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# An NH Moiety Is Not Required for Anion Binding to Amides in Aqueous Solution

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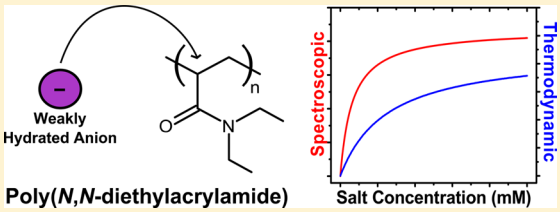
**ABSTRACT:** Herein, we use a combination of thermodynamic and spectroscopic measurements to investigate the interactions of Hofmeister anions with a thermoresponsive polymer, poly(*N*,*N*-diethylacrylamide) (PDEA). This amide-based polymer does not contain an NH moiety in its chemical structure and, thus, can serve as a model to test if anions bind to amides in the absence of an NH site. The lower critical solution temperature (LCST) of PDEA was measured as a function of the concentration for 11 sodium salts in aqueous solutions, and followed a direct Hofmeister series for the ability of anions to precipitate the polymer. More strongly hydrated anions ($CO_3^{2-}$, $SO_4^{2-}$, $S_2O_3^{2-}$, $H_2PO_4^-$, $F^-$, and $Cl^-$) linearly decreased the LCST of the polymer with increasing the salt concentration. Weakly hydrated anions ($SCN^-$, $ClO_4^-$, $I^-$, $NO_3^-$, and $Br^-$) increased the LCST at lower salt concentrations but salted the polymer out at higher salt concentrations. Proton nuclear magnetic resonance (NMR) was used to probe the mechanism of the salting-in effect and showed apparent binding between weakly hydrated anions ($SCN^-$ and $I^-$) and the $\alpha$ protons of the polymer backbone. Additional experiments performed by attenuated total reflection Fourier transform infrared (ATR−FTIR) spectroscopy found little change in the amide I band upon the addition of salt, which is consistent with very limited, if any, interactions between the salt ions and the carbonyl moiety of the amide. These results support a molecular mechanism for ion-specific effects on proteins and model amides that does not specifically require an NH group to interact with the anions for the salting-in effect to occur.




## INTRODUCTION

The physical behavior of numerous surfactants, polymers, and biomolecules in aqueous salt solutions is ion-specific and follows a Hofmeister series.[1−9] The series has been known for over a century and was originally developed to rank cations and anions by their ability to influence the solubility of egg white proteins.[10,11] This ordering, however, was found to recur for a wide variety of proteins and extends well beyond protein precipitation studies.[12] The Hofmeister effect has attracted the attention of many researchers over the last several decades in an attempt to generate a molecular level understanding of this phenomenon.[2,13−18] Although there has been progress, it remains a challenge because there are multiple mechanisms responsible for the Hofmeister series and the general interactions are very weak. Indeed, Hofmeister series behavior is typically found at millimolar salt concentrations and higher.[19] As such, in depth spectroscopic studies are required to elucidate the molecular level basis of the interactions, along with complementary macroscopic thermodynamic behavior.

Recent reports have made significant contributions toward developing an understanding of the underlying molecular mechanisms of ion interactions with small organic molecules, model amides, and protein backbones as well as their side chains in aqueous solutions.[13,20−27] Specifically, large polarizable ions, such as $I^-$ and $SCN^-$, can bind directly to protein backbones.[24−26] Nuclear magnetic resonance (NMR) measurements and molecular dynamics (MD) simulations demonstrate that such weakly hydrated anions interact directly with the backbone methylene or other CH groups adjacent to the amide moieties. These same techniques further indicate that the NH group of the amide also participates in anion binding. As such, we wished to directly test if it is necessary to have an NH moiety present for anion binding.

