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Simple Interpretation of Ionization and Helix–Coil Stability Shift When a Polyelectrolyte Adsorbs

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Direct measurements of the ionization of poly-L-glutamic acid (PGlu) as it adsorbs onto a polymer brush of opposite electrical charge indicate a transition from expected enhanced ionization when the surface coverage is sparse to considerable suppression below the value in bulk solution when the average charge–charge spacing exceeds a critical level. As additional PGlu molecules adsorbed, the additional charge that they brought to the surface was balanced by their suppressed ionization, so that the average spacing between charged segments remained nearly constant. Charge–charge repulsion therefore limits the extent of ionization because the equilibrium between the ionized and protonated states responds to the magnitude of repulsion. Already present as a physical process in bulk solution, this becomes particularly prominent near a surface because the adsorbed state tolerates a higher charge concentration than the bulk solution tolerates without becoming insoluble. The α -helix conformation of this polypeptide in the adsorbed state is predictably destabilized (at low surface coverage) or stabilized (at high surface coverage). This study shows that generic features of charge regulation reduce surface ionization below the bulk value. This occurs when the charge–charge spacing reaches a critical level, about 7 Å in a polar aqueous environment, and shows that charge regulation occurs without the need to postulate changes of the local dielectric constant.

Introduction

Electrical charge of dissolved molecules is central to our understanding of aqueous solutions. It is therefore of fundamental significance to predict this quantity. The past decades have seen burgeoning experimental and theoretical interest in understanding the interactions between charged objects in aqueous solution,^{1–6} but this activity has focused on the situation where the electrical charge on a molecule is *permanent*.

This study focuses instead on ionization, which is electrical charge derived from the gain or loss of protons such that the electrical charge on a molecule is variable. Researchers have been aware for a long time, mainly on the basis of theoretical considerations, that local ionization near a surface could differ significantly from its value in bulk solution,^{7–10} because surface electric field influences ionization. There is a large body of recent theoretical work based on this idea.^{11–13} But progress has been impeded by the paucity of direct experimental evidence. The perturbed ionization and perturbed pK_a values of protein residues

are known and, traditionally, have been analyzed in terms of local chemical specificity and local dielectric constant.¹⁴ Regarding homopolymers, the study presented below suggests that generic features of charge–charge interaction come into play without the need to postulate changes of the local dielectric constant.

We are not aware of any previous direct measurements of the contrast between ionization in bulk solution and at a surface.

Experimental Section

The experiments were performed at the University of Illinois. Using Fourier transform infrared spectroscopy in attenuated total reflection (FTIR-ATR) mode, the mass adsorbed was quantified, using experimental protocols and calibration methods described elsewhere.¹⁵ The amount ionized was determined from the calibrated intensity of infrared vibrations characteristic of –COOH and –COO[–] functional groups on the polypeptide, PGlu. The temperature was 25.0 °C. Measurements were made in D₂O, rather than H₂O, because D₂O is so much more transparent to infrared radiation in the regions of interest to us.

In the discussion below, we will refer to solution pD, rather than pH, since the experiments were performed in D₂O not H₂O.

To produce the adsorbing surface, chains of polyvinylpyridine were end-grafted to form a brush configuration on a silicon ATR crystal that was previously coated with a hydrophobic self-assembled monolayer and then quaternized by chemical reaction with a methyl group, using methods described recently.¹⁶ The quaternized polyvinylpyridine, QPVP ($M_w = 12\,500\text{ g}\cdot\text{mol}^{-1}$, $M_w/M_n = 1.17$), was determined by IR spectroscopy to be 98% quaternized. The characteristic infrared vibrations of the charged unit were used to quantify, in situ during each experiment, the charge of the QPVP at the surface.

The positive ion in the buffer solutions was Na⁺. The pD was buffered at 5.5 by a buffer composed of 10 mM mixtures of Na₂HPO₄ and NaH₂PO₄·H₂O.

