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Barnacle adhesion on natural and synthetic substrates: Adhesive structure and composition

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ABSTRACT

Sessile marine creatures are known for their adhesion on diverse range of materials from naturally occurring rocks to synthetic material surfaces. The current work compares morphological variation in the adhesion of barnacles (*Amphibalanus reticulatus*) on a natural patterned surface such as the shells of a green mussel (*Perna viridis*) and a synthetic polymeric surface such as polymethylmethacrylate. The elemental analysis in conjunction with characterization of the protein in both these cements brings out the biochemical differences as a function of the attached substrate.

1. Introduction

Being a sessile creature, the barnacle spends its entire lifetime attached to the surface carefully chosen during its cyprid larval stage. The major challenge it faces during such an irreversible adhesion process to a surface is the dynamic ambience that includes intertidal zones in which it has to overcome from being swept away by the ocean waves. The adhesion happens via the connection of its hard calcareous base-plate to a fixed hard foreign surface through a softer organic adhesive layer. The interface is hardly a few microns thick and the variation in stiffness across the interface is quite drastic due to the presence of this adhesive layer [1,2]. The existence of such large mismatch in stiffness can possibly disrupt the connection by the application of forces parallel to the cement layer [1]. However, the adhesion of the barnacle to a surface is much stronger than one would expect and it is surprising to note that such a thin adhesive interface functions effectively withstanding over millions of cycles of ocean currents. The performance of the adhesive could be possibly explained if one understands the two major characteristics such as: (a) its structural attributes as a function of different surfaces it attaches to, and (b) its biochemical characteristics. The previous experimental investigations have demonstrated that the barnacle adhesive is capable of adapting its structure based on the substrate material to which it attaches and the factors including surface roughness, texture, wettability, modulus, and thermal conductivity plays a vital role in defining the interfacial morphology [2–4]. The adhesion studies have been reported on mostly synthetic polymeric and metallic surfaces. However, the adhesion on biological surfaces including other marine creatures has not been explored. It is known that marine creatures with textured surfaces suffer relatively less barnacle attachment [5,6]. Though barnacles are commonly found attached to mussel shells, it is often observed that they can be easily peeled off from these surfaces which demonstrates the foul-release property of the latter. However, the adhesive present in such an interface has not been investigated till date. Hence, this preliminary study reports the morphological and biochemical characteristics of this adhesive. The dominant foulers of the Indian marine waters, *Amphibalanus reticulatus*, attached to the periostracum of the green mussel shells, *Perna viridis*, are chosen for this study. This adhesive is compared with the adhesive collected from barnacles attached to an artificial surface such as polymethylmethacrylate (PMMA).

2. Experimental

A. reticulatus attached to PMMA surfaces and *P. viridis* shells were collected from the marine waters of the Bay of Bengal, Chennai, South India. The contact angle of pristine PMMA surface and unfouled periostracum of *P. viridis* were measured by sessile drop method using an Easy Drop Contact Angle Measuring System (Krüss, Germany) with MilliQ water of surface tension

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72.8 mNm^{-1}. Those barnacles with a base-plate diameter of 1–1.5 cm were detached from the surfaces and washed with deionized water. The cement left on the base-plate (primary cement) was gently scraped off and transferred immediately to separate Eppendorf tubes and stored at −20 °C until the biochemical characterization studies were performed. The interfacial morphology of the barnacle adhesive was imaged using FEI Quanta 200 (USA) scanning electron microscope (SEM). The specimens were coated with 6 nm thick Au/Pd prior to SEM examination.

The vacuum dried cement was used for elemental analysis using a Perkin–Elmer Elemental analyzer (2400) series with the combustion oven set at 950 °C and the reduction oven at 500 °C [7]. The total carbohydrate and protein present in these two cements were determined using a weighed quantity (2–5 mg) of finely ground cement. The former was estimated by phenol–sulfuric acid method using bovine serum albumin protein (HiMedia™ Laboratories, Mumbai, India) as the standard [8]. The latter was estimated by Lowry's method with the help of Folin's Colin reagent (Merck, Mumbai, India). The percent of lipids present was determined using finely ground vacuum dried cement (2–5 mg) which was extracted with 2:1 (*v*/*v*) chloroform–methanol mixture [9].

