Probe Diffusion in Concentrated Polyelectrolyte Solutions: Effect of Probe Charge on Large Deviations from Stokes−Einstein Behavior

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ABSTRACT: The probe diffusion of green fluorescent protein (GFP) in a strongly interacting host polyelectrolyte solution of sodium polystyrenesulfonate (NaPSS) was studied using fluorescence recovery after photobleaching (FRAP). Along with providing a wide range of background polyelectrolyte conditions for probe molecules by varying the NaPSS concentration from dilute to highly concentrated and using two different NaPSS molecular weights, 1×10^6 and 7×10^4 Da, we also varied the net negative charge of the GFP by varying the pH. The probe diffusion coefficient D was significantly greater (up to 15-fold) than expected from the Stokes−Einstein (S−E) relation in concentrated solutions of high molecular weight NaPSS, but only moderately greater (2-fold or less) for lower molecular weight polymer. The deviations from S−E behavior increased with increasing pH, i.e., increasing negative GFP charge. We conjecture that the strong deviation from S−E behavior is due not only to microviscosity and electrostatic effects but also (for high molecular weight NaPSS) to the viscoelasticity of the concentrated polymer solution. D was fitted to the stretched exponential equation $D/D_0 = \exp(-\alpha c^{\nu})$ at different pHs. In high molecular weight NaPSS solution the exponent ν decreased with increasing pH, whereas for low molecular weight NaPSS solution it increased. Comparison with previous results shows that along with the concentration, size, flexibility, and charge of the polyelectrolyte background, probe charge plays a significant role in the diffusion of probe molecules.

Introduction

Diffusion of macromolecules is an interesting and important phenomenon in many fields of research and practical application. Diffusion in crowded and strongly interacting systems is particularly important in practice and as a challenge to our understanding. In refs 1−18 of our accompanying paper,[1] henceforth referred to as paper 1, we listed many studies done over the past several years on a variety of systems including neutral and charged background polymer solutions and neutral and charged probe molecules, with a focus on how probe−background interaction can influence probe diffusion. This interaction could arise because of charge on probe molecules or background polymer chains or both or simply by entering the nondilute regime of background polymer concentration.

In paper 1 we studied the effect on probe diffusion of varying the electrostatic interaction between background sodium polystyrenesulfonate (NaPSS) molecules by varying the ionic strength of the solution, while keeping the probe charge slightly negative and constant. In the present communication we report the effects of changing probe charge by varying the solution pH, while keeping constant the ionic strength, and therefore the effective net charge of the background polymer.

Along with the electrostatic effects on probe diffusion, we are also interested in the effect of crowding, which is generally present in biological systems where the macromolecular volume fraction can be as high as 30% (refs 20−22 of paper 1). We obtain similar crowding by varying the background polyelectrolyte concentration from dilute to semidilute to highly concentrated, from 5 to 100 g/L for high molecular weight NaPSS solutions and from 10 to 500 g/L for low molecular weight NaPSS solutions.

We analyzed the probe diffusion coefficient D obtained by fluorescence recovery after photobleaching (FRAP) according to the Stokes−Einstein (S−E) equation and tested the parameter $D\eta/D_0\eta_0$, where D_0 is the diffusion coefficient of the probe in solvent and η_0 is the solvent viscosity, for deviations from the ideal S−E value of unity. In addition, we fitted the normalized diffusion coefficient D/D_0 in both high and low molecular weight NaPSS solutions at different pHs to the stretched exponential equation.

Materials and Methods

Sample Preparation. Buffer solutions of pH 7.05 and 8.2 were made with 10 mM Tris (tris[hydroxymethyl]aminomethane)−HCl and those of pH 5.5 with 10 mM ammonium acetate−HCl in triply distilled deionized water.

