Atomic force microscopy of the morphology and mechanical behaviour of barnacle cyprid footprint proteins at the nanoscale

In Yee Phang^{1,2}, Nick Aldred³, Xing Yi Ling⁴, Jurriaan Huskens⁴, Anthony S. Clare³ and G. Julius Vancso^{1,2,*}

¹*Department of Materials Science and Technology of Polymers, MESA⁺ Institute for Nanotechnology, PO Box 217, 7500 AE Enschede, The Netherlands*

²*Dutch Polymer Institute, PO Box 902, 5600 Eindhoven, The Netherlands*

³*School of Marine Science and Technology, Newcastle University, Newcastle upon Tyne NE1 7RU, UK*

⁴*Molecular Nanofabrication Group, MESA⁺ Institute for Nanotechnology, University of Twente, PO Box 217, 7500 Enschede, The Netherlands*

Barnacles are a major biofouler of man-made underwater structures. Prior to settlement, cypris larvae explore surfaces by reversible attachment effected by a ‘temporary adhesive’. During this exploratory behaviour, cyprids deposit proteinaceous ‘footprints’ of a putatively adhesive material. In this study, footprints deposited by *Balanus amphitrite* cyprids were probed by atomic force microscopy (AFM) in artificial sea water (ASW) on silane-modified glass surfaces. AFM images obtained in air yielded better resolution than in ASW and revealed the fibrillar nature of the secretion, suggesting that the deposits were composed of single proteinaceous nanofibrils, or bundles of fibrils. The force curves generated in pull-off force experiments in sea water consisted of regions of gradually increasing force, separated by sharp drops in extension force manifesting a characteristic saw-tooth appearance. Following the relaxation of fibrils stretched to high strains, force–distance curves in reverse stretching experiments could be described by the entropic elasticity model of a polymer chain. When subjected to relaxation exceeding 500 ms, extended footprint proteins refolded, and again showed saw-tooth unfolding peaks in subsequent force cycles. Observed rupture and hysteresis behaviour were explained by the ‘sacrificial bond’ model. Longer durations of relaxation (>5 s) allowed more sacrificial bond reformation and contributed to enhanced energy dissipation (higher toughness). The persistence length for the protein chains (L_P) was obtained. At high elongation, following repeated stretching up to increasing upper strain limits, footprint proteins detached at total stretched length of 10 μm .

Keywords: atomic force microscopy; barnacle cyprid footprint; force spectroscopy; bioadhesion; glycoprotein

1. INTRODUCTION

Anti-biofouling research has intensified the study of barnacle biology and is likely to continue to do so until an effective solution to this billion-dollar problem is discovered (Yebra *et al.* 2004; Aldred & Clare 2008). The deleterious effects of biofouling by micro-organisms and encrusting forms, such as barnacles, are well documented and an environmentally compatible solution is sought (Yebra *et al.* 2006; Aldred & Clare 2008). The role of barnacles in the process of marine biofouling has continually motivated research and in-depth investigation of these organisms (Christie & Dalley 1987; Anderson *et al.* 2003; Aldred & Clare 2008). In addition

to their importance in marine biofouling, barnacles have also received intermittent interest as inspiration for the development of biomimetic glues (Otness & Medcalf 1972; Kamino 2001). Indeed, adult barnacles can adhere tenaciously to most natural surfaces and serve as an interesting model for investigating permanent bioadhesion in an aqueous medium. Adult barnacle cement is now known to be composed of at least five subunits (Kamino & Shizuri 1998; Kamino *et al.* 2000; Kamino 2001, 2006; Mori *et al.* 2007) and undergoes a complex non-covalent curing process. Only recently has a functioning synthetic mimic of one of the peptides been developed (Nakano *et al.* 2007).

In contrast, the cypris larvae of barnacles, which explore and colonize immersed surfaces, have received

*Author for correspondence (g.j.vancso@utwente.nl).

Figure 1. Micrograph of a barnacle, *Balanus amphitrite*, (a) cypris, (b) antennule of a cypris, (c) microstructure of the antennular attachment disc, and (d) a deposited footprint.

very little attention with regard to their adhesion mechanisms, despite their key role in the process of biofouling (Aldred & Clare 2008). Cyprids are particularly interesting owing to their unusual ability to engage in surface exploration through rapid, reversible temporary adhesion (Crisp *et al.* 1985; Lagersson & Hoeg 2002). Cyprids (figure 1a) are highly discriminating in their choice of settlement site (Yule & Walker 1987), which they explore, prior to settlement, using a pair of specialized antennules (figure 1b; Lagersson & Hoeg 2002). The antennules are terminated at the third segment with adhesion organs (figure 1c) covered with micro- to nanoscale cuticular villi, reminiscent of the pulvilli of flies (Niederregger *et al.* 2002). During surface exploration, cyprids use these structures to attach temporarily and walk across surfaces in a 'bipedal' fashion (Walker & Yule 1984). It has been suggested that this temporary adhesion is facilitated, in part at least (Walker & Yule 1984; Clare *et al.* 1994), by a proteinaceous secretion present on the antennular disc, putatively derived from hypodermal glands in the second antennular segment (Nott & Foster 1969). Depending on the surface being explored, small volumes of this proteinaceous material may be deposited by the cypris as a trail of footprints. The proteinaceous material deposited by cyprids onto surfaces will, hereafter, be referred to as footprint. The size and morphology of deposited footprints have been shown to vary depending on the wettability of the surface being explored (Phang *et al.* 2008, 2009). In addition to its presumed role in adhesion, the footprint secretion also functions as a settlement cue for subsequently exploring larvae (figure 1d; Matsumura *et al.* 1998; Dreanno *et al.* 2006a,b).

Using atomic force microscopy (AFM), Phang *et al.* (2008) studied the macro- and micro-morphology of footprints deposited by *Semibalanus balanoides*. The footprint deposits of *S. balanoides* were composed of bundles of proteinaceous nanofibrils with heights varying between 7 and 150 nm. Here, we report on the fine morphology and nanomechanical behaviour of footprint protein deposits from *Balanus amphitrite* with a view to better understanding the role of the footprint material in adhesion.

AFM is well suited to imaging the surface morphology of soft matter across the nanometres to hundreds of micrometres length scale and is, therefore, ideally suited to the investigation of cypris footprint material (Drake *et al.* 1989; Engel & Muller 2000). In addition,

Scheme 1. The pickup of footprint molecules with interconnected sacrificial bonds (represented by crosses) by an AFM tip.

