Understanding the structure of the adhesive plaque of *Amphibalanus reticulatus*

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The barnacle, *Amphibalanus reticulatus*, is a common fouler in the Indian marine waters and is found to attach to a wide variety of natural and man-made surfaces. The shells of the barnacles remain attached to the substrate irrespective of whether the barnacle is alive or dead and details of dried shells are relatively less explored. The dried adhesive plaque of the barnacles attached to polymethylmethacrylate (PMMA) substrates were isolated and subjected to several structural characterization studies like X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and atomic force microscopy (AFM). The results report the presence of calcite nano-crystallites and amide II groups corresponding to the adhesive protein. The characteristic concentric ring pattern of barnacle base-plate structure, under higher magnification using SEM, appears to be formed of alternate calcite bricks and cement duct openings with an increasing separation distance between adjacent rings. The shear strength studies of barnacles of varying size indicate a direct correspondence to the base-plate diameter.

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1. Introduction

The adhesion and colonization of marine creatures, especially barnacles, onto structures submerged in shallow waters of the ocean initialize biofouling and it continues to be a long standing problem for the naval industry [1–6]. The hard, calcified shells of the barnacles not only do reduce the efficiency of the ships, but also is highly difficult to be scraped away. Dry docking followed by scraping and sand blasting are the currently employed methods for cleaning the vessel hulls [7].

Barnacle attachment begins with a non-feeding larval stage called as the cyprid larva which undergoes a temporary settlement by the secretion of proteinaceous adhesive [8,9]. Later it undergoes metamorphosis to develop into an adult which is protected by calcareous parietal and basal shells. Simultaneously the permanent settlement occurs by the secretion of water insoluble adhesive called the barnacle cement which can cure inside water [10]. The body of the barnacle is in contact with the inner walls of the shell and the epithelial cells present on the surface of the body deposit both the shell and the base-plate minerals which are analyzed to be composed of calcite, a polymorph of calcium carbonate, Ca\(^2+\) and HCO\(_3^-\) ions, which are needed for the precipitation of calcite crystals, along with the organic compounds that are needed for binding the crystals are secreted by these epithelial shells during metamorphosis. Thus the base-plate is composed of more than 90% calcite and 1–5% of organic matter by mass. At the time of molting, a part of the exoskeleton is demineralized and shed and new stable and thick mineralized walls are built completely surrounding the barnacle [11]. The biomineralized basal region is cemented firmly to the substrate by the adhesive called barnacle cement.

Studies on barnacle shell can lead to biomimetic materials which can be used for several biomedical applications including bone implants. Due to the higher porosity of calcite crystals, they could favor better bonding with bone and hence can be an alternative to nacre which has aragonite crystals of higher density [5]. Barnacle adhesives can also be considered as potential candidates for dental filling application as they are secreted and cured inside water and they can adapt their structure according to the substrate of attachment [12].

Various characterization studies to understand the fundamental adhesion mechanism of live barnacles have been performed by many groups [8,12–19]. However, given the complexities involved in understanding the adhesion issues with respect to live barnacles in concomitant with the interdisciplinary nature of the problem, the present study is restricted to understanding the structure of the basal regions (adhesive plaque, which includes both the base-plate and cement) of the dead barnacles attached to a non-metallic substrate to start with. Such a study would also give insights on the material that adheres to a hard surface so strongly, even after the creature is dead. The results from the extensive structural characterization of the adhesive plaque are correlated with the existing knowledge on the cementing

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mechanism to provide a comprehensive understanding of the distribution of duct networks, microstructure of concentric rings of the plaque and chemical imprint left on the substrate after the detachment of the barnacle which have not been carried out before to the best of our knowledge.

For the present investigation only the shells and basal region of the dead barnacles attached to medium surface energy material such as polymethylmethacrylate substrate are chosen.

