

## Barnacle cement nanostructure imaged under physiological conditions

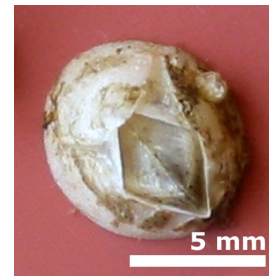
Biofouling caused by the growth of crenate barnacles (*Balanus crenatus*) on ships, off-shore platforms, etc. is an important expense factor for the naval industry. Barnacle cement is also interesting since it cures under water, without the need for air drying, and is able to bind to materials with a wide range of mechanical properties or textures. The cement is mainly composed of proteins (about 70 wt%), and is extruded from pores under the base plate of the barnacle so that it flows into any gaps between the base plate and the surface and cures within a few hours [1].

The adhesion of barnacles to most hard surfaces is very strong, (approx.  $9.3 \times 10^5 \text{ N/m}^2$  [1]), and frequently the forces needed to detach them are larger than the mechanical strength of the barnacle shell, so that they can not be removed without crushing them. On certain softer elastic surfaces, it is much easier to detach the barnacles. Poly(dimethylsiloxane) (PDMS), is of interest as an antibiofouling coating, since although the barnacles are able to grow on it, they are not able to bind to it as strongly as to hard surfaces due to its low surface energy (20-25 mN/m) [2]. This allows the PDMS to be used as a fouling-release coating.

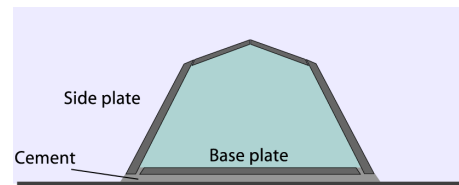
Recent scanning electron microscopy (SEM) investigations on cement from *Balanus improvisus* [3,4] have suggested that the nano-scale structure depends on the surface it is attached to. The cement from barnacles growing on a PDMS substrate appeared to be formed as a loose, fibrillar network, in contrast to the cement from barnacles grown on Al foil (as a model of a surface that the barnacles easily attach to and form strong adhesion), which formed a much more smooth, dense layer of round globular structures, around 50-200 nm in size. The properties of the barnacle cement may adapt to the surface the barnacle is grown on. For SEM imaging in vacuum, however, the cement must be fixed, dehydrated and coated with a conductive layer such as gold.

The atomic force microscope (AFM) is capable of high resolution imaging in liquid [5] without the need for fixing or drying, since images are formed by scanning a sharp tip over the sample to build up a true three-dimensional map of the surface. The AFM has been used here to image the nano-scale structure of the cement from intact *Balanus crenatus* base plates under physiological conditions.

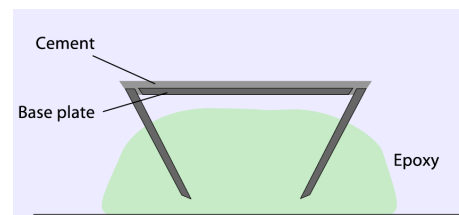
a) Barnacle growing on PDMS support



b) Schematic of barnacle shell structure



c) Schematic of barnacle mounted for imaging



**Fig 1.** Barnacle preparation for imaging. The picture in a) shows the living barnacle growing on the PDMS support. The schematic diagrams in b) and c) show how the top plates and the barnacle animal were removed, and the side and base plates mounted in epoxy. The cement surface of the base plate was kept covered with water throughout the procedure, and the entire sample was immersed for imaging.

## Materials and methods

Barnacles were grown on PDMS test plates immersed in the North Sea (Meldorf bay, Schleswig-Holstein, Germany) from July to October. After collection, the barnacles were kept alive in aerated seawater and fed with *Artemia salina* nauplii. Some barnacles were detached by peeling away the flexible PDMS support and allowed to settle on aluminium foil for around 24 hours. Foil was used since the thin layer could be peeled off the barnacle after attachment without crushing the barnacle shell. Only living, well-settled (difficult to remove) adult barnacles greater than 5 mm in diameter were used in this study.

Barnacles were prepared just before imaging as “fresh dead” samples. The scutum and tergum plates were removed and the animal pulled out using tweezers. The rest of the barnacle shell was left intact (including base and side plates) to prevent breakage of the base plate. The sample preparation is shown schematically in Figure 1. All the experiments were conducted in salinity-matched artificial seawater (Tropic Marine, salinity 26.7 ‰).

