Wet Adhesion

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Intrinsic Surface-Drying Properties of Bioadhesive Proteins**

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Abstract: *Sessile marine mussels must "dry" underwater surfaces before adhering to them. Synthetic adhesives have yet to overcome this fundamental challenge. Previous studies of bioinspired adhesion have largely been performed under applied compressive forces, but such studies are poor predictors of the ability of an adhesive to spontaneously penetrate surface hydration layers. In a force-free approach to measuring molecular-level interaction through surface-water diffusivity, different mussel foot proteins were found to have different abilities to evict hydration layers from surfaces—a necessary step for adsorption and adhesion. It was anticipated that DOPA would mediate dehydration owing to its efficacy in bioinspired wet adhesion. Instead, hydrophobic side chains were found to be a critical component for protein–surface intimacy. This direct measurement of interfacial water dynamics during force-free adsorptive interactions at solid surfaces offers guidance for the engineering of wet adhesives and coatings.*

The performance of man-made adhesives and water are fundamentally in conflict.[1] The presence of stable hydration layers around both the adhesive polymer and the surface results in strong repulsive hydration forces that undermine adhesion. A renewed appreciation of wet adhesion by sessile marine organisms, however, is invigorating efforts to better reconcile the two.[2] An understanding of the adaptive mechanisms by which mussels, for example, overcome repulsive hydration forces to adhere to any type of surface underwater could enable the design of a new generation of surface-drying wet adhesives with the potential to influence applications ranging from the enhancement of biomedical implants to underwater coatings.[3,4] While recognizing that actual mussel adhesion involves a complex choreography at multiple length scales encompassing protein chemistry, interfacial energy, and plaque mechanics, we believe that a reductionist approach to how adhesive proteins cope with surface hydration could be instrumental for improving the performance of wet-adhesive polymers.

Mussels attach to solid surfaces by a holdfast structure known as the byssus (Figure 1), which consists of a bundle of threads, each equipped with a distal adhesive plaque. The plaques contain at least eight different types of mussel foot proteins (Mfps), which collectively determine the adhesive properties of the plaque. Most Mfps are intrinsically disordered in aqueous solution,[5] and all contain posttranslationally modified 3,4-dihydroxyphenylalanine (DOPA), though to different degrees (0.1–30 mol %).[3] Studies with either the atomic force microscope (AFM) or surface forces apparatus (SFA) have suggested that under compressive contact, DOPA plays a critical role in the adhesion of Mfps to wet surfaces.[6,7]

AFM or SFA measurements, however, can less readily assess the ability of Mfps to spontaneously "dry" a surface, because the applied forces themselves effectively break through or disrupt the repulsive hydration layer. Conversely, an experimental approach that operates under force-free conditions can assess how well Mfps or synthetic polymers interact with hydrated surfaces on the basis of their intrinsic properties, and offer an experimentally unexplored, yet critical, perspective to wet adhesion. Given the apparent wet-adhesive ability of DOPA and the molecular diversity of DOPA-containing mussel adhesive proteins, we posed the following questions: Do the protein constituents released by the mussel foot "dry" the surface before adhering to it? How important is DOPA in different Mfps for overcoming repulsive hydration forces ubiquitously present on wet surfaces?

We addressed these questions by studying the diffusion dynamics of water hydrating a solid surface that is suspended in bulk water. The perturbation of the surface-water diffusion dynamics by macromolecules in solution is a measure of their ability to break through the hydration layer of the solid surface to adsorb to the surface. These measurements are enabled by a spectroscopic approach termed Overhauser dynamic nuclear polarization (ODNP) relaxometry, which quantifies the diffusion dynamics of surface local water.[10,11]