2. Experimental

PMMA coupons of size 10 cm × 15 cm were immersed in marine water in the Bay of Bengal (Chennai port, India) for a period of 11 months. Among the different attached species of barnacles, Amphibalanus reticulatus was chosen for the investigation as it is the dominant species found in Indian marine waters. The coupons were collected from the ocean after 11 months, subsequently allowed to dry up and the dead barnacles were removed. The shells and the basal plates along with the barnacle secreted cement (which has been cured in water) were remaining on the coupons, which are of research interest. The barnacles exhibited a moderately strong adhesion to the PMMA substrates. Any attempt to dislodge the barnacles resulted in the crushing of the shell along with the basal plate. Hence it was possible to collect the basal plate of the barnacle along with the cement by crushing the shell and carefully removing the shell from the basal plate. The crushed basal region was used for X-ray diffraction (XRD) studies. In other cases, when the fouled substrates were cut, a few barnacles got detached due to the mechanical vibration of cutting. The basal parts of those shells were also studied with scanning electron microscopy (SEM) and atomic force microscopy (AFM).

The surface energy of PMMA substrate before exposure was measured as 47.09 mN/m² using Owen–Wendt–Rable–Kaelble method on the basis of static contact angles. The liquids used were de-ionized water, formamide and di-iodomethane.

2.1. X-ray diffraction

The composition of the adhesive plaque thus collected was determined using XRD using the X-ray diffractometer, DB Discover, Bruker AXS, (Madison, USA), with Cu–Kα radiation (λ = 0.15406 nm). To check the consistency of the composition, a single barnacle was mounted upside down using clay without removing its shell and the diffraction studies were performed in the scan range from 10° to 90°.

2.2. Tapping mode atomic force microscopy

The detached surfaces were hard, dry and smooth enough to carry out tapping mode AFM imaging. The instrument used was Digital Instruments scanning probe microscope fitted with NanoScope IV controller and Dimension 3100 controller (Santa Barbara, California, USA). The phase imaging was performed using phosphorous doped silicon tips within the scan size 1 μm × 1 μm. Isolated scan lines were erased and the image quality was improved using flattening process thereby subtracting the background. The spring constant of the cantilever is 40 N/m.

2.3. Scanning electron microscopy

Those barnacles which detached due to mechanical vibration during cutting were chosen for SEM. The microstructure of the detached basal region (top region as well as bottom region of the base-plate) and the substrate, both in the secondary electron mode and backscattering mode, was studied using FEI Quanta 200 (USA) scanning electron microscope fitted with lithium doped silicon energy dispersive X-ray spectrometer (EDS) of AMETEK Process and Analytical Instruments.

2.4. Fourier transform infrared spectroscopy

The barnacles were carefully dislodged first from the substrate and the adhesive plaque was removed with a scalpel and washed in de-ionized water. This was placed in a vacuum oven at 80 °C for 12 h, followed by cooling it in liquid nitrogen and pulverization. Pellet of the powder was made with KBr and Fourier transform infrared spectroscopy (FTIR) spectra were measured using a Perkin Elmer (Spectrum one, USA) spectrometer at a resolution of 4 cm⁻¹ and in the frequency range of 4000–400 cm⁻¹.

2.5. Barnacle adhesion strength tests

Barnacle adhesion shear strength tester was constructed as per ASTM Standards D 5618 – 94 (Reapproved 2000): ‘Standard Test Method for Measurement of Barnacle Adhesion Strength in Shear’.

Barnacles having base-plate diameter between 4 and 11 mm attached to PMMA substrates were selected for testing and those barnacles which were not in direct contact with the other barnacles were subjected to shear force studies. A shear force was applied to the barnacle base at a rate of approximately 4.5 N/s until the barnacle got detached. Care was taken to apply the force parallel to the surface. The force required for detaching individual barnacles was noted and compared.

3. Results

The PMMA coupon fouled by barnacles considered for the present study is shown in Fig. 1a and the top and bottom views of one of the adult barnacles considered for microscopic observations are exemplified in Fig. 1b and c. In the following sections, results obtained on the barnacles using several characterization techniques are presented which provide a comprehensive understanding of the structural features of the adhesive plaque. The final section provides a quantitative statistical data on the adhesion strength of the barnacles attached to the substrate.