AFM allows the micro- and nanoscale mechanical properties of macromolecules to be investigated *in situ* (Drake *et al.* 1989; Mitsui *et al.* 1996; Ikai *et al.* 1997; Rief *et al.* 1997a,b; Oberhauser *et al.* 1998; Carrion-Vazquez *et al.* 2000; Engel & Muller 2000; Horber & Miles 2003; Zhang & Zhang 2003; Arce *et al.* 2004; Kellermayer 2005; Phang *et al.* 2007; Aldred *et al.* 2008). Previously, AFM has been used to study the adhesion of low-motility organisms such as bacteria (Razatos *et al.* 1998), diatoms (Higgins *et al.* 2003; Dugdale *et al.* 2005, 2006a,b) and algae (Callow *et al.* 2000; Mostaert *et al.* 2006; Mostaert & Jarvis 2007); however, barnacle adhesives (especially those of the cypris) are less conducive to AFM, being heterogeneous and difficult to locate on the surface (Sun *et al.* 2004; Phang *et al.* 2006). The advantage of AFM force spectroscopy (scheme 1) is that it allows the study of nanomechanical properties, such as strength, elasticity and toughness of biomacromolecules, at the single-chain level (Bustamante *et al.* 2000; Marszalek *et al.* 2001; Best *et al.* 2003; Beyer & Clausen-Schaumann

2005). In most natural materials, especially natural bioadhesives, so-called sacrificial bonds can prevent the breakdown of the primary structure of the molecule under an applied stress. Sacrificial bonds are those linkages that fail by design, before severe damage is done to the main structure (usually the backbone of the biomacromolecule; Fantner *et al.* 2006). When a force is applied to bioadhesives, sacrificial bonds break and energy becomes dissipated by chain fracture. The hidden chain length that is protected by the sacrificial bond unravels under these circumstances. Incorporation of sacrificial bonds into bioadhesives creates a very tough system capable of dissipating large amounts of energy. Consequently, many naturally occurring bioadhesives are unusually tough by synthetic adhesive standards (Groshong 2007). In fact, sacrificial bonds are abundant in nature and can be found in many biomaterials such as bone (Smith *et al.* 1999; Fantner *et al.* 2005), spider silk (Becker *et al.* 2003), natural adhesives from algae (Callow *et al.* 2000; Mostaert *et al.* 2006; Mostaert & Jarvis 2007), diatom mucilage (Higgins *et al.* 2003; Dugdale *et al.* 2005, 2006a,b) and adult barnacle cement (Sun *et al.* 2004).

In this paper, the morphology and nanomechanical properties of cyprid footprints were investigated with AFM. Once the footprints were located, force curves were measured from the composite nanofibrils. When footprint proteins became attached to the AFM tip, they could be stretched in a reversible way, allowing investigation of their dynamic properties. Force–extension curves were recorded continually until contact failure occurred at the weakest point in the system. Different pulling rates and delay times were applied between pulling cycles to study the reforming ability of sacrificial bonds in the footprint material. Finally, simulation of force curves by the worm-like chain (WLC) polymer elasticity model is described.

2. MATERIAL AND METHODS

2.1. Animals

Balanus amphitrite cyprids were batch cultured in the laboratory. Nauplii were released by adult *B. amphitrite* and raised on a diet of *Skeletonema costatum* according to Hellio *et al.* (2004). Metamorphosis into cyprids occurred within 5 days. Cyprids were stored at 6°C to prevent settlement and used for AFM experiments within 5 days following the final nauplius moult to the cyprid (Rittschof *et al.* 1984).

2.2. Surface preparation

Glass microscopy coverslips were sonicated in ethanol for 5 min and then immersed in piranha solution (a mixture of concentrated sulphuric acid and 33% hydrogen peroxide in a 3:1 ratio) for 15 min. The surfaces were rinsed with Milli-Q water (Ultrapure Water system) and dried under N₂. Amino (NH₂)-terminated surfaces were obtained by gas-phase evaporation of 3-aminopropyl triethoxysilane (APTES) in a desiccator under vacuum (Vandenberg *et al.* 1991). APTES was obtained from Sigma Aldrich and used as received.

Surfaces were incubated for several hours and then carefully rinsed with 99 per cent ethanol and Milli-Q water.

2.3. Atomic force microscopy experiments

AFM measurements were carried out using a Dimension D3100 atomic force microscope equipped with a NanoScope IVa controller and a hybrid scanner (H-153) with *x*-, *y*-, *z*-feedbacks from Veeco (Veeco/Digital Instruments (DI), Santa Barbara, CA, USA). Triangular shaped silicon nitride cantilevers (Veeco/DI) with typical tip diameter of 20 nm were used throughout the study and cantilever spring constants were calibrated using the thermal noise method (Hutter & Bechhoefer 1993). The spring constant values obtained were in the range of 80–90 pN nm^{−1}. The cyprids were stored, prior to use, in 33 parts per thousand artificial sea water (ASW; Tropic Marin) and were then deposited onto prepared surfaces by micro-pipette. The modified glass coverslips were mounted in polystyrene Petri dishes prior to experiments. Typically, cyprids would attach to the glass surface and begin exploration when stimulated by small water currents. Explored areas of the modified glass were marked on the base of the Petri dishes and cyprids were then removed. Surfaces were flushed with large amounts of filtered ASW to minimize contamination. Petri dishes were then transferred to the AFM and the search for footprints was focused on the marked regions. Footprint images were obtained with contact mode in ASW. Force–separation curves were subsequently obtained on preselected locations of the footprints in ASW. Forward trigger (to the surface) value was set to be 50 nm and once this trigger value was achieved, the tip retracted from the surface. Once the footprint protein was attached to the tip, the scan range was manually adjusted via the piezo-scanner displacement and the trigger was no longer applied. When the step motor was used to raise the scanner manually, a large noise could be observed in the force curves. This was used to serve as the indication of the upward and downward movement of the scanner. After the force spectroscopy performed in ASW, higher resolution images of footprints were obtained in air. There are no significant differences between the images in air and ASW. For imaging in air, samples were rinsed again with ASW and with Milli-Q water and dried in a stream of nitrogen gas. Silicon cantilevers (PointProbe Plus non-contact high resonance frequency from Nanosensors, Wetzlar, Germany) were used for intermittent contact (tapping) mode operation to obtain high-resolution images of the samples in the dry state. Scan rates were varied from 0.3 to 1 Hz and the free amplitude set-point value was around 1.5 V. Nanoscope software version 613b26 was used for data analysis.

