

## SHORT COMMUNICATION

### Confocal microscopy-based goniometry of barnacle cyprid permanent adhesive

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#### SUMMARY

**Biological adhesives are materials of particular interest in the fields of bio-inspired technology and antifouling research. The adhesive of adult barnacles has received much attention over the years; however, the permanent adhesive of the cyprid – the colonisation stage of barnacles – is a material about which very little is presently known. We applied confocal laser-scanning microscopy to the measurement of contact angles between the permanent adhesive of barnacle cyprid larvae and self-assembled monolayers of OH- and CH<sub>3</sub>-terminated thiols. Measurement of contact angles between actual bioadhesives and surfaces has never previously been achieved and the data may provide insight into the physicochemical properties and mechanism of action of these functional materials. The adhesive is a dual-phase system post-secretion, with the behaviour of the components governed separately by the surface chemistry. The findings imply that the cyprid permanent adhesion process is more complex than previously thought, necessitating broad re-evaluation of the system. Improved understanding will have significant implications for the production of barnacle-resistant coatings as well as development of bio-inspired glues for niche applications.**

Key words: adhesion, adhesive, barnacle, biofouling, cement, cyprid.

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#### INTRODUCTION

Recent decades have seen a proliferation of research into biological adhesives with interest stemming from a variety of applied fields. Knowledge of the mechanisms and principles underpinning adhesion would benefit the development of biomimetic glues, with potential applications from human surgery to hi-tech electronics. There is also considerable investment in novel technologies for preventing the adhesion of sessile organisms, particularly in the marine environment (Callow and Callow, 2011). One of the major challenges in understanding how natural adhesives function is describing the passive (i.e. no behavioural input from the organism), thermodynamically governed spreading of adhesive on a surface. Quantitative modelling of this behaviour would be a significant step towards development of synthetic materials with similar characteristics. However, meaningful characterisation of the interactions between biological adhesives and surfaces is challenging, particularly in aquatic environments. Biological adhesives are often proteinaceous (Stewart et al., 2011) and their constituents may be complexed, crosslinked and/or present in such small quantities that conventional biochemical analysis is impossible. Therefore, the relative contributions of structure and composition can be difficult to determine.

Barnacles are major marine fouling pests and potential model species in the development of novel, bio-inspired adhesives. The majority of research has focussed upon the adhesive of adult barnacles, with some progress also being made towards better understanding the temporary adhesive system of the cypris larva. The permanent adhesive of cyprids, which is used to attach

irreversibly to a chosen surface prior to metamorphosis (Aldred and Clare, 2008), remains almost completely unstudied. This is surprising for two reasons: first, cyprid permanent adhesion is a logical point of intervention for antifouling strategies, and second, the adhesive system itself is intriguing and potentially of use as a model for synthetic analogues.

During attachment, cyprids release permanent adhesive from a pair of large kidney-shaped cement glands within the body, which coalesces as a single adhesive plaque (Walker, 1971). Walker presented a compelling argument that the glands contain two cell types whose contents differ in composition. This result was confirmed by Okano et al. (Okano et al., 1996); however, Ödling et al. (Ödling et al., 2006) suggested that these may be the same cells with contents at different phases of exocytosis. Circumstantial evidence also suggested that the components may cure, when mixed, by a process of quinone tanning. Since 1971 there have been only three further studies of cyprid permanent adhesive (Okano et al., 1996; Ödling et al., 2006; Phang et al., 2006), although none of these shed significant new light on the composition of the adhesive components, the thermodynamics or the mechanics of adhesion to surfaces. Here, the behaviour of naturally released adhesive was observed on two well-characterised self-assembled monolayers (SAMs) of alkanethiols to determine simultaneously the structure of the secreted adhesive deposit and the effect of Gibbs surface energy (Petrone et al., 2011) on adhesive interactions with the surfaces.