3.1. X-ray diffraction

X-ray diffractogram of the basal region is shown in Fig. 2. The XRD peaks obtained are quite sharp indicating that the pulverized cement along the detached basal region of barnacles composed of crystalline phases. The resultant peaks were subjected to background subtraction and the peaks were indexed. The peaks indicate the presence of rhombohedral calcite (CaCO₃) crystals and an amorphous hump below 15°. It should be noted that the XRD results correspond to both barnacle cement and the base-plate. The presence of calcite is also observed for single adult barnacle indicating that the parietal shells are also composed of the same. The strongest line (104) was selected and the full width half maximum (FWHM) was evaluated after obtaining a Pseudo–Voigt profile function fit. The average crystallite size obtained is around 40 nm. From the broadening of the peak, the lattice strain is also calculated to be nearly 0.53%.

3.2. Tapping mode atomic force microscopy

The tapping mode AFM phase image is shown in Fig. 3. AFM imaging was carried out at various regions of the cement-free base-plate to observe the distribution and size of the calcite crystals. In order to obtain a cement-free base-plate, the adhesive plaque was wiped with acetone to ensure that the imaging is performed only on the base-plate. From Fig. 3, it is observed that the distribution and size of the nano-calcite crystals remain the same throughout the base-plate. When observed in the scan range 1 μm × 1 μm, it is observed that the base-plate is made of crystals with a wide range of size distribution from 20 nm till 250 nm.
3.3. Scanning electron microscopy

Scanning electron microscopic observations of the detached basal surfaces and substrates are shown from Fig. 4a–h. Adult barnacles were detached carefully without damaging their base-plates from PMMA substrate and were wiped using ethanol to remove any dirt. The specimens appear dense at the center point of secretion of cement, translucent away from the center and denser again at the edges. When viewed with naked eye, concentric ring patterns are observed originating from the center throughout the basal plate and streaks are observed radial in all directions. Before observation the samples were sputter-coated with gold-palladium about 5 nm to prevent charging.

SEM observation of the surfaces of the barnacles base-plate from bottom side appears very smooth without indicating much features in secondary electron (SE) mode at a lower magnification (the SE images are not indicated here). But in back scattered electron mode (BSE) concentric ring patterns are clearly visible starting from a particular point which is considered to be the beginning of permanent adhesion where the cyprid had undergone metamorphosis (Fig. 4a and b). When the rings were observed at a higher magnification it was understood that they are not continuous but instead made of brick-like structures separated by uniformly shaped cracks as clearly observed in Fig. 4c. The notable feature is that the width of each brick is same in every ring.

The top view of the barnacle base-plate as viewed under SEM (refer Fig. 1b for top view of the barnacle; here, only the top view of base-plate is considered) is indicated in Fig. 4d and e. Branching structures which divide and interconnect with the neighboring branches are seen continuously throughout the base-plate. These structures also appear to

![Fig. 1. PMMA coupon fouled by barnacles chosen for the present study is indicated in (a); The top view and bottom view of an adult barnacle is shown in (b) and (c) respectively.](image1)

![Fig. 2. XRD pattern for the powdered cement and base-plate. The indexed peaks correspond to that of crystalline calcite.](image2)

![Fig. 3. Tapping mode AFM phase image of the base-plate at 1 µm x 1 µm, indicating the nano-calcite crystals.](image3)
Fig. 4. Surface topography of barnacle base-plate; (a) BSE image indicating an overall view indicating concentric ring patterns; (b) BSE image showing the alternate arrangement of calcite bricks and duct ends forming the rings. It also shows the uniformity in thickness of each brick; (c) Magnified BSE images of calcite brick and duct end; (d) and (e) The BSE images of the top view of base-plate. Here the complex cementing channels are visible. (f) Phase contrast on the base-plate due to the cement; (g) Corresponding SE image of panel (f) where the cement is appearing as a thin layer over the base-plate; (h) SE image of the chemical imprint of the adhesive left on the substrate.
form concentric rings similar to that present at the bottom region. The separation distance between consecutive rings was measured both from the bottom side and top side and is found to be the same. The distance between adjacent rings is found to be increasing when observed from the center to the periphery. For example, in Fig. 4b the separation distance between the first ring and the second ring is 122 µm, the second and third is 133 µm and the third and fourth ring is 147 µm.

Phase contrast is observed in BSE images of the barnacle-base detached from PMMA (Fig. 4f). Small-area EDS was done at the regions of phase contrast to identify the difference in composition. The areas where EDS were done are labeled in Fig. 4f. Considerable variation in the weight percentage of carbon, nitrogen, calcium and oxygen is observed at the brighter and darker regions.

