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The effect of charged residue substitutions on the thermodynamics of protein-surface interactions

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Abstract

The interactions of proteins with surfaces are important in both biological processes and biotechnologies. In contrast to decades of study regarding the biophysics of proteins in bulk solution, however, our mechanistic understanding of the biophysics of proteins interacting with surfaces remains largely qualitative. In response, we have set to explore quantitatively the thermodynamics of protein-surface interactions. In this work, we explore systematically the role of electrostatics in modulating the interaction between proteins and charged surfaces. In particular, we use electrochemistry to explore the extent to which a macroscopic, hydroxyl-coated surface held at a slightly negative potential affects the folding thermodynamics of surface-attached protein variants with different composition of charged amino acids. Doing so, we find that attachment to the surface generally leads to a net stabilization, presumably due to excluded volume effects that reduce the entropy of the unfolded state. The magnitude of this stabilization, however, is strongly correlated with the charged-residue content of the protein. In particular, we find statistically significant correlations with both the net charge of the protein, with greater negative charge leading to less stabilization by the surface, and with the number of arginines, with more arginines leading to greater stabilization. Such findings refine our understanding of protein-surface interactions, providing in turn a guiding rationale to achieve the functional deposition of proteins on artificial surfaces for implementation in, for example, protein-based biotechnologies.

KEYWORDS

biophysics, biosensors, electrochemistry, electrostatics, monolayers, proteins

1 | INTRODUCTION

Interactions between proteins and the surfaces of membranes are ubiquitous in biology, where they contribute to many important processes, including membrane-induced activation of proapoptotic proteins,[1,2] recruitment of cytosolic proteins to cellular membranes for signaling purposes,[3,4] insertion of viral proteins into the cell membrane during infection,[5] and membrane shaping by membrane-curving proteins to facilitate endocytosis, intracellular trafficking, cytokinesis, and the shaping of organelles.[6] Conversely, the dysfunctional interaction of proteins with surfaces sometimes underlies pathologies, including the membrane-induced amyloidogenesis that occurs in some neurodegenerative diseases.[7,8] Protein-surface interactions are likewise important in many

biotechnologies, although here, too, the propensity of proteins to unfold on and irreversibly adsorb to artificial surfaces often limits such applications.[9–11] Understanding the mechanisms defining the interactions of proteins with surfaces would thus further both our understanding of biology and our ability to adapt biomolecules into artificial surfaces, with the latter in turn improving our success in implementing proteins in biosensors and other biotechnologies.[12,13]

To improve the mechanistic understanding of protein-surface interactions, we have previously characterized experimentally the thermodynamic consequences of biomolecule-surface interactions. Specifically, we have measured the extent to which site-specific attachment to macroscopic surfaces of well-defined chemistry alters the folding free energies of a number of proteins and nucleic acids.[14–19] In these studies, we found that excluded volume effects induced by the surface reduce the entropy of the unfolded state,[20] therefore significantly stabilizing surface-bound biomolecules (Figure 1a).[14,18] Conversely, we expect chemical and electrostatic interactions with the surface to be destabilizing, as the flexible unfolded state is free to adopt conformations that optimally satisfy such interactions (Figure 1b).[21,22] For example, given that the flexible unfolded state is free to expand away from the surface in response to an external electric field, we expect that electrostatic interactions between like-charged biopolymers and surfaces will be destabilizing. The net effect of the interaction of a given protein with a given surface thus results from the balance between excluded volume effects and the contribution from chemical or electrostatic interactions. Consistent with this, we have previously shown that electrostatic repulsion between the homogeneously negatively charged backbone of the DNA and negatively charged surfaces can be sufficiently destabilizing such that it overcomes the stabilizing contribution of the excluded volume.[14,17]

Although not as highly charged as DNA, proteins are, nevertheless, almost always charged, and thus we expect that electrostatics will also play an important role in their interactions with surfaces.[16,18] To explore this, here we have experimentally measured the extent to which interaction with a charged surface alters the folding free energy of protein variants differing in net charge and number of charged residues. Specifically, we have studied six charge-residue variants of the well-characterized, single-domain, two-state folding protein L.[18,23,24] Our rationale is that, by measuring the folding free energy of these variants when attached to a charged surface and comparing this to their stability when free in bulk solution, we will learn about the extent to which electrostatic interactions with the surface alter the protein's folding free energy.

2 | RESULTS

In our studies, we determine the extent to which attachment to a charged surface alter the stability of a set of six lysine-free, single cysteine variants of protein L, each with a different composition of charged amino acids (Table 1). To do so, we compare the proteins' folding free energy when surface-attached and when free in bulk solution. Taking the difference between these two stabilities corrects for any effects the amino acid substitutions may have on the intrinsic stability of the protein, thus providing an estimate of the thermodynamic consequences of surface-attachment itself.

