the proteinaceous phase (green). c-g) Isosurface renderings of the volume projection from a viewed
from various angles. The bacterial growth (green) can be seen accumulated on phase of material
that is not labelled and the bacteria appear separated from the proteinaceous phase (blue). h)
Cyprid cement plaque from Fig. 1c labelled for proteins showing the loose reticulum and dense
outer skin. i) Plaque from h labelled with a lipophilic probe staining the lipidaceous phase of the
plaque along with the cyprid antennules and bacterial growth, j is composite of two fluorescence
channels h & i. Scale bars: a, b= ~10 µm, c= ~7 µm, d= ~4µm, e=~5µm, f=~3µm, g=~2µm, h-
j= ~15µm.

Supplementary Figure 2: Structure of the cyprid adhesive plaque and cement gland
observed by inverting fluorescence data. a-c) Cyprid cement plaques embedding the cyprid
antennules labelled for proteins (fluoresceinamine) and bacterial growth labelled for nucleic
acids (Hoechst). The images were first converted to grayscale and inverted using the ‘invert’
function in Adobe Photoshop. Brightness and contrast were then adjusted in the images. The
proteinaceous phase of the plaque is composed of a fibrilar reticulum (yellow arrows)
surrounded by a dense proteinaceous skin (arrow heads). The lipidaceous phase is not labelled
and appears as a gap (red arrows) between the bacterial growth (green arrows) and the
d) CLSM volume projection of a cyprid cement gland labelled with lipid specific (FM 1-43 FX)
and protein specific (fluoresceinamine) probes (same as Fig. 3a). The data is inverted as
described above. Two separate columnar cells containing two different granule types are
observed in the sagittal section of the gland. The lipidaceous granules (red arrows) appear to be
larger in size compared to the proteinaceous granules (blue arrows). The proteinaceous granules
appear to be packed more tightly than the lipidaceous granules and the contents of the lipid
granule containing cells are concentrated more towards the lower ventral half around the cement collecting duct. Scale bars: a= ~5 µm, b= ~10 µm, c= ~15 µm.

**Supplementary Figure 3: Coherent Raman Imaging Using Broadband CARS.** Schematic diagram of Broadband Coherent Anti-Stokes Raman scattering (BCARS) microscope: Polarizing beam splitter: PBS, Dispersionless filter: DpF, Waveplate: WP, Short-pass filter: SP.

**Supplementary Figure 4: Coherent Raman (BCARS) Imaging of the Cyprid Adhesive Plaque.** a) Single-pixel spectra from various points of interest in the adhesive plaque. Attachment disk (purple solid line), 2nd and 4th antennular segments (green and blue solid lines, respectively), inner proteinaceous phase (red solid line), and outer lipidaceous phase (solid black line). b) Pseudocolored BCARS chemical image with 500 nm lateral resolution taken at 30 ms / pixel acquisition times at 7 µm from the coverslip surface. RGB image chemical contrast comes from total C-H stretch at 2920 cm⁻¹ (red channel), the ratio of C-H to Amide III of 2920:1262 cm⁻¹ (green channel), and the ratio of C-H to nucleotide bases of 2920:1486 cm⁻¹ (blue channel). Arrows point to pixel locations where spectra were obtained in a. c) Axial stack of pseudocolored BCARS chemical images from z= 1-13 µm above the surface of the coverslip and corresponding bright field image at either end of the z-stack. Identical imaging parameters as (b) although with different spectral contrasts used for RGB channels (R: 1486:1522 cm⁻¹ G: 1540:1262 cm⁻¹ B: 2920:2850 cm⁻¹). Scale bar: b = 10 µm, c = 20 µm.