3.4. Fourier transform infrared spectroscopy

The spectral characterization of the adhesive plaque (base-plate) of barnacles attached to PMMA substrates is shown in Fig. 5. The presence of carbonate ion is seen as a peak at 1421 cm\(^{-1}\) of the spectrum which matches with the findings of Berglin and Gatenholm [13]. The characteristic peaks of calcite are observed at 876 and 713 cm\(^{-1}\) as reported previously by the same authors. This method is not purely an interfacial analysis, since more of the bulk determination is from the adhesive plaque. Care was taken during sample preparation so that the carbonate peak might not originate from the parietal shells which are also composed of calcite [20]. The spectrum of barnacle base-plate on PMMA also reveals the presence of carboxylic acids stretch at 3435 cm\(^{-1}\) and amine stretch at 2514 cm\(^{-1}\).

3.5. Barnacle adhesion strength tests

The adhesion strength of the barnacles attached to the PMMA substrate as a function of the size of the barnacles is exemplified in Fig. 7. The adhesion strength seems to increase with the size of the barnacles with an average starting value of around 3 kPa for smaller barnacles and reaching a maximum for barnacles having an average basal plate diameter of 8–9 mm. The average maximum adhesion shear strength is found to be around 6 kPa. However, a decreasing trend is seen henceforth with shear strength values of around 3 kPa for largest size of barnacles tested (i.e., with base-plate diameter of around 10–11 mm).

4. Discussion

4.1. Biomineralization

The presence of calcite as crystalline form for the basal shell was reported by Otness and Medcalf [21]. Regarding the biomineralization of solidified cement they put forward a hypothesis suggesting that the precipitation of calcium carbonate occurred by the reaction of calcium ions with the anionic groups present in the organic matrix. Apatite crystals [\(\text{Ca}_{10} \left(\text{PO}_4\right)_6 \left(\text{OH}\right)_2\)] were considered as the sources of calcium ions necessary for this reaction [21]. Hence as per this hypothesis the base-plate is a composite material where calcite crystals are embedded in an organic matrix. The amorphous hump in the X-ray diffractogram that is observed below 15° can correspond to either the cement, or the organic matrix of the base-plate or both, as the detached basal region has traces of cement left which is evident from SEM images.

Weigner and Dove [22] suggested that having formed under controlled conditions, the biomineralized crystals have properties such as texture, crystallinity, size, lattice strain and shape in addition to the presence of organic matter. The lattice strain as well as size is determined from XRD and AFM. The presence of texture also is evident from the XRD. The comparative higher intensity reflections from (006), (0012) and (1112) planes and the lower intensity reflections from (110), (113) and (202) planes in contradiction to the standard data obtained from JCPDS-
ICD data indicates the presence of texture in the biomineralized calcite phase. Since AFM imaging was done on the cement-free base-plate, the individual calcite crystals are observed clearly at a lower scan size. The phase imaging mode helped in distinguishing clearly the calcite crystals and the organic phase holding these crystals.

The size of calcite crystals obtained from XRD could be probably due to the collective effect of all crystals from several base-plates whereas the actual distribution of calcite crystals of various sizes can be observed in the AFM image. Thus AFM image gives a direct visual evidence for the size of calcite crystals which is in correspondence with XRD results.

4.2. Cementing mechanism

It has been reported that during its growth the barnacle base-plate enlarges in a horizontal direction whereas the parietal plate grows in a downward direction [12]. Since the cement cells are the modified epidermal cells of the barnacle, the secretion of cement is linked to its molting cycle. The secretion takes place between consecutive molting at the periphery of the base-plate and the low viscous cement flows and spreads itself to fill any gap between the base-plate and the substrate. When the base-plate grows further, new active cement glands are formed again at the periphery while the old ones stop secreting and they lay embedded in the hardened cement still carrying the non-solidified liquid adhesive inside them. Thus the cured cement appears are discrete concentric rings throughout the base-plate [10].