2.4. Worm-like chain polymer elasticity model

Polymer elasticity was described by the WLC model (Giannotti & Vancso 2007). The WLC model describes the relationship between the chain extension *x* and entropic force *F*(*x*) generated in the form: $F(x) = (kT/L_p)[0.25(1 - x/L_C)^{-2} - 0.25 + x/L_C]$, where *k* is

Figure 2. (a) AFM micrograph (tapping mode amplitude image taken in air) of a cypris, *Balanus amphitrite*, footprint on NH_2 -terminated silanized glass. (b) High-magnification image of the highlighted area in (a) that shows the extended conformation of nanofibrils across the surface and (c,d) high-magnification images of single nanofibrils (highlighted area in (b)). The arrows in (c) indicate spreading of single chain across the NH_2 -terminated silanized glass.

Boltzmann's constant, T the temperature, L_P the persistence length of the molecule and L_C the contour length (Kellermayer 2005).

3. RESULTS AND DISCUSSION

3.1. Morphology of *Balanus amphitrite* footprints

AFM for surface imaging was used to observe the morphology of the adhesive footprints of *B. amphitrite* cyprids. Footprints of the proteinaceous secretion deposited by cyprids during surface exploration were probed under ASW on silane-modified glass surfaces. However, to obtain better resolution of morphological detail, footprint images (figure 2a) were taken using tapping mode AFM in air (height image, z -range 100 nm). Footprints were elliptical in shape, as has been described previously, with diameters of approximately 30 μm (Phang *et al.* 2008, 2009). The total surface coverage of an average footprint was $4.1 \pm 0.6 \times 10^{-10} \text{ m}^2$ ($n = 19$). The footprint size roughly corresponded to the diameter of the antennular disc of the cypris (figure 1c; Phang *et al.* 2008). The microtexture of the footprint was porous in nature with bundles of fibrils (bright features in figure 2b) and individual nanofibrils (indicated by arrows

in figure 2c) observable across the contacted surface as shown in figure 2. In figure 2a–d, a series of AFM micrographs, of increasing magnification, are presented to show the morphological details of footprint deposits. A high-resolution AFM image (figure 2b; magnified section labelled by the box in figure 2a) shows the conformation of the nanofibrils. Figure 2c shows a high-resolution scan of the boxed region in figure 2b. Several extended fibrils, as identified by the white arrows, probably correspond to single adhesive protein chains (figure 2c) or bundles of a small number of chains.

As shown in figure 2b, the individual fibrils of the footprint protein adhesive (thickness approx. 20 nm, including broadening by tip convolution) exhibited anisotropic properties across the area of the footprint deposit. The fibrils generally exhibited circumferential orientation (parallel with the footprint circumference; figure 2a). These nanofibrils were connected in their perpendicular direction by thinner fibrous structures (figure 2b). Several possibilities can be suggested to explain this morphology. It is possible that during attachment of the antennules, the footprint material was injected into the interface between the adhesive disc and the surface, resulting in radial shear of the proteins. Or when the walking larva detached from the surface, cohesive failure within the footprint material and the presence of the cuticular villi on the base of the

Figure 3. (a) Force spectra recorded from a footprint of a *Balanus amphitrite* cyprid. Mechanical unfolding of footprint nanofibrils, which shows a saw-tooth characteristic with multiple progressive unfolding peaks (grey arrow) and monotonic entropic elasticity in the relaxation curve. (b) The footprint response under different mechanical strains (extension).

adhesive disc could have manifested this arrangement. The larger protein aggregates arising vertically from the surface of the deposit probably correspond to areas of adhesive failure between the deposit and the antennular disc surface (figure 2b). Similar fibrillar adhesives are commonly found in nature, such as in diatoms, fly larvae, frogs, silkworms, spiders and starfish (Higgins *et al.* 2003; Fantner *et al.* 2005; Graham *et al.* 2005; Vollrath & Porter 2006; Hennebert *et al.* 2008).

3.2. Reversible unfolding–refolding, elasticity and dynamics of footprint nanofibrils

In order to understand the nanomechanical properties of footprints under tensile deformation, AFM-based force spectroscopy was used to stretch footprint proteins. Corresponding experiments were carried out by first allowing the tip to be ‘immersed’ in the footprint for 1 s. Once the footprint molecules were attached to the tip, the cantilever was retracted from the surface. The force–extension curves of microfibrils ‘picked up’ by the tip (figure 3) exhibited typical ‘saw-tooth’ fingerprints (Rief *et al.* 1997a; Oberhauser *et al.* 1998). The force curves consisted of sections of gradually increasing force, separated by sharp drops in the force. Similar force–extension curves can also be observed in single-protein AFM force spectroscopy, where the sharp drops in force correspond to the breaking up of domains within single proteins (Rief *et al.* 1997a; Best *et al.* 2003). However, in the present case, the initial force–extension curves did not represent single-protein pulling. Rather, it is likely that we observed the

stretching of bundles of protein aggregates and nanofibrils, connected via sacrificial bonds (Smith *et al.* 1999; Dugdale *et al.* 2005, 2006a,b; Fantner *et al.* 2005, 2006; Groshong 2007). Once a sacrificial bond was broken, the shielded polypeptide chain of the protein unfolded, resulting in a sharp reduction in tension. The rupture forces of the sacrificial bonds ranged between 220 and 580 pN. At a given maximum extension, without breaking tip–protein–surface contact, the relative motion of the tip was reversed and the fibrils were allowed to relax by fully removing the stress. As the tip re-approached the surface, nanofibrils exhibited a monotonic relaxation (black curve in figure 3a) showing entropic-elastic behaviour. The shaded area under the force–separation curves represents the energy required to break the sacrificial bonds (approx. 120×10^{-18} J). This process was designed to simulate the interactions that take place when an external force separates sections of the footprints from the surface. The rupture of sacrificial bonds functions as a natural damping process to dissipate large quantities of energy. Some of the observed saw-teeth could be related to a break-up of the protein internal superstructure (the mechanical denaturing of footprint protein). However, owing to the complex unfolding pathway, it is not possible to assign such intermolecular events with certainty.

