

Charged Polypeptide Diffusion at a Very High Ionic Strength

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ABSTRACT: Two charged polypeptides of opposite charge, poly(glutamic acid) (negative charge) and polylysine (positive charge), were end-labeled with Alexa fluorescent dyes, and their translational diffusion coefficient (D) values in dilute solutions ($\sim 10^{-4}$ mg mL⁻¹) were studied at the biological pH with fluorescence correlation spectroscopy as a function of the ionic strength (C_s) mediated by the addition of NaCl. At a moderate ionic strength, D increased consistently with expected chain contraction because of electrostatic screening. At a very high ionic strength, D of poly(glutamic acid) increased more rapidly, following the empirical power law $R_H \sim C_s^{-1/2}$ over a limited range of C_s , where the changes in D were interpreted as changes in the hydrodynamic radius, R_H . However, D of polylysine at first decreased but eventually passed through a maximum followed by a decrease. These large increases implied that R_H decreased considerably, in turn implying a strong contraction of the chain conformations even though the polymer remained soluble and showed no evidence of aggregation. For polylysine, the unexpected minimum R_H value may be related to the salting-in phenomenon. © 2005 Wiley Periodicals, Inc. *J Polym Sci Part B: Polym Phys* 43: 3497–3502, 2005

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INTRODUCTION

Charged amino acids such as glutamic acid and lysine are common hydrophilic elements of proteins and are highly involved in the weak interactions that determine protein structure and function. Synthetic polypeptides of these amino acids are appealing model systems for studying the polyelectrolyte problem. From this perspective, the goal of understanding polyelectrolyte diffusion in solution^{1,2} is fundamental not only in academic polymer science but also in the numerous functions and technological applications of polyelec-

trolytes that are predicated on their possession of a definite level of mobility. Furthermore, in addition to their widespread technological uses (e.g., in water purification, steric stabilization, hair care, and food science), synthetic polyelectrolytes serve as touchstone model systems for understanding more complex issues involving the science and applications of polypeptides and DNA.

The simplest case to consider is that of polyelectrolytes consisting of a single type of repeat unit. Various theories have considered their diffusion.^{3,4} Here we consider one common presumption: when the ionic strength is sufficiently high, the screening of electrostatic interactions increases to the point that polyelectrolytes are expected to behave as nonpolar polymers.^{3,4}

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This reasonable hypothesis seems to predict that (all other things equal) diffusion should not depend on the ionic strength provided that the ionic strength is sufficiently high. To our surprise, the experiments described here showed instead a strong dependence. In interpreting the significance of these unexpected findings, we conclude tentatively against trivial explanations. The observed acceleration does not appear to reflect salt-induced phase separation because the polyelectrolytes remained soluble in all proportions. Moreover, the available computer simulations predict that monovalent salts, used in these experiments to control the ionic strength, do not cause substantial changes in polyelectrolyte conformations⁵ (multivalent ions are needed to observe this effect). At the same time, the slow-down of polylysine does appear to reflect the so-called salting-in behavior already known from the literature.⁶ Although further experiments are needed to explore the generality of these striking observations, they seem worth reporting at this time.

Poly(L-glutamic acid) (PGlu) with molar mass $M \approx 10,000$ g/mole and poly(L-lysine) (PLys) with $M \approx 20,000$ g/mole were selected as the model systems; although they are oligomers from the perspective of polymer science, their hydrodynamic radius (R_H) is in the range common for proteins. For polyelectrolyte study, they were selected for several reasons. First, the backbones are believed to be hydrophilic, or at least less hydrophobic than vinyl-based polyelectrolytes; this is beneficial as it alleviates the complexity of hydrophobic interactions. Second, they are weak polyelectrolytes, and this means that their charge density can be varied simply by changes in the pH (although this study was restricted to the biological pH at which they were fully charged). Most important, the fact that polypeptides carry an amino group or carboxylic acid group at one sole end is convenient for labeling with a fluorescent dye. This monofunctionality guaranteed a single fluorescent dye after labeling, which was important for data interpretation.

Bearing in mind the attractive conceptual simplicity of avoiding complexities of polymer-polymer interactions, we selected a polymer concentration that was extremely dilute. Much attention in the literature has been given to the ordinary-extraordinary transition, often interpreted to reflect the diffusion of some kind of chain aggregation; a sufficiently dilute concentration avoided this issue. The fluorescence methods used in this

study have single-molecule sensitivity, thus enabling experiments in which the polymer concentrations were unequivocally dilute ($\sim 10^{-4}$ mg mL⁻¹).