The probe—green fluorescent protein (GFP)—and the background polyelectrolyte—sodium polystyrenesulfonate (NaPSS)—are described in detail in paper 1. As in that paper, we refer to NaPSS of molecular weight 7×10^4 Da as low molecular weight NaPSS and to NaPSS of molecular weight 1×10^6 Da as high molecular weight NaPSS.

Polyelectrolyte solutions were prepared by thoroughly mixing appropriate amounts of the powder form of NaPSS in the desired buffers. The probe concentrations were kept low enough to avoid interactions among GFP molecules that might affect their fluorescence. 2.5 μL of GFP molecules at 1 mg/mL was added to 50 μL of polyelectrolyte solution and mixed to uniformity with a vortex mixer.

Fluorescence Recovery after Photobleaching. Details of the FRAP experimental procedure and data analysis are described in paper 1.

Viscometry. Viscosities of the polyelectrolyte solutions were measured using a Cannon-Ubbelohde viscometer at a temperature of 20 ± 0.2 °C. Details are described in paper 1.

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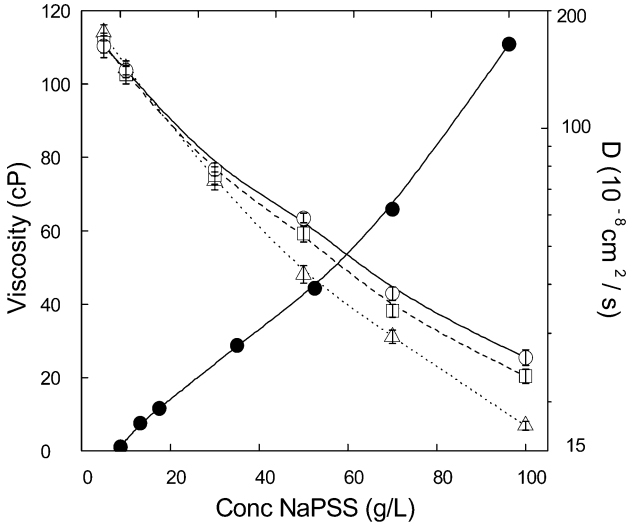

Figure 1. Diffusion coefficients of probe GFP molecules (right axis) in high molecular weight NaPSS solution at pH 5.5 (△), pH 7.05 (□), and pH 8.2 (○) and NaPSS solution viscosity (left axis) (●) as a function of NaPSS concentration (g/L).

Results

Calculation of Probe Charge. GFP is a naturally fluorescing molecule found in the jellyfish *Aequorea Victoria*.[2] The enhanced GFP used as a probe in this study is a mutated form with Phe64Leu and Ser65Thr mutations.[3] This protein contains 238 amino acids, of which the numbers with ionizable side chains are histidine (10), lysine (20), arginine (6), aspartic acid (18), glutamic acid (16), tyrosine (10), and cystine (2). In addition, the C-terminal carboxy and N-terminal amino groups also contribute charge.

We used the Henderson−Hasselbalch equation,[4]

$$\mathrm{pH} = \mathrm{p}K_\mathrm{a} + \log\left(\frac{[\text{conjugate base}]}{[\text{conjugage acid}]}\right) \qquad (1)$$

where $\mathrm{p}K_\mathrm{a}$ is the negative logarithm of the acid dissociation constant, to calculate the net charge on the protein at a given pH by calculating the charges on the individual amino acid side chains and the amino and carboxy terminal groups at that pH and adding the results.

The net charges on the protein obtained by this method were +0.9 at pH 5.5, −7.1 at pH 7.05, and −8.9 at pH 8.2. The isoelectric point was calculated to be 5.65, compared to the experimental value of 5.1.[5] We believe this is reasonably good agreement considering the simplicity in the model, which neglects environmental effects and charge interactions among the titratable groups. We see that, at all three pHs at which our experiments were performed, GFP is negatively charged with a magnitude that increases with increasing pH.