Poly(*N*,*N*-diethylacrylamide) (PDEA) was employed as a model system to probe the necessity of the amide NH site. As seen in Figure 1, this thermoresponsive polymer is structurally similar to poly(*N*-isopropylacrylamide) (PNIPAM). The latter is much more widely employed for lower critical solution temperature (LCST) measurements, although both undergo

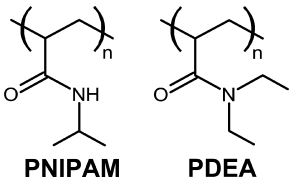



**Figure 1.** Schematic diagrams of the structures for PNIPAM and PDEA.

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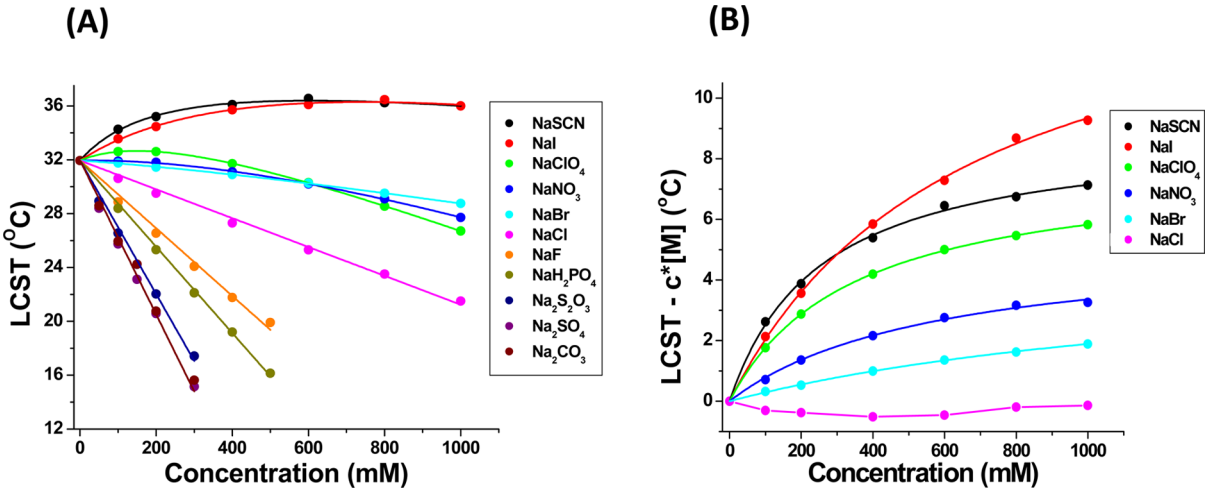


**Figure 2.** (A) LCST values of PDEA as a function of the salt concentration for 11 sodium salts. The solid lines are the best fits to eq 1. (B) Residual LCST data for PDEA of 6 salts after subtraction of the linear component. The solid lines are best fits to the binding isotherm portion of eq 1, except for NaCl, where the data points are connected only as a guide to the eye.

the same inverse phase transition. In fact, PDEA displays an LCST value of ~32 °C,[28] which is quite close to that of PNIPAM (~31 °C).[29] An important chemical difference for PDEA, however, is that it does not have an NH moiety on its amide group, as is the case for PNIPAM or polypeptides. Therefore, PDEA is only a hydrogen bond acceptor in aqueous solutions. To glean molecular level information for ion-specific interactions with PDEA, we have employed NMR and attenuated total reflection Fourier transform infrared (ATR–FTIR) spectroscopy along with LCST measurements as a function of salt identity and concentration. It was found that the ability of a specific anion to modulate the LCST of this polymer in aqueous solution followed a direct Hofmeister series. As such, more strongly hydrated anions salted the polymer out of solution and the more weakly hydrated anions salted the polymer in solution. Using proton NMR, it was shown that the binding site on PDEA involves the backbone $\alpha$ protons. Moreover, the apparent equilibrium dissociation constants for weakly hydrated anions were comparable to those for PNIPAM in the presence of the same ions.[2] ATR–FTIR was used to probe the amide I band of PDEA and showed no appreciable changes in peak frequency or intensity in the presence of various salts. This is consistent with the notion that the cations do not strongly interact with the carbonyl moiety of the amide.[22] The overall results of this study demonstrate that an NH moiety is not required for the salting in of polymers or peptides by ions in aqueous salt solutions.