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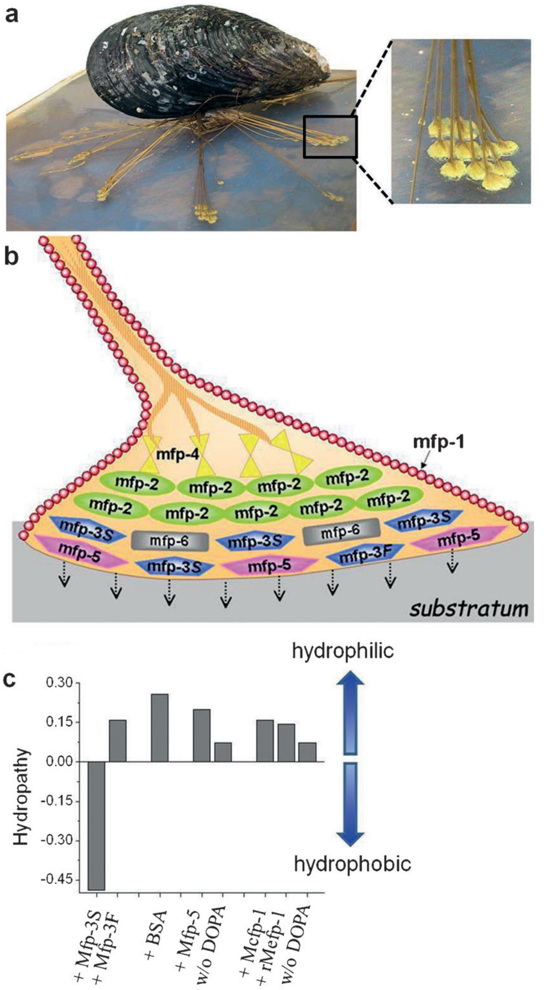

Figure 1. a) A mussel (*Mytilus californianus*) attached to the substratum by a byssus: essentially a bundle of adhesive-tipped threads. b) Diagram of one of the adhesive tips or plaques in (a) enlarged to show the approximate location of known Mfps. c) Hopp and Woods mean hydropathy values of proteins per amino acid as determined by using ExPaSy tools. Hopp and Woods hydropathy[8] was selected over others because it is based on experimentally determined partition coefficients of amino acids, including DOPA.[9]

Specifically, we investigated the molecular intimacy between Mfps and the surface of spin-labeled polystyrene (SLPS) and silica ($SLSiO_2$) nanobeads suspended in water. We propose that those Mfps that exhibit the greatest initial intimacy with a given solid surface are the initial facilitators, or vanguards, of wet adhesion under force-free conditions, and pave the way for other Mfp proteins to finalize adhesive-bond formation and sealing. Three different Mfp families were analyzed in this study: Mfp-1, Mfp-3, and Mfp-5 (see the Supporting Information). Mfp-3 is an interfacial protein encompassing two electrophoretically distinguishable polymorphic families, that is, the slow- (S) and fast-moving (F) isoforms.[12] On the basis of the Hopp and Woods hydropathy index,[8] Mfp-3S (DOPA: 10-15 mol %) is significantly more hydrophobic than Mfp-3F, Mfp-1, and Mfp-5 (Figure 1c).

ODNP exploits a ^{1}H NMR spectroscopic signal enhancement of water at 0.35 T by driving electron spin resonance (ESR) saturation at its Larmor frequency of approximately 10 GHz with the detection of ^{1}H NMR signal amplification at approximately 15 MHz. Only ^{1}H in fast-moving H_2O molecules, relative to about 10 GHz, will induce concerted electron–^{1}H spin flip-flops that give rise to ^{1}H NMR signal enhancement, and in this way can be exploited to quantify local water diffusivity near nitroxide-radical-based spin labels commonly used for ESR analysis (see the Supporting Information).[10,11] The motion of hydration water is expressed with a translational diffusion correlation time ($\tau_{\text{surface water}}$), which represents the time needed for water to diffuse across a distance b (typically 5–15 Å), depending on the local diffusion coefficient (D) according to $\tau = b^2/D$.[10,11,13] Crucially, ODNP, when combined with NMR relaxometry, can separate contributions of freely, diffusively translating hydration water (k_σ, picosecond timescale) from those of bound water (k_{low}, nanosecond timescale).[14] Protein adsorption will decrease the diffusivity of the surface hydration water as a result of increased molecular collision, as reflected by decreased k_σ values, and in instances of strong adsorption, also by increased k_{low} values. We further applied ESR line-shape analysis to obtain the rotational diffusivity of the spin label itself in the given local environment.[15]