Finally, we should comment about our decision to interpret the data with the Stokes-Einstein equation, so that the measured translational diffusion coefficient (D) was inversely proportional to the inferred R_H value. There is an alternative school of theory regarding polyelectrolyte solutions that considers the coupling between polyelectrolytes and small ions; in this view, fluctuations of the relatively mobile small ions near the polyelectrolyte speed up the motion of polyelectrolyte segments nearby, rendering the entire polyelectrolyte more mobile. The relation of the macroion diffusion to the Stokes law diffusion coefficients of the individual ions has been considered.^{4,7,8} As the fluorescence technique that we employed measures not fluctuations in the density of the solution but rather fluctuations (on the scale of hundreds of nanometers) in the positions of fluorescent dyes, it seems difficult to establish at this time an unequivocal relation to experiment at this time, so this interesting theoretical approach was not pursued in interpreting the data presented here.

EXPERIMENTAL

PGlu (Sigma) was end-coupled by the condensation reaction of the terminal amine group of PGlu with the succinimidyl ester group of Alexa-488 (Molecular Probes), which is a bright and exceptionally photostable derivative of Rhodamine B. The reaction was performed in a sodium bicarbonate buffer at pH ~ 8 . To remove uncoupled dye, the reaction mixture was purified first by gel electrophoresis and then by passage through a NAP-5 desalt column (Amer Bioscience). The yield was estimated to be around 3% on the basis of the fluorescence correlation spectroscopy (FCS) measurements. The low yield was due to the hydrolysis of the succinimidyl ester group in an aqueous solution. Phosphate buffer solutions (PBS; pH = 4 and 7.9) and sodium chloride solutions were prepared in deionized water (Nano-Pure II Barnstead). The molecular weight of the polymer was stated by the manufacturer to be 17,000 by viscosity measurements and to be 8850 by multi-angle laser light scattering. The relatively high polydispersity could not be avoided because, to date, we have been unable to find a

PGlu of a narrower molecular weight distribution.

Polylysine (Sigma) was end-labeled with Alexa-594 (Molecular Probes) by a reaction with carboxylic acid group on the basis of the procedure recommended by Molecular Probes. The purification procedure was similar to that for PGlu.

FCS in the mode of two-photon excitation was used to measure translational diffusion with a homebuilt apparatus described previously.^{9,10} Near-infrared light (800 nm) from a femtosecond laser (Tsunami, Spectra-Physics) with a 50-fs pulse width and an 82-MHz repetition rate was directed into a Zeiss Axiovert 135 TV microscope. A water-immersion objective lens (Zeiss Capochromat; 63 \times , numerical aperture NA = 1.2) was chosen to focus the laser beam, producing a Gaussian profile with a width of ~ 0.38 μm and a depth of ~ 4 μm . To minimize photodamage, the power at the sample stage was kept low, ≈ 5 mW. The fluorescent signal was collected by the same objective and split into two channels by a beam splitter. Each channel was directed to a single-photon-counting module (Hamamatsu). The fluorescence fluctuation was recorded with an FCS acquisition board (ISS, Champaign, IL), and its software was used to calculate the two-channel cross correlation. The D values were obtained by the fitting of the cross-correlation function, $G(\tau)$, to the following equation:

$$G(\tau) = \frac{1}{2\langle N \rangle} \left(1 + \frac{8D\tau}{w_0^2} \right)^{-1} \left(1 + \frac{8D\tau}{z^2} \right)^{-1/2} \quad (1)$$

where w_0 and z are the width and height of the focus spot, respectively; $\langle N \rangle$ is the average number of the molecules in the excitation area; and τ is the time lag in the autocorrelation function. Equation 1 is standard in the use of this technique.¹¹

To confirm successful polymer labeling, the diffusion of the labeled PGlu after purification was tested and compared with that of free Alexa-488. To test the influence of the ionic strength on the diffusion, the purified sample containing labeled polymer was first desalted for 5 h with a dialysis membrane (molecular weight cutoff = 3000) and then diluted to about 4 nM dye. A PBS buffer solution (pH 7 or 9) was gradually added to 10 mM, followed by the stepwise addition of NaCl (Aldrich) with stirring. Thus, the same sample was used at many salt concentrations, up to the maximum concentration of 2 M NaCl. The same was done with PLys, except the pH was around 4.