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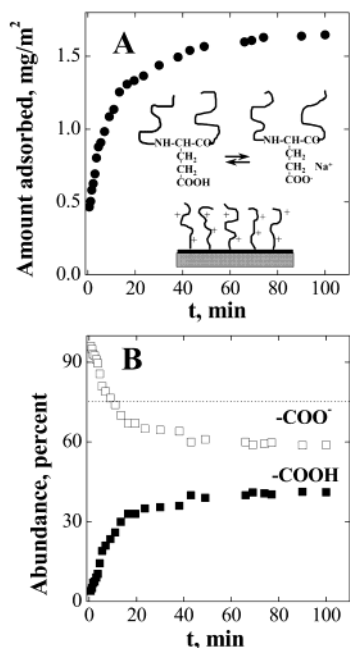


Figure 1. Changes of ionization during the course of adsorption in an illustrative experiment as poly-L-glutamic acid, PGLu, was allowed to adsorb onto brushes of quaternized polyvinylpyridine, QPVP, at pH = 5.5. Panel A shows the mass adsorbed as a function of time elapsed and includes a schematic diagram showing the QPVP brush surface and the chemical structure of the PGLu repeat unit, which was in equilibrium between the ionized and nonionized structures. Panel B shows the relative abundance of the ionized and nonionized PGLu plotted against elapsed time. Filled squares indicate COOH; open squares indicate COO⁻. The dotted horizontal line indicates the fraction of COO⁻ measured for PGLu in bulk solution at this buffer condition.

The poly-L-glutamic acid, PGLu, was purchased from Aldrich-Sigma and used as received. The PGLu (weight-average molecular weight $M_w = 14\,000\text{ g}\cdot\text{mol}^{-1}$, ratio of weight-average to number-average molecular weight $M_w/M_n = 1.15$) was exposed to end-tethered QPVP at several different levels of surface density, from 0.6 to 2.4 $\text{mg}\cdot\text{m}^{-2}$, as specified below. In adsorption experiments, its concentration in solution was usually 0.005 $\text{mg}\cdot\text{mL}^{-1}$. Figure 1 includes a schematic diagram showing the QPVP brush surface and the chemical structure, which was in equilibrium between the ionized and nonionized structures, of the PGLu repeat unit.

For experiments in which ionization in bulk solution was measured, the surface was an ATR crystal of oxidized silicon. As this surface is negatively charged at the pH (pD) studied, the negatively charged PGLu did not adsorb, so that the infrared spectra reflected the state of PGLu in solution.

Results and Discussion

A central observation that motivates this study is illustrated in Figure 1. As the negatively charged polypeptides adsorbed onto the initially bare surface of opposite charge (Figure 1A), their ionization began at a level *more* ionized than that in the bulk solution but ended *less* so (Figure 1B). How can one explain why the *sign* of the deviation from behavior in bulk solution changed as the surface became more densely covered?

Coverage-Dependent Ionization. It is known that polyelectrolyte complexes comprised of charges of opposite sign, such as (PGLu)/(QPVP), collapse densely against a solid surface.¹⁷ This is also consistent with the observed insolubility of solution mixtures of these polymers. In the