To capture interfacial interactions by ODNP and ESR spectroscopy, we prepared SLPS beads ($d \approx 50$ nm; see the Supporting Information). An acidic solution of pH 3.0 was chosen to prevent DOPA oxidation and better mimic the delivery conditions of the Mfps in mussels.[6,7] ESR line-shape analysis confirmed the presence of a single population of slow and anisotropically moving spin labels with an average rotational correlation time of $\tau_R = 4.2$ ns, in contrast to that of the isotropically moving free spin labels of $\tau_R = 20$ ps (Figure 2a; see also Figure S1 in the Supporting Information). Although PS is considered hydrophobic, the benzene rings on the PS bead surface are reported to form hydrogen bonds with water molecules, thereby increasing wettability.[16] The ODNP-derived $\tau_{\text{surface water}}$ on SLPS surfaces was found to be 335 ps (Figure 2b), which corresponds to a more than 10-fold retardation as compared to bulk water ($\tau = 33$ ps).[11] This considerably slower diffusion baseline implies that water is constrained within the hydration layer of the PS surface; the magnitude of retardation suggests spin labels may be partially obscured by a soft polymer coat, which is consistent with the somewhat immobilized spin-label motion derived from ESR line shapes.

Next, the surface hydration dynamics were tracked following the addition of various Mfps (2.5 mg mL^{-1}; Figure 2b), as well as bovine serum albumin (BSA), 4.5 kDa polyethylene glycol (PEG), and poly-L-lysine. In the presence of native Mcfp-1, recombinant Mefp-1 with DOPA, recombinant Mefp-1 without DOPA, native Mfp-5, recombinant Mfp-5 without DOPA, PEG, and poly-L-lysine, the $\tau_{\text{surface water}}$ and k_σ or k_{low} values did not change beyond the margin of error, and nor did their ESR spectra (see Figure S2), thus indicating that none of the macromolecules achieved significant intimacy with the SLPS surface. Mfp-1 and Mfp-5, with or without DOPA, are positively charged at pH 3 and display chiefly hydrophilic domains (Figure 1c). Apparently, the strong hydration shell surrounding these proteins (as well as

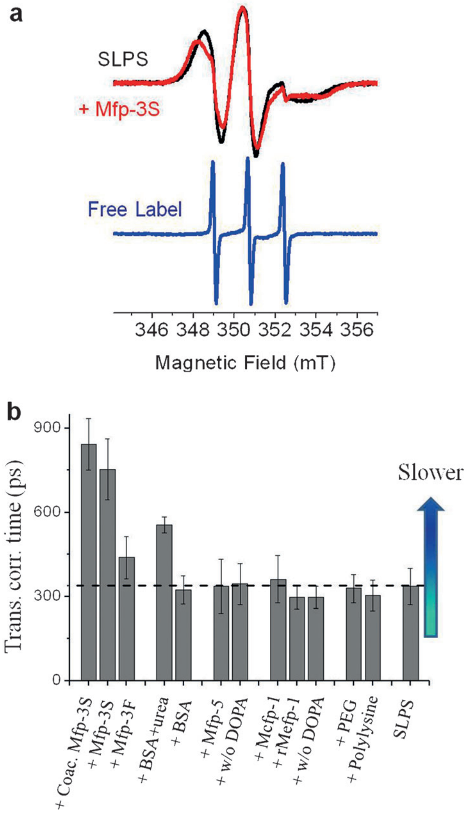

Figure 2. a) ESR spectra of SLPS before (black) and after the addition of Mfp-3S (slow; red) in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at pH 3.0 and 20 °C, and ESR spectrum of free 4-carboxy-2,2,6,6-tetramethylpiperidin-1-oxyl, in MES buffer at pH 3.0 (blue). b) Translational correlation time ($\tau_{\text{surface water}}$) of hydration water at the surface of SLPS before and after the addition of Mfp-3S, Mfp-3F (fast), BSA denatured with 5 M urea, Mfp-5, rMfp-5 without DOPA, rMefp-1, rMefp-1 without DOPA, 4.5 kDa PEG, poly-L-lysine in MES buffer (pH 3.0), and coacervated Mfp-3S in MES buffer (pH 5.5), each at a concentration of 2.5 mg mL^{-1}. The horizontal dashed line marks the reference τ value of the bare SLPS surface. Higher τ values reflect slower motion of hydration water. Error bars represent the standard deviation.

hydrophilic PEG and cationic poly-L-lysine) prevents them from approaching the SLPS surface under force-free conditions, irrespective of whether DOPA functionalities are present or not.

