Proton-Coupled Protein Binding: Controlling Lysozyme/Poly (acrylic) Acid Interactions with pH.

ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · APRIL 2014

Impact Factor: 3.3 · DOI: 10.1021/jp500310w · Source: PubMed

3 AUTHORS:

Ananta Ghimire
University of Connecticut
3 PUBLICATIONS **4** CITATIONS
SEE PROFILE

Rajeswari Kasi
University of Connecticut
52 PUBLICATIONS **801** CITATIONS
SEE PROFILE

Challa V Kumar
University of Connecticut
186 PUBLICATIONS **5,745** CITATIONS
SEE PROFILE

THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

pubs.acs.org/JPCB

Proton-Coupled Protein Binding: Controlling Lysozyme/Poly(acrylic acid) Interactions with pH

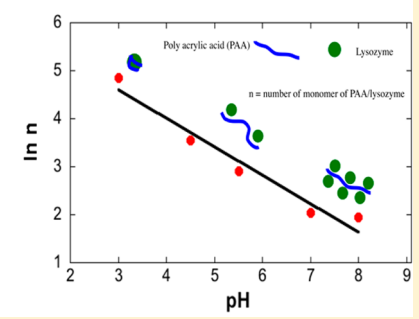
Ananta Ghimire,[†] Rajeswari M. Kasi,*[,†,‡] and Challa V. Kumar*[,†]

[†]Department of Chemistry, U-3060, University of Connecticut, Storrs, Connecticut 06269-3060, United States

[‡]Polymer Program, The Institute of Materials Science, University of Connecticut, Storrs, Connecticut 06269-3060, United States

Supporting Information

ABSTRACT: Rational design of protein−polymer composites and their use, under the influence of the stimulus, for numerous applications requires a clear understanding of protein−polymer interfaces. Here, using poly(acrylic acid) (PAA) and lysozyme as model systems, the binding interactions between these macromolecules were investigated by isothermal titration calorimetry. The binding is proposed to require and be governed by "charge neutralization of the protein/polymer interface" and predicted to depend on solution pH. Calorimetric data show strong exothermic binding of lysozyme to PAA with a molar ΔH and $T\Delta S$ values of −107 and −95 kcal/mol, respectively, at pH 7 and room temperature. Both ΔH and $T\Delta S$ decreased linearly with increasing pH from 3 to 8, and these plots had slopes of −17.7 and −17.5 kcal/mol per pH unit, respectively. The net result was that the binding propensity (ΔG) was nearly independent of pH but the binding stoichiometry, surprisingly, increased rapidly with increasing pH from 1 lysozyme binding per PAA molecule at pH 3 to 16 lysozyme molecules binding per PAA molecule at pH 8. A plot of stoichiometry vs pH was linear, and consistent with this result, a plot of ln(average size of the protein/polymer complex) vs pH was also linear. Thus, protonation−deprotonation plays a major role in the binding mechanism. "Charge neutralization" of the lysozyme/PAA interface controls the binding stoichiometry as well as the binding enthalpies/entropies in a predictable fashion, but it did not control the binding affinity (ΔG). The pH dependence of lysozyme binding to PAA, demonstrated here, provides a stimuli-responsive system for protein binding and release from the polymer surface.



INTRODUCTION

The thermodynamics of the interactions of lysozyme with poly(acrylic acid) has been studied by isothermal titration calorimetry, here, and the data show strong coupling of protonation with protein binding.[1,2] Understanding of the protein−polymer interactions is of current interest, and these interactions play a key role in a number of applications, where polymers are brought in contact with the biological world. The covalent conjugation of proteins with polymers, for example, involves the formation of a physical complex between the two components, prior to chemical bonding, and formation of this complex may be controlled by hydrogen bonding, electrostatic, hydrophobic, and a variety of other interactions.[3] Understanding these interactions is of fundamental value to predict how to design polymers that bind to proteins in a predictable manner.

Very few studies, however, are reported on the thermodynamics of such interactions,[4−7] and despite these studies, our understanding of factors contributing to these interactions at the molecular level is still rudimentary.[4−6,8] The central question remains: can a universal molecular model be developed to quantitatively predict the best conditions to assist or inhibit the formation of the protein/polymer complex? However, answering this question is a challenging proposition due to the variability in the composition, structure, size, and solubility associated with both proteins and polymers, not to mention the role of solvent, ions, temperature, and pH on the binding equilibrium. To accelerate progress in this direction, the study of protein−polymer interactions could be approached using a thermodynamic method in which binding parameters are quantitatively assessed on the basis of physical parameters such as pH, ionic strength, or temperature and subsequent analysis of these data as a function of one variable at a time could reveal a firm molecular basis for these interactions.

The pH of the medium provides a good handle to evaluate the role of protonation and deprotonation that might occur prior to or during the binding event. It influences the interaction of proteins and polymers by changing the net charge on the protein, such as lysozyme, and the net charge on ionizable polymers such as poly(acrylic acid) (PAA). Therefore, pH provides a good handle in evaluating the roles of electrostatic interactions and the protonation−deprotonation equilibrium on the binding process.

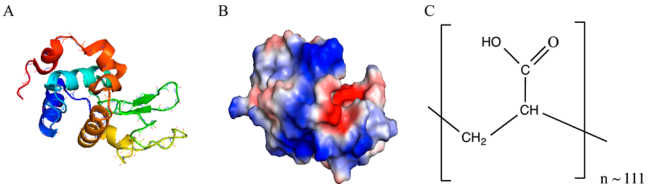
Using the thermodynamic approach, the binding of PAA to methemoglobin (Hb), for example, was examined by isothermal titration calorimetery (ITC), where binding is

Received: January 10, 2014
Revised: March 29, 2014
Published: April 16, 2014

driven by an increase in entropy.[9] Approximately 14 Hb molecules bind to 1 molecule of PAA (MW 450 000), and direct electrostatic interactions between weakly negatively charged Hb with strongly negatively charged PAA are unfavorable. Therefore, other interactions were thought to be responsible for the binding,[10] but metal ions present in the solution were found to assist "charge neutralization" of the protein−polymer interface and promote the binding of these similarly charged partners. These observations led to the metal-ion-coupled protein binding, which appears to be more widely applicable than originally proposed.[11]

In contrast to Hb, the binding of a positively charged protein such as lysozyme with the negatively charged PAA (Chart 1) may be dominated by favorable electrostatic interactions, especially under alkaline pH conditions rather than under acidic pH's. This simple electrostatic model was tested here by examining the binding of lysozyme with PAA by ITC and the binding thermodynamics evaluated as a function of pH.