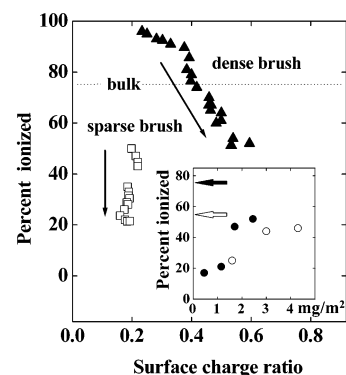


Figure 2. Percent of ionized PGLu adsorbed segments plotted against an inventory of surface charge, the surface charge ratio, which is defined as the ratio of negative charges from ionized PGLu to positive charges from the QPVP brush. Illustrative data are shown for a dense brush (2.4 $\text{mg}\cdot\text{m}^{-2}$, filled triangles) and for a sparse brush (1.3 $\text{mg}\cdot\text{m}^{-2}$, open squares). The dotted horizontal line shows the fraction of COO⁻ measured for PGLu in bulk solution at this pH = 5.5. The arrows indicate elapsed time during which the surface coverage increased. The inset shows percent ionization at saturated surface coverage plotted against the amount adsorbed (filled circles, pH = 5.5; open circles, pH = 4.65) as the brush surface coverage was varied over a wide range, 0.6 to 2.4 $\text{mg}\cdot\text{m}^{-2}$. Filled and open horizontal arrows in the inset denote the ionization characteristic of PGLu in bulk solution at the indicated pH, pH = 5.5 or 4.65.

adsorbed state of high local concentration, the COO⁻ charge of PGLu could in principle either form a complex with QPVP, (COO⁻)(QPVP⁺), or be neutralized by addition of a proton to form COOH. How can one distinguish between these possibilities?

To predict the point at which the latter might become significant, there is an analogy to make with the Bjerrum length that plays a fundamental role in theoretical analysis of electrostatic interactions in bulk aqueous solutions.¹⁻⁶ Energy balance shows that electrostatic repulsion equals thermal energy when $q^2/\epsilon_0\epsilon_r r_B = k_B T$. (Here q is the electric charge, ϵ_0 is the dielectric constant in a vacuum, ϵ_r is the dielectric constant of the medium relative to vacuum, k_B is the Boltzmann constant, T is the absolute temperature, and r_B is a critical length, the Bjerrum length.) At 25 °C in water, $r_B = 7.1\text{ Å}$. It has been much discussed that the unfavorable situation $r < r_B$ should result in “condensation” of permanent charges of opposite sign.^{1-6,18} To distinguish ion condensation from the present case, we emphasize that ion condensation between permanent charges is not observable in the vibrational spectrum, an observation which shows that a specific bond is not formed. However, the chemical association of H⁺ with COO⁻, to form COOH, is obvious in the infrared spectrum. Elsewhere we have shown illustrations of this infrared spectrum obtained in attenuated total reflection.¹⁹

An inventory of net surface charge is shown in Figure 2. Ionization of the carboxylic acid group is plotted against the ratio of negative charge from ionized PGLu to positive charge from the QPVP brush surfaces; the arrows indicate the direction of increasing time, during which continued adsorption caused the surface coverage to increase. Whereas a high density of surface charge (“dense brush”; see discussion below) was correlated with the sign change shown in Figure 1, a low density of surface charge (“sparse brush”) was correlated with negative deviations starting already at the least amount adsorbed. On the basis of the discussion below, we will quantify the brush surface

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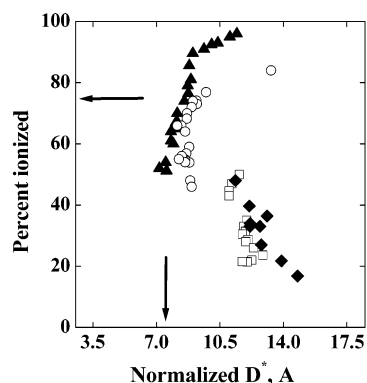


Figure 3. Percent of ionized PGlu adsorbed segments at $pD = 5.5$ plotted as a function of the estimated average charge–charge spacing between adsorbed PGlu segments, D^* . The data were normalized for the amount of PGlu adsorbed as described in the text and refer to the QPVP brush coverages $2.4 \text{ mg}\cdot\text{m}^{-2}$ (filled triangles), $1.3 \text{ mg}\cdot\text{m}^{-2}$ (open circles), $0.9 \text{ mg}\cdot\text{m}^{-2}$ (open squares), and $0.6 \text{ mg}\cdot\text{m}^{-2}$ (filled diamonds). The horizontal arrow points to the ionization characteristic of PGlu in bulk solution. The vertical arrow points to the Bjerrum length at which the charge–charge repulsive energy between monovalent ions equals the thermal energy in bulk aqueous solution.

