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Surface Attachment Enhances the Thermodynamic Stability of Protein L

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Abstract

Despite the importance of protein-surface interactions in both biology and biotechnology, our understanding of their origins is limited due to a paucity of experimental studies of the thermodynamics behind such interactions. In response we have characterized the extent to which interaction with a chemically well-defined macroscopic surface alters the stability of protein L. To do so, we site-specifically attached a redox-reporter-modified protein variant to a hydroxyl-terminated monolayer on a gold surface and then used electrochemistry to monitor its guanidine denaturation and determine its folding free energy. Comparison with the free energy seen in solution indicates that interaction with this surface stabilizes the protein by 6 $kJ \cdot mol^{-1}$, a value in good agreement with theoretical estimates of the entropic consequences of surface-induced excluded volume effects, suggesting that chemically specific interactions with this surface (e.g., electrostatics) are limited in magnitude.

Graphical Abstract

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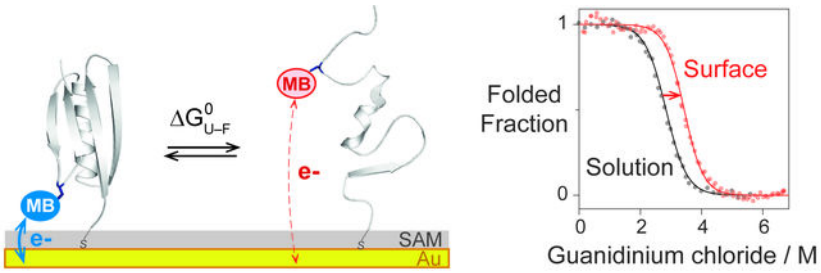

Surface-protein interactions exhibit rich and complex biophysics, which we probe here by measuring the stability of protein L when attached to a hydroxyl-coated monolayer. We report protein stabilization arising due to surface-induced excluded volume effects that restrict the conformational entropy of the unfolded state, and macromolecular crowding effects on the surface that are markedly different than those in solution.

Keywords

proteins; biophysics; thermodynamics; protein engineering; electrochemistry

Interactions between biomolecules and surfaces play key roles in many aspects of biology and biotechnology. The functional interaction of proteins with surfaces occurs in, for example, cell adhesion[1] and signal transduction.[2] Undesired effects, including adsorption and surface-induced aggregation[3], can also occur at biological surfaces and are even more common on artificial surfaces, where they often carry grave consequences for biotechnologies that attempt to add protein functionality to non-biological systems.[4] The development of artificial surfaces upon which proteins remain folded and functional could thus significantly advance a wide range of technologies, including biosensors, implants, and other biomedical devices.[5]

The study of protein-surface interactions also holds fundamental biophysical implications, as the delicate interplay of contributions that determine a protein's thermodynamic stability when it interacts with a surface likely differs from the effects that define its stability in bulk solution. Theory and simulations suggest, for example, that interaction of a protein with a surface limits the conformations available to the unfolded state, thus reducing its entropy and pushing the folding equilibrium towards the native state.[6] Simulations also suggest that chemical interactions with the surface, in contrast, will always stabilize the unfolded state because its flexibility allows it to conform to or move away from the surface, thus better accommodating, for example, hydrogen bonding, hydrophobic interactions, or electrostatic attraction and repulsion between a protein's charged residues and a charged surface.[7,8] Which of these effects dominates will presumably depend on the details of the system. Relevant parameters likely include the chemistry and charge of the surface, the length, charge distribution, and hydrophobicity of the protein,[9] as well as its specific orientation on the surface, which will determine the extent to which any given interaction with the surface is supported and which unfolded conformations are restricted.[10,11] However, while these theoretical and computational arguments provide a detailed and fairly consistent view of the thermodynamics of protein-surface interactions, it has proven challenging to test them

experimentally. Consequently, our understanding of the biophysics that dictate how and why proteins interact with surfaces remains limited.