Methods for measuring the interactions of micro-scale adhesive deposits with surfaces are currently scarce, although contact angle

analysis is the benchmark for investigating the interactions of liquids and surfaces at the macro scale. Although their value as a predictor of adhesion potential is debateable – there are surfaces with similar contact angles of probe liquids to which biological adhesion strengths differ (Ederth et al., 2011) – contact angles are certainly useful for pragmatic characterisation of biointerfaces; if an adhesive cannot spread on a surface, for example, it is unlikely to adhere effectively. Although contact angles of proxy liquids have been used to predict the behaviour of bioadhesives on surfaces with varying degrees of success, measurements of contact angles between actual bioadhesives and surfaces have never previously been achieved.

Conventional wetting theory predicts that the underwater spreading of a liquid adhesive with a lower surface tension than water should be restricted across high-energy -OH terminated SAMs, but spread freely on low-energy -CH<sub>3</sub> terminated SAMs (providing that the change in surface chemistry has a broadly similar effect on the substrate–seawater and substrate–adhesive interfacial tensions). In this case, adsorption of a hydrophobic adhesive to a hydrophobic surface is energetically favourable, because it reduces the number of ordered water molecules required to surround these two phases and increases the entropy in the system. Biological adhesives across diverse groups of marine organisms, however, have unique and poorly understood attributes that allow them to contradict this simple rule.

The composition and curing mechanism of the cyprid adhesive are currently unknown and it is unlikely that conventional analytical approaches will elucidate these in the short term. Confocal-microscopy-based contact angle goniometry, however, provides a novel method to simultaneously provide both compositional information (*via* histological staining) and functional biophysical data through direct measurement of interactions of the adhesive with a known surface. Therefore, the findings of this study pertaining to cyprid adhesive specifically, as well as the broader method development, represent significant progress towards hypothesis-driven design of antifouling materials and bio-inspired glues.

## MATERIALS AND METHODS

### Larval culture

*Balanus amphitrite* Darwin 1854 (= *Amphibalanus amphitrite*) nauplii obtained from field-collected adults were reared in mass culture to the cyprid stage on *Skeletonema costatum*. Cyprids were collected by a sieve cascade from cultures after 4 days, cleaned of debris and held at 6°C until use in experiments.

### Surface preparation

SAMs from HS(CH<sub>2</sub>)<sub>15</sub>CH<sub>3</sub> (1-hexadecanethiol) (Fluka Chemie, Bruchs, Switzerland) and HS(CH<sub>2</sub>)<sub>16</sub>OH (16-hydroxy-1-hexadecanethiol) [gift from Biacore AB (now GE Healthcare), Uppsala, Sweden] thiols were prepared exactly as described in Petrone et al. (Petrone et al., 2011). The thickness, advancing water contact angle and Gibbs surface energy for CH<sub>3</sub>- and OH-terminated SAMs are reported in Table 1.

### Sample preparation and microscopy

Cyprids were deposited onto the SAMs in a 1 ml drop of artificial seawater (33 p.p.t.) and allowed to settle at 23°C. Attached but unmetamorphosed cyprids were fixed with 4% formaldehyde [freshly prepared from paraformaldehyde made up in phosphate buffered saline (PBS), pH 7.4] for 40 min. Fixation had no effect on adhesive morphology. The surfaces with attached cyprids were then washed twice with PBS for 5 min each. Following fixation the specimens were permeabilised with 0.1% Triton X-100 for 5 min.

Table 1. Thickness, advancing water contact angle and Gibbs surface energy for CH<sub>3</sub>- and OH-terminated self-assembled monolayers (SAMs)

SAM	Thickness (Å)	Advancing contact angle (deg)	Gibbs surface energy (mJ m <sup>-2</sup> )
-CH <sub>3</sub>	18.4±0.2	107±1	19.6
-OH	21.3±0.2	39±2	47.4

Means are presented ±s.d.