The side plates were dried gently, and the sample immediately mounted with the base plate facing upwards on a glass slide using epoxy resin (left to part-cure for 5 minutes before use). This allowed the sample to be mounted stably, while keeping the base plate immersed in a droplet of artificial seawater. After a few minutes, when the epoxy had fully cured, the whole sample was immersed in the same solution for AFM imaging.

AFM imaging was carried out in intermittent contact mode in liquid using a NanoWizard<sup>®</sup> AFM (JPK Instruments, Germany) mounted on an inverted optical microscope (Axiovert 200, Zeiss, Germany). All imaging was performed under the artificial seawater solution using triangular silicon nitride cantilevers with a nominal cantilever spring constant of 0.3 N/m. Oscillation amplitudes of around 15-20 nm were used because the barnacle cement was so sticky.

Several areas on each barnacle were scanned, concentrating on the region near the edge of the barnacle, where the most recent cement should be located. This

was important for the barnacle settled on aluminium foil, where the recent cement should show any differences from the change of attachment substrate. The images were processed only by a first-order planefit to remove the overall sample tilt and erasing isolated scan lines where tip-sample adhesion caused streaking. No line-by-line fitting was performed, so that the texture of the surface retained an accurate representation of the sample texture.

## Imaging of the barnacle cement

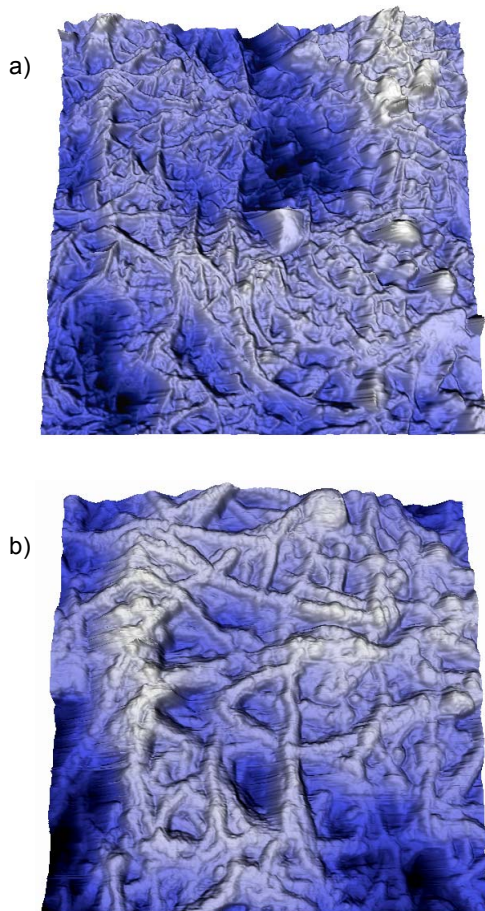
Typical AFM images of the barnacle cement mounted as described above on epoxy and imaged under artificial seawater using intermittent contact mode are shown in Figures 2 and 3. This topography information is displayed here as a 3-D view projection, to best show the surface texture.

### *Barnacles on PDMS*

Figure 2 shows representative images from the cement of barnacles grown on the PDMS surface. The scan areas are 4 x 4 microns in (a) and 1.5 x 1.5 microns in (b). The scale bar is 1 micron for both images. The total height ranges are 409 nm (a) and 256 nm (d).

The texture of the cement surface from the barnacles grown on PDMS in Figure 2 is a tangled mat of fibres. These fibres form a loose meshwork, so that the surface is quite rough compared with the 50-100 nm diameter of the fibres (see also Figure 4). The fibres appear to be made up from generally globular structures, although the details of the substructure are not clear. The cement surface was sticky, and force spectroscopy using the standard silicon nitride tip showed strong adhesion, with some events pulling out parts of the surface over a range of microns (see also Figure 5).

The forces during imaging were much lower, so the tip did not interact so strongly with the surface, but the tip quickly became coated with a layer of material, so the effective tip size increased. Higher resolution images could possibly be obtained using cantilevers coated to reduce the adhesion.