To address the above questions we have recently developed an experimental technique to measure the folding free energy of surface-attached nucleic acids and proteins, which we compare to the stability in bulk solution to obtain quantitative information on the thermodynamics of protein-surface interactions.[7,12] Here, we harness this approach to measure the thermodynamics underlying the interactions of protein L, a well-studied 65-residue protein,[13] with a chemically well-defined, hydroxyl-coated macroscopic surface. To attach the protein to this surface we modified the amino terminus of a lysine-free variant via amide bond formation to 7-thiol-heptanoic acid.[12] Using this linker we site-specifically attached the protein to a gold electrode that we then treated with mercaptohexanol to form a hydroxyl-terminated, six-carbon self-assembled monolayer. This monolayer forms a polar, hydroxyl-terminated surface that is employed in a wide range of biotechnologies[14] (in contrast, proteins tend to unfold on and adhere to bare gold).[15] The attachment chemistry we employ aims to place the protein one atom above the surface of the monolayer so as to minimize any perturbation of it, ensuring that the chemistry of the surface is homogeneous and well defined.

We determined the unfolding free energy of the surface-attached protein by modifying it with a methylene blue (MB) redox reporter and performing guanidine hydrochloride (GuHCl) denaturation that we monitored using square-wave voltammetry.[7,12] The (maleimide-modified) reporter was attached to a cysteine introduced at a position that is close to the electrode surface when the protein is folded (Fig. 1A). As expected,[12] the peak current measured at a square-wave frequency of 600 Hz decreases as the protein unfolds and the reporter moves, on average, farther from the surface (Fig. 1A,B). The resulting denaturation curve (Figs. 1C, 2) exhibits the sigmoidal shape expected for two-state folding, as is also observed for protein L when free in bulk in solution.[16] Critically, the denaturation is reversible, suggesting that the signal we are tracking follows protein denaturation and not degradation of the protein-modified monolayer (Fig. 1C).

Fitting the denaturation curve of the surface-attached protein to the linear free energy relationship[17] describing a two-state chemical denaturation we find that its unfolding free energy and *m*-value are 26.7±0.8 kJ·mol^{-1} and −7.7±0.2 kJ·mol^{-1}·M^{-1} respectively. To determine the extent to which interaction with the surface impacts these values we used circular dichroism to monitor the denaturation of the same reporter-and-linker-modified protein in solution. Doing so, we obtain an unfolding free energy of 21±1 kJ·mol^{-1} and a GuHCl *m*-value of −7.2±0.4 kJ·mol^{-1}·M^{-1}. Thus, while the GuHCl *m*-value appears to be unaffected by surface confinement, the stability of the protein is higher on the surface than in bulk solution by 6±1 kJ·mol^{-1} (Fig. 2A, Table S1).

To confirm that our experiments are accurately reporting on the thermodynamics of the surface-attached protein we measured the extent to which sodium sulfate alters its stability. Our assumption here is that sulfate, a stabilizing salt of the Hofmeister series, should alter the free energy of the surface-attached and free protein equally. Consistent with this, 450

mM Na_2SO_4 stabilizes the protein by 7±1 kJ·mol^{-1} when surface attached and 6±1 kJ·mol^{-1} when free in solution (Fig. 3, Table S1).

The 6±1 kJ·mol^{-1} stabilization associated with surface attachment presumably arises due to the entropic restriction that the surface imposes on the unfolded state. Specifically, theoretical and computational studies suggest that attaching the end of an unfolded polymer to a surface leads to excluded-volume effects that restrict the number of conformations available, reducing its entropy and thus favoring folding.[10,18] Knotts et al.,[8] for example, report just such entropic stabilization in atomistic simulations of protein G (a 56-residue protein of similar structure to protein L)[13] when tethered to a non-interacting surface via its amino terminus. Zhou and Dill[6] also simulated the confinement of a 200-residue unfolded Gaussian chain onto a surface to estimate a conformational restriction equivalent to ~12 kJ·mol^{-1}, which, as expected, is higher than the value we observe for our shorter chain. Finally, Chen and Luo[19] used simulations to estimate the loss in conformational entropy associated with attaching a self-avoiding chain to a non-interacting surface. For the equivalent of a 65-amino acid unfolded protein, they find a stabilization of 8.6 kJ·mol^{-1}, a value reasonably close to the 6±1 kJ·mol^{-1} that we observe experimentally.