The response of footprint proteins to different magnitudes of mechanical strain (extension) was examined by subjecting the attached footprint nanofibrils to different stretch lengths in successive elongation–relaxation experiments. Figure 3b shows three

Figure 4. Series of force–extension curves created by manually raising the piezoelectric scanner by two preset values of 278 and 556 nm until the attached nanofibrils were found to separate from the cantilever or substrate (approx. 10 μm).

force–separation curves for the same protein bundle with saw-tooth characteristics. The peak forces varied from 250 to 1000 pN, possibly owing to the stretching of two or more protein chains in parallel. When the fibrils were extended to the contour length of 560 nm in the first cycle (curve 1 in figure 3*b*), a sharp drop in force was observed, probably because of complete unfolding or detachment of segments of proteins from the nanofibrils. The reforming of footprint protein domains during the retraction cycle occurred quickly. Reforming of footprint protein domains was indicated by repeated unfolding peaks observed (grey colour line; figure 3*b*) in the subsequent cycles of the same protein(s). Figure 3*b* (curve 3) shows the third stretching–relaxation cycle, with a sharp increase in force at the end of a 1100 nm extension. The force–extension curves showed that the footprint nanofibrils could be stretched and relaxed in a repetitive manner. The pull-off force curves showed similar features, after repeated extend–retract cycles, to those in the first cycle. Footprint nanofibrils underwent bond reformation when allowed to relax as the tip moved towards the surface. In the next force–separation curve, the rupturing of sacrificial bonds was again observed, suggesting that footprint protein(s) possesses ‘self-healing’ ability, similar to that reported in titin, silk, diatom adhesives and other proteins (Rief *et al.* 1997*a*; Becker *et al.* 2003; Dugdale *et al.* 2005, 2006*a,b*). The footprint appearance observed in figure 2 supported the hypothesis that the fibrils are composed of complex and interconnected smaller macromolecular subunits. Hence, it is not surprising that several molecules were stretched simultaneously in pulling experiments. Owing to the complexity of the system, force–separation experiments did not yield regular and reproducible curves with uniform unfolding force as have been produced for rabbit muscle titin molecules and engineered proteins (Li *et al.* 2002).

In the experiments described so far, the piezoelectric scanner eventually reached its vertical z -direction limit

(approx. 2600 nm) during stretching of the nanofibrils. In order to further increase elongation, the scanner was raised manually by two preset values of 278 and 556 nm. Figure 4 shows the corresponding force–extension curves. Curve 1 in figure 4 displays a force–separation curve with two distinct patterns, namely saw-tooth characteristics at low extension (up to 700 nm) and predominantly elastic behaviour at high extension (>700 nm). Once all sacrificial bonds were broken, the tensile energy was stored elastically at higher strain (Fantner *et al.* 2005). When the tip was moved away from the surface, the saw-tooth characteristic gradually disappeared (figure 4, curve 5). Curve 5 shows a monotonic chain elasticity type behaviour throughout the entire stretched length in the extension and retraction cycles. A transition flattening (plateau) force was observed at a stretch length of 3000 nm. This transition remained visible in figure 4, curve 5, until the final detachment of the nanofibrils (at curve 16). The maximum force values observed in subsequent curves varied owing to variation in segment detachment, rupture of sacrificial bonds and structure break-up in the complex molecular bundles. At curve 16, complete rupture of contact was observed. Prior to rupture, the pull-off force rose sharply to approximately 2000 pN followed by a sharp drop of tension to the baseline. In the next cycle, no signal from the stretching of footprint molecules was observed in the force–separation curve. Thus, the nanofibrils detached completely before a total stretching length of 10 μm .

3.3. Refolding dynamics of footprint protein segments

During temporary attachment, the cyprid may experience significant mechanical stress in the form of hydrodynamic shear. The corresponding stress on the sacrificial bonds in footprints may eventually detach the cyprid from the surface. The stochastic nature of

Figure 5. (a) The refolding dynamics of nanofibrils allowed different relaxation times (delay time from 0 to 10 s) and (b) energy dissipation and hysteresis length calculated from the force–extension curves with different delay time.

the magnitude and periodicity of hydrodynamic forces may also allow sacrificial bonds to reform in the footprint material before failure occurs within the protein or at the interface. To mimic the stress–relaxation behaviour of footprint nanofibrils, isolated nanofibrils were subjected to repeated AFM extension–relaxation cycles and allowed to relax over different delay periods. Figure 5a shows a series of force–separation curves with different delay times. The first force curve obtained immediately following a stretch–relax cycle, without delay, is shown at the top of the force–extension diagram in figure 5a. This force–extension curve shows predominantly elastic behaviour, with only a few force peaks during extension, and smooth relaxation to the unstretched state. When a delay of 500 μ s was applied between the pulling cycles, several force peaks were observed in the resulting force–extension trace. This trend continued for longer delays, i.e. enhancement of the saw-tooth characteristic and hysteresis was observed with increasing delay time. Moreover, the

maximum pull-off distance also increased with increasing delay. The last pulling cycle shown in figure 5a was captured without delay immediately following the previous cycle, i.e. 10 s delay. Some recovery of the sacrificial bonds can be observed, but the magnitude of energy dissipation (area between the force curves) was reduced to those force curves with 50 ms to 2 s surface delay. These traces did not disappear, even after several pulling cycles, implying that more energy was dissipated when a longer delay time was applied. Thompson *et al.* (2001) reported similar observations on the stretching of collagen. They proposed that the longer delay provided more time for sacrificial bonds to reform and thus more energy could be dissipated in the next pulling cycle.

Figure 5b shows the energy dissipated during the pull cycle calculated from the hysteresis (equivalent to the area between curves) of the force–extension curves and the length of the extension section for which hysteresis behaviour was demonstrated. This length is referred

Table 1. List of number of force curves, total number of peaks, maximum hysteresis length, total energy dissipation and the ratio of number of curves against total number of peaks for the data presented in figure 5*b*.

delay (ms)	number of curves, <i>n</i>	total number of peaks, <i>p</i>	hysteresis length (nm)	total energy dissipation ($\times 10^{-18}$ J)	<i>p/n</i>
0.5	9	14	430 ± 50	30 ± 10	2
5	26	102	440 ± 110	50 ± 30	4
50	29	106	430 ± 100	40 ± 22	4
500	28	133	510 ± 120	60 ± 30	5
1000	31	132	520 ± 100	60 ± 20	4
2000	31	147	530 ± 120	60 ± 30	5
5000	30	319	700 ± 110	180 ± 90	11
10 000	23	320	800 ± 300	230 ± 140	14
0 (after 10 000)	45	241	670 ± 80	60 ± 30	5