For protein analysis, the cement was subjected to fractionation by the addition of 1.5 M Tris-HCl buffer (pH 8.5) containing 7 M of guanidine hydrochloride and 20 mM of ethylene diamine tetra acetic acid (3 mg/ml) and then reduced with 0.5 M dithiothreitol (DTT) for 1 h at 60 °C under continuous agitation [10]. The resulting suspension was centrifuged and the protein fraction in the supernatant was used for electrophoretic separation under reduced conditions. The protein fraction was then concentrated using Amicon (Millipore Ltd., India) ultrafilter centrifuge tubes of 30 kD molecular weight cut-off. Each fraction was dialyzed against 0.1% acetic acid at 4 °C and lyophilized. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12–15% gel. Following electrophoresis, the gels were stained either with a mixture of 0.1% Coomassie Brilliant Blue (CBB) in methanol, glacial acetic acid and water at a ratio of 5:1:5, or stained with silver after treating with methanol–water at a ratio of 4:5. The molecular weight of the protein was calculated using the reference values of standard proteins from Biorad laboratories using Quantity ones® software in Gel Doc system (Biorad Laboratories, India). All the chemicals used for this study (unless otherwise specified) were purchased from SRL (Mumbai, India).

3. Results

The contact angles of the pristine PMMA surface and the periostracum of *P. viridis* are 79.7° ± 1.9 and 55.0° ± 0.6 respectively. The photographs indicating the primary cement on the base-plates are shown in Fig. 1. The adhesive from PMMA is thin-layered and reveals the radial channels of the base-plate (Fig. 1a). Whereas on *P. viridis*, it is thick and viscid and can be peeled off from the base-plate (Fig. 1b). The detachment of barnacles from both these surfaces resulted in cohesive as well as adhesive failure at different locations of the interface. The SEM image reveals a drastic variation in the microstructure of the cement detached from the surface of *P. viridis*. The adhesive-failed region of the cement has a number of parallel lines throughout its surface (Fig. 2b). These lines are negative replica of the surface structure of periostracum of *P. viridis* as indicated in Fig. 2a. The cohesive-failed region of the cement is shown in Fig. 2c and the cement here is non-porous and layered in nature. The pristine surface of PMMA before exposure to marine waters is shown in Fig. 2d. The morphology along the adhesive failed region appears smooth and electron-transparent revealing the cement-duct openings beneath it (Fig. 2e). The cement morphology across the cohesive-failed region on PMMA is sponge-like and the pore-size ranges from 0.1 to 4 μm (Fig. 2f).

The elemental analysis shows that the carbon, hydrogen and nitrogen (C: 50.63%; H: 4.71% and N: 14.31%) content in the viscid cement secreted on periostracum is higher than that from PMMA (C: 33.5; H: 3.76% and N: 9.29%). Similarly, the carbohydrate, protein and lipid (19%, 89.4% and 0.33% respectively) are high in the cement detached from periostracum when compared to that from PMMA (4%, 58.1% and 0.10% respectively). The SDS-PAGE analysis of the viscid cement from *P. viridis* indicates the presence of proteins with 108, 84, 64, 32 and 22 kD molecular weight whereas the spongy cement from PMMA surface shows proteins with 102, 93, 84, 81, 64, 58, 32 and 20 kD molecular weight (Figure not included).

4. Discussion

From the earlier reports it can be concluded that it is easier to dislodge a barnacle from its attached surface as the adhesive thickness across the barnacle-substrate increases [4,11]. The photographs of the dried cement left on the base-plates of the barnacles explain perhaps one of the reasons why peeling off becomes easier from the green mussel shells. Further exploration of the adhesive-failed and cohesive-failed region of cement remnant on the base-plates provides more information on the surface-topography-dependent adhesive morphology. The adhesive-failed region of cement from PMMA and *P. viridis* shell brings out the fine-tuning of the cement according to the patterns on the supporting surface. The alternate parallel linear elevations