coverage in terms of equivalent monolayers of densely packed segments. For the condition of saturated surface coverage, the inset of Figure 2 shows the ionization plotted against the amount adsorbed as the brush coverage was varied systematically. The data are similar at two values of pH. As the overall positive charge density at the surface varied by a factor of 4 in these experiments, with the QPVP concentration varying between 0.6 and $2.4 \text{ mg}\cdot\text{m}^{-2}$, it is evident that the overall surface charge can hardly explain the observed drop of ionization of the carboxylic acids. Instead, we looked at how local charge–charge interactions may result in charge regulation.

The following argument was used to calculate from the available data the average spacing between ionized groups on the adsorbed PGlu macromolecules. Considering that the adsorbed state is collapsed, it is reasonable to express the mass adsorbed in terms of the number of equivalent monolayers. In the study of monolayers at the air–water interface, it is conventional to calculate from surface coverage ($\text{mass} \times \text{area}^{-1}$) the average area per molecule.²⁰ In addition, it was necessary to take into account that the surface coverage in these experiments exceeded that of an equivalent monolayer in some cases and was less than this in other cases. To estimate the number of equivalent monolayers of adsorbed PGlu segments, the actual surface coverage was therefore normalized by the reference number $1 \text{ mg}\cdot\text{m}^{-2}$, knowing that full surface coverage, for molecular units whose size is comparable to that of the PGlu repeat unit, is approximately $1 \text{ mg}\cdot\text{m}^{-2}$.²⁰ On the basis of this normalization, the square root of the effective area per ionized group gave an estimate of the normalized spacing between ionized segments on the PGlu molecules. While the quantitative calculation is based on such strong assumptions that the resulting numbers are uncertain, we emphasize that the relative numbers are meaningful. Any other reasonable normalization to account for different surface coverage would also result in the qualitative conclusions discussed below.

In Figure 3, the observed ionization is plotted against this charge–charge spacing, D^* , for two cases where we expect different effective dielectric constants near the

surface. For a surface densely covered by brushes (high dielectric constant), we observe that enhanced ionization at low surface coverage turned to suppression at a well-defined point, $D^* \approx 7 \text{ \AA}$ for the assumptions made here. More important than the specific number is the more general conclusion that, as additional PGlu molecules adsorbed, the additional charge that they brought to the surface was balanced by their suppressed ionization, so that the average spacing between charged segments remained nearly constant (control experiments also verified the persistence of this effect at other values of pD .) This pattern described the ionization of PGlu adsorbed onto dense brushes and is consistent with the picture that charge was reduced to minimize electrostatic interactions.

Seeking to test the generality of this idea, we also sought to decrease the charge–charge separation by raising the solution concentration at constant pD . Our initial attempts to do this by raising the polyelectrolyte concentration were thwarted by its precipitation when concentration increased. To increase solubility, we sought to ask this same question of smaller molecules bearing a carboxylic acid, but even for the smallest of these, formic acid, we could not reach sufficiently high concentration in solution. Thus, it appears that the very high local concentrations of charge, studied in this paper, are produced in the adsorbed state but not in solution.

Influence of the Local Dielectric Constant. An alternative line of interpretation might be to the effect that the local dielectric constant decreased during the course of adsorption. However, the monotonic decrease of ionization, starting when the surface coverage was dilute, argues against explaining the effect this way. In addition, the inset of Figure 2 shows the ionization plotted against the amount adsorbed as the brush density was varied systematically under conditions of saturated PGlu adsorption. The data are similar at two values of pD , demonstrating the generality of this effect.

The differences between findings obtained using dense and sparse brushes likely stem from a reduced effective dielectric constant in the latter case. Even larger suppression of ionization, with enhanced charge–charge spacing, was observed for the sparse brushes. From the discrepancy, a factor of approximately 2, the magnitude of the local dielectric constant can be roughly estimated.