to as ‘hysteresis length’ and is related to the break up of all sacrificial bonds during extension. Table 1 lists all the experimental parameters used in the analysis reported in figure 5*b*. The negligible energy dissipation and hysteresis length from the predominantly elastic behaviour is not included in table 1. When the delay time was increased from 2 to 10 s, sharp increases in the number of local peaks showing the saw-tooth pattern, hysteresis length and amount of dissipated energy were observed. This indicated the onset of sacrificial bond reformation. It was noted that similar trends were observed in several extension-delay cycles obtained for different footprints. The energy dissipations for 2 and 10 s delays were $60 \pm 30 \times 10^{-18}$ J and $230 \pm 140 \times 10^{-18}$ J, respectively. The hysteresis length varied from 530 ± 120 to 800 ± 300 nm for 2 and 10 s delay, respectively. A sharp increase in energy dissipation owing to force cycle delays from 2 to 5 s indicates that the required recovery time for the sacrificial bonds is in the range of several seconds. This recovery rate is faster than the reported 10 s rebonding time for bone adhesive (Fantner *et al.* 2005), 30 s for diatom *Eunotia sudetica* adhesive (Gebeshuber *et al.* 2002), and more than 100 s for bone collagen in calcium buffer (Thompson *et al.* 2001). The necessity for a rapid recovery of strength in cyprid footprints is important when one considers the size of the cyprid (*B. amphitrite* = approx. 500 μ m length) compared with that of a diatom or algal spore (generally an order of magnitude smaller). Even considering the streamlined ‘aerofoil’ shape of cyprids, the drag force exerted on it would likely be significantly greater than that of a smaller organism and they would, therefore, require a correspondingly stronger adhesion system. With only a tiny amount (approx. 2×10^{-18} l on NH_2 -terminated glass surface) of footprint material used in temporary attachment (for each step), the footprints must be able to reform and recover quickly.

In order to check possible loading rate effects, the average rupture force observed in one pulling cycle was calculated and plotted as a function of stretching rate (piezo retraction rate). The average rupture force

Figure 6. Stretch-rate dependence experiments with and without relaxation between the pulling cycles. Grey open circles, 0 s ramp delay; black open circles, 1 s ramp delay.

was determined as the arithmetic mean of each local rupture at a sacrificial bond observed in the saw-tooth pattern obtained from the force–extension curves similar to those reported in figure 3. Figure 6 shows the results of the stretch-rate experiments observed without delay and following 1 s delay between cycles. No differences in pull-off force with respect to stretching rate could be observed. The absence of stretching rate dependence indicates that the system fluctuates between the bound and unbound states on a time scale that is much faster than that of the pulling experiment (Evans & Ritchie 1997). Again, this is a necessary scenario, given the abundance of instantaneous forces that would act on an attached larva in the turbulent intertidal environment.

3.4. Segment elasticity of footprint protein chains

The force–extension relationship of footprint nanofibrils was described using the WLC model (Giannotti & Vancso 2007) in this manner (for a quantitative expression, see §2). Fitting the WLC model involves the persistence length (L_P) and contour length (L_C) data of the footprint protein, which are measured under tension. It is recognized that in an ideal situation this model would be applied to stretching of a single-chain segment; however, the WLC model can also be used to describe the stretching of chain segments in parallel and this is the likely scenario in many of the present experiments. Figure 7 shows a representative force–extension curve with saw-tooth characteristics, including the fitted lines (grey) obtained from the WLC model. The L_P and L_C values are included in figure 7 to illustrate the relationship between the information obtained from the force–separation curves and simulation results from the WLC model. The value of L_P is shown and numbered for each curve fitted. The value of L_C is slightly higher than the extension. This is because the simulated L_C represents the theoretical fully extended molecule length. In reality, the force–extension experiments never achieved the fully extended conformation before the segment detached because of the lower force value of the tip–protein interaction.

Figure 7. The description of saw-tooth unfolding peaks by the WLC model (line). The obtained contour length (L_C ; circles) is plotted versus persistence length (L_P) to illustrate the relationship between the information obtained from the force–separation curves and simulation results from the WLC model.

Figure 8. Histogram of persistence length (L_P) obtained from fitting the peaks through the WLC model.

The L_P obtained for the individual saw-teeth (figure 7) varied between 0.06 and 0.22 nm. L_P is closely related to the number of segments stretched (Dugdale *et al.* 2006*a,b*). Persistence length values $L_P < 0.14$ nm were considered ‘non-physical’ because they are smaller than an atomic radius (Abu-Lail & Camesano 2002; Dugdale *et al.* 2006*a*). However, such non-physical values did occur and could be due, in this case, to the stretching and overlapping of multiple parallel molecules in the footprint nanofibrils (Dugdale *et al.* 2006*a*; Sarkar *et al.* 2007). The unfolding peak force increased from peak 1 to peak 3, but the corresponding L_P values were reduced. Reduction of L_P indicated that a new protein molecule was stretched with the existing backbone segment and therefore higher force was required. In contrast, the reduction of unfolding peak forces from peaks 3 to 5 gave an increase in simulated L_P . This is because protein molecule(s) detached from the fibrils, fewer chains were stretched in parallel and reduced force was required to extend the fibrils. The L_P remained relatively constant from peak 6 onwards. The unfolding force for peaks 9 to 11 was 760 pN, which is higher than the 640 pN to unfold peaks 6 to 8. The L_P from peak 6 to peak 11 implied that the stretched footprint nanofibrils were segments originating from one common backbone. The different unfolding forces could relate to the mechanical hierarchy present in the footprint nanofibrils and also to the

fluctuating number of sacrificial bonds attached in different configurations to the tip (Fantner *et al.* 2006). Figure 8 shows the histogram of the persistence length (L_P) obtained from 56 different force curves with 665 peaks fitted via the WLC model. The majority of the data concentrate at a low L_P value (Gaussian peak at $L_P = 0.11$ nm), with a broad distribution that varied from 0.02 to 2 nm. The variation of the L_P values supported the previous assessment that, in most of the experiments, a number of parallel chains were pulled simultaneously, rather than a single strand of footprint proteins.

4. CONCLUSIONS

Footprints of barnacle cypris larvae (*B. amphitrite*) were studied using AFM. Images revealed a complex and interconnected morphology of footprint proteins, consisting of aggregated and interconnected nanofibrils. Molecular-scale mechanical properties of the fibrillar aggregates were measured by force spectroscopy experiments. Characteristic saw-tooth force–extension curves and entropy-elastic stretch behaviour were observed depending on the degree of extension and deformation history. Hysteresis behaviour was observed in repeated elongation–relaxation cycles. We interpreted this behaviour in terms of a sacrificial bond model arising from intra-/intermolecular loading and unloading. Delay time prior to testing of individual fibrils was found to be important. For the section of force–extension curves exhibiting entropy-like spring behaviour, the WLC model was applied to estimate the effective persistence and contour lengths. The change in persistence length with repeated testing was an indication of the breaking of sacrificial bonds between proteinaceous segments connected either in a parallel or serial fashion in the protein nanofibrils. The time needed to reform sacrificial bonds, estimated from experiments with controlled delay, was 2–5 s. The reformation of sacrificial bonds provides a dynamic binding mechanism for cyprids to resist water currents and instantaneous stress in a marine environment.