We determine the folding thermodynamics of surface-attached proteins using a previously described electrochemical approach.[16,18] This involves using site-directed

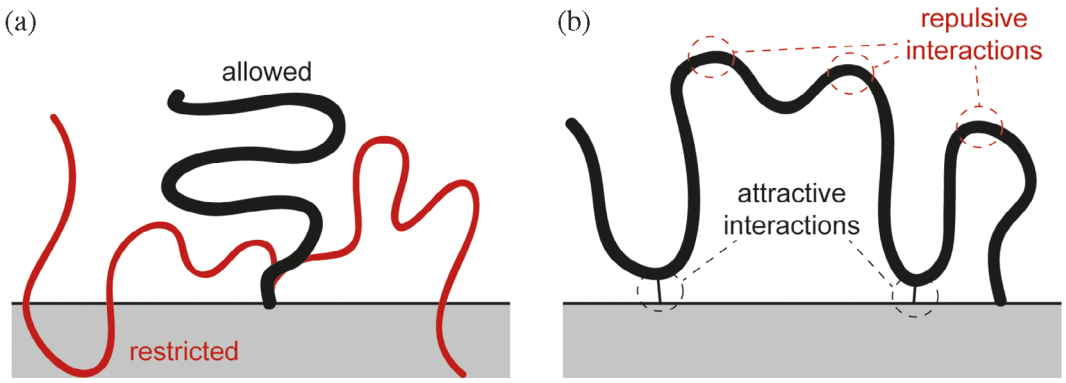

FIGURE 1 Thermodynamic contributions to protein-surface interactions. (a) Surface-attached biopolymers are stabilized by excluded volume effects reducing the conformational entropy of their unfolded state. (b) Conversely, any chemical or electrostatic interactions with the surface presumably stabilize the unfolded state, as its greater flexibility allows it to maximize attractive effects while simultaneously minimizing any repulsive effects, and thus destabilize the protein

mutagenesis to introduce a unique cysteine, and to remove all lysines to render the amino terminus the sole free amino group. We then modify the cysteine's thiol with a methylene blue redox reporter and the amino terminus with a seven-carbon alkane thiol, the latter of which we use to site-specifically attach the protein to a gold electrode (Figure 2a). Finally, we treat the electrode's surface with 6-mercaptohexanol to form a homogeneous, hydroxyl-terminated self-assembled monolayer, which adopts a slight negative charge (estimated to be −50 mV) at the potential applied to interrogate the methylene blue redox reporter.[25] Using square-wave voltammetry, we then monitor the protein's conformational state during a guanidinium chloride "melt" (Figure 2b), from which we extract its folding free energy. In parallel, using circular dichroism we monitor the protein's conformational state during the analogous chemical denaturation experiment in bulk solution, from which we determine its folding free energy when free in bulk solution.[26]

In earlier work, we showed that attachment to the hydroxyl-terminated surface employed here stabilized a lysine-free "parent" protein L variant, in which we substituted all lysines by arginines to maintain the same net charge and charge distribution as the wild type protein.[18,19] To explore the origins of this stabilization, here we have characterized five additional protein L variants (Tables 1 and S1, Figure S1), each varying in the composition and distribution of charged amino acids, and measured the extent to which attachment to the same hydroxyl-terminated surface alter their folding free energy. We selected these variants to cover a range of net charges while also retaining the solubility, stability, and reversible refolding (both when surface-attached and

TABLE 1 Variants of protein L differing in the number and pattern of charged-residues

Protein L	Net charge	Number of Arg[b]	Number of Asp + Glu	ΔG_U (sol)[a] (kJ mol^{-1})	ΔG_U (sur)[a] (kJ mol^{-1})	$\Delta\Delta G_U$[a] (sol-sur) (kJ mol^{-1})
Rx6Q	−10	0	10	13.6 ± 0.7	12 ± 3	−2 ± 3
Rx2E	−8	4	12	20 ± 1	24.4 ± 0.8	+4 ± 2
parent	−4	6	10	20.0 ± 0.7	26.7 ± 0.8	+7 ± 1
Ex2R	0	8	8	16.4 ± 0.6	27 ± 1	+10 ± 2
DxN/Ex3Q	0	6	6	17.2 ± 0.9	24 ± 2	+7 ± 2
DxR/Ex2R	+2	9	7	16 ± 2	23.9 ± 0.9	+8 ± 2
Nx2D[c]	−6	6	12	15 ± 1	Not determined	Not determined
Nx3D/QxE[c]	−8	6	14	9 ± 2	Not determined	Not determined

[a]Uncertainties reported correspond to 95% confidence intervals.
[b]All variants were rendered lysine-free to ensure site-specific surface attachment through the amino terminus.
[c]These two variants did not produce an unfolding sigmoid on the surface, and thus we could not determine their unfolding free energies.

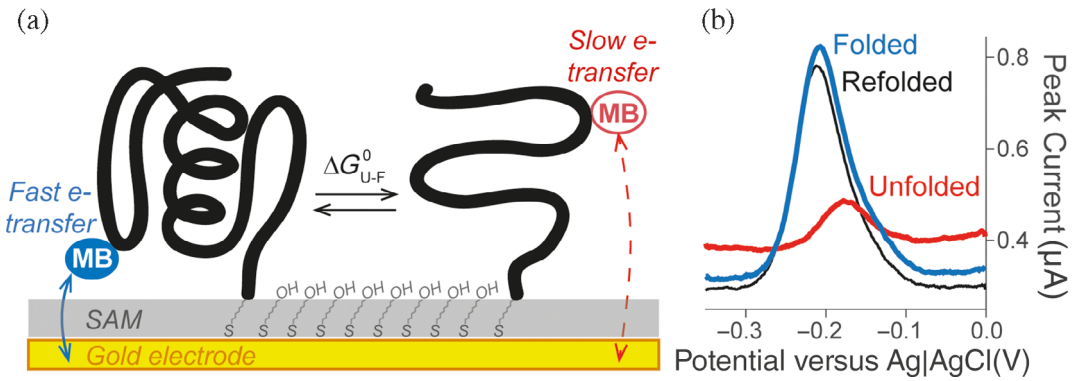

FIGURE 2 Experimental measurement of the folding thermodynamics of surface-attached proteins. (a) To measure the extent to which surface attachment affects a protein's folding free energy, we modified its single cysteine with a redox reporter (methylene blue [MB]) and tethered its amino terminus to a gold electrode coated with a self-assembled-monolayer of 6-mercaptohexanol. Upon unfolding, the methylene blue moves further away from the surface and the electron transfer rate decreases. (b) The square-wave peak current measured at 600 Hz thus decreases as unfolding progresses, allowing us to monitor the folding of the surface-attached protein

when free in bulk solution) necessary to characterize their folding thermodynamics.