Shifted Helix–Coil Transition. Evidence has been building for some time that location at a surface can shift the stability of α -helix and α -sheet conformations of polypeptides, but the literature has been conflicting, since even the sign of the shift has depended on the system at hand.^{21–24}

The PGlu system is an attractive model system in which to study this. The molecule also displays a helix–coil transition, but many of the usual complexities that affect the helix–coil transition in more complex molecules become secondary to the influence of helix charge.²⁵ If charge dominates, it follows that when ionization is enhanced relative to the bulk solution, such that a PGlu helix carries a relatively large amount of negative charge compared to that in the solution at the given pD , the helix should be destabilized. Conversely, it should be stabilized under conditions where ionization is suppressed.

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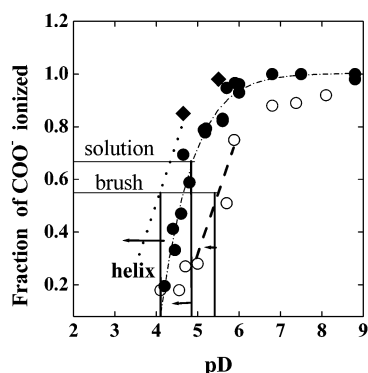


Figure 4. Percent of ionized PGlu adsorbed segments plotted against pD (we refer to solution pD rather than pH, since the experiments were performed in D₂O not H₂O) for three distinct cases. All data refer to the same dense brush substrate, 2.0 mg·m⁻² of QPVP. Filled circles denote PGlu in bulk solution. Filled diamonds denote PGlu at a sparse surface coverage of 0.2 mg·m⁻². Open circles denote PGlu at a high surface coverage of 2.1 mg·m⁻². The point of transition between random-coil and α -helix configurations, determined from the infrared amide I bands of PGlu, is denoted in the three cases by solid vertical lines to show pH and solid horizontal lines to show the percent ionized at the point of helix-coil transition. The data show a distinctive helix destabilization at low surface coverage and stabilization at high surface coverage that correlate well with the major changes of surface ionization shown in the other figures.

In our experiments, the point of transition between random-coil and α -helix configurations of adsorbed PGlu was determined from the infrared amide I bands. As plotted in Figure 4, we found a distinctive transition from helix destabilization at low surface coverage to stabilization at high surface coverage. This confirms the major role of surface ionization state, determined by surface charge density, in determining the helix-to-coil transition of this simple polypeptide.

Outlook

While our quantitative determination of D^* is based on such strong assumptions that the identity of its minimum value with the Bjerrum length may be fortuitous, we emphasize that the relative values shown in Figure 3 do

not depend on the details of the normalization. In addition, while PGlu is a convenient system for study as a model polypeptide, there is no reason to believe that it is a special case, and indeed we have performed control experiments with poly(methacrylic acid), PMA, that confirm the quantitative generality of the patterns shown in Figures 1–3. This suggests the generality of the findings presented here.

These findings also have some potential relevance to the burgeoning recent interest in predicting the structural stability of proteins and other molecules of biological importance. It is easy to imagine that their stability in the environment of an associated lipid membrane might be very different from that in bulk solution. This would have evident implications for drug delivery.

In summary, these experiments point for the first time to the importance of charge-charge repulsion in limiting the extent of ionization when a molecule adsorbs from aqueous solution. This repulsion is already present in bulk solution but becomes particularly prominent near a surface because the adsorbed state results in such dense local charge concentration. In this way we have provided an explicit connection between the traditional idea that the surface electric field should enhance ionization and the observation that ionization was suppressed below that characteristic of the bulk solution. The point at which the local dielectric constant fell below that characteristic of bulk aqueous solution also became directly evident. Predictable changes of surface ionization with changing surface coverage gave insight into the observed helix-coil transition of our model polypeptide and may also find application in many other areas, from colloidal processing to food technology, where ionizable molecules adsorb at aqueous surfaces.

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