That the experimentally measured stabilization associated with surface attachment is slightly below prior Chen and Luo's estimate of the entropic consequences of attachment suggests that any enthalpic contributions to the thermodynamics of the interactions with the surface are: i) rather small and ii) destabilizing. Such contributions presumably arise due to interactions with the surface of the hydroxyl-terminated monolayer that are better accommodated in the flexible unfolded state[20] such as, for example, hydrogen bonding, hydrophobic interactions and electrostatic effects.[7,9,12] We believe that at least the latter should be present to some extent, as both the surface and the protein are charged. Specifically, our protein L variant has 10 negatively charged residues and 6 positively charged residues (pI = 4.5), whereas the electrode surface is also likely charged given that, to interrogate our system, we apply potentials that differ from the reported potential of zero charge of a mercaptohexanol-on-gold monolayer.[21] The final net balance of these contributions depends on the details of the system, as illustrated by the fact that while protein L is significantly stabilized upon surface interaction, FynSH3, a protein of similar size but with a different amino acid composition, is marginally destabilized on this same surface.[12]

All the experiments described above were performed in the "dilute" regime in which neighboring molecules are, on average, too far apart to interact. If, instead, we increase the packing density (by increasing the protein concentration employed for deposition) we find that the estimated *m*-value and thus the fitted stability of the protein appear to decrease (Fig. 4). We have previously observed a similar trend in the unfolding of a surface-attached DNA stem-loop, which we attributed to two anti-cooperative effects.[22] First, at low denaturant concentrations interactions between densely packed, folded neighbors destabilize the first few molecules to unfold. As unfolding progresses, however, the increasing number of unfolded neighbors leads to excluded volume effects that oppose the unfolding of any additional molecules. The dependence of the stability of any one molecule on the conformational state of its neighbors will progressively broaden the unfolding transition and

cause the estimated *m*-value (related to the slope, and thus the width, of the unfolding transition) to be lower than the *m*-value seen under (orders of magnitude less crowded) bulk solution conditions. In support of this argument we note that, at the highest packing densities we have characterized, the equivalent concentration of protein L molecules within one protein diameter (approximately 3 nm) of the surface is greater than 7 mM (50 mg/mL), a value that is close to the protein's solubility limit.[23] In the dilute regime, in contrast, neighboring molecules are relatively far apart and thus intermolecular effects, if any, are expected to diminish. Consistent with this, the estimated *m*-value reaches (to within experimental error) the value seen in solution (Fig. 4C) when the average intermolecular separation is of similar magnitude to the dimensions of the fully extended polypeptide chain.

Here we have investigated the extent to which attachment to a macroscopic, hydroxyl-terminated surface alters the folding thermodynamics of protein L and find that interactions with this surface stabilize the protein by 6±1 $kJ \cdot mol^{-1}$. This presumably arises due to surface-induced excluded volume effects that reduce the conformational entropy of the unfolded state, a contribution that had been long predicted for surface-attached proteins but that had previously not been observed experimentally. The GuHCl *m*-value of the surface-attached protein, in contrast, appears effectively indistinguishable from that of the free protein. Consistent with this, sulfate, a stabilizer in the Hofmeister anion series, alters the stability of the surface-attached protein in a manner that mimics the behavior seen for the protein when free in solution, thus indicating that we are accurately probing the folding thermodynamics of the surface-attached protein. Finally, in exploring the effects of crowding we find the estimated *m*-value and stability both decrease at high packing densities, an observation that we attribute to interactions between closely packed molecules and that highlights the fact that the biophysics of surface-confined proteins can differ significantly from those in bulk solution.