**Supplementary Figure 5: Lipidaceous Contents of the Cyprid Cement Gland Confirmed Using Fluorescent Lipophilic Stains.** a-e) Cyprid cement gland labelled with Nile-Red a) XY view from TPEFM volume projection of a Nile-Red labelled gland. Only the lipidaceous granules label with Nile-Red (arrow) and are seen in close proximity to the median cement collecting
duct. b) Lipid granule containing cells are present only in the ventral lower half of the gland. c) Isosurface rendered TPEFM volume projection of Nile-Red labelled lipid granules superimposed on the volume projection of the gland. d-e) YZ, lateral (d) and frontal (e) orthogonal views of the isosurface rendered TPEFM volume projection of a Nile-Red labelled glad. Nile-Red labelled lipid granules are superimposed onto a volume projection of the gland showing the presence of lipidaceous granules (white arrows) along the entire length of the ventral lower half of the gland. Since proteinaceous granules are not labelled with Nile-Red, the upper dorsal half of the gland appears empty (green arrows). f-h) Cyprid cement gland labelled with Oil-Red-O, f) TPEFM volume projection of the cement gland labelled with Oil-Red-O (ORO). Similar to Nile-Red, ORO labelled lipidaceous granules present in the ventral lower half of the cement gland. i-j) Cyprid cement gland labelled with Pro-Q Emerald® lipopolysaccharide stain. Similar to Nile-Red and ORO, ProQ Emerald stained the lipidaceous granules (white arrow) alone, which are observed in the ventral lower half of the cement gland. Scale bars: a-e= ~20 µm, f-h =~ 15 µm, i-j= ~20 µm. Also see Supplementary Movie M9.

Supplementary Figure 6: Proteinaceous contents of the cyprid cement gland confirmed using Pro-Q Diamond® fluorescent phosphoprotein stain a) TPEFM volume projection of a cement gland labelled with ProQ-Diamond (ProQ-D) shows that the proteinaceous granule containing cells, which constitute bulk of the cement gland, label for phosphoproteins (white arrows), while the lipidaceous granules do not label and appear as vacuoles (yellow arrows) surrounding the median cement collecting duct. b) Isosurface rendering of the volume projections from a, bulk of the cement gland contains the phosphoproteinaceous granules while the lipidaceous granules are not labelled and only appear as vacuoles in the rendered surface. c)
Transverse section of a ProQ-D labelled cement gland showing phosphoprotein granules approximately 50 µm into the gland. d) Isosurface rendering of the same. e) Approximately 25 µm below the transverse section in c, towards the cement collecting duct. The granules in the lipid granule containing cells do not label and appear as empty vacuoles (yellow arrows). f) Isosurface rendering of e, showing absence of granularity in the cells adjoining the median collecting duct. Scale bars: a-d= ~20 µm, e-f= ~15 µm. Also see Supplementary Movie M8.

Supplementary Figure 7: Proteinaceous phase of cyprid cement plaque confirmed using fluorescent stains. a-c) Cyprid cement plaque labelled with Pro-Q Diamond® phosphoprotein stain a) TPEFM volume projection of a cyprid cement plaque embedding a pair of antennules and labelled with ProQ-D. XY, YZ and XZ orthogonal views show that bulk of the plaque is proteinaceous and, in fact, composed of phosphoproteins. b-c) Isosurface rendering of the plaque from a viewed from above (b) and from underneath the plaque-surface interface (c). Adhesive disc: a.d. and red arrow heads. 4th antennular segment: IV. d-f) Cyprid cement plaque labelled with coomassie brilliant blue. d) TPEFM volume projection of an adhesive plaque labelled with coomassie blue (CBB) in XY, YZ and XZ orthogonal views. Similar to ProQ-D staining, bulk of the plaque is proteinaceous (white arrow), surrounded by a thin layer of material that appears not to be fluorescent (red arrows). e-f) Isosurface rendered plaque from d viewed from various angles. g) Similar to ProQ-D and CCB, TPEFM volume projection of an adhesive plaque labelled with fluoresceinamine labelling primary amines in the bulk of the proteinaceous phase. h) Isosurface rendering of the plaque from g. Scale bars: a= ~20 µm, b-c= ~15 µm, d-h= ~20 µm. Also see Supplementary Movie M6, M7.
Supplementary Figure 8: Lipidaceous precursors in the cyprid adhesive plaque confirmed using lipophilic stains. a-b) Cyprid cement plaque labelled with lysochrome Oil-Red-O a) Since Oil-Red-O is a lysochrome (lipid soluble dye) only the lipidaceous phase (arrow) of the cyprid cement is labelled along with the membranes of the cyprid antennules. b) Isosurface rendering of the plaque from a shows that the lipidaceous phase forms a ‘hollow cast’ (white arrows) around an empty space unlike the solid core that is the characteristic of the proteinaceous phase (as seen in Extended Data Fig. 7). The empty space would be filled by the proteinaceous phase of the plaque both secreted by the attachment disc (yellow arrowheads). c-f) Cyprid cement plaques labelled with Pro-Q Emerad® lipopolysaccharide stain. Cyprid cement plaques stained with Pro-Q E labels the lipidaceous phase of the cement (arrows) similar to ORO staining. Isosurface renderings of the data (d, f) show that the lipidaceous phase forms the characteristic ‘hollow cast’. Attachment disc: arrow heads. Scale bars: a= ~10 µm, b= ~7 µm, c-d= ~20 µm, e-f= ~10 µm. Also see Supplementary Movie M4, M5.