This research is supported by Dutch Polymer Institute grant no. DPI-510 (to G.J.V.). N.A. and A.S.C. thank the US Office of Naval Research for financial support (grant N00014-05-1-0767 to A.S.C.). X.Y.L and J.H. thank

the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (NWO-CW) for financial support (Vidi Vemieuwingsimpuls grant 700.52.423 to J.H.). I.Y.P. thanks SYNTHEsys for funding work with Prof. Jens Høeg at the University of Copenhagen.

REFERENCES

- Abu-Lail, N. I. & Camesano, T. A. 2002 Elasticity of *Pseudomonas putida* KT2442 surface polymers probed with single-molecule force microscopy. *Langmuir* **18**, 4071–4081. (doi:10.1021/la015695b)
- Aldred, N. & Clare, A. S. 2008 The adhesive strategies of cyprids and development of barnacle-resistant marine coatings. *Biofouling* **24**, 351–363. (doi:10.1080/08927010802256117)
- Aldred, N., Phang, I. Y., Conlan, S. L., Clare, A. S. & Vancso, G. J. 2008 The effect of a serine protease, Alacalase®, on the adhesives of barnacle cyprids (*Balanus amphitrite*). *Biofouling* **24**, 97–107. (doi:10.1080/08927010801885908)
- Anderson, C., Atlar, M., Callow, M., Candries, M., Milne, A. & Townsin, R. L. 2003 The development of foul-release coatings for seagoing vessels. *J. Mar. Des. Oper.* **B4**, 11–23.
- Arce, F. T., Avci, R., Beech, I. B., Cooksey, K. E. & Wigglesworth-Cooksey, B. 2004 A live bioprobe for studying diatom-surface interactions. *Biophys. J.* **87**, 4284–4297. (doi:10.1529/biophysj.104.043307)
- Becker, N., Oroudjev, E., Mutz, S., Cleveland, J. P., Hansma, P. K., Hayashi, C. Y., Makarov, D. E. & Hansma, H. G. 2003 Molecular nanosprings in spider capture-silk threads. *Nat. Mater.* **2**, 278–283. (doi:10.1038/nmat858)
- Best, R. B., Brockwell, D. J., Toca-Herrera, J. L., Blake, A. W., Smith, D. A., Radford, S. E. & Clarke, J. 2003 Force mode atomic force microscopy as a tool for protein folding studies. *Anal. Chim. Acta* **479**, 87–105. (doi:10.1016/S0003-2670(02)01572-6)
- Beyer, M. K. & Clausen-Schaumann, H. 2005 Mechanochemistry: the mechanical activation of covalent bonds. *Chem. Rev.* **105**, 2921–2948. (doi:10.1021/cr030697h)
- Bustamante, C., Macosko, J. C. & Wuite, G. J. L. 2000 Grabbing the cat by the tail: manipulating molecules one by one. *Nat. Rev. Mol. Cell Biol.* **1**, 130–136. (doi:10.1038/35040072)
- Callow, J. A., Crawford, S. A., Higgins, M. J., Mulvaney, P. & Wetherbee, R. 2000 The application of atomic force microscopy to topographical studies and force measurements on the secreted adhesive of the green alga *Enteromorpha*. *Planta* **211**, 641–647. (doi:10.1007/s004250000337)
- Carrion-Vazquez, M., Oberhauser, A. F., Fisher, T. E., Marszalek, P. E., Li, H. B. & Fernandez, J. M. 2000 Mechanical design of proteins studied by single-molecule force spectroscopy and protein engineering. *Prog. Biophys. Mol. Biol.* **74**, 63–91. (doi:10.1016/S0079-6107(00)00017-1)
- Christie, A. O. & Dalley, R. 1987 Barnacle fouling and its prevention. In *Crustacean issues 5: barnacle biology* (ed. A. J. Southward), pp. 419–433. Rotterdam, The Netherlands: Balkema.
- Clare, A. S., Freet, R. K. & McClary, M. 1994 On the antennular secretion of the cyprid of *Balanus amphitrite*, and its role as a settlement pheromone. *J. Mar. Biol. Assoc. UK* **74**, 243–250. (doi:10.1017/S0025315400035803)
- Crisp, D. J., Walker, G., Young, G. A. & Yule, A. B. 1985 Adhesion and substrate choice in mussels and barnacles. *J. Colloid Interface Sci.* **104**, 40–50. (doi:10.1016/0021-9797(85)90007-4)
- Drake, B., Prater, C. B., Weisenhorn, A. L., Gould, S. A. C., Albrecht, T. R., Quate, C. F., Cannell, D. S., Hansma, H. G. & Hansma, P. K. 1989 Imaging crystals, polymers, and processes in water with the atomic force microscope. *Science* **243**, 1586–1589. (doi:10.1126/science.2928794)
- Dreanno, C., Kirby, R. R. & Clare, A. S. 2006a Smelly feet are not always a bad thing: the relationship between cyprid footprint protein and the barnacle settlement pheromone. *Biol. Lett.* **2**, 423–425. (doi:10.1098/rsbl.2006.0503)
- Dreanno, C., Matsumura, K., Dohmae, N., Takio, K., Hirota, H., Kirby, R. R. & Clare, A. S. 2006b An alpha(2)-macroglobulin-like protein is the cue to gregarious settlement of the barnacle *Balanus amphitrite*. *Proc. Natl Acad. Sci. USA* **103**, 14 396–14 401. (doi:10.1073/pnas.0602763103)
- Dugdale, T. M., Dagastine, R., Chiovitti, A., Mulvaney, P. & Wetherbee, R. 2005 Single adhesive nanofibers from a live diatom have the signature fingerprint of modular proteins. *Biophys. J.* **89**, 4252–4260. (doi:10.1529/biophysj.105.062489)
- Dugdale, T. M., Dagastine, R., Chiovitti, A. & Wetherbee, R. 2006a Diatom adhesive mucilage contains distinct supramolecular assemblies of a single modular protein. *Biophys. J.* **90**, 2987–2993. (doi:10.1529/biophysj.105.079129)
- Dugdale, T. M., Willis, A. & Wetherbee, R. 2006b Adhesive modular proteins occur in the extracellular mucilage of the motile, pennate diatom *Phaeodactylum tricornutum*. *Biophys. J.* **90**, L58–L60. (doi:10.1529/biophysj.106.081687)
- Engel, A. & Muller, D. J. 2000 Observing single biomolecules at work with the atomic force microscope. *Nat. Struct. Biol.* **7**, 715–718. (doi:10.1038/78929)
- Evans, E. & Ritchie, K. 1997 Dynamic strength of molecular adhesion bonds. *Biophys. J.* **72**, 1541–1555. (doi:10.1016/S0006-3495(97)78802-7)
- Fantner, G. E. *et al.* 2005 Sacrificial bonds and hidden length dissipate energy as mineralized fibrils separate during bone fracture. *Nat. Mater.* **4**, 612–616. (doi:10.1038/nmat1428)
- Fantner, G. E. *et al.* 2006 Sacrificial bonds and hidden length: unraveling molecular mesostructures in tough materials. *Biophys. J.* **90**, 1411–1418. (doi:10.1529/biophysj.105.069344)
- Gebeshuber, I. C., Thompson, J. B., Del Amo, Y., Stachelberger, H. & Kindt, J. H. 2002 In vivo nanoscale atomic force microscopy investigation of diatom adhesion properties. *Mater. Sci. Technol.* **18**, 763–766. (doi:10.1179/026708302225003857)
- Giannotti, G. I. & Vancso, G. J. 2007 Interrogation of single synthetic polymer chains and polysaccharides by AFM-based force spectroscopy. *ChemPhysChem* **8**, 2290–2307. (doi:10.1002/cphc.200700175)
- Graham, L. D. *et al.* 2005 Characterization of a protein-based adhesive elastomer secreted by the Australian frog *Notaden bennetti*. *Biomacromolecules* **6**, 3300–3312. (doi:10.1021/bm050335e)
- Groshong, K. 2007 Unbreakable: the tough secrets of nature's glue. *New Scientist* **194**, 43–45. (doi:10.1016/S0262-4079(07)61444-4)
- Hellio, C., Marechal, J. P., Veron, B., Bremer, G., Clare, A. S. & Le Gal, Y. 2004 Seasonal variation of antifouling activities of marine algae from the Brittany coast (France). *Mar. Biotech.* **6**, 67–82. (doi:10.1007/s10126-003-0020-x)
- Hennebert, E., Viville, P., Lazzaroni, R. & Flammang, P. 2008 Micro- and nanostructure of the adhesive material secreted by the tube feet of the sea star *Asterias rubens*. *J. Struct. Biol.* **164**, 108–118. (doi:10.1016/j.jsb.2008.06.007)
- Higgins, M. J., Molino, P., Mulvaney, P. & Wetherbee, R. 2003 The structure and nanomechanical properties of the adhesive mucilage that mediates diatom-substratum