The rich chemical complexity of proteins renders protein-modified surfaces technologically attractive, but this same complexity can also render their interactions with surfaces complicated. Given this, considerable effort has been dedicated to improving protein implementation in biotechnologies, with examples including empirical searches of adsorption-resistant materials,[25] the design of surfaces mimicking biological membranes,[26] or qualitative studies on biomolecule adsorption.[27] In the absence of quantitative mechanistic insights into the origins of protein-surface interactions, success in achieving a consistent implementation of surface-attached proteins has, historically, been irregular, with only a few applications reaching, for example, clinical application.[28] Given this, quantitative experimental measurements of the thermodynamic consequences of protein immobilization on artificial surfaces, such as the ones presented here, will likely leave us better placed to identify and dissect systematically the key enthalpic and entropic contributions that determine how and why protein-surface interactions occur. We believe that doing so will expand the current understanding of the biophysics underlying these interactions, which will ultimately allow us to predict and engineer protein stability on surfaces and to co-optimize proteins and surfaces for the successful implementation of proteins on biotechnological platforms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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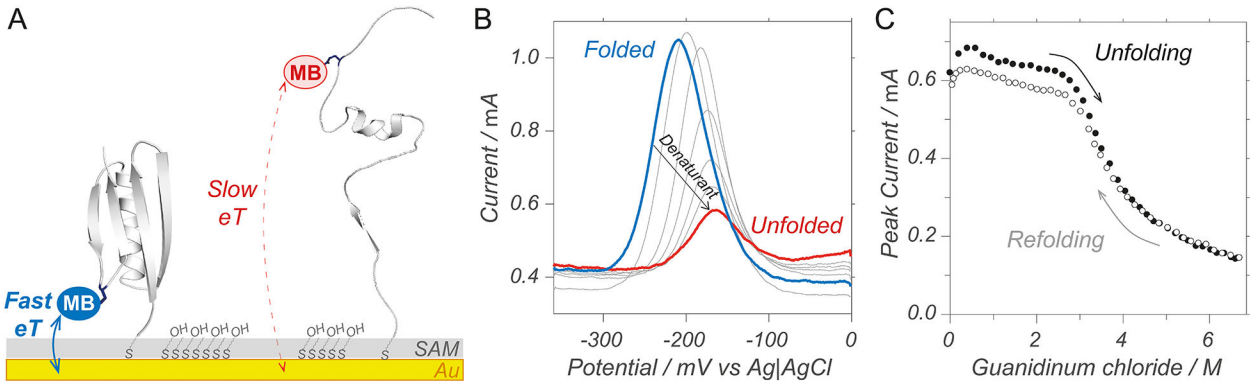

Figure 1.

(A) To study the surface-interaction thermo-dynamics of protein L we generated a lysine-free variant of the protein attached via its amino terminus to the surface of a 6-mercaptohexanol mono-layer deposited on gold. Unfolding reduces the efficiency with which a methylene blue (MB) redox reporter conjugated to a cysteine at position 54 approaches this surface. (B) Square-wave voltammograms measured at 600 Hz thus exhibit greater peak currents in the folded than in the unfolded state.[12] The shift in redox potential observed upon GuHCl addition appears due to the effect of chloride on the potential of the Ag|AgCl reference electrode (Fig. S3). (C) Plotting the peak current of these voltammograms as a function of GuHCl concentration yields the sigmoidal transition expected for a two-state unfolding. Critically, this transition is reversible, suggesting that it is monitoring the unfolding and refolding of the protein and not the degradation of the monolayer.