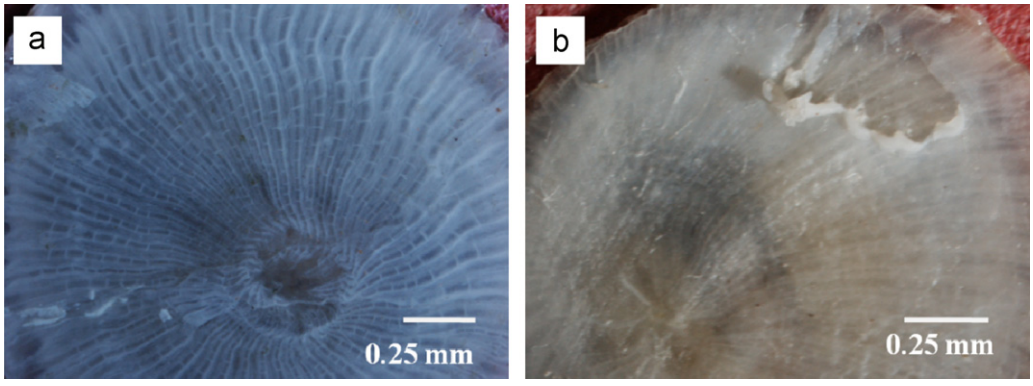

Fig. 1. (a) Translucent rigid cement on barnacle base-plate detached from PMMA substrate and (b) thick viscid cement on barnacle base-plate removed from the periostracum of *Perna viridis*.

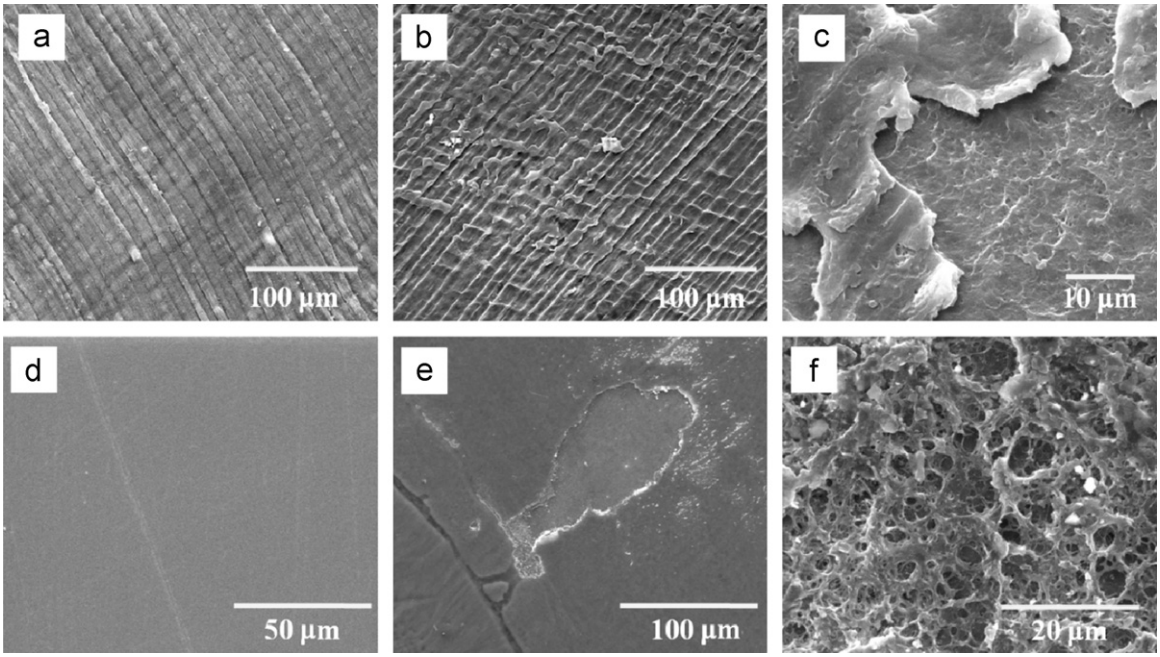

Fig. 2. (a) Periostracum of *Perna viridis*, (b) interfacial cement layer replicating the features of periostracum, (c) cohesive-failed region of cement detached from periostracum, (d) pristine surface of PMMA, (e) adhesive-failed interfacial cement layer replicating PMMA surface and (f) sponge-like cement on base-plate detached from PMMA.