The alternative brick-like structures and hollow regions observed in SEM can be correlated to the above cementing mechanism and growth of barnacles. As the new cement glands are formed at the periphery of the base-plate during each molting cycle, production of brick-like calcite structures occurs so as to support the cement ducts and also to ensure the continuity of the base-plate as it grows. Also it has been shown that the cement duct-ends get exposed out when the barnacle is detached from the substrate and flushing cement will come out for reattachment through these duct-ends [10,23,24]. Hence it can be inferred that the hollow regions seen at the edges of each brick is the opening of the cement ducts. At the very left edge of each brick and duct opening, a peculiar microstructure is observed as if the fluid cement had flown in a direction towards the centre and appeared to be solidified during the flowing process (see Fig. 4c).

The uniformly spread adhesive imprint on the substrate is another interesting feature observed. At some region the adhesive appears to be torn which would have happened during detachment of the shell from substrate. The torn adhesive left on the substrate gives rise to phase contrast on the base-plate. In the secondary electron images the topography of the layer of adhesive sticking onto the base-plate is clearly visible (Fig. 4g). The regions appear darker in BSE image (Fig. 4f) due to the absence of any heavy elements in it. Since the adhesive has been reported as a multi-protein complex, the evolution of nitrogen and carbon peaks in EDS spectrum can be related to the same [19]. The base-plate appears brighter than the adhesive due to the dominance of calcium and oxygen which again is in agreement with the XRD results indicating the presence of calcium carbonate.

The complex cement duct network arrangement throughout the base-plate and its re-secretion process after detachment was studied by spectral characterization. The presence of amide II bands in the infrared spectrum [28] reveals the presence of amide II group. The presence of amide II bands is the major band of the protein infrared spectrum [28]. Parker reported that the primary amine and secondary amine stretching regions were expected to be in the range of 3435 cm$^{-1}$ and 713 cm$^{-1}$ of calcite [13]. The other spectral peaks of barnacle base-plate observed at 3435 cm$^{-1}$, 1144 cm$^{-1}$, 2923 cm$^{-1}$ and 2914 cm$^{-1}$ corresponds to the N–H, C–N, C–O and O–H bonds respectively in amide II group. The absence of characteristic band at 1740 cm$^{-1}$ and 1380 cm$^{-1}$ for ester carbonyl group and methyl group respectively in the spectrum confirms the absence of PMMA substrate contamination in the adhesive plaque. Based on the spectral peak for calcite observed with the amide peaks, it is revealed that traces of protein from the adhesive are still left out even after the detachment from the substrate. It has been reported that this underwater adhesive is a multi-protein complex and the underwater attachment is a multifunctional process suggesting that each component has some responsibility for each one of the functions [27]. Hence adhesive protein further has to be analyzed for determining each component of the protein complex and to determine the specific role of each protein of barnacle adhesive cement in attachment with the substrates.

4.4. Adhesion strength

While the increase in adhesion strength with increase in size of the barnacles is understood based on the increase in area of adhesion with the substrate, the reasons for the decrease in strength for bigger barnacles henceforth are not clear. It is obvious that the adhesion strength is a function of the kind of molecular bonding the adhesive can have with the substrate material and hence the values of the interfacial strength depend on strength of these bonds. However, a more rigorous study is required to understand the molecular mechanisms of adhesion coupled with precise quantification of the interfacial strength, which has not been considered for the present study (Fig. 7).

5. Conclusion

Extensive structural characterization of the adhesive plaque (the base-plate of the barnacle, A. reticulatus, along with the cement) by
XRD, FTIR, and SEM has indicated the existence of mainly calcite crystals, a polymorph of calcium carbonate. FTIR studies also indicate the presence of amide II groups, which correspond to the presence of protein. SEM studies of the base-plate reveals that the concentric ring patterns, related to the growth of barnacles, are made of alternate brick-like structures of uniform width separated by cement duct-endings. Small-area EDS at various positions in the base-plate shows the major components to be calcium, carbon and oxygen revealing perfect agreement in the results obtained by all characterization techniques. At some regions relatively larger amount of carbon and nitrogen is seen indicating the presence of cement giving rise to phase contrast. The phase image of tapping mode AFM studies suggests that the base-plate consists of nano-calcite crystals of a wide range of size. The adhesion strength of the barnacles attached varies from 3–6kPa as a function of its base-plate diameter.

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