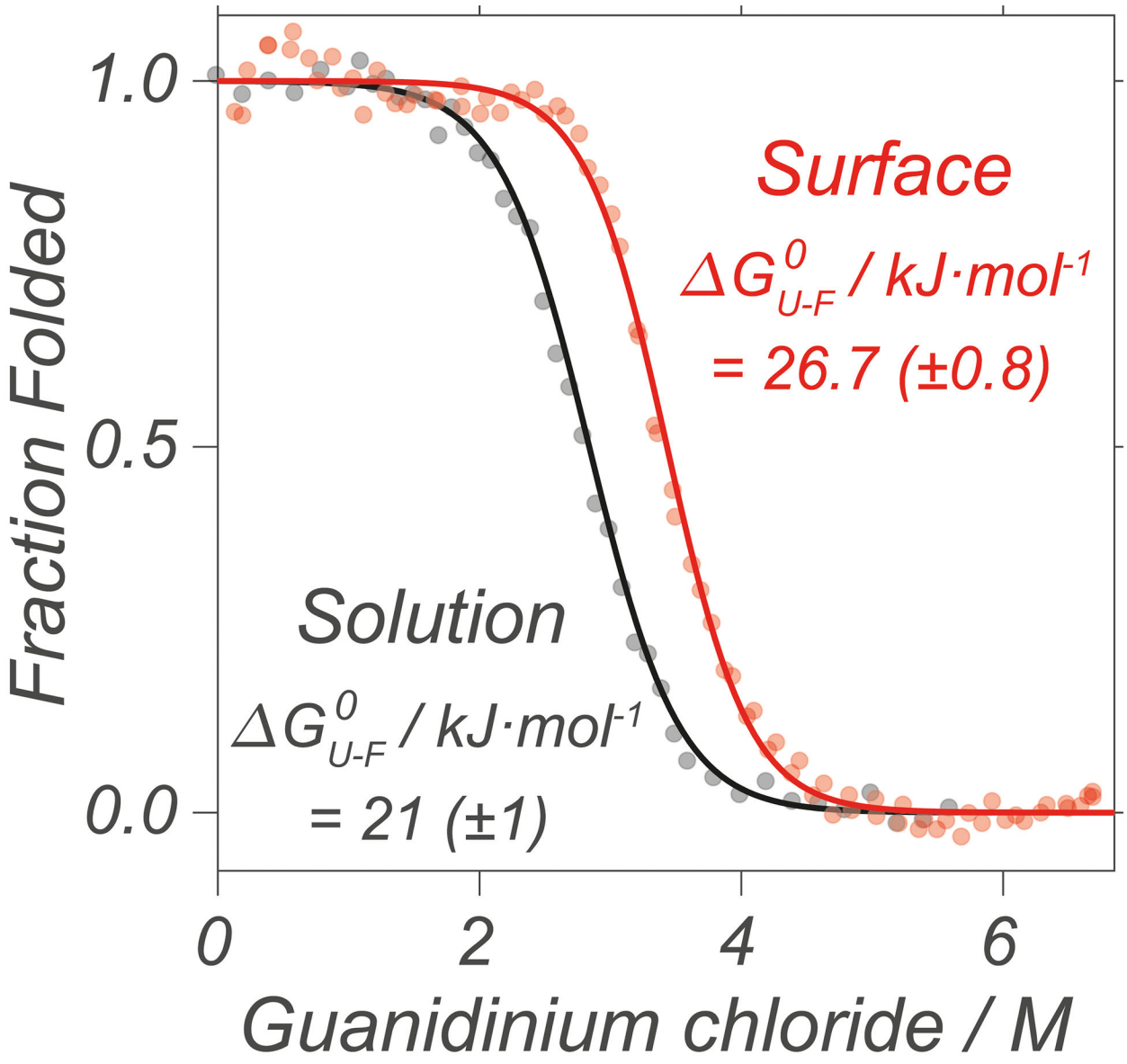

Figure 2.

Attachment to the hydroxyl-terminated surface explored here increases the stability of protein L from 21±1 to 26.7±0.8 kJ·mol^{-1}, as shown by the comparison between GuHCl denaturation curves obtained for the (MB-and-linker-modified) protein when free in bulk solution and when site-specifically attached to the surface (the experimental data for the surface denaturation, in red, includes consecutive unfolding and refolding events). Solid lines represent fits to a two-state equilibrium-unfolding model.[17] Shown are experimental values converted to fraction folded for a more straightforward comparison between the two experiments; see Figs. 1C and S6 for equivalent raw data.