While surface attachment is generally stabilizing, the extent of this stabilization correlates with the net charge of the protein. In particular, as the variants become more negatively charged, attachment to the surface becomes less stabilizing. For example, whereas surface attachment stabilizes the -4-charged parent sequence by 7 $\pm$ 1 kJ mol^{-1} (Figure 3a), a variant in which two arginines are replaced by glutamates to produce a net charge of -8 is stabilized by only 4 $\pm$ 2 kJ mol^{-1} (Figure 3b). Further increasing the negative charge to -10 via the substitution of all positively charged residues by neutral glutamines completely eliminates the stabilizing effect of surface attachment on the stability of the protein, producing a free energy change that, at -2 ± 3 kJ mol^{-1}, is within error of zero (Figure 3c). Conversely, a slightly positively charged variant (net charge +2) is stabilized by surface attachment by 8 $\pm$ 2 kJ mol^{-1} (Figure 3f). This variant thus appears to be slightly more stabilized by the surface than the parent sequence.

While the above studies investigated the extent to which changes in net charge alter the stability of surface-attached protein L, we have also investigated the extent to which the specific pattern of charged residues modulates this effect. To do so, we generated two variants of zero net charge that differ in the number and positioning of charged residues. Specifically, in one variant we introduced four charge-ablating glutamate-to-glutamine and aspartate-to-asparagine substitutions, while in the second we introduced two charge-reversing glutamate-to-arginine substitutions (Tables 1 and S1). Consistent with the trend described above, attachment to our surface stabilizes both of these net-neutral proteins. The magnitude of this stabilization, however, differs. Specifically, surface attachment stabilizes the charge-ablated variant by 7