Probe Diffusion in Concentrated NaPSS Solution. Figure 1 shows solution viscosity and probe diffusion coefficient as functions of high molecular weight NaPSS concentration at three pHs corresponding to three different probe charges. As expected, D decreased with increase in NaPSS concentrations owing to increase in solution viscosity. D also increased with increasing pH in concentrated NaPSS solutions (50−100 g/L) but did not vary with pH in solutions of lower NaPSS concentration.

Figure 2 shows similar plots for diffusion and viscosity in low molecular weight NaPSS. We used higher polymer concentrations, up to 500 g/L (see the inset), to match the viscosity of high molecular weight NaPSS solutions. However, we could only reach about half the maximum value, 52.5 cP, before it became difficult to dissolve the low molecular weight NaPSS. As in the high molecular weight NaPSS solution, D decreased with increasing solution viscosity. The probe diffusion coefficient increased with increasing pH from 30 to 200 g/L NaPSS but became independent of pH for 400 and 500 g/L.

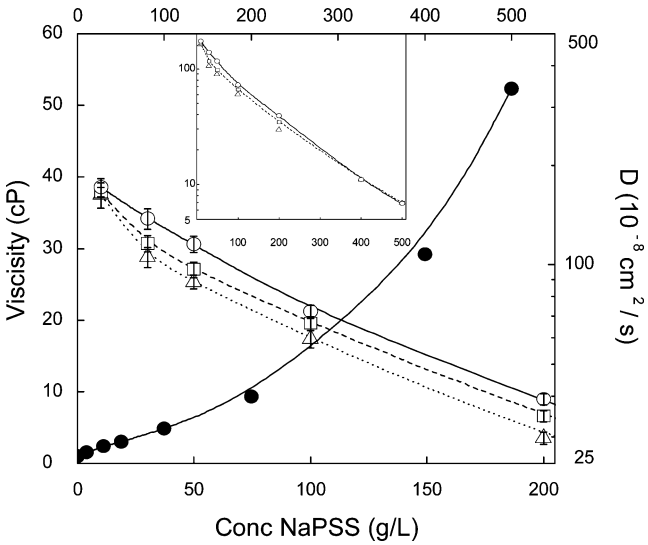

Figure 2. Diffusion coefficient of GFP (right axis) in low molecular weight NaPSS solution at pH 5.5 (△), pH 7.05 (□), and pH 8.2 (○) and NaPSS solution viscosity (left axis) (●) as a function of NaPSS concentration. The inset shows D for probe molecules up to concentration of 500 g/L.

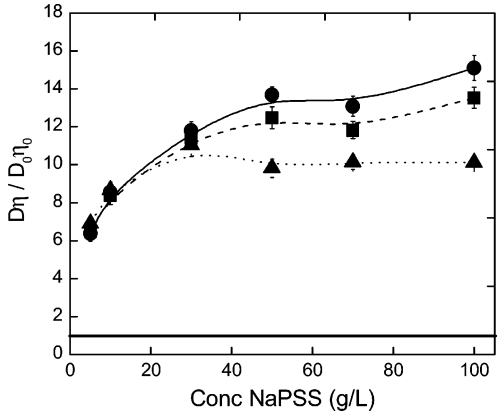

Figure 3. Deviations from S−E ratio $D\eta/D_0\eta_0$ for GFP in high molecular weight NaPSS solution at pH 5.5 (▲), pH 7.05 (■), and pH 8.2 (●) as a function of NaPSS concentration. The S−E prediction for the ratio is the horizontal line at 1.0.