and depressions of the periostracum of the green mussel shell (Fig. 2b) and the smooth featureless topography of PMMA are replicated by the interfacial cement layer (Fig. 2e). Some of the previous reports show that the barnacles (*Chthamolus fragilis*) present on the leaf surfaces of the plant, *Spartina alternifora* carefully replicated the epithelial cells and the other features present on the surface [12]. A much earlier report on the growth of barnacle specie, *Balanus eburneus*, on phonographic records shows that the barnacle growth was mostly oriented along the direction of the grooves of the record [13]. The base-plate of the barnacles was grooved in similar morphology of the grooves on the record. On the smooth side of the record it was found that the barnacles grew in random directions and no patterns were observed among different shell parts. From the observations of the present study, it can be suggested that the liquid cement secreted by the barnacles initially wets the surface and completely fills the surface contours and fissures of both the base-plate as well as the substrate. Once solidified, this can ensure rigid mechanical interlock with the surfaces [14]. The solidified cement appears layered in its structure which is evident from Fig. 2c. Sun et al. [15] proposed from AFM indentation experiments that barnacle cement exists in layers and it is the outermost softest cement layer that governs the shear strength of adhesion. This was followed by reports on visual evidences of the presence of sheet-like adhesive layer with underlying cross-links [2,11].

The cohesive-failed region suggests the differences in the microstructure of the bulk cement as a function of the supporting substrate material. Morphological cross-linking is more in the cement detached from PMMA when compared to the relatively denser cement detached from *P. viridis*. The porous nature of the cement from the former is relatively absent in the latter. This suggests the curing of the cement secreted on the periostracum is poorer in comparison to the cement secreted on metals where fibrous or globular microstructures are well-developed [2,11] (Fig. 2c). The absence of any cross-linked structures can also be the reason behind the ease in peeling the barnacle off from the periostracum. Even though the contact angle measurements suggest that periostracum is more hydrophilic than the PMMA surface, the attachment is stronger on the latter. Apart from wettability, there are other factors reported which can likely influence the adhesion. For example, the microtopography of the periostracum of mussel shells has shown to be capable of exhibiting foul-resistance as well as foul release properties [6]. In addition, the diethyl ether fraction extracted from periostracum has also shown to inhibit the attachment of barnacle larvae [16]. Other factors including modulus can also influence the strength of barnacle adhesion. The elastic modulus of periostracum of *Mytilus galloprovincialis* has been reported to be 100 MPa [17] whereas that of PMMA is typically 3.7 GPa [18] which could have possibly influenced the interfacial strength.

SDS-PAGE analysis indicates the existence of polypeptides of different molecular weight in the barnacle cement. The polypeptides of the cement from the specie, *Megabalanus rosa*, have been identified by Kamino and his colleagues and the functional roles of six proteins have been recognized. The protein cp-19 kD, which is a minor component of the cement is found to have functional roles in binding with the surface. Another minor polypeptide, cp-20 kD showed no post-translational modifications and found to be calcite-specific in its adsorption behavior [19]. Two major proteins, 100 kD and 52 kD were identified to exhibit bulk functions [10]. Homologous cDNA of some of the proteins have been discovered in several other genera of acorn barnacles [1]. The cement of *A. reticulatus* also exhibits presence of polypeptides of similar molecular masses as present in *M. rosa*. However, the functional roles of these polypeptides are yet to be identified. The predominant bands at ~100 kD, 80 kD and ~20 kD suggests that these have major roles in the adhesion process. The layered nature of the cement and the interfacial cement layer replicating the substrate features suggests that each layer has a different functional role. It is not clear, at this point, if just one polypeptide or combinations of several polypeptides are involved in the formation of each layer.

To summarize, the replication of the surface features of a biological material such as the green mussel shell and a synthetic surface such as PMMA by the barnacle cement is investigated in this study. The existence of barnacle cement as layers and their

significances are discussed. The differences in the elemental composition and the polypeptide profiles are also compared. Thus, the present study brings out the need for understanding the biochemical and functional aspects of the cement proteins in conjunction with the interfacial morphology to resolve the curing process of barnacle cement happening underwater.

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