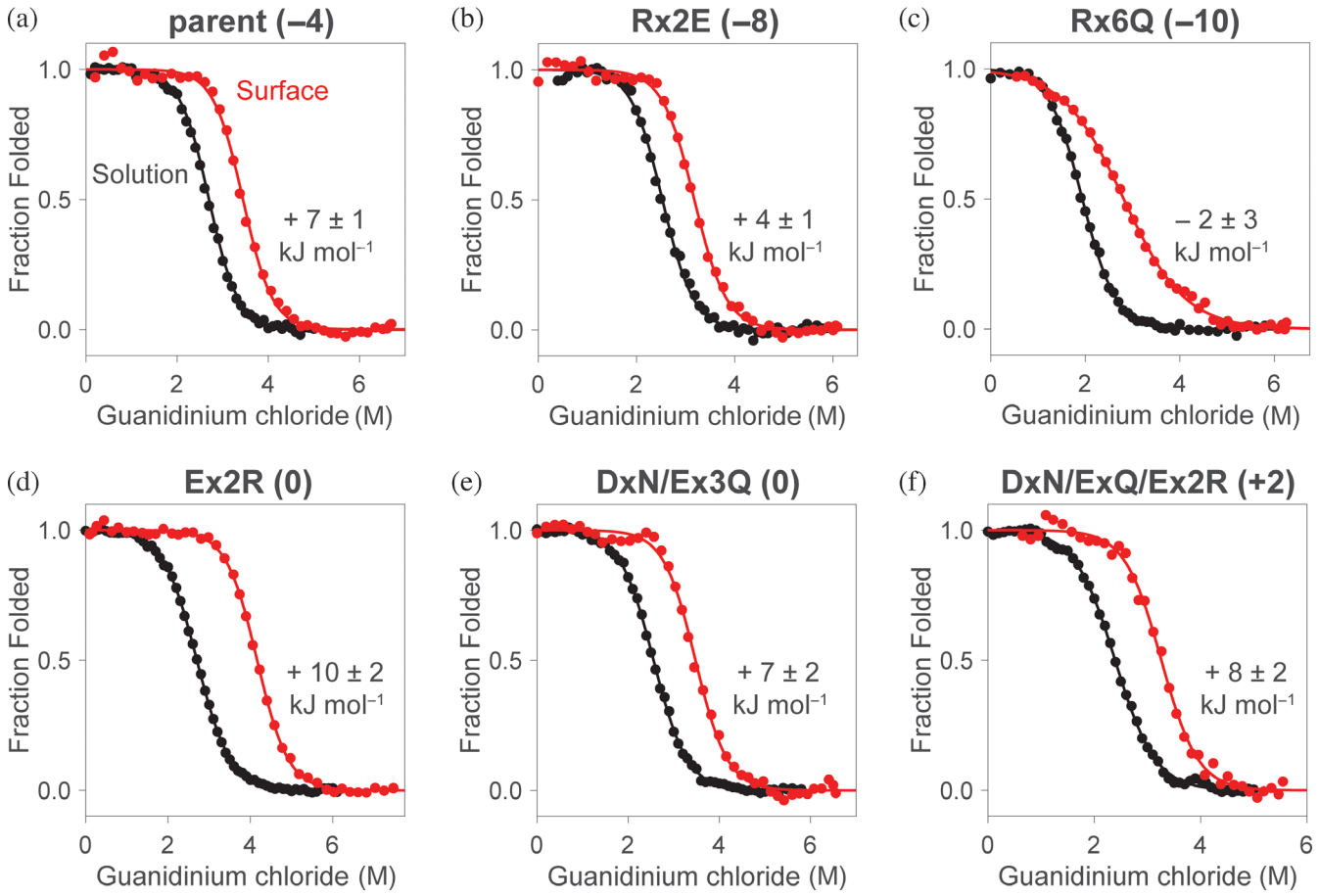

FIGURE 3 Effect of surface attachment on the stability of protein L charge variants. Comparison of the denaturation of protein L charge variants (net charge indicated in parenthesis) when surface-attached (red) and when free in solution (black) informs on the extent to which attachment to the surface affects their stabilities. While surface attachment stabilizes the parent sequence (a), the magnitude of this stabilization is reduced for a more negatively charged variant (b), and effectively eliminated for a homogeneously (and highly) negatively charged variant (c). Consistent with this, surface attachment stabilizes two neutral variants (d, e) and a net positively charged variant (f) as much or more than it does the parent sequence

± 2 kJ mol^{-1} (Figure 3e), whereas it stabilizes the charge-reversal variant by 10 ± 2 kJ mol^{-1} (Figure 3d), which is the largest surface-induced stabilization we have observed. It thus appears that the number and/or spatial distribution of charged residues also contributes to defining the extent to which attachment to this surface affect protein stability.

In a belief that negative results are also informative, we also note our unsuccessful attempts to characterize the effect of surface attachment on the stability of two other protein L variants, one of net charge −6 (Nx2D) and one of net charge −8 (Nx3D/QxE). That is, despite refolding reversibly in bulk solution, neither variant did so when surface-attached (Figure S2). Of potential relevance, these variants contain more charged residues (18 and 20, respectively; Table 1) than any of the other variants we characterized. This perhaps indicates that unfolded states presenting a large number of charged residues form significant electrostatic interactions with a charged surface, thus favoring adhesion. To explore this, we measured the rates of electron transfer from these two variants in the absence and presence of denaturant using the approach of Komorsky-Lovrić and Lovrić.[27] Doing so, we observe that, while under native conditions the transfer rates of the parent sequence and both variants are quite similar, the transfer rates of the denatured variants (30 s^{-1}; Figure S3) are ∼6 times more rapid than those of the denatured parent sequence (5 s^{-1}).[18] This suggests that, under denaturing conditions, these highly charged chains remain close to the electrode surface. If correct, this is consistent with our initial hypothesis (Figure 1b) that both attractive and repulsive electrostatic or chemical interactions would be better accommodated by the more flexible unfolded state, thus destabilizing proteins on surfaces.

The extent to which attachment to our hydroxyl-coated surface alters the stability of our negatively charged and neutral variants correlates with their overall net charge ($R^2 = 0.76$; Figure 4). Moreover, this correlation with net charge is seen despite the fact that charge patterning also appears to contribute to the effect; compare, for example, the differing stabilization seen for our two net neutral variants. We presume that this relationship between protein net-charge and surface-induced stabilization arises due to repulsive electrostatic interactions between the negatively charged variants and the surface, which assumes a negative charge at the redox potential of methylene blue. Specifically, and as noted above, the potential of zero charge for our surface is such that, at the redox potential of methylene blue, the surface adopts a potential of −50 mV.[25]

The charge-stabilization correlation we observe for our protein L variants may also hold for other proteins.

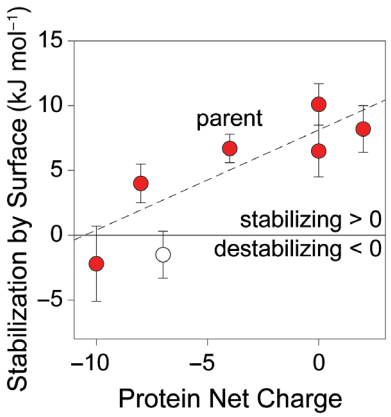

FIGURE 4 The extent to which surface-attachment alters the stability of protein L variants correlates with their net charge. While surface attachment stabilizes the parent protein L sequence (net charge −4), two net uncharged variants and a slightly positively charged variant are equally or more stabilized. In contrast, as the proteins become more negative, the net effect of surface attachment becomes less stabilizing and, eventually, negligible. The extent to which surface attachment affects the stability of protein L shows a linear correlation ($R^2 = 0.76$) with the overall net charge of the protein. This overall trend qualitatively holds for other proteins, such as the unrelated protein FynSH3 (white circle; excluded from the linear regression, data taken from ref. 16), which, at 61 residues, is of similar size to the 65-residue protein L

Specifically, we have previously measured the extent to which attachment to the same hydroxyl-coated surface affects the stability of FynSH3, an unrelated monomeric protein of 61 residues (i.e., only four residues shorter than Protein L), under similar experimental conditions.[16] The charge-stabilization relationship we observe for protein L would predict that, given FynSH3's −8 net charge, surface attachment should only stabilize this protein by 2 kJ mol^{-1}, a value reasonably close to the slightly destabilizing 1.5 ± 1.8 kJ mol^{-1} we have previously measured (Figure 4, open circle). The effects we observe may thus hold across unrelated proteins.