## MATERIALS AND METHODS

**LCST Measurements.** LCST measurements of PDEA were performed by measuring the turbidity change of polymer solutions in capillary tubes with a charge-coupled device (CCD) camera that was interfaced with digital image processing software (MPA 100 Optimelt automated melting point system, Stanford Research Systems). The light scattering intensity was measured as a function of the temperature at a ramp rate of 1.0 °C per minute. The transition temperatures were determined as the onset of the increase in light scattering intensity relative to the baseline as described previously.[5,30]

**Sample Preparation.** PDEA with a molecular weight of 260 kDa was purchased from Polymer Source, Inc. (Dorval, Quebec, Canada). Sodium salts were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. NaSCN was at least 98% pure, while the other salts used, $Na_2SO_4$, $Na_2CO_3$, $Na_2S_2O_3$, $NaH_2PO_4$, NaF, NaCl, NaBr, $NaNO_3$, $NaClO_4$, and NaI, were at least 99% pure. The 18.2 MΩ cm purified water from a Thermo Scientific Nanopure Barnstead purification system was used to prepare salt and polymer solutions. For LCST and NMR measurements, the polymer was precipitated from hot aqueous solution to remove unpolymerized monomers and short-chain oligomers. Redissolved aqueous polymer solutions were aliquoted and vacuum-dried from a 10 mg/mL stock solution. The appropriate concentrations of salt solutions were added to the vacuum-dried pellets at temperatures below the LCST, followed by vortexing until the polymer was fully dissolved. The final PDEA concentration employed in both LCST and NMR measurements was 1 mg/mL.

**NMR.** All spectra were acquired on a 400 MHz NMR spectrometer equipped with a 5 mm TXI probe (Bruker, Billerica, MA) at a temperature of 5 °C. For chemical shift assignments of PDEA, [$^1$H,$^1$H]-NOESY (100 ms mixing time) and [$^1$H,$^1$H]-TOCSY (100 ms mixing time) were employed.[31] TopSpin software (Bruker) was used for data processing. $^1$H NMR spectra were acquired using Watergate for water suppression[32] for all experiments involving the titration of PDEA with salts. It was verified that this suppression profile caused no measurable peak shifts to any of the polymer proton resonances that were investigated in these PDEA studies. Sample spectra were externally referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, Cambridge Isotope Laboratories) in pure $D_2O$ (99.9% D, Cambridge Isotope Laboratories) in NMR tubes adapted with coaxial inserts (Wilmad-LabGlass). DSS was always in the inner tube of the concentric tubes, while the polymer sample was in the outer tube. As such, the DSS reference was never exposed to the polymer or varying salt concentrations.

**FTIR.** Infrared spectra were measured with a Nicolet 470 FTIR spectrometer using an attenuated total reflection (ATR) attachment (Pike Miracle, Madison, WI).[22] The ATR crystal (diamond-coated ZnSe, Pike Technologies, Madison, WI) was employed in a single bounce geometry to make the measurements. The infrared radiation was detected with a liquid nitrogen-cooled MCT detector (Thermo Electron Corp., Madison, WI). All spectra were averaged over 128 scans and collected in a range from 650 to 4000 $cm^{-1}$ with a spectral resolution of 2 $cm^{-1}$. The samples for ATR–FTIR were prepared by dissolving vacuum-dried PDEA in the desired salt solution. The final polymer concentration was 10 mg/mL. To completely dissolve the polymer, the samples were stored overnight at 4 °C. During acquisition of spectra, the sample stage was kept at 5 °C, which was at least 10 °C below the LCST of the polymer under all conditions. Sample spectra were measured a minimum of 3 times each. Moreover, an otherwise identical salt solution without PDEA was used to make background measurements, which were made immediately preceding


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sample measurements. This background was subtracted from each sample spectrum using OMNIC software.