In contrast, the addition of hydrophobic Mfp-3S at pH 3 resulted in significant retardation of surface water, with an increase in $\tau_{\text{surface water}}$ from 335 to 752 ps (Figure 2b). This result was independently corroborated by the observation of k_σ and k_{low} values, which showed increased contributions from slow, bound hydration water and decreased contributions from fast, diffusing hydration water, thus implying significant protein adsorption to the SLPS surface (see Figure S3).

Mfp-3S is unique among the Mfps in that it self-coacervates at higher pH values.[17] Coacervated Mfp-3S retards the translational diffusion of surface water from $\tau_{\text{surface water}} = 335$ to 842 ps at pH 5.5, which resembles the effect of Mfp-3S in solution (Figure 2a; see also Figure S1). We propose that Mfp-3S, in essence, breaks down the repulsive hydration layer of the SLPS surface by exposing a hydrophobic face (containing tryptophan and tyrosine) that is weakly hydrated, and thus interacts more aggressively with the hydrated polymer surface before adsorbing to it (Figure 3). This localized interaction seems relatively unaffected by whether the Mfp-3S is delivered as a dilute solution or as a concentrated fluidic coacervate, thus suggesting a strong intrinsic attraction of Mfp-3S to surfaces. In contrast, highly polar Mfp-3F weakly, but measurably, retards the translational diffusion of surface water from $\tau_{\text{surface water}} = 335$ to 438 ps, even though Mfp-3F should be stably hydrated like Mfp-1 and Mfp-5. This anomalous behavior may be due to a higher content of flexible amino acid residues in Mfp-3F, which would make it slightly more prone to undergo interaction with surfaces,[18] or may be due to homologous hydrophobic tryptophan-containing sequences, for example, GWN/G and PWP, found in both Mfp-3F (3 per protein) and Mfp-3S (4 per protein). Of particular interest is a comparison of results obtained with Mfps and BSA. BSA is frequently used as a "standard protein" and a blocking agent to prevent nonspecific hydrophobic binding of antigens and antibodies to nontarget surfaces. In the presence of native BSA (2.5 mg mL^{-1}), the SLPS surface-water diffusivity remains unaltered. However, partially unfolded BSA (exposed to 5 mM urea) moderately slows the water dynamics on the SLPS surface from 335 to 555 ps (Figure 2b), which implies that initially buried hydrophobic segments may be recruited to facilitate interaction with the SLPS surface. Still, Mfp-3S interacts much more intimately with SLPS surfaces than unfolded BSA; its effect is comparable with that of 12-fold concentrated BSA (see Figures S4 and S5).

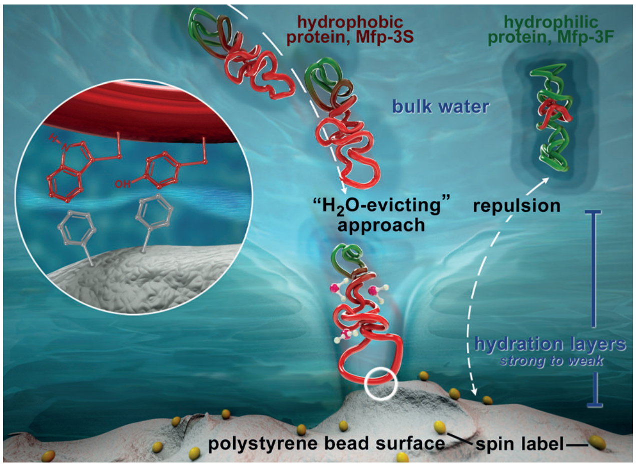

Figure 3. Mfp adhesion to surfaces requires the hydration layers to be broken through. Under force-free solution conditions, the strong hydration layers (gray-blue) surrounding Mfp-3F and the PS surface pose a double hydration barrier to adhesive interactions between these structures. In contrast, the weak hydration layers (light blue) of Mfp-3S facilitate the intimate approach of Mfp-3S to the PS surface by readily evicting the initially surface bound hydration water molecules and subsequently mediating hydrophobic interactions as depicted in the magnified illustration in the circle.