The intercept of the linear relationship between stabilization and net charge matches our expectations of the physics of this system. Specifically, this value provides an estimate of the interaction free energy at zero net charge, when the electrostatic contributions are minimized and thus any other contributions dominate. In particular, we expect a significant contribution from the surface-induced excluded volume restriction of the unfolded state's conformational entropy.[14,18,20,28,29] Consistent with this, the 9 ± 1 kJ mol^{-1} value we observe is consistent with the 8.6 kJ mol^{-1} estimate of this effect for an unfolded 65-bead (equivalent in length to protein L) self-avoiding random walk polymer

tethered by one end to an inert surface.[30] To provide a physical intuition of the magnitude of this effect, we can also express it in terms of the fraction of conformations restricted by the surface. In the case of an ideal, inert chain, the loss of entropy is proportional to the fraction of restricted conformations (P_R) as defined by the equation $\Delta S = -R \ln (1 - P_R)$.[31] The 9 kJ mol^{-1} stabilization we observe is thus equivalent to a decrease of 30 kJ K^{-1} mol^{-1} in the entropy of the unfolded state (at 298 K), which is in turn equivalent to a restriction of 97% of its conformations.

In contrast to the intercept of the correlation between stabilization and net charge, the slope, which reflects the energetics of the interaction per unit of charge on the protein, is larger than expected. Specifically, the slope's value of 0.8 ± 0.2 kJ mol^{-1} of elemental charges is equivalent to the energy required to move one elemental charge across a 9 mV potential drop. Under the experimental conditions we have employed (−50 mV net surface potential; 3 M ionic strength at the onset of the guanidinium-induced unfolding), this is equivalent to bringing a charge from the 2.6 nm radius of gyration of the unfolded state to within just one residue length (0.4 nm) of the surface,[32] with the latter distance being notably shorter that the 1.5 nm radius of gyration of folded protein L.[33,34]

Given the unexpectedly large slope on the correlation between the protein net charge and the surface-induced stabilization, we also explored other parameters that might correlate with the stabilization of our variants, such as the number and type of charged residues, the intrinsic protein stability in bulk solution, or the change in accessible surface area (Figure S4). In doing so, we observe an even stronger correlation ($R^2 = 0.98$) between the number of arginine residues (the only positively charged residues in these variants) and the stabilization induced by surface attachment (Figure 5). We do not, however, find an opposing correlation between this stabilization and the number of negatively charged residues (Figure S4b). Taken together, these data suggest a potential alternative hypothesis. Given the negative potential of our experimental surface, an electric double layer of ions forms, in which interleaved layers of cations and anions approach the surface to neutralize its charge.[32] Under our experimental conditions, guanidinium is the primary cation present. Given this, we speculate that the guanidinium headgroups in the protein's arginine side chains (the only positively charged side chains in our variants) may also be participating in the formation of the dense layer of guanidinium cations on the surface. This "stapling" of the arginines to the surface would likely reduce the entropy of the unfolded state and, in turn, stabilize the protein. Put in quantitative terms, the slope of 1.4 kJ mol^{-1} per residue translates into a loss of 43% of the unfolded conformations for each additional arginine side chain.

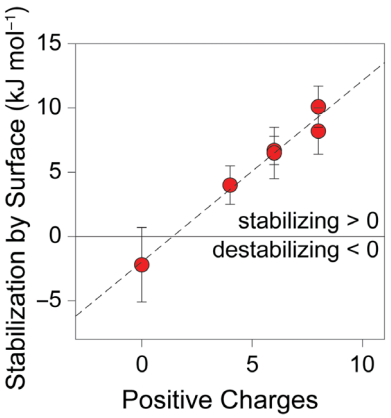

FIGURE 5 The effect of surface-attachment on the stability of protein L variants is strongly correlated with the total number of positively charged residues. Protein variants with a higher number of arginines (which are the only positively charged residues in these lysine-free variants) exhibit greater stabilization upon surface attachment, with a correlation coefficient (R^2) of 0.98 across the set of protein L variants we have characterized

3 | DISCUSSION

Here we have explored the extent to which altering the number and pattern of charged residues affects the stability of protein L when interacting with a macroscopic, charged surface. In doing so, we have found that the composition of charged amino acids significantly affects the stability of surface-attached proteins in a manner that is, at a first approximation, compatible with direct coulombic interactions. Specifically, while proteins are generally stabilized by attachment to a surface due to excluded volume effects, our data shows a correlation between the magnitude of this stabilization and the net charge of the protein, with more negatively charged variants increasingly less stabilized by the surface. This suggests a destabilizing electrostatic repulsion between negatively charged proteins and the likewise negatively charged surface. We also observe, however, a more significant correlation between surface-induced stabilization and the number of arginine residues, which are the only positively charged side chains in our constructs. This suggests an alternative hypothesis: that the conformational restriction and concomitant reduction in the entropy of the unfolded state arising due to the migration of the protein's positively charged arginines into the electric double layer could be underlying the observed stabilization effects.

