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

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ABSTRACT

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.

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 scrapping 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 pate. 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/m^2 using Owen–Wendt–Rable–Kaeble 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, D8 Discover, Bruker AXS, (Madison, USA,) with Cu–K_α radiation ($\lambda = 0.15406$ 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 ($CaCO_3$) 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.