Surfaces were placed in Petri dishes containing 1.4 mmol l<sup>-1</sup> fluoresceinamine (Sigma-Aldrich), a protein (primary amine)-specific fluorophore, made up in PBS. The Petri dishes were placed on an orbital shaker for 30 min. Specimens were then washed twice with PBS for 5 min each and counter-stained with DAPI (4',6-diamidino-2-phenylindole) (Life Technologies, Carlsbad, CA, USA) at a final concentration of 300 nmol l<sup>-1</sup> for 5 min. Surfaces were washed twice with PBS for 5 min each and then imaged immediately using a Nikon TiE Eclipse inverted microscope equipped with a Nikon CFI Plan-Apo 60X (1.2 NA, Melville, NY, USA) water immersion objective and a Nikon C1Si spectral confocal scan head and spectrometer (Gohad et al., 2012). Surfaces were imaged using 404 and 488 nm laser lines and appropriate excitation and emission filter sets. Unstained surfaces were used as auto-fluorescence controls and were not found to be auto-fluorescent in either of the wavelengths used for imaging.

### Image analysis

Confocal Z-stacks of the adhesive plaques were analysed using Nikon NIS Elements image analysis software. Orthogonal views of the Z-stacks were used to calculate the contact angles using the 'angle measurement' tool in NIS Elements. Contact angle values were then exported to Microsoft Excel for further analysis. Internal contact angles were measured for seven adhesive plaques on each SAM type (no more than three plaques per individual surface), with six random measurements taken and pooled per plaque.

## RESULTS AND DISCUSSION

Imaging cyprid adhesive plaques through SAMs revealed several previously undescribed features. First, the adhesive released from the cyprid antennules did not always merge into one continuous plaque as is most often seen on natural substrata. In fact, on the CH<sub>3</sub> SAM the adhesive often remained separate as two discrete deposits, each embedding the terminal portion of a single antennule.

The second observation (Figs 1, 2) was that the adhesive was clearly not homogeneously mixed. Rather, two distinct strata were visible: the central phase fluorescing strongly with the protein stain and the outer phase showing little or no fluorescence. The distinction between phases on the OH SAM (Fig. 1) was less obvious than on the CH<sub>3</sub> SAM. On the OH SAM, when an outer layer was visible, it was very thin, suggesting that less of the outer material was released in proportion to the inner proteinaceous material, that the inner proteinaceous material spread further on the OH SAM, reducing the thickness of a finite quantity of outer material, or that the two were mixed to some degree on the OH SAM and not on the CH<sub>3</sub> SAM. Some replicate surfaces were also counter-stained with DAPI, highlighting bacteria on the outer surface of the plaque and improving visibility of the non-proteinaceous outer layer of adhesive.

Stratification of the cyprid permanent adhesive was first noted by Walker (Walker, 1971) in ultrathin sections of the material used

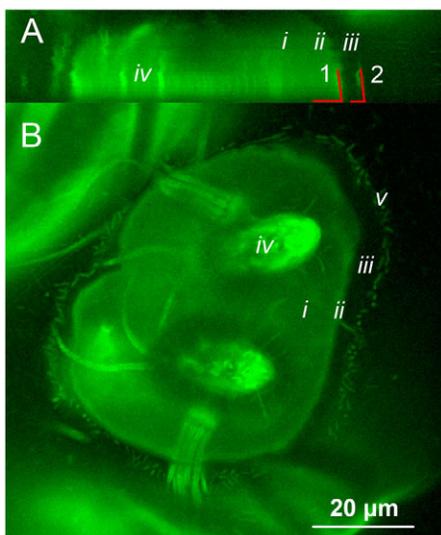


Fig. 1. (A) Orthogonal section through a pair of cyprid antennular discs embedded in a single, merged adhesive plaque on an OH-terminated self-assembled monolayer (SAM). Contact angles are also delineated in red. (B) The same pair of antennules and adhesive disc viewed from beneath. *i*, the central adhesive phase, fluorescing strongly on exposure to fluoresceinamine; *ii*, the interface between two phases in the adhesive; *iii*, the external, less fluorescent phase; *iv*, an attachment disc present on the third antennular segment; *v*, bacteria.