Regardless of the specific mechanisms underlying these correlations, these results, along with our prior studies on the effect of crowding and intermolecular interactions,[18,19] provide a more detailed view on the contributions that determine the interaction of proteins with surfaces, illustrating how the biophysics of proteins in the vicinity of interfaces differ from those in bulk solution. From a more practical perspective, these studies also provide some rationale to guide the deposition of proteins on artificial surfaces. We have shown, for example, that optimizing the protein packing density on the surface, or selecting certain types of cosolutes and surface chemistries, can affect the stability and interactions of surface-attached proteins.[18,19] Now, in this work, we show that by optimizing the composition of charged residues we can modulate the effect of the surface on the stability and folding reversibility of surface-attached proteins. Our results suggest, for example, that proteins with a high net charge become more destabilized on like-charged surfaces, and that an excess of charged residues is detrimental for the reversibility of the folding. We believe that these observations will facilitate the functional deposition of proteins on artificial surfaces, in turn improving our success at implementing proteins in biotechnologies, such as biocompatible materials and biosensors.

4 | MATERIALS AND METHODS

4.1 | Reagents

Sodium chloride, sodium hydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide, guanidinium chloride, tris(2-carboxyethyl)phosphine (TCEP), and dithiothreitol (DTT) were used as received from Fisher Scientific (Waltham, MA). Dimethyl sulfoxide, ethanol, sulfuric acid, and 6-mercapto-1-hexanol (97%) were used as received from Sigma Aldrich (St. Louis, MO). BL21 (DE3) Competent *Escherichia coli* cells were purchased from New England Biolabs (Ipswich, MA). Plasmids encoding for recombinant proteins were purchased from GeneScript Biotech (Piscataway, NJ). Maleimide-modified Methylene Blue (ATTO MB2, Product No. AD MB2) was purchased from ATTO-TEC (Siegen, Germany) and dissolved at 1 mg per 50 μl of dimethyl sulfoxide prior to use.

4.2 | Protein purification

We employed six lysine-free, single cysteine variants of protein L, each with a different composition of charged amino acids (Table S1, Figure S1). We purified these following previously described protocols,[18,19] but introducing some modifications for the more aggregation-prone variants. In brief, we transformed *E. coli* BL21 (DE3) competent cells with plasmids encoding for the proteins, induced overexpression with isopropyl β-D-1-thiogalactopyranoside for 4 hr at 37°C, harvested the cells by centrifugation, resuspended the pellet in buffer (20 mM sodium phosphate, pH 7.0, 130 mM NaCl) with 1 mM DTT, and stored the resuspended pellets at −20°C. We then lysed the cells by sonication, and purified the proteins from the cell lysate by 10 min of thermal shock at 75°C followed by size exclusion chromatography (HiPrep 26/60 Sephacryl-S100; GE Healthcare Life Sciences, Marlborough, MA). For the positively charged and the two neutral variants, which tend to form inclusion bodies and aggregate, we completely resuspended the cells in buffer with 6 M guanidinium chloride and 1 mM DTT. We then performed a fast refolding by slowly diluting the resuspended pellet in a 100-fold larger volume of buffer with 1 mM DTT, separated the aggregates by centrifugation, and purified the resulting supernatant by size-exclusion chromatography. For the positively charged variant, we performed an additional ion exchange chromatography (HiTrap Q FF; GE Healthcare Life Sciences) to remove nucleic acid contamination. Pure protein fractions were identified by sodium dodecylsulphate-polyacrylamide gel electrophoresis and used as is for solution experiments.

4.3 | Protein modification for surface attachment and electrochemical monitoring

To measure the proteins' unfolding free energy on the surface, we followed previously described protocols to modify their free cysteine with a redox reporter methylene blue, and their amino terminus with a surface linker.[16,18] Specifically, we reacted ∼1 ml of 0.1–1 mM protein (previously incubated with 1 mM TCEP) with a fivefold excess of maleimide-modified methylene blue redox reporter (in buffer 20 mM sodium phosphate, pH 7.0, 130 mM NaCl, overnight, at room temperature, protected from light, under gentle stirring). We then removed the unreacted methylene blue by performing dialysis, first against buffer with 8 M urea and then against buffer without urea. We then diluted the dialysate in buffer with 1.2 M ammonium sulfate and separated the unreacted protein via hydrophobic interaction chromatography (HiTrap Phenyl HP; GE Healthcare Life Sciences) with a gradient from 1 to 0 M ammonium sulfate. Next, we reacted the methylene-blue modified protein (10–100 μM) with a 50-fold excess of surface linker

(7-mercaptosulfosuccinimidyl heptanoate synthesized in house[16]) in buffer (same conditions as above, except for the positively charge variant, in which this and subsequent steps were performed in the presence of 6 M guanidinium chloride to avoid aggregation), followed by dialysis to remove the excess of unreacted linker. The resulting methylene blue-and-linker-modified protein was then aliquoted and stored at −80°C until use.