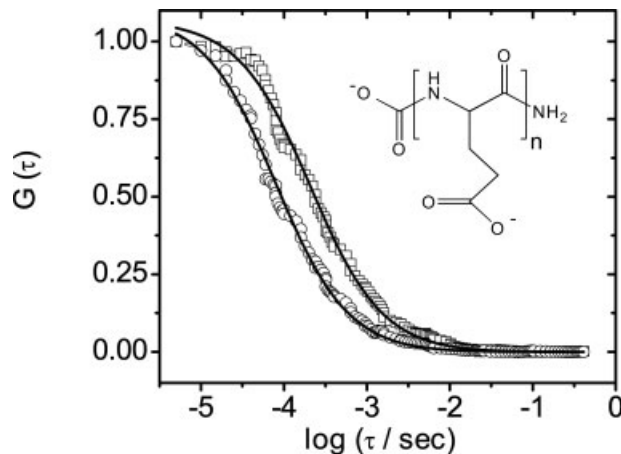


Figure 1. $G(\tau)$, normalized to unity at short times, plotted as a function of $\log \tau$ for fluorescently labeled poly(glutamic acid) at (\square) a dilute salt concentration and (\circ) a salt concentration of 2 M at pH 9. The measurements imply that D was 100 and 220 $\mu\text{m}^2/\text{s}$, respectively. The inset shows the chemical structure of PGlu.

The circular dichroism experiment employed a Jasco J-720 spectropolarimeter at the Laboratory for Fluorescence Dynamics at the University of Illinois.

RESULTS AND DISCUSSION

Figure 1 compares the autocorrelation functions of the labeled PGlu at a very low salt concentration and at a salt concentration of 2 M. The autocorrelation function is plotted against $\log \tau$. The physical idea behind inferring D from such data is that as the volume was known and the diffusion time was known from the time for the autocorrelation function to decay, it is reasonable to suppose that D equals the distance squared divided by the time. A quantitative elaboration of this idea, standard in FCS, also takes into account the Gaussian shape of the spot illuminated by the laser beam, as specified in eq 1.¹¹ In Figure 1, the solid lines show the fit of the data to eq 1. The fit to a single diffusion process is satisfactory despite the polymer's polydispersity; no physical significance is attributed to the appearance of slight oscillations in the autocorrelation functions. From control experiments (not shown here), it is also evident that the diffusion of the labeled polymer was slower by a factor of three than that for the free dye, and this indicated successful labeling of the polymer. We can reasonably conclude that diffusion of the dye represents center-of-mass diffusion of the polymer.

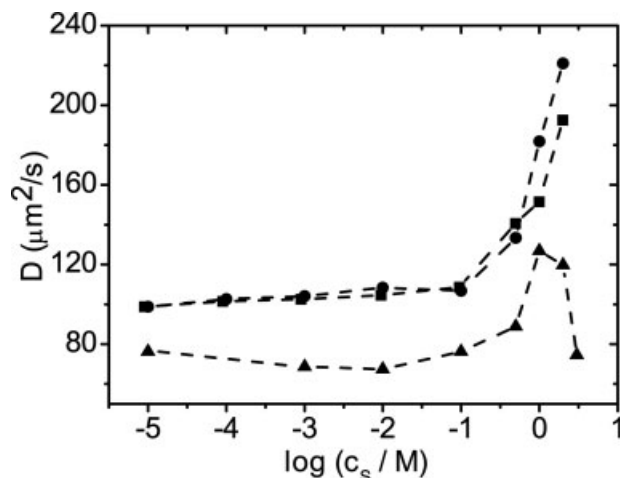


Figure 2. Center-of-mass diffusion coefficient (D) of (■) poly(glutamic acid) at pH 7, (●) poly(glutamic acid) at pH 9, and (▲) polylysine at pH 4, inferred from FCS measurements illustrated in Figure 1, plotted against $\log c_s$. The lower salt concentrations reflect the PBS buffer. NaCl was added to produce higher salt concentrations. The dashed lines are guides for the eye. The points plotted at each salt concentration are the averages of about eight repeated experiments.

As salt was added, the polymer diffused more rapidly. In Figure 2, by the global fitting of about eight repeated experiments performed at each salt concentration, the implied self-diffusion coefficient is plotted against the logarithm of the salt concentration in the extremes of no added NaCl (under this condition, the ionic strength was 10^{-5} M because of the PBS buffer) and 2 M added salt. The increase of D when NaCl was first added is easy to understand because electrostatic screening of segments along the polymer chain is known to result in chain contraction. However, to our surprise, this tendency failed to saturate when the ionic strength was high: it accelerated instead. As the ionic strength was increased from 1 to 2 M, D increased more than twofold, from 100 to $220 \mu\text{m}^2 \text{s}^{-1}$. The contrast with polylysine (whose data are included in Fig. 2) is discussed later.