Chart 1. (A) 3D Structure of Lysozyme,[12] (B) Distribution of the Cationic (Blue) and Anionic (Red) Residues on the Surface of Lysozyme, and (C) Poly(acrylic acid) (MW 8000)[a]



[a]Lysozyme structure was from the Protein Data Bank (hydrodynamic radius, 18.9 Å).[13]

Lysozyme is an enzyme with antibacterial properties, and it is capable of hydrolyzing bacterial cell walls. It is used as a food preservative, as an additive in infant milk formula to resemble protein composition of human milk, and as an analgesic for cancer patients.[14] It is a small protein of mass 14 kDa with an isoelectric point of ∼11 and carries a charge of ∼ +14[15] at pH 3.0 and ∼ +7 at pH 8.0. The active site of the enzyme is lined with two key carboxyl groups, while the enzyme surface has several positively charged residues for interactions with anionic substrates such as the rigid cell walls.

PAA is a negatively charged polymer, and its degree of ionization increases with pH as 20, 50, 65, 90, and 100% at pH values of 3.0, 4.5, 5.5, 7.0, and 8.0, respectively.[16] PAA is fully charged at alkaline pH and adopts a stretched-out conformation, while under acidic conditions it is extensively protonated and collapses into a coil.[17] Therefore, the charge on PAA changes drastically with pH 3−8, while the charge on lysozyme changes only marginally over this range.

The hypothesis that electrostatic interactions dominate when the binding partners are oppositely charged and "charge neutrality of the protein−polymer interface" drives binding is tested here. Since pH of the medium determines the net charge on lysozyme and the polyelectrolyte, the binding interactions are expected to be sensitive to the pH. Previous work indicated the dependence of these interactions on ionic strength of the medium and that surface charge plays a critical role in the binding interaction.[18,19] We also test the proton-coupled protein binding model where protonation−deprotonation could contribute to changes in the protein/polymer interfacial charge and drive/inhibit the binding. The data showed that "charge neutrality" controls the binding stoichiometry of the protein/polymer complex, while the binding free energy was nearly independent of pH. A predictable model is given to explain the pH dependence of the binding equilibrium, the size of the protein/polymer complex, as well as the binding stoichiometry. Our observations are enumerated below.

EXPERIMENTAL SECTION

Materials. Chicken egg white lysozyme and poly(acrylic acid) (PAA, MW 8000, aqueous solution of 45% by wt) were obtained from Sigma-Aldrich (St. Louis, MO). Phosphate buffers (PB, 10 mM) with pH adjusted to 3.0, 4.5, 5.5, 7.0, and 8.0 were used to keep the same type of ions in the solution. Phosphate buffer of pH 7.0 was prepared, for example, by dissolving 0.5836 g/L of $NaH_2PO_4{\cdot}H_2O$ and 1.5466 g/L of $Na_2HPO_4{\cdot}7H_2O$ in deionized water (DI). The pH of the solution was adjusted with phosphoric acid or sodium hydroxide, as needed. The amounts of $NaH_2PO_4{\cdot}H_2O$ and $Na_2HPO_4{\cdot}7H_2O$ are varied to achieve the desired pH buffers (Table S1, Supporting Information).

Isothermal Titration Calorimetry (ITC). Thermodynamic parameters were obtained using a nanocalorimeter (VP-ITC from Microcal Inc., Piscataway, NJ) by following methods established in our laboratory.[20] Lysozyme solution was prepared by dissolving 4.41 mg/mL of lysozyme in DI water. Stock solution of PAA (0.5 mM) was prepared by dissolving 11.55 mg/mL in DI water, as described earlier.[21] All solutions were dialyzed against desired phosphate buffers of appropriate pH (Table S2, Supporting Information).

The calorimeter was thermally equilibrated for 1 h, and the sample cell was loaded with lysozyme (1.46167 mL) while the syringe was filled with PAA solution (300 μL). Titration was carried out by adding 18 μL of PAA solution in successive injections to lysozyme solution at 300 s time intervals between injections, at a stirrer speed of 260 rpm. Prior to the titration, both solutions were degassed approximately for 30 min and changes in power required to maintain the same temperature between the sample and reference cells recorded and plotted. The corresponding changes in the power for the dilution of the protein and the polymer solutions were measured separately and both subtracted from the protein/polymer titration data.

The observed change in power data were converted to integrated enthalpy changes as a function of protein to polymer concentrations (Origin software v. 5.0, Microcal Inc., Piscataway, NJ). Heat released or absorbed (Q) was calculated with eq 1,[22,23] using the molar heat of protein binding (ΔH), the volume of the sample cell (V_o), the initial concentration of the ligand (X_t), the PAA concentration (M_t), the binding constant (K_b), and the number of binding sites (n) as monomers of PAA bound to one lysozyme.

$$Q = \frac{nM_t\Delta HV_o}{2}\left[1 + \frac{X_t}{nM_t} + \frac{1}{nK_bM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nK_bM_t}\right)^2 - \frac{4X_t}{nM_t}}\right] \quad (1)$$

The experimental isotherms were fitted to the "single set of noninteracting, identical" binding site model, which consistently gave satisfactory fits for data collected at all pH's. The