- adhesion and motility. *J. Phycol.* **39**, 1181–1193. (doi:10.1111/j.0022-3646.2003.03-027.x)
- Horber, J. K. H. & Miles, M. J. 2003 Scanning probe evolution in biology. *Science* **302**, 1002–1005. (doi:10.1126/science.1067410)
- Hutter, J. L. & Bechhoefer, J. 1993 Calibration of atomic-force microscope tips. *Rev. Sci. Instrum.* **64**, 1868–1873. (doi:10.1063/1.1143970)
- Ikai, A., Mitsui, K., Tokuoka, H. & Xu, X. M. 1997 Mechanical measurements of a single protein molecule and human chromosomes by atomic force microscopy. *Mater. Sci. Eng. C* **4**, 233–240. (doi:10.1016/S0928-4931(97)00006-4)
- Kamino, K. 2001 Novel barnacle underwater adhesive protein is a charged amino acid-rich protein constituted by a Cys-rich repetitive sequence. *Biochem. J.* **356**, 503–507. (doi:10.1042/0264-6021:3560503)
- Kamino, K. 2006 Barnacle underwater attachment. In *Biological adhesives* (eds A. M. Smith & J. A. Callow), pp. 145–180. New York, NY: Springer.
- Kamino, K. & Shizuri, Y. 1998 Structure and function of barnacle cement proteins. In *New developments in marine biotechnology* (eds Y. LeGal & H. O. Halvorson), pp. 77–80. New York, NY: Plenum Press.
- Kamino, K., Inoue, K., Maruyama, T., Takamatsu, N., Harayama, S. & Shizuri, Y. 2000 Barnacle cement proteins—importance of disulfide bonds in their insolubility. *J. Biol. Chem.* **275**, 27 360–27 365.
- Kellermayer, M. S. Z. 2005 Visualizing and manipulating individual protein molecules. *Physiol. Meas.* **26**, R119–R153. (doi:10.1088/0967-3334/26/4/R02)
- Lagerström, N. C. & Hoeg, J. T. 2002 Settlement behavior and antennular biomechanics in cypris larvae of *Balanus amphitrite* (Crustacea: Thecostraca: Cirripedia). *Mar. Biol.* **141**, 513–526. (doi:10.1007/s00227-002-0854-1)
- Li, H. B., Linke, W. A., Oberhauser, A. F., Carrion-Vazquez, M., Kerkvliet, J. G., Lu, H., Marszalek, P. E. & Fernandez, J. M. 2002 Reverse engineering of the giant muscle protein titin. *Nature* **418**, 998–1002. (doi:10.1038/nature00938)
- Marszalek, P. E., Li, H. B. & Fernandez, J. M. 2001 Fingerprinting polysaccharides with single-molecule atomic force microscopy. *Nat. Biotechnol.* **19**, 258–262. (doi:10.1038/85712)
- Matsumura, K., Nagano, M., Kato-Yoshinaga, Y., Yamazaki, M., Clare, A. S. & Fusetani, N. 1998 Immunological studies on the settlement-inducing protein complex (SIPC) of the barnacle *Balanus amphitrite* and its possible involvement in larva–larva interactions. *Proc. R. Soc. Lond. B* **265**, 1825–1830. (doi:10.1098/rspb.1998.0508)
- Mitsui, K., Hara, M. & Ikai, A. 1996 Mechanical unfolding of alpha(2)-macroglobulin molecules with atomic force microscope. *FEBS Lett.* **385**, 29–33. (doi:10.1016/0014-5793(96)00319-5)
- Mori, Y., Urushida, Y., Nakano, M., Uchiyama, S. & Kamino, K. 2007 Calcite-specific coupling protein in barnacle underwater cement. *FEBS J.* **274**, 6436–6446.
- Mostaert, A. S. & Jarvis, S. P. 2007 Beneficial characteristics of mechanically functional amyloid fibrils evolutionarily preserved in natural adhesives. *Nanotechnology* **18**, 044010. (doi:10.1088/0957-4484/18/4/044010)
- Mostaert, A. S., Higgins, M. J., Fukuma, T., Rindi, F. & Jarvis, S. P. 2006 Nanoscale mechanical characterisation of amyloid fibrils discovered in a natural adhesive. *J. Biol. Phys.* **32**, 393–401. (doi:10.1007/s10867-006-9023-y)
- Nakano, M., Shen, J. R. & Kamino, K. 2007 Self-assembling peptide inspired by a barnacle underwater adhesive protein. *Biomacromolecules* **8**, 1830–1835. (doi:10.1021/bm0612236)
- Niederegger, S., Gorb, S. & Jiao, Y. K. 2002 Contact behaviour of tenent setae in attachment pads of the blowfly *Calliphora vicina* (Diptera, Calliphoridae). *J. Comp. Physiol.* **187**, 961–970. (doi:10.1007/s00359-001-0265-7)
- Nott, J. A. & Foster, B. A. 1969 On structure of antennular attachment organ of cypris larva of *Balanus balanoides* (L.). *Phil. Trans. R. Soc. Lond. B* **256**, 115–134. (doi:10.1098/rstb.1969.0038)
- Oberhauser, A. F., Marszalek, P. E., Erickson, H. P. & Fernandez, J. M. 1998 The molecular elasticity of the extracellular matrix protein tenascin. *Nature* **393**, 181–185. (doi:10.1038/30270)
- Otness, J. S. & Medcalf, D. G. 1972 Chemical and physical characterization of barnacle cement. *Comp. Biochem. Physiol. B* **43**, 443–449. (doi:10.1016/0305-0491(72)90305-7)
- Phang, I. Y., Aldred, N., Clare, A. S., Callow, J. A. & Vancso, G. J. 2006 An in situ study of the nanomechanical properties of barnacle (*Balanus amphitrite*) cyprid cement using atomic force microscopy (AFM). *Biofouling* **22**, 245–250. (doi:10.1080/08927010600857686)
- Phang, I. Y., Aldred, N., Clare, A. S. & Vancso, G. J. 2007 Development of effective marine antifouling coatings—studying barnacle cyprid adhesion with atomic force microscopy. *Nanos* **01**, 35–39.
- Phang, I. Y., Aldred, N., Clare, A. S. & Vancso, G. J. 2008 Towards a nanomechanical basis of temporary adhesion in barnacle cyprids (*Semibalanus balanoides*). *J. R. Soc. Interface* **5**, 397–401. (doi:10.1098/rsif.2007.1209)
- Phang, I. Y., Chaw, K. C., Choo, S. S. H., Kang, R. K. C., Lee, S. S. C., Birch, W., Teo, S. L. M. & Vancso, G. J. 2009 Marine biofouling field tests, settlement assay and footprint micromorphology of cyprid larvae of *Balanus amphitrite* model surfaces. *Biofouling* **25**, 139–147. (doi:10.1080/08927010802592925)
- Razatos, A., Ong, Y. L., Sharma, M. M. & Georgiou, G. 1998 Molecular determinants of bacterial adhesion monitored by atomic force microscopy. *Proc. Natl Acad. Sci. USA* **95**, 11 059–11 064. (doi:10.1073/pnas.95.19.11059)
- Rief, M., Gautel, M., Oesterhelt, F., Fernandez, J. M. & Gaub, H. E. 1997a Reversible unfolding of individual titin immunoglobulin domains by AFM. *Science* **276**, 1109–1112. (doi:10.1126/science.276.5315.1109)
- Rief, M., Oesterhelt, F., Heymann, B. & Gaub, H. E. 1997b Single molecule force spectroscopy on polysaccharides by atomic force microscopy. *Science* **275**, 1295–1297. (doi:10.1126/science.275.5304.1295)
- Rittschof, D., Branscomb, E. S. & Costlow, J. D. 1984 Settlement and behavior in relation to flow and surface in larval barnacles, *Balanus amphitrite* Darwin. *J. Exp. Mar. Biol. Ecol.* **82**, 131–146. (doi:10.1016/0022-0981(84)90099-6)
- Sarkar, A., Caamano, S. & Fernandez, J. M. 2007 The mechanical fingerprint of a parallel polyprotein dimer. *Biophys. J.* **92**, L36–L38. (doi:10.1529/biophysj.106.097741)
- Smith, B. L., Schaffer, T. E., Viani, M., Thompson, J. B., Frederick, N. A., Kindt, J., Belcher, A., Stucky, G. D., Morse, D. E. & Hansma, P. K. 1999 Molecular mechanistic origin of the toughness of natural adhesives, fibres and composites. *Nature* **399**, 761–763. (doi:10.1038/21607)
- Sun, Y. J., Guo, S. L., Walker, G. C., Kavanagh, C. J. & Swain, G. W. 2004 Surface elastic modulus of barnacle adhesive and release characteristics from silicone surfaces. *Biofouling* **20**, 279–289. (doi:10.1080/08927010400026383)
- Thompson, J. B., Kindt, J. H., Drake, B., Hansma, H. G., Morse, D. E. & Hansma, P. K. 2001 Bone indentation recovery time correlates with bond reforming time. *Nature* **414**, 773–776. (doi:10.1038/414773a)