4.4 | Chemical denaturation experiments on the surface

To measure the unfolding free energy of our proteins when surface-attached, we first deposited them on the surface of a gold electrode coated with an alkane-thiol self-assembled-monolayer following previously described protocols. First, we performed a series of mechanical and electrochemical cleaning steps[35] on 2 mm gold disk electrodes (CH Instruments, Inc., TX), before functionalizing its surface with the methylene blue-and-linker-modified variant of protein L[18] (for the positively charged variant, we carried out this step in the presence of 6 M guanidinium chloride). Finally, we formed a hydroxyl-terminated self-assembled-monolayer by incubating (overnight, room temperature) the electrodes in buffer with 5 mM 6-mercapto-1-hexanol. After a series of chemical and electrochemical preparation steps, we used cyclic voltammetry to measure the packing density of the proteins on the surface, ensuring we sample a "dilute regime" in which the distance between individual molecules is, on average, larger than the protein's contour length to avoid intermolecular crowding effects.[18]

We then used electrochemistry to track the unfolding of the proteins over the course of guanidinium chloride melts.[16,18] To do so, we performed two consecutive unfolding and refolding guanidinium denaturation experiments using an automatic titrator (Microlab® 500 Series; Hamilton Co., NV, and a BASi C3 Cell Stand; Bioanalytical systems, Inc., West Lafayette, IN), allowing 10 min for complete mixing and equilibration. We buffered our solutions with 20 mM sodium phosphate at pH 7.0, 500 mM NaCl, including 50 μM of 6-mercapto-1-hexanol to prevent monolayer degradation. At each guanidinium concentration, we monitored protein folding by measuring square-wave voltammetry scans between 0 and −400 mV, at a square-wave amplitude of 600 Hz and a frequency of 25 mV (CHI684 potentiostat and CHI660D multiplexer; CH Instruments, Inc.), and then extracted the square-wave peak current as a function of denaturant concentration by using in-house coded Matlab® processing scripts. Reported uncertainties for all measurements performed on surface-attached proteins correspond to 95% confidence intervals derived from experimental replicates carried out on a minimum of four independently fabricated electrodes.

4.5 | Chemical denaturation experiments in bulk solution

We used circular dichroism to monitor the chemical denaturations and determine protein unfolding free energy in bulk solution. For this, we employed an initial volume of 1.7 ml of 5 μM unmodified protein L variant in buffer 20 mM sodium phosphate, pH 7.0, 0.1 mM DTT, 500 mM NaCl. We denatured the protein by adding a guanidinium chloride solution of the same protein and buffer concentration (using a JASCO AST-530 automatic titrator; JASCO, MD) in a quartz cuvette of 1 cm path length, allowing 1 min for mixing and equilibration. At each guanidinium concentration, we measured a wavelength scan between 210 and 250 nm (scan speed 50 nm min^{-1}; bandwidth 4 nm; digital integration time 4 s; performed on a J-1500 Spectrophotometer; JASCO) and followed the chemical denaturation by monitoring the ellipticity at 222 nm. Reported uncertainties for these bulk-solution-phase studies correspond to 95% confidence intervals derived from the error of the fits.

4.6 | Analysis of chemical denaturations

To determine the stability of our protein variants, we analyzed the chemical denaturations by using a two-state folding equilibrium model.[26] In particular, we performed a least-square minimization of our circular dichroism (for bulk solution proteins) and square-wave voltammetry (for surface-attached proteins) data to the following equation using in-house developed Matlab® analysis scripts:

$$I_{\text{obs}} = \frac{I_{\text{F}}(0) + m_{\text{F}}[\text{GuHCl}] + (I_{\text{U}}(0) + m_{\text{U}}[\text{GuHCl}])\, e^{\frac{-\Delta G^0_{\text{U}}(0) - m_G[\text{GuHCl}]}{RT}}}{1 + e^{\frac{-\Delta G^0_{\text{U}}(0) - m_G[\text{GuHCl}]}{RT}}} \quad (1)$$

where the observed signal, I_{obs}, follows a hyperbolic tangent function of denaturant concentration, [GuHCl], the protein's unfolding free energy in the absence of denaturant, $\Delta G^0_U(0)$, the linear dependence of the protein's unfolding free energy on denaturant concentration, m_G, the intrinsic signal of the folded and unfolded states, $I_F(0)$ and $I_U(0)$, and the linear dependence of these on denaturant concentration, m_F and m_U.