dx.doi.org/10.1021/jp500310w | J. Phys. Chem. B 2014, 118, 5026−5033

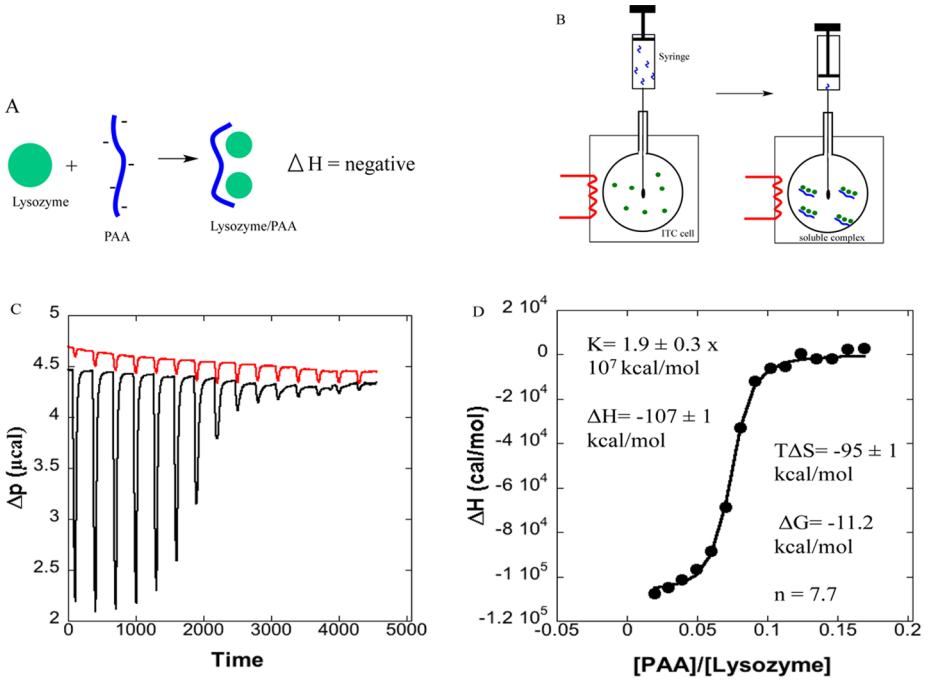


Figure 1. (A) Schematic representation of lysozyme binding to PAA. (B) Addition of a solution of the polymer (56 μM, in syringe) to the protein solution (65 μM, in cell) in the calorimeter. (C) Change in power vs time plot for the titration (black curve) and the corresponding heat of dilution data are given by the red curve, recorded in PB pH 7.0 and 25 °C. (D) Enthalpy change vs [PAA]/[lysozyme]. The black line is the best fit to the experimental data according to eq 1.

values of the binding constant (K_b), binding stoichiometry (n), enthalpy change (ΔH), and entropy change (ΔS) were obtained from the best fits to eq 1. All experiments were at 298 K and have been repeated at least twice to estimate errors.

Dynamic Light Scattering (DLS). Hydrodynamic radii of lysozyme and lysozyme/PAA complexes were measured using a CoolBatch+ dynamic light scattering apparatus, with a Precision detector (Varian Inc. Palo Alto, CA). Samples were prepared at stoichiometric ratios obtained from ITC studies. Samples were then centrifuged and filtered using a 0.2 μm syringe filter to remove the larger aggregates, if any, and were kept in a 0.5 × 0.5 cm^2 cuvette for DLS studies. A laser source of 658 nm wavelength at 90° geometry was used to excite the sample, all samples were equilibrated for 100 s at 25 °C, and four trials were averaged at constant temperature. Precision Elucidate Version 1.1.0.9 and Deconvolve Version 5.5 from the manufacturer were used to estimate the corresponding average hydrodynamic radii of the protein/polymer complexes at 298 K.

Circular Dichroism (CD). Protein structural changes, if any, upon complexation of lysozyme with PAA, at particular pH values, were monitored using a JASCO J-710 Spectropolarimeter in a 0.05 cm path length cuvette from 200 to 260 nm at a scan speed of 50 nm/min. The average of four accumulations was collected for each sample, and data were plotted using Kaleidagraph 4.1.3 (Synergy Software, Reading, PA). The spectra were normalized with respect to protein concentration and path length and data analyzed by well established methods.[24]

Zeta Potential Measurements. A ZetaPLus (Brookhaven Instruments, Holtsville, NY) with an SR-516 type electrode was used to measure the zeta potentials of samples prepared in 10 mM PB (4 mL) at stoichiometric ratios determined from ITC. Three runs were averaged for each sample, and zeta potentials were calculated using Smoluchowski fits to the data with software provided by the manufacturer.

RESULTS AND DISCUSSION

Understanding the details of protein−polyelectrolyte interactions is important in designing advanced materials for various applications, but only few studies have been conducted and these are yet to be fully understood.[25] The simple electrostatic model predicts that the interaction requires "charge neutralization" of the protein/polymer interface. This hypothesis was tested here using lysozyme and PAA by ITC, circular dichroism, and light scattering studies as a function of solution pH.

ITC Studies at pH 7. The binding of lysozyme to PAA in 10 mM phosphate buffer at pH 7.0 was carried out by adding a solution of 56 μM PAA solution in the syringe to a solution of lysozyme (65 μM) in the calorimeter (Figure 1B). A total of 15 injections of 18 μL each were made, and the heat produced during each injection has been recorded as the change in power (Δp) vs time (Figure 1C). The integrated area under each peak, therefore, is the heat produced during each injection.

Subsequent to each injection, the amount of the free protein available to bind to PAA progressively decreases, and the magnitude of the heat released also decreases progressively. The residual heat effects observed after the binding is saturated, seen after 11 injections in Figure 1C, originate from the dilution of PAA, lysozyme/PAA complex, and the mechanical effects. The enthalpy of dilution of PAA, measured during the control experiment, was subtracted from the observed titration data, while the enthalpy of dilution of lysozyme was found to be negligible, under these conditions. There was no aggregation or precipitation during or after the titration, and the net heat produced during the titration was plotted as a function of the molar ratio of PAA to lysozyme (Figure 1D). The

dx.doi.org/10.1021/jp500310w | J. Phys. Chem. B 2014, 118, 5026−5033

thermodynamic parameters were obtained from the best fit to the experimental data using eq 1, which describes ligand binding to a single, identical, noncooperative binding site. The goodness of the fit was routinely tested using a number of criteria provided by the software, and best fits are chosen to extract the binding parameters.

The binding stoichiometry (n) deduced from the best fits from Figure 1D was that approximately 14 lysozyme molecules bind to 1 PAA molecule or 8 monomers of PAA are occupied by 1 lysozyme. The protein packs around the polymer as beads-on-a-string, at this pH, and this value of n approximately corresponds to the midpoint of the titration curve, as expected. This agreement confirms the validity of the best fit to the experimental data, and evaluation of the binding parameters was then conducted.