- Vandenberg, E. T., Bertilsson, L., Liedberg, B., Uvdal, K., Erlandsson, R., Elwing, H. & Lundstrom, I. 1991 Structure of 3-aminopropyl triethoxy silane on silicon-oxide. *J. Colloid Interface Sci.* **147**, 103–118. (doi:10.1016/0021-9797(91)90139-Y)
- Vollrath, F. & Porter, D. 2006 Spider silk as archetypal protein elastomer. *Soft Matter* **2**, 377–385. (doi:10.1039/b600098n)
- Walker, G. & Yule, A. B. 1984 Temporary adhesion of the barnacle cyprid—the existence of an antennular adhesive secretion. *J. Mar. Biol. Assoc. UK* **64**, 679–686. (doi:10.1017/S0025315400030344)
- Yeber, D. M., Kiil, S. & Dam-Johansen, K. 2004 Antifouling technology—past, present and future steps towards efficient and environmentally friendly antifouling coatings. *Prog. Org. Coat.* **50**, 75–104. (doi:10.1016/j.porgcoat.2003.06.001)
- Yeber, D. M., Kiil, S., Weinell, C. & Dam-Johansen, K. 2006 Presence and effects of marine microbial biofilms on biocide-based antifouling paints. *Biofouling* **22**, 33–41. (doi:10.1080/08927010500519097)
- Yule, A. B. & Walker, G. 1987 Adhesion in barnacles. In *Crustacean issues 5: barnacle biology* (ed. A. J. Southward), pp. 389–402. Rotterdam, The Netherlands: A. A. Balkema.
- Zhang, W. & Zhang, X. 2003 Single molecule mechanochemistry of macromolecules. *Prog. Polym. Sci.* **28**, 1271–1295. (doi:10.1016/S0079-6700(03)00046-7)