We also carried out parallel measurements on silica nanobeads suspended in a buffered aqueous solution (see the Supporting Information). The ODNP-derived $\tau_{\text{surface water}}$ value is 357 ps, thus confirming that the spin label intimately experiences the retarded surface hydration water, whereas the spin label ($\tau_R = 2.5$ ns) shows surface immobilization. Notably, ODNP- and ESR-derived surface dynamics did not change upon the addition of any of the tested adhesive proteins or polymers (for example, Mfps, BSA, P188) beyond the margin of error; we can therefore conclude that none achieved intimacy with the silica surface (see Figures S6 and S7). Quartz crystal microbalance (QCM-D) measurements confirmed that there was no measurable spontaneous adsorption of Mfp-3S (20 $\mu g\,mL^{-1}$) from solution onto silica surfaces and thus independently verified the ODNP results (see Figure S8). Other hydrophilic surfaces, such as surfaces of unilamellar liposomes freely suspended in solution, were also tested for spontaneous surface adsorption. Mfp-3S did not adsorb to any of the hydrophilic membrane surfaces tested (see Figure S9).

Silica surfaces are surrounded by much stronger hydration barriers, to the extent that Mfps studied by SFA and AFM require an applied pressure to adhere to silica.[18,19] Notably, silica surfaces are employed in size exclusion chromatography for protein separation, as permanent interactions between proteins and silica surfaces are weak, thus further supporting the notion that *spontaneous adsorption* of proteins to silica surfaces is hindered by the strongly repulsive silica hydration layer. That mussels have no problem sticking to silica-based glass[20] surfaces appears to contradict this prediction. However, sessile mollusks have multiple options to counteract strong surface hydration, including high foot pressure derived from suction,[21] the opportunity to piggyback on microbial biofilms already adsorbed to surfaces, and perhaps even the existence of additional Mfp-3 variants specifically adapted for silica surfaces. The emerging theme is that wet bioadhesion may require imposed "clamping" forces except when surface water is effectively evicted by a molecular agent.

The findings presented herein are consistent with the dominant biological role of hydrophobic interactions, which are considered the strongest noncovalent interactions between apolar/weakly polar molecules in physiological saline media. For example, recent SFA measurements show that all three hydrophilic Mfps (Mfp-1, Mfp-3F, and Mfp-5) adhere 10 times more strongly to hydrophobic surfaces than to hydrophilic surfaces.[22] We suggest that this behavior is observed because, in general, "dry" contact surfaces are a prerequisite for strong adhesion underwater and are facilitated at hydrophobic interfaces whose hydration layers are less strongly attracted to the surfaces and hence cost less energy to remove once forces bring the adsorbates into contact. Our studies support the interpretation that the "drying" ability of Mfps relies on constituent hydrophobic side chains, not their DOPA functionalities, and is dramatically dampened when these Mfps approach a highly repulsive hydration layer, as found on silica surfaces as opposed to modestly hydrophilic PS surfaces.

Interestingly, Mfp-3S is abundant in plaque footprints harvested from Plexiglas surfaces according to in situ analysis by MALDI-TOF mass spectrometry.[12] We propose that mussels squirt Mfp-3S onto polymer surfaces as a "molecular vanguard" in anticipation of adhesive action; in this way, they prepare the surface by breaking down the hydration-layer barriers (Figure 3). Specific Mfps adapted for highly hydrophilic surfaces, such as silica, have yet to be identified. Once the molecular vanguards have achieved intimacy with a wet surface, stronger and more lasting adhesive interactions can be introduced by recruiting DOPA-rich Mfp-3F and Mfp-5. Our results suggest that hydrophobic molecular vanguards (whose solubility is maintained by strategically placed hydrophilic residues) weaken surface hydration forces, enhance the adhesive–surface encounter, more reliably and more readily prepare a dry adhesion interface, and pave the way for stronger and lasting adhesion underwater.

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