The affinity constant (K_b) of lysozyme for PAA at pH 7 was found to be $(1.9 \pm 0.3) \times 10^7$ M^{-1} which is orders of magnitude larger than the affinity constants noted for lysozyme binding to PAA (5.1×10^4 M^{-1}) or poly(vinyl) sulfonic acid (2.7×10^3 M^{-1}).[8] The previously reported binding constant of lysozyme for PAA, however, was measured at 50 mM PB which is much higher than 10 mM PB used here and this increase in affinity at lower ionic strength suggests the strong role of electrostatic interactions in the binding mechanism. We tested this hypothesis by examining lysozyme/PAA formation as a function of ionic strength, at pH 7.0. At high lysozyme concentrations (100 μM), the addition of PAA (50 μM, n = 7.7, 10 mM PB) resulted in precipitation of the complex (transmittance <4%; Figure S1, Supporting Information). As the ionic strength was raised at this pH, T (%) increased as the complex redissolved and transmittance reached 99% at 50 mM PB. These data indicated that the protein/polymer complex is insoluble at low ionic strengths due to extensive protein binding to the polymer, resulting charge neutralization on both components, rendering the complex less soluble. As the ionic strength is raised, some of this binding is inhibited, resulting in improved solubility of the resulting protein/polymer complex. In support of this explanation, when the experiment was repeated at much lower lysozyme concentration (65 μM, PAA 56 μM, n = 7.7), there was no precipitate, as was the case during the ITC titrations. These observations are consistent with previously reported theoretical studies on the ionic strength dependence of lysozyme binding to anionic surfaces.[18]

The binding was strongly exothermic, and the corresponding ΔH and $T\Delta S$ values estimated from the above data were -107 ± 1 and -95 ± 1 kcal/mol, respectively. The binding was entirely driven by enthalpy at an entropic penalty. In contrast, the binding of Hb to PAA was driven entirely by entropy at an enthalpic penalty. The two proteins differ in terms of their net charge, positive vs negative, and favorable electrostatic interactions between lysozyme and PAA drive this process against entropy losses. The entropy loss could be due to decreased flexibility of the polymer backbone in the complex, loss in its rotations, and losses in the rotational motions of the amino acid side chains of the protein that are present at the protein/polymer interface. Nevertheless, the simple electrostatic model appears to be adequate in describing the binding scenario.

If "charge neutrality" of the protein/polymer interface is important for the binding, then these parameters reflect on the enthalpy changes due to the charge neutralization and other contributions such as desolvation, H-bonding, van der Waals interactions, etc. From the known charge of +8 on lysozyme, -8 on the PAA segment occupied by each lysozyme molecule at pH 7, the enthalpy change per unit charge of PAA has a minimum of $-107/8$ or -13 kcal/mol per charge. As the pH of the medium is decreased, the charge on PAA diminishes and the charge on lysozyme increases. Therefore, the electrostatic contributions to ΔH are expected to decrease due to the decrease in the degree of ionization of PAA as well as lysozyme when the pH is lowered. We systematically examined the binding thermodynamics as a function of pH, from a value well below the isoelectric point of PAA to alkaline pH where PAA is fully ionized.

The pH Dependence Study. The isoelectric point of lysozyme is 11.0. It carries +8 charge at pH 7, and its charge increases marginally to +14 at pH 3. On the other hand, the degree of ionization of PAA decreases with a decrease in pH, from 8 to 3. If electrostatic interactions are important in the binding mechanism, then the binding affinities, enthalpies, entropies, and stoichiometry should vary systematically, in a predictable manner, over this pH range. Therefore, we carried out a number of titrations at pH 3.0, 4.5, 5.5, and 8.0 (Figure 2 and Figures S2 and S3, Supporting Information), and the reproducibility of the data are established in multiple runs.

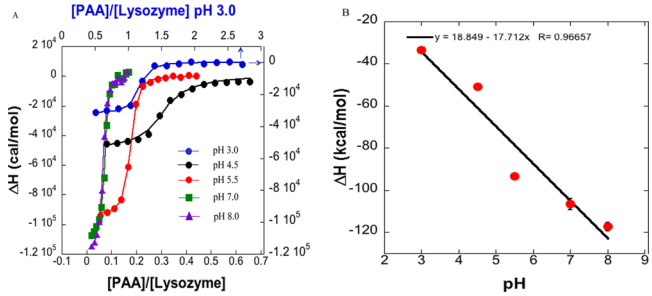


Figure 2. (A) Enthalpy change vs [PAA]/[lysozyme] at pH 3.0, 4.5, 5.5, 7.0, and 8.0. Solid lines are the best fits according to eq 1. (B) ΔH as a function of pH for lysozyme/PAA interactions at 25 °C (10 mM PB).

The ITC curves were corrected for dilution of PAA, using the corresponding control data sets. There was no precipitation or aggregation during or after the titrations, and the resulting data have been fitted to eq 1. All data sets were fitted satisfactorily to this model, and this fact further supports the validity of the binding model. Qualitative examination of the data in Figure 2A shows that the binding is exothermic at all pH's examined here (pH 8.0, 7.0, 5.5, 4.5, and 3.0), while the magnitude of ΔH decreased. Quantitative values for the binding parameters were deduced from the best fits to these data sets (K_b, ΔH, ΔS, and n), which are compiled in Table 1.

ΔH changed from -34 to -117 kcal/mol when the pH increased from 3 to 8, respectively, which demonstrated a strong contribution of electrostatic interactions to the binding. ΔH became more and more negative with an increase in pH. The plot of ΔH vs pH was linear with a slope of -17.7 kcal/mol per unit pH (Figure 2B). As the polymer charge increased with pH, the binding was driven more and more by enthalpy, and binding was more enthalpically favorable at higher pH than at lower pH. At pH 8.0, where PAA was completely ionized and stretched out, the electrostatic interaction and the corresponding magnitude of binding enthalpy was maximized. At pH 3.0, due to extensive protonation of the carboxylate groups of PAA,

dx.doi.org/10.1021/jp500310w | J. Phys. Chem. B 2014, 118, 5026−5033

Table 1. Values of K_b, ΔH, ΔG, ΔS, and n for PAA Binding to Lysozyme at Different pH's and the Corresponding Estimated Electrostatic Charges on PAA and Lysozyme[15,16]

	pH 3.0	pH 4.5	pH 5.5	pH 7.0	pH 8.0
K_b (M^{-1})	$(1.7 \pm 0.7) \times 10^7$	$(6.4 \pm 0.2) \times 10^5$	$(2.1 \pm 0.1) \times 10^7$	$(1.9 \pm 0.4) \times 10^7$	$(1.3 \pm 0.1) \times 10^6$
ΔH (kcal/mol)	-34 ± 1	-51 ± 1	-93 ± 2	-107 ± 1	-117 ± 1
$T\Delta S$ (kcal/mol)	-24	-43	-83	-95	-108
ΔG (kcal/mol)	-10 ± 1	-8 ± 1	-10 ± 1	-11 ± 1	-10 ± 1
n	129.0	35.2	18.2	7.7	7.0
charge on PAA	-22	-55	-72	-100	-111
charge on lysozyme	+14	+11.5	+9.5	+8	+7

the magnitude of ΔH decreased, even when the charge on lysozyme was increased to +14.