Test of Stokes−Einstein Relation. In Figures 3 and 4 we have plotted the ratio $D\eta/D_0\eta_0$ against high and low molecular weight NaPSS concentrations at different pHs to display the deviations from the ideal S−E value of 1.0. Deviations from the S−E expectation increased with increasing NaPSS concentrations but saturated beyond 50 g/L at all pHs. Deviations from the S−E expectation were greater at higher pHs, except at the highest concentrations of low molecular weight NaPSS. This observable charge effect differed from that observed in paper 1, where we did not observe any systematic dependence of deviation from the S−E value with change in added salt concentration, i.e., with change in screening of background polymer charge. The

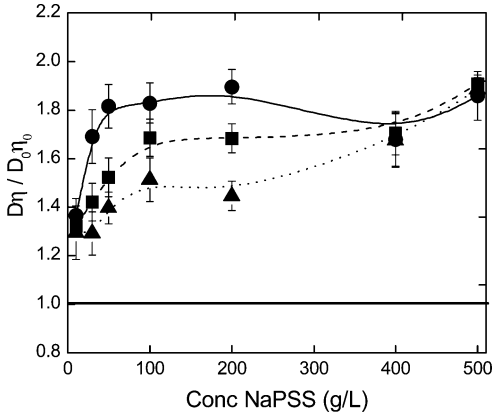

Figure 4. Deviations from S−E ratio $D\eta/D_0\eta_0$ for GFP in low molecular weight NaPSS solution at pH 5.5 (▲), pH 7.05 (■), and pH 8.2 (●) as a function of NaPSS concentration. The S−E prediction for the ratio is the horizontal line at 1.0.

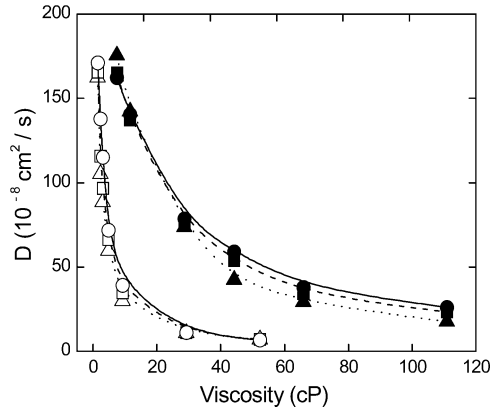

Figure 5. Diffusion coefficient of probe GFP (open symbols for low molecular weight NaPSS and closed symbols for high molecular weight NaPSS) at pH 5.5 (▲), pH 7.05 (■), and pH 8.2 (●) as a function of solution viscosity.

deviations from S−E behavior were much smaller—only about 2-fold—in low molecular weight NaPSS than in high molecular weight NaPSS where the deviation was as high as 15-fold.

This difference in diffusion behavior between high and low molecular weight NaPSS solutions is manifest in Figure 5. At the same solution viscosity, D was much higher in the high molecular weight solutions. At the same viscosity, D was also higher for higher pHs.

Stretched Exponential Behavior of Probe Diffusion. Figures 6 and 7 show nonlinear least-squares fits of the normalized diffusion coefficient D/D_0 as a function of NaPSS concentration to the stretched exponential equation[6] (eq 4 in paper 1). The fitted parameters are given in Table 1. In high molecular weight NaPSS solutions v decreased from 1.02 ± 0.08 at pH 5.5 to 0.83 ± 0.05 at pH 8.2. In low molecular weight NaPSS solutions the trend was opposite: an increase from 0.68 ± 0.03 at pH 5.5 to 0.86 ± 0.03 at pH 8.2.

Discussion and Conclusions

The results reported in this paper represent significant new findings about probe diffusion in concentrated, strongly interacting polyelectrolyte solutions. Perhaps most striking, the 15-fold positive deviation from ideal S−E behavior for GFP at high pH in high molecular weight NaPSS is the highest we are aware of for diffusion of a globular protein or other compact polymer molecule. It shows that if the probe itself has significant

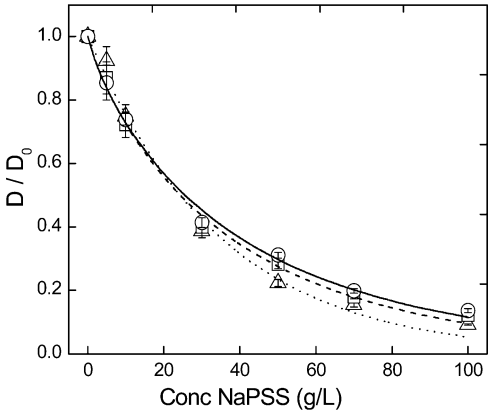

Figure 6. Normalized diffusion coefficient of GFP in high molecular weight NaPSS solution at pH 5.5 (△), pH 7.05 (□), and pH 8.2 (○) as a function of NaPSS concentration. The lines are fits to the stretched exponential equation.