Supplementary Movies

a) Supplementary Movie 1: CLSM volume projection and isosurface rendering of a cyprid permanent adhesive plaque stained with fluoresceinamine, labeling proteins.

b) Supplementary Movie 2: CLSM volume projection and isosurface rendering of a two individual cyprid adhesive plaques (one from each antennule) stained with fluoresceinamine, labeling
proteins. Bacteria accumulate on the true outer phase of the plaque, which doesn’t stain for proteins and appears as a gap between the proteinaceous phase and bacterial growth.

c) **Supplementary Movie 3:** CLSM volume projection and isosurface rendering of cyprid permanent adhesive imaged early in the secretion process. As the lipidaceous phase is secreted first it doesn’t label with fluoresceinamine and the bacteria accumulated on it can be seen.

d) **Supplementary Movie 4 and 5:** TPEFM volume projection and isosurface rendering of cyprid adhesive plaques stained with Pro-Q Emerald labelling lipopolysaccharides. Pro-Q Emerald only stains the outer lipidaceous phase forming the characteristic ‘hollow cast’ as seen in the isosurface renderings.

e) **Supplementary Movie 6 and 7:** TPEFM volume projection and isosurface rendering of cyprid adhesive plaques stained with Pro-Q Diamond, labeling phosphoproteins. Bulk of the cyprid adhesive plaque is proteinaceous, composed of phosphoproteins and forms a solid core as seen in the isosurface renderings.

f) **Supplementary Movie 8:** TPEFM volume projection and isosurface rendering of cyprid cement glands stained with Pro-Q Diamond, staining phosphoproteins. Secretory cells containing phosphoproteinaceous cement granules constitute bulk of the cement gland. While the lipidaceous granules in the cells found in ventral lower half of the gland are not labeled by Pro-Q Diamond and appear as empty vacuoles.

g) **Supplementary Movie 9:** TPEFM volume projection and isosurface rendering of cyprid cement gland stained with Nile-Red, staining lipids. Lipidaceous granules are present in the cement gland secretory cells which are found only in the ventral lower half of the gland in close proximity to the median cement collecting duct.

h) **Supplementary Movie 10:** A combined time-lapse sequence of excised cement glands undergoing cement granule exocytosis when treated with norepinephrine imaged in differential interference contrast (DIC) mode.
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Author Contributions

NVG and NA were responsible for conception of the experiments. NVG designed and prepared the experimental systems and protocols. NA aided NVG in the experimental work. NVG and NA wrote the manuscript. CH performed BCARS microscopy and data analysis and YJL aided CH in BCARS microscopy. BO provided cypris larvae. ASM, MC, ASC and DR provided expertise and critical evaluation.

Author Information

Certain equipment, instruments, or materials are identified in this paper in order to adequately specify the experimental details. Such identification does not imply recommendation by the National Institute of Standards and Technology nor does it imply the materials are necessarily the best available for the purpose. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to NVG (neerajg@clemson.edu).