Binding was enthalpy driven and achieved at an entropic penalty, under all pH conditions, and the magnitude of ΔS increased with an increase in pH (Figure 3A). The

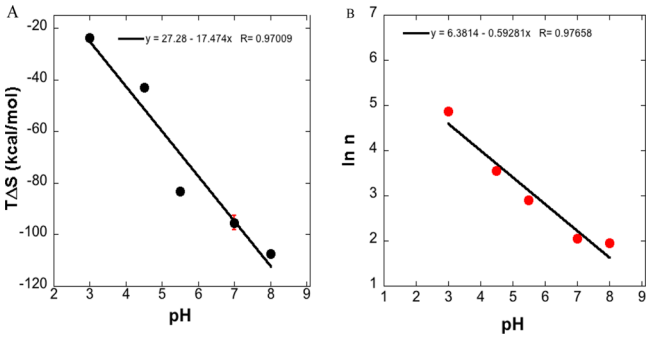


Figure 3. (A) Change in entropy as a function of pH and (B) plot of ln(n) as a function of pH for lysozyme/PAA interactions at 25 °C (10 mM PB).

corresponding $T\Delta S$ plot was linear with a slope of -17.5 kcal/mol, and this clearly shows a strong compensation for a strong decrease in ΔH. The large increase in the magnitude of ΔS with an increase in pH cannot be explained solely by considering that entropy changes are due to the two partners coming together, as this happens at all pH values examined. An alternate source for this large negative slope of the $T\Delta S$ vs pH plot could be due to protonation of functional groups at the protein/polymer interface, particularly at low pH values.

As the pH drops, the likelihood of protonation of remaining carboxylate groups on PAA increases, and such protonation will result in the loss of entropy due to the transfer of the proton from the bulk to the lysozyme/PAA interface. Proton-coupled protein binding can explain the large negative slope of the $T\Delta S$ vs pH plot, and this explanation is also applied to our previous study of Hb binding to PAA, where the two partners are negatively charged. The binding was promoted by protonation of the functional groups at the protein/polymer interface, and this explanation was supported by the observed increase in binding stoichiometry (n) discussed below.

The n values obtained from the best fits of the above data indicated an exponential decrease with an increase in pH. That is, the number of monomers of PAA occupied by 1 lysozyme molecule decreased from 129 at pH 3.0 to 7 at pH 8.0. Note that the charge on the binding partners changes as a function of pH, but at pH 3, lysozyme carries a net charge of only +14, while the net charge on PAA is much larger, -22. At the observed stoichiometry of 1 lysozyme binding to 129 monomers of PAA at pH 3, the protein/polymer complex should have excess negative charge which can destabilize it. This excess negative charge can be neutralized if carboxylate groups at the protein/polymer interface are protonated, as promoted by the negative charge field of the polymer. A plot of ln n as a function of pH is linear (Figure 3B) with a negative slope, which relates the increase in the binding stoichiometry with a decrease in pH.

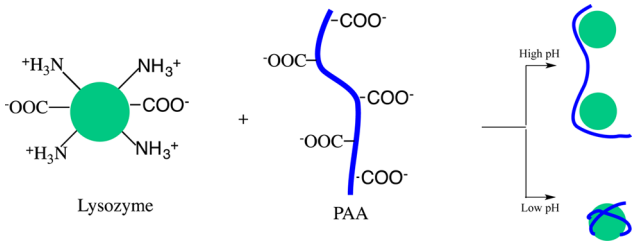
The above explanation is in agreement with the observed stoichiometric charge ratio, that is, the ratio of "observed stoichiometry multiplied by the expected degree of ionization of PAA" to "expected charge on lysozyme, at a given pH". This gives the charge ratio of the lysozyme/PAA complex formed at that particular pH, and it will be equal to "~1" if charge neutrality is to be maintained in the complex. The zeta potentials of mixtures of lysozyme (65 μM) and PAA (56 μM) at pH 7 and 8 are nearly zero (Figure S4A, Supporting Information). The oppositely charged components bind in the stoichiometric ratio such that each component cancels the charge on the other, and charge neutrality is achieved, which is consistent with previous simulation studies.[18] However, this does not preclude protonation and deprotonation of residues at the protein/polymer interface. To test this possibility, we examined the change in pH when lysozyme solution (1 mM in DI, pH 7.0) was mixed with a solution of PAA (0.4 mM in DI, pH 7.0) at the observed stoichiometric ratio (n = 7.7), in the absence of any buffer ions. The net pH increased by 0.7 units, upon mixing, which indicated the depletion of bulk H^+ due to their absorption at the protein/polymer interface. Thus, protein binding and proton absorption at the interface are significant for these systems but masked by buffer ions present in the system.

As the pH decreased, the stoichiometric charge ratio increased and reached 1.8 at pH 3.0. That is, a larger number of PAA monomers bind per lysozyme than anticipated from the estimated charges on the individual binding partners and the complex should have excess negative charge. However, the small zeta potential value of +2 for the lysozyme/PAA complex (pH 3.0) prepared at a molar ratio of lysozyme (20 μM) to PAA (350 μM) (10 mM PB, Figure S4B, Supporting Information) clearly indicates a significant degree of protonation of the lysozyme/PAA complex at this pH. Thus, protonation and binding are clearly connected. This interpretation is supported by the reported stoichiometric charge ratio of 1.4 for lysozyme binding to poly(sodium (sulfamate-carboxylate) isoprene).[26] Essentially, 1 lysozyme molecule binds to 1 PAA molecule at low pH, while a large number of lysozymes bind to 1 PAA at high pH's (Scheme 1), or the enzyme is densely packed on the polymer.