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REFERENCES

1. Serra-Batiste M, Ninot-Pedrosa M, Bayoumi M, Gairí M, Maglia G, Carulla N. Aβ42 assembles into specific β-barrel pore-forming oligomers in membrane-mimicking environments. Proc Natl Acad Sci U S A. 2016;113:10866–10871.
2. Wicky BIM, Gupta K, Kwan TOC, Robinson CV, Clarke J. Spontaneous oligomerization of BAK/BAX is suppressed by hetero-dimerization with MCL-1. bioRxiv. 2019;756874.
3. Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature. 1984;308:693–698.
4. Cho W, Stahelin RV. Membrane-protein interactions in cell signaling and membrane trafficking. Annu Rev Biophys Biomol Struct. 2005;34:119–151.
5. Yang ST, Kiessling V, Simmons JA, White JM, Tamm LK. HIV gp41-mediated membrane fusion occurs at edges of cholesterol-rich lipid domains. Nat Chem Biol. 2015;11:424–431.
6. Simunovic M, Šarić A, Henderson JM, Lee KYC, Voth GA. Long-range organization of membrane-curving proteins. ACS Cent Sci. 2017;3:1246–1253.
7. Butterfield SM, Lashuel HA. Amyloidogenic protein–membrane interactions: Mechanistic insight from model systems. Angew Chem Int Ed Engl. 2010;49:5628–5654.
8. Lashuel HA, Overk CR, Oueslati A, Masliah E. The many faces of alpha-synuclein: From structure and toxicity to therapeutic target. Nat Rev Neurosci. 2013;14:38–48.
9. Butler JE, Ni L, Brown WR, et al. The immunochemistry of sandwich ELISAs—VI. Greater than 90% of monoclonal and 75% of polyclonal anti-fluorescyl capture antibodies (CAbs) are denatured by passive adsorption. Mol Immunol. 1993;30:1165–1175.
10. Dubiel EA, Martin Y, Vermette P. Bridging the gap between physicochemistry and interpretation prevalent in cell–surface interactions. Chem Rev. 2011;111:2900–2936.
11. Meyers SR, Grinstaff MW. Biocompatible and bioactive surface modifications for prolonged in vivo efficacy. Chem Rev. 2012;112:1615–1632.
12. Rana D, Matsuura T. Surface modifications for antifouling membranes. Chem Rev. 2010;110:2448–2471.
13. Kurnik M, Pang EZ, Plaxco KW. An electrochemical biosensor architecture based on protein folding supports direct real-time measurements in whole blood. Angew Chem Int Ed. 2020;59:18442–18445.
14. Watkins HM, Vallee-Belisle A, Ricci F, Makarov DE, Plaxco KW. Entropic and electrostatic effects on the folding free energy of a surface-attached biomolecule: An experimental and theoretical study. J Am Chem Soc. 2012;134:2120–2126.
15. Watkins HM, Simon AJ, Ricci F, Plaxco KW. Effects of crowding on the stability of a surface-tethered biopolymer: An experimental study of folding in a highly crowded regime. J Am Chem Soc. 2014;136:8923–8927.
16. Kurnik M, Ortega G, Dauphin-Ducharme P, Li H, Caceres A, Plaxco KW. Quantitative measurements of protein–surface interaction thermodynamics. Proc Natl Acad Sci U S A. 2018;115:8352–8357.
17. Watkins HM, Ricci F, Plaxco KW. Experimental measurement of surface charge effects on the stability of a surface-bound biopolymer. Langmuir. 2018;34:14993–14999.
18. Ortega G, Kurnik M, Dauphin-Ducharme P, et al. Surface attachment enhances the thermodynamic stability of protein L. Angew Chem Int Ed. 2019;58:1714–1718.
19. Ortega G, Kurnik M, Gautam BK, Plaxco KW. Attachment of proteins to a hydroxyl-terminated surface eliminates the stabilizing effects of polyols. J Am Chem Soc. 2020;142:15349–15354.
20. Zhou HX, Dill KA. Stabilization of proteins in confined spaces. Biochemistry. 2001;40:11289–11293.

21. Knotts TA 4th, Rathore N, de Pablo JJ. An entropic perspective of protein stability on surfaces. Biophys J. 2008;94:4473–4483.
22. Levine ZA, Fischer SA, Shea JE, Pfaendtner J. Trp-cage folding on organic surfaces. J Phys Chem B. 2015;119:10417–10425.
23. Wikström M, Sjöbring U, Kastern W, Björck L, Drakenberg T, Forsen S. Proton nuclear magnetic resonance sequential assignments and secondary structure of an immunoglobulin light chain-binding domain of protein L. Biochemistry. 1993; 32:3381–3386.
24. Yi Q, Scalley ML, Simons KT, Gladwin ST, Baker D. Characterization of the free energy spectrum of peptostreptococcal protein L. Fold Des. 1997;2:271–280.
25. Rentsch S, Siegenthaler H, Papastavrou G. Diffuse layer properties of thiol-modified gold electrodes probed by direct force measurements. Langmuir. 2007;23:9083–9091.
26. Santoro MM, Bolen DW. Unfolding free energy changes determined by the linear extrapolation method. 1. Unfolding of phenylmethanesulfonyl alpha-chymotrypsin using different denaturants. Biochemistry. 1988;27:8063–8068.
27. Komorsky-Lovrić Š, Lovrić M. Measurements of redox kinetics of adsorbed azobenzene by "a quasireversible maximum" in square-wave voltammetry. Electrochim Acta. 1995;40:1781–1784.
28. Friedel M, Baumketner A, Shea J-E. Effects of surface tethering on protein folding mechanisms. Proc Natl Acad Sci U S A. 2006;103:8396–8401.
29. Friedel M, Baumketner A, Shea JE. Stability of a protein tethered to a surface. J Chem Phys. 2007;126:095101.
30. Chen YC, Luo MB. Dynamic Monte Carlo study on the probability distribution functions of tail-like polymer chain. J Zhejiang Univ Sci B. 2005;6:1130–1134.
31. Pickett SD, Sternberg MJ. Empirical scale of side-chain conformational entropy in protein folding. J Mol Biol. 1993;231: 825–839.
32. Bard AJ, Faulkner LR. Electrochemical methods: Fundamentals and applications. Hoboken, New Jersey, USA: John Wiley & Sons, 2001.
33. Plaxco KW, Millett IS, Segel DJ, Doniach S, Baker D. Chain collapse can occur concomitantly with the rate-limiting step in protein folding. Nat Struct Biol. 1999;6:554–556.
34. Yoo TY, Meisburger SP, Hinshaw J, et al. Small-angle X-ray scattering and single-molecule FRET spectroscopy produce highly divergent views of the low-denaturant unfolded state. J Mol Biol. 2012;418:226–236.
35. Xiao Y, Lai RY, Plaxco KW. Preparation of electrode-immobilized, redox-modified oligonucleotides for electrochemical DNA and aptamer-based sensing. Nat Protoc. 2007;2:2875–2880.

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