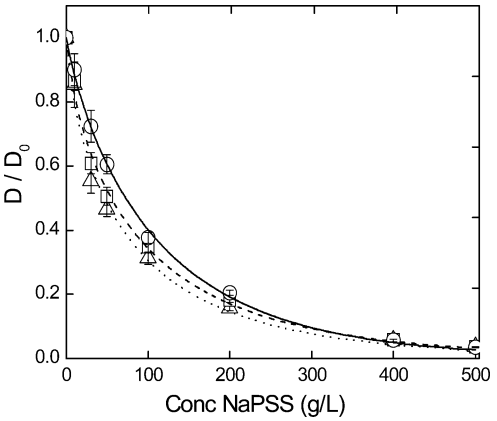

Figure 7. Normalized diffusion coefficient of GFP in low molecular weight NaPSS solution at pH 5.5 (△), pH 7.05 (□), and pH 8.2 (○) as a function of NaPSS concentration. The lines are fits to the stretched exponential equation.

Table 1. Nonlinear Regression Parameters for Stretched Exponential Equation $D/D_0 = \exp(-\alpha c^v)$

	NaPSS ($M_w = 1 \times 10^6$)		NaPSS ($M_w = 7 \times 10^4$)	
pH	α	v	α	v
5.5	0.027 ± 0.008	1.02 ± 0.08	0.047 ± 0.01	0.68 ± 0.03
7.05	0.045 ± 0.008	0.87 ± 0.05	0.038 ± 0.007	0.73 ± 0.04
8.2	0.047 ± 0.007	0.83 ± 0.04	0.017 ± 0.002	0.86 ± 0.03

charge, then Coulombic interactions between probe and background play a major role in probe diffusion. The increasing deviations from ideal S−E behavior observed as the probe charge becomes more negative contrast with the results in paper 1, where increased repulsion between background low molecular weight NaPSS chains, caused by lowering the salt concentration, does not affect probe diffusion.

It is useful to put the work reported in this paper into the context of the broader results we have obtained. This work is a continuation of paper 1 and earlier work from our laboratory,[7,8] in which we have used FRAP to study the probe diffusion of small protein molecules—GFP and bovine serum albumin labeled with fluorescein isothiocyanate (BSA-FITC)—in a variety of macromolecular solutions including neutral Ficoll and negatively charged NaPSS and DNA. Our aim has been to garner insights into how the combined effects of macromolecular crowding, solution viscosity, and electrostatic interactions influence probe diffusion, a complex of issues pertinent to understanding the dynamics of protein motion (and

the motion of other relatively small, compact biopolymers such as tRNA) in cellular environments.

High concentrations of background macromolecules influence probe diffusion both directly through their occupancy of solution volume—crowding—and indirectly through their effect on solution viscosity. Our studies have shown clear distinctions between relatively small, compact macromolecules such as Ficoll and low molecular weight NaPSS, small but asymmetric persistence length DNA, and large flexible polymers such as high molecular weight NaPSS. The large or asymmetric polymers strongly increase solution viscosity even at relatively low volume occupancy, but do so in a manner that occupies space rather unevenly, leaving fluctuating regions through which probe molecules can diffuse considerably faster than would be predicted from the bulk viscosity. For example, to achieve a solution viscosity of 29 cP requires 80 g/L of persistence length DNA, but 400 g/L of low molecular weight NaPSS. This is one important source of large positive deviations from ideal Stokes−Einstein behavior.