The above thermodynamic data were further correlated with the estimated charges on PAA to establish quantitative relations between charge and the thermodynamic parameters. Plots of

dx.doi.org/10.1021/jp500310w | J. Phys. Chem. B 2014, 118, 5026−5033

Scheme 1. Formation of Distinct Protein/Polymer Complexes at High and Low pH's



ΔH and $T\Delta S$ as a function of the degree of ionization of PAA, or charge per binding site on the polymer occupied by lysozyme, are linear (Figure 4). For a particular PAA/lysozyme

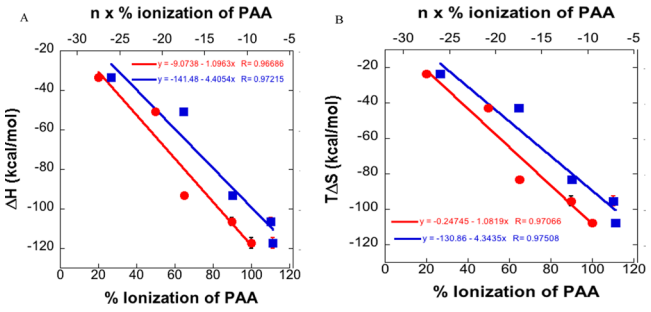


Figure 4. (A) Change in enthalpy as a function of degree of ionization of PAA (bottom axis, red) and total charge per n monomers (top axis, blue). (B) Plot of $T\Delta S$ as a function of degree of ionization of PAA (bottom axis, red) and total charge per n monomers (top axis, blue).

ratio, an order of magnitude difference in enthalpy between pH 3.0 and 8.0 indicates its charge dependency and dominance of the electrostatic nature of the binding interactions.

The change in K_b as a function of pH is shown in Figure S5 (Supporting Information). The binding constant varied from a minimum of 10^5 to a maximum of 10^7 M^{-1}, but it did not follow any specific pattern. The ΔG values obtained from the above ΔH and ΔS values did not show significant variation with pH, and remained fairly the same, within experimental error (Table 1). The mechanism of enthalpy−entropy compensation in protein/polymer binding explains the small variations in ΔG with pH, but this remains to be tested.[27] We further characterized the interactions between lysozyme and PAA by physical and biochemical methods to gain further understanding of particular contributors to the binding.

Circular Dichroism (CD) Studies. Another source of binding free energy could arise from the loss of protein structure due to hydrophobic interactions with the polymer backbone during the binding event. We examined these contributions, if any, by monitoring the CD spectra of each set of lysozyme and lysozyme/PAA complexes at particular pH's (3.0, 5.5, and 7.0), under similar conditions. The CD spectrum of unbound lysozyme at pH 7 (red line) followed that of the lysozyme/PAA complex (blue curve) at the same pH (Figure 5A). The lysozyme/PAA complex at pH 7 indicated some improvement in the intensities of its 211 and 222 nm UV CD bands, and this could be due to tightening of the protein structure in the complex, when compared to the unbound protein. Such small improvements in structure were noted when proteins bind to solid substrates.[9,28]

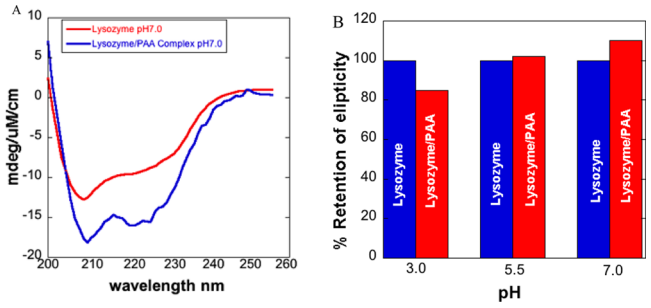


Figure 5. (A) Circular dichroism spectra of lysozyme (red curve) and lysozyme/PAA (blue curve) at pH 7.0. (B) The secondary structure retention of lysozyme/PAA (red) as compared to unbound lysozyme (blue) at pH 3.0, 5.5, and 7.0.

The extent of structure retention was estimated by recording the ratio of the intensities of the 222 nm minimum of lysozyme to that of the lysozyme/PAA complex, both spectra recorded at the same pH. The percent structure retention at pH 3.0, 5.5, and 7.0 was compared in the bar graph (Figure 5B). A small decrease in structure retention (~15%) at pH 3 was noted for the lysozyme/PAA complex, and this could be due to minor structural changes induced by binding at this low pH, promoted by protonation of some of its residues. Such a decrease in the CD signal was previously observed when lysozyme was complexed with poly(sodium (sulfamate-carboxylate) isoprene) at pH 7.0.[26] In the context of prior work, current data indicated that PAA has very little impact on the secondary structure of lysozyme at neutral pH but had only a minor influence at pH 3. Therefore, these very minor structural changes in lysozyme cannot account for the large changes noted in the thermodynamic parameters (ΔH and ΔS), and the protein/polymer complex retained most of its secondary structure. This outcome could be of practical value for biocatalytic applications.

Dynamic Light Scattering (DLS) Studies. The formation and sizes of the lysozyme/PAA complexes prepared at four different pH's (3.0, 4.5, 5.5, and 7.0) were determined by DLS. The radius of complex formed at pH 3.0 was around 68 nm, while those at pH 4.5, 5.5, and 7.0 were around 100, 115, and 150 nm, respectively. DLS data of lysozyme, PAA, and lysozyme/PAA complex are shown in Figure S6 (Supporting Information), and the linear increase of the hydrodynamic radius of the lysozyme/PAA complex as a function of pH is shown in Figure 6A. Two different factors may contribute to this observation: increase in negative charge on PAA stretching the polymer at high pH and increase in the stoichiometry of lysozyme binding to PAA at high pH.

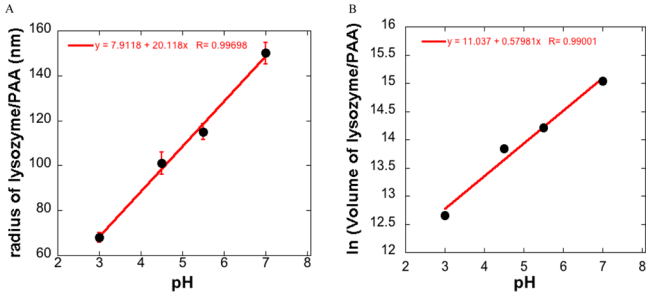


Figure 6. Plots of (A) radii and (B) ln(V) of the lysozyme/PAA complex as a function of pH.

dx.doi.org/10.1021/jp500310w | J. Phys. Chem. B 2014, 118, 5026−5033

The observed size of lysozyme/PAA does not correspond to one lysozyme binding to one PAA but is consistent with the observed stoichiometry from the ITC data and explains the linear increase in the hydrodynamic radius of the complex with pH. At pH 8.0, ITC studies show that ∼16 lysozyme molecules bind with 1 PAA molecule (16:1) or higher mass at higher pH, but this stoichiometry decreased to ∼1:1 at pH 3.0 or reduced the size of the lysozyme/PAA complex. Assuming that the density of the complex remained unchanged with pH, ITC studies suggest that the mass as well as the volume of the complex should increase with pH, which is independently derived from Figure 3A as well. Consistent with this picture, the polymer was suggested to collapse around the protein at low ionic strengths or higher charge densities on lysozyme.[18] This scenario is similar to low pH values where the charge on the protein is increased and the polymer collapses on the protein. This agreement between several independent approaches confirms the current findings.