It is reasonable to consider these polymers as isolated objects, as the average distance between them was on the order of 200 nm. R_H was calculated with the Stokes–Einstein equation:

$$R_H = \frac{k_B T}{6\pi\eta_s D} \quad (2)$$

where k_B is the Boltzmann constant, T is the absolute temperature, and η_s is the solvent viscosity. In making these calculations, we took note of

the changes in the solution viscosity with the salt concentration, which are known from the literature. In Figure 3, the inferred R_H values are plotted against the logarithm of the ionic strength. The salt concentration always exceeded that of the polymer and PBS buffer; therefore, screening was determined by NaCl. Although R_H in a low-salt solution was approximately 3 times that of the unattached dye, as salt was added, it decreased monotonically to the point, at the very highest ionic strength, of being indistinguishable from that of the unlabeled dye.

Control experiments showed that PGlu was soluble at concentrations up to 1 mg mL^{-1} at 4 M NaCl; therefore, it seems unlikely that phase separation produced the shrinkage of polymer conformations that these data imply. Further evidence against phase separation is the fact that the FCS experiments showed no evidence of aggregation; if the second virial coefficient were negative as expected near a point of phase separation, bursts of fluorescence intensity and slow diffusion symptomatic of aggregation should have been observed, but they were not. Yet it is interesting that even if PGlu of this molecular weight were to collapse into a dense globule from which all solvent were excluded (density = 1 g cm^{-3}), the radius of this globule would be 0.76 nm, which within the experimental uncertainty equals the measured value at a high salt concentration.

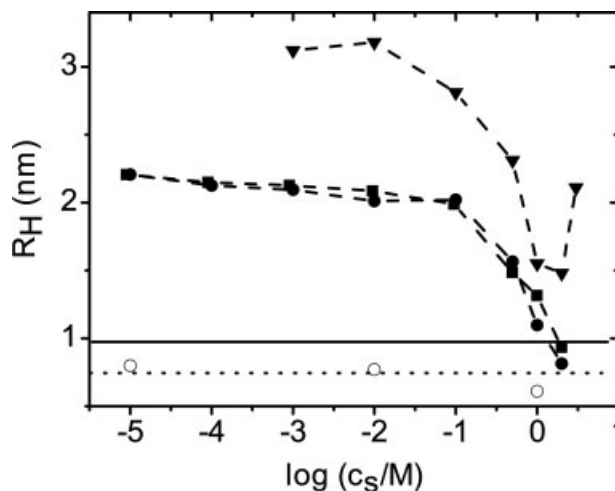


Figure 3. R_H of (■) poly(glutamic acid) at pH 7, (●) poly(glutamic acid) at pH 9, (▲) polylysine at pH 4, and (○) free Alexa-488 dye plotted against $\log c_s$. The horizontal, dotted line shows the hypothetical R_H value if PGlu of this molecular weight were to collapse into a dense globule from which all solvent was excluded (density = 1 g cm^{-3}). The horizontal, solid line shows this same comparison for PLys.

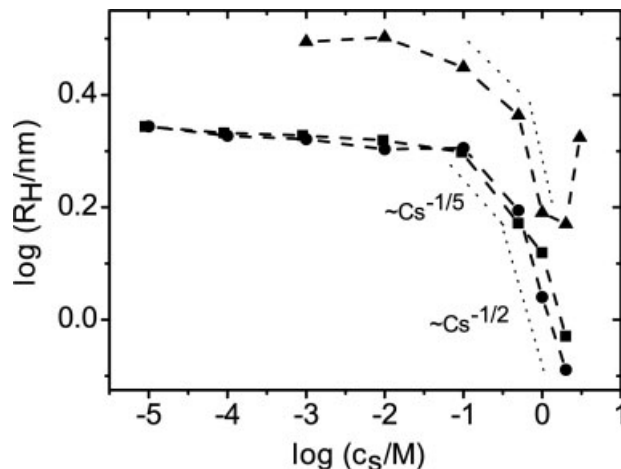


Figure 4. R_H of (■) poly(glutamic acid) at pH 7, (●) poly(glutamic acid) at pH 9, and (▲) polylysine at pH 4 plotted against c_s on log–log scales. Reference lines with power-law slopes of $-1/5$ and $-1/2$ are included as guides for the eye.