Matters become even more complicated when electrostatic interactions enter the picture. Both DNA and NaPSS are fully negatively charged. By changing the ionic strength with added salt, Coulombic interactions between polyelectrolyte molecules and between charged segments on the same chain can be modulated. In particular, reducing the ionic strength swells large flexible chains such as high molecular weight NaPSS leading both to higher solution viscosity and to larger fluctuating voids, thus increasing the positive deviations from S−E expectations. Such chain swelling does not occur to such an appreciable extent in low molecular weight NaPSS and may saturate even in high molecular weight polyelectrolyte at high polymer concentrations.

If the probe is uncharged or only weakly charged, as was the case for GFP in paper 1, then its diffusion will reflect mainly the behavior of the background polymer. However, if the probe has significant charge, as was the case in this paper for GFP at higher pH and also for BSA-FITC,[7,8] then Coulombic interactions between probe and background also affect diffusion. If probe and background have charges of the same sign, as they do in the systems we have studied, then electrostatic repulsion should provide an additional driving force for diffusion. This effect may saturate at very high background polymer concentrations, where chain segments are so close together that the probe experiences an essentially uniform electrostatic potential. The increase in D of GFP with increasing pH, and then it is leveling off at the highest NaPSS concentrations, is consistent with this picture.

In persistence length DNA of approximately the same molecular weight as low molecular weight NaPSS, an extraordinary diffusion phase emerges at low ionic strength.[9,10] This poorly understood diffusional behavior of the DNA appears to couple to the diffusion of the probe, presumably by an electrostatic mechanism, leading to large positive deviations from S−E expectations.[7,8]

Another contribution to the large deviations from S−E behavior, in addition to microviscosity and electrostatic interactions, may be the viscoelasticity of high molecular weight concentrated polyelectrolyte solutions. Because of their large size and charge, some polyelectrolytes are known to behave as viscoelastic rather than Newtonian liquids.[13] Dunstan et al.,[14] who studied probe diffusion in a viscoelastic liquid by dynamic light scattering, observed fast diffusion coefficients for probe molecules that were larger than in pure solvent. It seemed that this could only be possible because of the viscoelastic nature of the background polymer solution, in which elastic energy stored in the network is transferred to drive probe motion.

The deviations from S−E behavior that we have found in this and our previous studies are the largest ever observed, to our knowledge, for globular protein molecules: 5-fold for GFP in concentrated DNA solutions at low electrolyte concentrations,[7] 10-fold for GFP near its isoelectric point in concentrated solutions of high molecular weight NaPSS at low electrolyte concentrations (paper 1), and about 15-fold for GFP in concentrated high molecular weight NaPSS solutions at the highest pH investigated in this study. Larger deviations from S−E expectations have been observed for random coil polymers,[11] but in those cases the diffusion is thought to occur by a reptation mechanism.[12] Reptation is not a possibility for globular proteins, which are compact and essentially incompressible.

The stretched exponential parameters in Table 1 behave differently for high and low molecular weight NaPSS solutions. For high molecular weight NaPSS the exponent v is 1.0 for pH 5.5 and decreases to 0.83 with increase in pH to 8.2, whereas for low molecular weight NaPSS v is 0.68 at pH 5.5 and increases to 0.86 at pH 8.2. A survey[15] of probe diffusion in a wide range of polymer background environments showed that v varies between 0.5 and 1, with the lower and higher values corresponding to hydrodynamic and electrostatic interactions, respectively. This corresponds with our observations in low molecular weight NaPSS solutions but is opposite to the behavior observed in high molecular weight NaPSS. We currently have no explanation for this discrepancy; but given the complex set of interactions, which appear to govern probe diffusion in highly concentrated, highly charged, high molecular weight polymers, lack of a completely consistent explanation is not surprising.

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