for transmission electron microscopy (TEM). However, it was considered unlikely that the adhesive components could be truly phase-separated on the grounds that sequential release of adhesive components would be a far more complex procedure than simply mixing the components internally and releasing them together. This rationale led Walker to suggest that seawater, or components thereof, might be important in the curing of the adhesive and that the apparent stratification was in fact evidence of incomplete polymerisation. However, artefacts inherent to conventional TEM sample preparation could lead to erroneous conclusions, and the degree of peptide crosslinking, if this even occurs in the adhesive, would not affect sample fluorescence in the present case. Use of dehydrating solvents and transitional fluids such as acetone or propylene oxide, as well as components of the embedding resin itself, may also have influenced the different adhesive phases unpredictably. Our results on fresh samples suggest that the two putative components of cyprid adhesive, contained separately within the cement glands, are either released in sequence or else, if released together, the components must then phase-separate in a manner dependent upon the composition of the attachment substratum.

Unfortunately, the complexity of the adhesive structure restricted the potential of the contact angle method in this context. Despite fluorescing brightly, the contact angle between the inner proteinaceous material and the SAM was often very difficult to delineate with accuracy. In many cases, the interface between the two adhesive phases was not well defined, and thus only contact angles between the outer non-proteinaceous phase and the SAM were measured in all cases (i.e. the outer phase–SAM–seawater interface; Fig. 1A, Fig. 2A, angle 2).

Restricted spreading on the CH<sub>3</sub> SAM, alluded to by the frequent presence of separate adhesive deposits, was supported by contact angle measurements. On average, significantly larger internal contact angles were recorded between the outer adhesive phase and the CH<sub>3</sub> SAM compared with the OH SAM (one-way ANOVA,

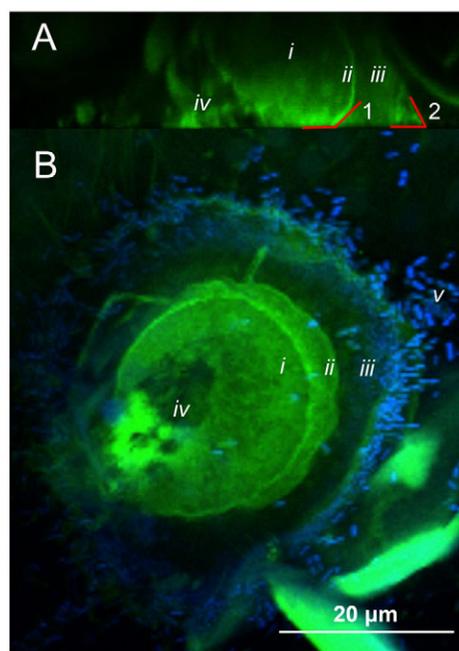


Fig. 2. (A) Orthogonal section through a single cyprid antennular disc/adhesive plaque and terminal antennular processes attached to a CH<sub>3</sub>-terminated SAM. Contact angles are also delineated in red. (B) The same antennule and adhesive disc viewed from beneath. *i*, the central adhesive phase, fluorescing strongly on exposure to fluoresceinamine; *ii*, the interface between two phases in the adhesive; *iii*, the external, less fluorescent phase; *iv*, the attachment disc of the third antennular segment; *v*, bacteria, counterstained using DAPI.

$F=124.4$ ,  $P<0.001$ ; Fig. 3). The mean ( $\pm$ s.d.) angle of contact between the outer adhesive phase and the OH SAM ( $67.9\pm 10.8$  deg) suggested that spreading is more favourable over the OH SAM than over the CH<sub>3</sub> SAM ( $86.7\pm 4.4$  deg). Variability in the contact angle data was such that there was a degree of overlap between the two surfaces, explaining why angles in the images selected for Figs 1 and 2 appear to contradict this trend. Although impossible to quantify on the OH SAM, the angle between the proteinaceous inner phase and the CH<sub>3</sub> SAM was often obtuse (Fig. 2A, angle 1), suggesting restricted spreading of the inner proteinaceous phase on the hydrophobic surface.