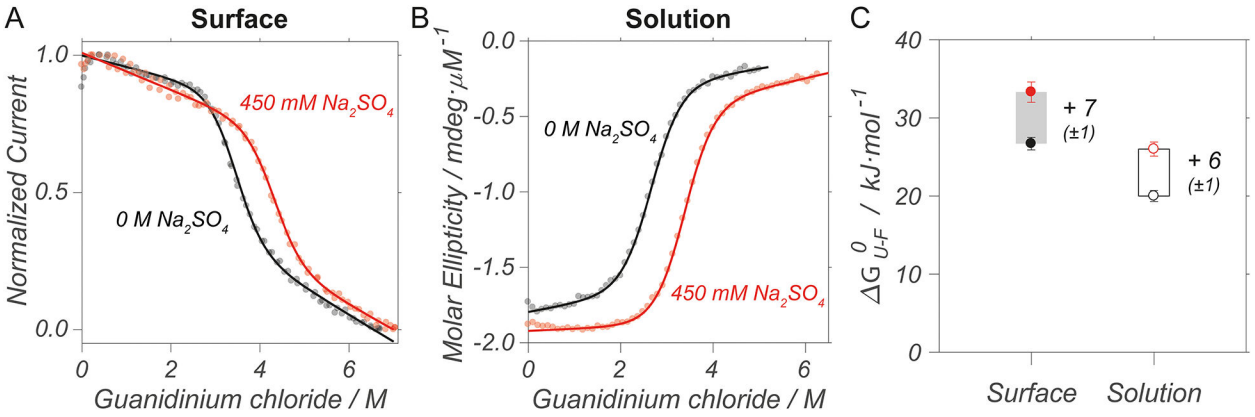

Figure 3.

(A) GuHCl denaturations in the absence (black) and presence (red) of 450 mM sodium sulfate indicate that this salt stabilizes surface-attached protein L. Points correspond to the normalized square-wave voltammetric peak currents, whereas solid lines represent best fits to a two-state folding equilibrium with sloped baselines. (B) Sulfate also stabilizes the unmodified protein when free in solution, as shown by circular-dichroism-monitored melts. (C) The extent of stabilization for the surface-attached protein (grey bar) is effectively indistinguishable from that seen when the protein is free in solution (white bar). Absolute unfolding free energies are shown for surface-bound (filled circles) and free-solution (open circles) protein L in the presence (red) and absence (black) of sodium sulfate.

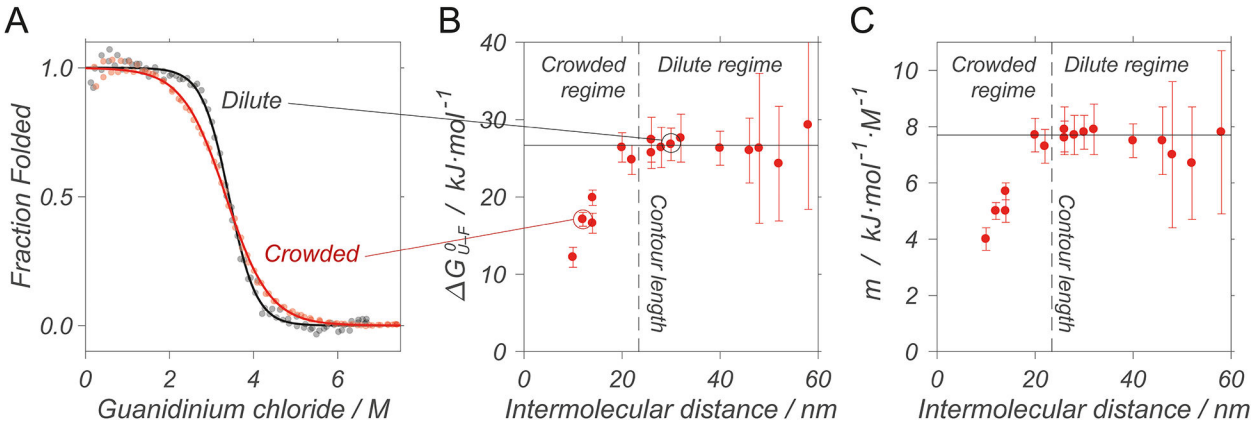

Figure 4.

(A) In the dilute regime (black), in which neighboring molecules on the surface do not interact, the calculated unfolding free energies of surface-attached protein L remain constant and we recover the *m*-value seen for the protein when free in solution (symbols correspond to experimental observations converted into fraction folded for ease of comparison; see Fig. S7 for the corresponding raw data). At higher packing densities (red), however, interactions between neighboring molecules cause the estimated unfolding free energies (B) and *m*-values (C) to become strong functions of packing density (shown here as a function of mean intermolecular separation). For scale, the broken vertical line represents the approximate contour length of a single 65-residues unfolded chain.[24] Here, error bars reflect 95% confidence intervals derived from the fitting error of individual electrodes (because of the difficulty of obtaining perfectly repeatable packing densities). The large error bars seen at large intermolecular distances arise due to the small currents produced by the low number of protein molecules present on the surface at highly dilute conditions.