Other control experiments were motivated by the fact that PGLu is known to adopt a helical conformation in the uncharged state (at a pH below $pK_a = 4$). Speculating that the same might occur when the salt concentration is very high, we measured the circular dichroism of a sample with a concentration of 0.25 mg mL^{-1} , at NaCl concentrations up to 2 M. No evidence for the helical conformation was observed.

To test the generality of these observations, similar experiments were also performed with polylysine, which is positively charged. In this case, by a similar calculation, R_H decreases until 0.5 M and then increases.

The scaling theory of Dobrynin, Colby, and Rubinstein predicts $R_H \sim c_s^{-1/5}$, where c_s is the salt concentration.³ In Figure 4, R_H is plotted against c_s on log–log scales. The data are consistent with the theoretical prediction in the intermediate concentration range, $0.1 \leq c_s \leq 1 \text{ M}$. This confirmation of the theory is also consistent with earlier experiments¹² and simulations.¹³ The comparatively narrow concentration range might indicate errors in the PBS buffer concentration at a low nominal c_s and might also indicate that the degree of polymerization was not high enough to reach scaling behavior. Although no explanation is offered at this time, we note that R_H also decreased systematically with increasing c_s even before reaching the regime in which $R_H \sim c_s^{-1/5}$.

At the higher c_s values of primary concern in this article, R_H of the polymer started to decrease

more strongly, empirically as $R_H \sim c_s^{-1/2}$, although the concentration range is too small to demonstrate a power law. To the best of our knowledge, no theory has predicted this phenomenon. This is probably tied to the fact that available theories of polyelectrolyte diffusion are based on linear approximations of the Poisson–Boltzmann equation, but it is well known that this assumption must break down when c_s is very high. In addition, when the screening length is on the order of the size of individual polymer segments, additional short-range interactions such as van der Waals attractions between side chains may start to play an important role.¹⁴ Overall, this effect is believed to have altered the solvent quality for both polymers that we studied; it is reasonable that the effect was observed sooner for polylysine because its side chain is more hydrophobic. The size swelling of polylysine that one observes in Figure 3 at very high c_s may be related to the known salting-in effect.⁶

Considering this problem intuitively, we find it interesting to consider the relative salt concentration, within and outside a polyelectrolyte coil. From the measured R_H values and neglecting for the sake of argument counterion condensation that should lessen charge density below that for full ionization, we estimated the ionic strength within the coils around 2.5 M at radius $R \approx 2 \text{ nm}$. On the other hand, if because of counterion condensation only 20% of the polymer segments were charged, the ionic strength was around 0.5 M. At $\approx 0.5 \text{ M}$ NaCl, the charge density of the salt bulk solution approximately equaled that within the polyelectrolyte coil with counterion condensation. Physically, polymer coils beyond this crossover point no longer consisted of highly charged objects in a solution of lesser charge density but rather consisted of weakly charged particles inside a concentrated sea of ions. It is not clear why this charge mismatch should be expected to enhance polymer mobility (models of coupled diffusion^{4,7,8} may be relevant); however, the situation is reminiscent of recent measurements showing that the thickness of strong polyelectrolyte brushes shrinks at salt concentrations greater than 0.2 M.¹⁵

CONCLUSIONS

This study has failed to confirm the expectation that polyelectrolyte diffusion should be insensitive to ionic strength in the regime in which this is very high: the anticipated regime in which this

polyelectrolyte would behave simply as a nonpolar polymer was not observed. Yet the experiments were designed to present a simple system, free of potential complications: a polyelectrolyte consisting of a hydrophilic backbone, a dilute solution, and a monovalent salt. Tentatively, we conclude that the physics of having very high ionic strength somehow reduced chain dimensions more than anticipated by available theories and simulations. A more definitive study would measure concurrently how the radius of gyration of this polyelectrolyte varies with the salt concentration, but unfortunately, as no source could be found of samples with a wide range of molecular weights, we were not able to perform that measurement at this time.

Speculatively, it is interesting to consider a possible connection to proteins. In the natural human body, the average salt concentration is around 150 mM; it is possible that local salt concentrations may enter the region considered in this article. On the basis of the data reported here, the dimensions of short sequences of polypeptide might change by a factor of 2–3. If so, this would be considerable enough to change the protein conformation and hence the biological function.

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