Quantitative evaluation of the wetting behaviour of the cyprid adhesive, as intended in this study, would therefore require consideration of a four-phase system with two unknown phases or, alternatively, two separate three-phase systems, rather than the conventional three-phase wetting system with one unknown phase that had been expected. Extension of the classic relationships ('Young's equation'), which only account for a single unknown phase, required additional data presently unobtainable and thus analysis could not be taken further. However, with an improved understanding of the forces governing liquid spreading on surfaces and knowledge of the composition of the phases of the cyprid adhesive, contact angles for this material on a range of surfaces with different characteristics may ultimately be used to predict work of adhesion and identify the key physicochemical characteristics of a successful underwater glue.

Larger contact angles and, presumably, restricted spreading of cyprid cement on the low-energy CH<sub>3</sub> SAM suggest lower adhesion strength to that surface. This prediction is supported by a generation of marine coatings designed to resist barnacle attachment that are

formulated around the principle of minimising surface (Gibbs) free energy. With this in mind, future applications of the goniometry method could be to test contemporary fouling-resistance hypotheses, by observing the effects of amphiphilic, zwitterionic or mixed-charge surfaces on adhesive morphology. In single-phase adhesives, the contact angle of the adhesive would provide a more robust indication of the affinity of the adhesive for the test surface and these data may then be used predictively. For cyprid adhesive, the composition, behaviour and contribution to adhesion of each adhesive phase have yet to be determined. The very fact that cyprid permanent adhesive is not a single homogeneous material, uniform in composition, raises numerous intriguing questions regarding the production, storage, sequential release and hardening of the adhesive components. Crucially, the present findings demonstrate conclusively that there are multiple components that must, presumably, be stored separately in the cement glands. It appears that despite suggestions to the contrary (Ödling et al., 2006), Walker (Walker, 1971) was correct in his original observations.

### Conclusions

Long known to be a dual-component adhesive, but previously believed to be homogeneously mixed upon secretion, it was found that the permanent adhesive of *B. amphitrite* cyprids was actually a dual-phase material. The stratified morphology of the adhesive restricted the potential of the contact angle method for quantitative modelling, although it was clear that the outer non-proteinaceous phase of the adhesive spread preferentially on the high-energy, OH-terminated SAM compared with the low-energy CH<sub>3</sub>-terminated SAM. This behaviour is contrary to the predictions of conventional wetting theory, but supports previous observations with regard to the unusual wetting properties of the adhesives of spores of *Ulva linza* and byssus of *Mytilus edulis*. Although not quantified in this study, it appeared that the inner proteinaceous phase behaved in the opposite manner to the outer phase, with a higher internal contact angle (restricted spreading) on a CH<sub>3</sub> SAM. Therefore, it is tempting to postulate that the two components of the adhesive might serve to interact preferentially with surfaces of different chemistry, thus improving the adhesion of the cyprid to the unpredictable range of naturally occurring substrata. Alternatively, the outer layer could be a hydrophobic material designed to provide a conducive environment for adsorption of the proteinaceous adhesive to surfaces, unimpeded by the presence of water. The phase-separated nature of the adhesive components post-secretion casts doubt upon the quinone tanning hypothesis, and therefore the entire adhesive system of cyprids, from physiology to biochemistry of adhesion, requires significant re-evaluation.

To summarise, confocal microscopy-based contact angle goniometry provides a novel method for gathering morphological, compositional and temporal dynamic information pertaining to unmodified samples of biological adhesives. In this way, the method provides previously unobtainable 'real world' information regarding the interactions of these complex materials with surfaces. In this study, the technique highlighted significant inconsistencies with the presumed morphology and behaviour of cyprid adhesive, providing a basis for considerable future experimentation.

### AUTHOR CONTRIBUTIONS

N.A. was responsible for conception of the experiments and practical execution. N.V.G. was responsible for the microscopy, methods and technical application. L.P. produced SAMs and provided technical input. B.O. produced and provided cypris larvae. T.E., A.M., D.R. B.L. and A.S.C. provided supervisory expertise and critical evaluation.

### COMPETING INTERESTS

No competing interests declared.

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