The linear increase in hydrodynamic radius requires further evaluation. The volume (V) of the complex is proportional to the cube of its radius (r), which could be considered as its hydrodynamic radius. As shown in Figure 6B, a plot of ln V as a function of pH was also linear but a plot of V vs pH was not. Therefore, the increase in the number of lysozyme molecules bound per PAA results in increased radius as a function of pH, but contributions from the changes in the structure of the protein−polymer complex also need to be considered. At low pH, the 1:1 complex is expected to be compact, could be spherical but at alkaline pH, the PAA is stretched out, and a large number of lysozymes bind. Thus, a change in the shape of the complex also contributes to the increased radius. Such volume changes were noted in the pH dependent study of the complexation between chitosan (biopolymer of isoelectric point 6.4) and pepsin (isoelectric point 1). An increase in pH from 3.0 to 4.0 decreased the pepsin/chitosan complex size from 410 to 180 nm.[29] This size contraction was attributed to a decrease in positive charge on the biopolymer and an increase in the negative charge of pepsin. Thus, size measurements could be used to monitor the complex formation.

CONCLUSIONS

Stimuli responsive protein−polymer composites are useful for controlled delivery of biological cargo using pH as a stimulus. However, the molecular nature of the interactions of the protein with the polymer as a function of pH is still being developed. Here, lysozyme and PAA are used as models to investigate the thermodynamics of their interactions and to evaluate and establish rules that control the molecular details of enthalpy/entropy changes, stoichiometry, size of the complex, and role of pH.

The binding of lysozyme to PAA was an exothermic process from pH 3 to 8 and driven by enthalpy, with an entropic penalty. The exothermicity increased 3-fold, accompanied by a 5-fold increase in negative charge on PAA and 2-fold decrease in the positive charge on lysozyme, as the pH increased from 3 to 8. If the electrostatic interactions between the protein and the polymer are sole contributors to binding, then the exothermicity should have increased by a factor of (5/1)*(1/2) or 2.5-fold. However, this is not the case and the discrepancy between the observed and estimated values is due to other interactions that are not included in a simple pH dependent electrostatic model. For example, more H-bonds could be formed at lower pH than at higher pH and contribute to binding, as a function of pH. The $\Delta\Delta H$ value per unit charge, deduced from the above data, turns out to be −0.63 kcal/mol per unit charge on lysozyme and unit charge on PAA, and suggests the strong role of electrostatic interactions on binding enthalpies but not binding free energies. This scenario is in contrast to the binding of Hb to PAA, published earlier, where both binding partners are negatively charged and the electrostatic interactions are not favorable for binding.

The strong decrease in entropy (ΔS) as a function of pH is due to the loss in various degrees of freedom when the protein/polymer complex is formed as well as proton uptake/release by the complex. At high pH's, compared to low pH, the polymer is expected to be stretched out due to the repulsion between the ionized COOH groups and polymer binding to the protein neutralizes the charge locally, and binding could resemble protein beads attached to a polymer string.[30] This is a model proposed to account for the observations here, and needs to be tested in future studies. Protein-loaded polymer could be more rigid than the unbound polymer itself. The $\Delta(T\Delta S)$ value per unit charge, deduced from the above data, turns out to be −0.62 kcal/mol per unit charge on lysozyme and unit charge on PAA. At low pH values, a greater number of negative charges was accommodated in the complex, with the stoichiometric charge ratio being 1.8, which is greater than the anticipated value from the known degrees of ionization of the protein and the polymer, with a charge ratio of 1. Both protein binding and protonation of the COO− groups of the PAA in the protein−polymer complex can account for this discrepancy. Together, charge neutralization due to lysozyme binding and by protonation of the carboxylates proceeds, at least under acidic conditions. Thus, binding and protonation proceed until charge neutralization is achieved in the lysozyme/PAA complex. This conclusion needs to be further tested with other proteins and polymers, but current data provides a strong case for continued studies along these lines.

The above scenario of binding enthalpy and entropy dependencies on pH is consistent with the observed increase in the binding stoichiometry from 1 lysozyme molecule binding per PAA at pH 3 to 16 lysozyme molecules binding per PAA at pH 8.0.[31] However, overall binding affinities are nearly independent of pH. The physical insights gained from this study will be of significant value in the design of novel protein-delivery systems, where the protein can be loaded onto a polymer and unloaded, under pH control for the delivery of therapeutic proteins even when the binding affinities are nearly independent of pH.

ASSOCIATED CONTENT

Supporting Information

Additional information on zeta potential, ITC tittrations, and ionic strength dependence. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: kasi@ims.uconn.edu.
*E-mail: challa.kumar@uconn.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support was provided by the University of Connecticut Research Foundation faculty grant to R.M.K. and NSF funding to C.V.K. (DMR-1005609).