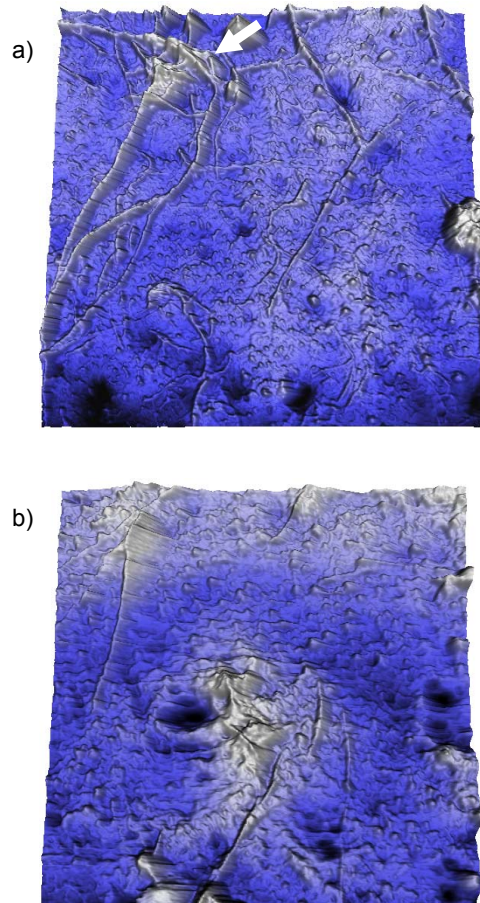
**Fig 2.** AFM topography images of barnacle cement from PDMS under liquid (height information displayed as a 3-D projection). The image areas are 4 x 4 microns in (a) and 1.5 x 1.5 microns in (b) (scale bar 1 micron for both). Total height ranges 409 nm (a) and 256 nm (d).

**Barnacles on aluminium**

Figure 3 shows representative images from the cement of barnacles grown on the aluminium surface. The scan areas are 4 x 4 microns in (a) and 1.5 x 1.5 microns in (b). The scale bar is 1 micron for both. The total height ranges are 231 nm in (a) and 105 nm in (b).

The cement from the barnacles allowed to settle on the aluminium surface showed a different texture from the PDMS surface. In Figure 3 some remnants of the larger fibre structures can still be seen (such as the example

marked with an arrowhead in (a), but now the surface is generally formed from a much smoother layer of globular features. The surface is very different from the open meshwork of the previous images, with the cement now filling most of the gaps.

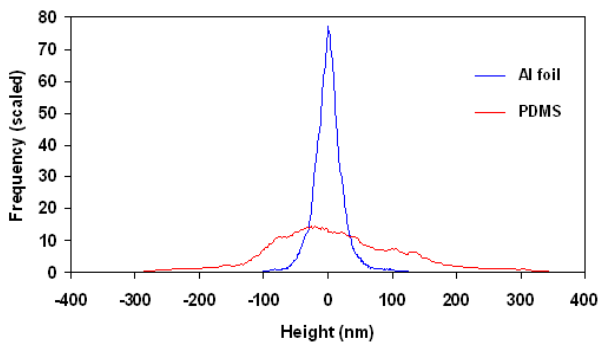


**Fig 3.** AFM topography images of barnacle cement from the aluminium surface. Image areas are 4 x 4 microns (a) and 1.5 x 1.5 microns (b) (scale bar 1 micron for both). Total height ranges 231 nm (a) and 105 nm (b). A small group of fibres is marked with an arrowhead in (a).

The height scales in the images in Figure 3 are generally lower than the similar size scan areas in Figure 2. The areas where there are large changes in height in Figure 3 appear more like isolated craters, rather than the general mesh seen in Figure 2. Similar features were seen in various scan areas around the edge of the barnacle.

**Height ranges**

Figure 4 shows graphically the difference between the height ranges of the structures. The height values for two typical images on Al and on PDMS are shown as a histogram. The images had the same scan size (5 x 5 microns) and number of points (512 x 512). The height range of the cement from the PDMS surface is much larger than on the Al foil surface.



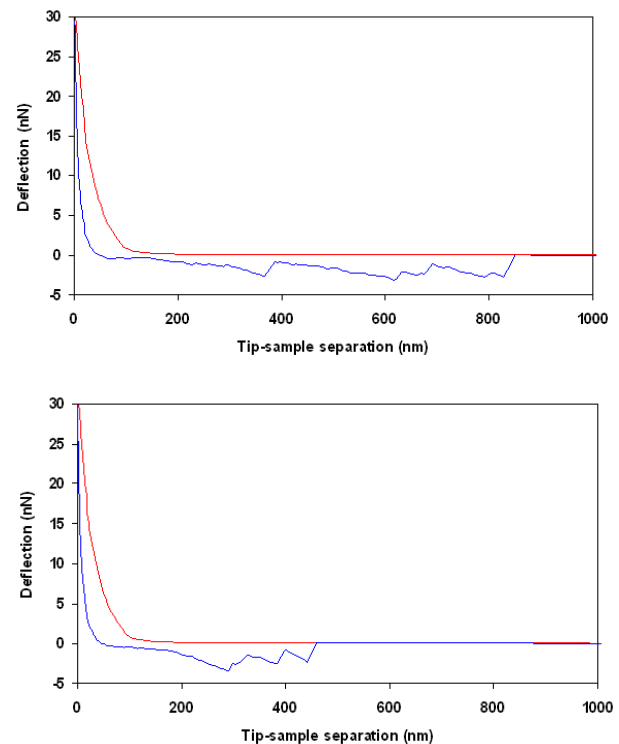
**Fig 4.** Histogram comparison of AFM height images on barnacles grown on the aluminium and the PDMS. Both images were 5 x 5 micron scans, with 512 x 512 points. The reduced topographic range on the barnacle cement from the Al foil is clear.