■ REFERENCES

(1) Veronese, F. M.; Morpurgo, M. Bioconjugation in pharmaceutical chemistry. *Farmaco* **1999**, *54*, 497−516.
(2) Hoste, K.; De Winne, K.; Schacht, E. Polymeric prodrugs. *Int. J. Pharm.* **2004**, *277*, 119−131.
(3) Park, J. M.; Muhoberac, B. B.; Dubin, P. L.; Xia, J. Effect of protein charge heterogeneity in protein−polyelectrolyte complexation. *Macromolecules* **1992**, *25*, 290−295.
(4) Xu, Y.; Mazzawi, M.; Chen, K.; Sun, L.; Dubin, P. L. Protein Purification by Polyelectrolyte Coacervation: Influence of Protein Charge Anisotropy on Selectivity. *Biomacromolecules* **2011**, *5*, 1512−1522.
(5) Azegami, S.; Tsuboi, A.; Izumi, T.; Hirata, M.; Dubin, P. L.; Wang, B.; Kokufuta, E. Formation of an Intrapolymer Complex from Human Serum Albumin and Poly(ethylene glycol). *Langmuir* **1999**, *15*, 940−947.
(6) Picó, G.; Bassani, G.; Farruggia, B.; Nerli, B. Calorimetric investigation of the protein-flexible chain polymer interactions and its relationship with protein partition in aqueous two-phase systems. *Int. J. Biol. Macromol.* **2007**, *40*, 268−75.
(7) Almeida, N. L.; Oliveira, C. L. P.; Torriani, I. L.; Loh, W. *Colloids Surf., B* **2004**, *38*, 67−76.
(8) Romanini, D.; Braia, M.; Angarten, R. G.; Loh, W.; Picó, G. Calorimetric and structural investigation of the interaction of lysozyme and bovine serum albumin with poly(ethylene oxide) and its copolymers. *J. Chromatogr. B* **2007**, *857*, 25−31.
(9) Thilakarathne, V. K.; Kasi, R. M.; Kumar, C. V. Tuning hemoglobin-poly(acrylic acid) interactions by controlled chemical modification with triethylenetetramine. *J. Phys. Chem. B* **2012**, *116*, 12783−12792.
(10) Szleifer, I. Polymers and proteins:interactions at interfaces. *Curr. Opin. Solid State Mater. Sci.* **1997**, *2*, 337−44.
(11) Deshapriya, I. K.; Kumar, C. V. Nanobiointerfaces: Charge control of enzyme/inorganic interfaces for advanced biocatalysis. *Langmuir* **2013**, *29*, 14001−14016.
(12) Blake, C. C. F.; Koenig, D. F.; Mair, G. A.; North, A. C. T.; Phillips, D. C.; Sarma, R. Structure of hen egg-white lysozyme: A three dimensional Fourier synthesis at 2 Å resolution. *Nature* **1965**, *206*, 757−761.
(13) Parmar, A. S.; Muschol, M. Hydration and hydrodynamic interactions of lysozyme: Effects of chaotropic versus kosmotropic ions. *Biophys. J.* **2009**, *97*, 590−598.
(14) Proctor, V. A.; Cunnigham, F. E. The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. *Food Sci. Nutr.* **1988**, *26*, 359−395.
(15) Allison, S. A.; Potter, M.; McCammon, A. Modeling the electrophoresis of lysozyme. II. Inclusion of ion relaxation. *Biophys. J.* **1997**, *73*, 133−140.
(16) Borisova, O.; Billon, L.; Zaremski, M.; Grassel, B.; Bakaeva, Z.; Lapp, A.; Stepanek, P.; Borisov, O. Synthesis and pH- and salinity-controlled self-assembly of novel amphiphilic block-gradient copolymers of styrene and acrylic acid. *Soft Matter* **2012**, *8*, 7649−7659.
(17) Connal, L. A.; Li, Q.; Quinn, J. F.; Tjipto, E.; Caruso, F.; Qiao, G. G. pH-Responsive Poly(acrylic acid) Core Cross-Linked Star Polymers: Morphology Transitions in Solution and Multilayer Thin Films. *Biomacromolecules* **2008**, *41*, 2620−2626.
(18) Carlsson, F.; Linse, P.; Malmsten, M. Monte Carlo Simulations of Polyelectrolyte-Protein Complexation. *J. Phys. Chem. B* **2001**, *105*, 9040−9049.
(19) Carlsson, F.; Hyltner, E.; Arnebrant, T.; Malmsten, M.; Linse, P. Protein-polymer cluster formation and re-dissolution, A Monte Carlo study. *J. Am. Chem. Soc.* **2003**, *125*, 3140−3149.
(20) Duff, M.; Kumar, C. V. Molecular Signatures of Enzyme−Solid Interactions: Thermodynamics of Protein Binding to α-Zr(IV) Phosphate Nanoplates. *J. Phys. Chem. B* **2009**, *113*, 15083−15089.
(21) Mudhivarthy, V. K.; Cole, K. S.; Novak, M. J.; Kipphut, W.; Deshapriya, I. K.; Zhou, Y.; Kasi, R. M.; Kumar, C. V. Ultra-stable hemoglobin-poly(acrylic) acid conjugates. *J. Mater. Chem.* **2012**, *22*, 20423−20433.
(22) Sturtevant, J. M. Biochemical Applications of Differential Scanning Calorimetry. *Annu. Rev. Biochem.* **1998**, *38*, 463−88.
(23) Breslauer, K. J.; Freire, E.; Straume, M. Calorimetry: A tool for DNA and Ligand-DNA studies. *Methods Enzymol.* **1992**, *211*, 533−567.
(24) Greenfield, N. J. Using circular dichroism spectra to estimate protein secondary structure. *Nat. Protoc.* **2006**, *1*, 2876−90.
(25) Hallberg, R.; Dubin, P. Effect of pH on the Binding of β-Lactoglobulin to Sodium Polystyrenesulfonate. *J. Phys. Chem. B* **1998**, *102*, 8629−8633.
(26) Karayianni, M.; Pispas, S.; Chryssikos, G. D.; Gionis, V. Complexation of Lysozyme with Poly(sodium(sulfamate-carboxylate)-isoprene). *Biomacromolecules* **2011**, *12*, 1697−1706.
(27) Breiten, B.; Lockett, M. R.; Sherman, W.; Fujita, S.; Al-Sayah, M.; Lange, H.; Bowers, C. M.; Heroux, A.; Krilov, G.; Whitesides, G. M. Water networks contribute to enthalpy/entropy compensation in protein-ligand binding. *J. Am. Chem. Soc.* **2013**, *135*, 15579−15584.
(28) Boeris, V.; Farruggia, B.; Nerli, B.; Romanini, D.; Pico, G. Protein-flexible chain polymer interactions to explain protein partition in aqueous two-phase systems and the protein-polyelectrolyte complex formation. *Int. J. Biol. Macromol.* **2007**, *41*, 286−294.
(29) Boeris, V.; Micheletto, Y.; Lionzo, M.; da Silveria, N. P. Pico, Guillemo. Interaction behavior between chitosan and pepsin. *Carbohydr. Polym.* **2011**, *84*, 459−464.
(30) Reches, M.; Snyder, P. W.; Whitesides, G. M. Folding of electrostatically charged beads-on-a-string as an experimental realization of a theoretical model in polymer science. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 17644−17649.
(31) Mattison, K. W.; Dubin, P. L.; Brittain, I. J. Complex formation between bovine serum albumin and strong polyelectrolytes: Effect of polymer charge density. *J. Phys. Chem. B* **1998**, *102*, 3830−3836.