**Force-distance measurements**

The atomic force microscope is capable of direct force and interaction measurements, as well as sample imaging. In this case there are several interesting questions that can be explored with the atomic force microscope. One question is about the mechanical properties of the barnacle cement, and whether differences can be seen for barnacles grown on different substrates, as predicted by some models of barnacle attachment. Another question is the measurement of the actual adhesion of the barnacle cement against different materials.

Figure 5 shows examples of AFM force curves on the barnacle cement from the PDMS surface. Approach curves are shown in red, retract curves in blue. The viscoelastic response of the cement can be seen in the compression part of the curves, and long-range adhesion in the retract part of the curves. The piezo height has been converted to separation values using the sensitivity measured on glass.

The force curves were performed using a linearized z-piezo, so the hysteresis in the compression part of the curve comes directly from the sample, and is not an effect of the piezo material. No hysteresis was seen on the force curves on the hard glass surface for the sensitivity measurements.



**Fig 5.** Examples of AFM force curves on the barnacle cement from the PDMS surface. Approach curves are shown in red, retract curves in blue. The viscoelastic response of the cement can be seen in the compression part of the curves, and long-range adhesion in the retract part of the curves. The piezo height has been converted to separation values using the sensitivity measured on glass.

To fully investigate the properties on different surfaces, many curves must be collected and statistics analysis used to separate the interactions. The material properties (elasticity on compression) and the adhesion are both accessible to the AFM measurements, and further work will bring a full comparison of the cement produced by the barnacles on different surfaces.



## Conclusions

The images here are the first high resolution images of barnacle cement imaged under physiological conditions. The barnacle cement was imaged without fixation under artificial seawater on intact barnacle base plates.

Barnacles grown on PDMS substrates were compared with ones that had been allowed to settle on aluminium foil for 24 hours, where a much stronger bond is possible.

The cement of barnacles grown on PDMS consisted of a loose fibrillar network structure, with fibres formed from globular structures around 50 nm in diameter. This cement should be relatively fresh, since the barnacles were disturbed during transport over the previous 24 hours, from bending of the flexible PDMS test pieces. This would stimulate cement production around the edge of the base plate where these images were collected. For barnacles left to settle on aluminium foil overnight, the fresh cement appeared to have filled in most of this mesh structure, and the surface was much smoother. Only a few of the larger filaments could still be seen, and the structure was dominated by smaller globular features.

This supports the hypothesis that the barnacle cement adopts a different structure on the PDMS support compared with other, more conventional substrates. The fibres observed previously [3] have now been observed in the hydrated state, on a sample that had not been fixed or dried. The fibrous structure seen in electron microscopy images is not an artefact of the dehydration process, but actually forms in a liquid environment.

This work will be extended to studies of the barnacle cement nanostructure depending on substrate elasticity, microtopography, and free surface energy in its natural liquid environment. The AFM is also able to measure quantitative forces for both adhesion and compression, to fully characterise the properties of the cement. In-situ measurements of fresh cement production will extend understanding of the properties of this material and how the structure changes during curing.

## Acknowledgements

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## Literature

- [1] Yule, A.B. & Walker, G. "Adhesion in barnacles". In: Barnacle Biology (ed: Southward, A.J.), 389-402, Balkema, Rotterdam, The Netherlands (1987).
- [2] Berglin, M. & Gatenholm, P. "The nature of bioadhesive bonding between barnacles and fouling-release silicone coatings". J. Adhesion Sci. Technol. 13, 713-727 (1999).
- [3] Wiegemann, M. & Watermann, B. "Peculiarities of barnacle adhesive cured on non-stick surfaces" J. Adhesion Sci. Technol. 17, 1957-1977 (2003).
- [4] Dougherty, W.J. "SEM observations on the interfacial surface of the cement of the adult barnacle, attached to natural and synthetic adherends". Tissue and Cell 22(4), 463-470 (1990).
- [5] Hörber, J.K.H. & Miles, M.J. "Scanning probe evolution in biology" Science 302, 1002-1005 (2003).