## RESULTS AND DISCUSSION

**Solubility of PDEA as a Function of Sodium Salts.** Figure 2A plots the LCST of PDEA as a function of the salt concentration for 11 sodium salts. The transition temperature decreases linearly with increasing the salt concentration for solutions containing the more strongly hydrated anions. The ability of a particular sodium salt to decrease the LCST had the following rank ordering: $CO_3^{2-} \sim SO_4^{2-} > S_2O_3^{2-} > H_2PO_4^- > F^- > Cl^-$. In contrast, when $Br^-$, $NO_3^-$, $ClO_4^-$, $I^-$, and $SCN^-$ were added to solution, the transition temperature changed in a nonlinear fashion. By analogy with previous results for PNIPAM,[2] these nonlinear data can be fit to a simple empirical equation consisting of a linear term plus a Langmuir isotherm term (eq 1).

$$\text{LCST (°C)} = T_0 + c[M] + \frac{B_{max}[M]}{K_d + [M]} \quad (1)$$

$T_0$, 31.9 °C, is the LCST in the absence of salt, and $c$, which has units of temperature/concentration (°C/[$M$]), is the slope of the linear term. The value of $c$ is related to the salting-out efficacy of a given salt and can be associated with an increase in surface tension at the polymer/water interface as well as excluded volume effects from the depletion of well-hydrated ions from the polymer/water interface.[33] The nonlinear term represents an apparent binding isotherm with a coefficient $B_{max}$, which corresponds to the maximum increase in the LCST of the polymer upon saturation binding of ions. $K_d$ is the apparent equilibrium dissociation constant of the anion−polymer interaction. It is worth noting that a rigorous treatment of these empirical fitting parameters has been recently undertaken and that a properly defined binding constant involves contributions from $c$ and $B_{max}$ as well as $K_d$.[17]

It should be noted that the data for more strongly hydrated anions can be fit to eq 1 assuming an infinitely weak binding interaction; e.g., the nonlinear term would approach zero as $K_d$ approaches infinity. After subtraction of the fitted linear term ($T_0 + c[M]$) from the data in Figure 2A for weakly hydrated anions, the residual changes in LCST can be plotted as a function of the salt concentration (Figure 2B). These residual values show saturation behavior and can be well-fit to Langmuir isotherms, as shown by the solid line fits to the data. The corresponding apparent $K_d$ values are listed in Table 1. It should be noted that, although the fits in Figure 2B are quite good, using phase transition measurements to approximate equilibrium dissociation constants are not proper isothermal measurements. Indeed, the phase transition temperature changes slightly as salt is added to the solution. Therefore, it is important to also investigate these interactions isothermally. Such work, which is described in the next section, has been performed using NMR at a constant temperature. Moreover, NMR can provide chemistry-specific information that can then be correlated with the thermodynamic measurements described above.

**Table 1. Abstracted Apparent Dissociation Constants, $K_d$ Values, of PDEA from Both NMR and LCST Data Fits[a]**

| $Na^+$ counteranion | $K_d$ (mM) from LCST | $K_d$ (mM) from $\alpha$ proton |
|---|---|---|
| $SCN^-$ | 250 | 70 |
| $I^-$ | 660 | 260 |
| $ClO_4^-$ | 350 | |
| $Br^-$ | >1000 | |
| $NO_3^-$ | >1000 | |

[a] $K_d$ values larger than 1000 mM represent binding weaker than the highest concentration of salt measured.

**NMR Titration of PDEA versus Sodium Salts.** In a set of proton NMR experiments, $Na_2SO_4$, NaCl, NaI, and NaSCN were chosen as representative Hofmeister salts to probe the interactions of PDEA with anions. Figure 3 plots the change in the chemical shift ($\Delta\delta$) of the $N$-$CH_2$ protons (black circles), methyl protons (green circles), and backbone $\alpha$ protons (red circles) as a function of the salt concentration. As seen from the data for $Na_2SO_4$ and NaCl (panels A and B of Figure 3), the relative chemical shift decreases linearly at all three positions on the polymer. The linear decreases in chemical shift resemble the linear decreases in the LCST values for the corresponding salts in Figure 2A. As such, these linearly decreasing proton chemical shift values are consistent with the idea that the ions do not interact strongly with the polymer. Indeed, the salting-out behavior in Figure 2A is interpreted to mean that the ions are depleted at the polymer/water interface.[33−36]

Panels C and D of Figure 3 plot the relative chemical shift changes of $N$-$CH_2$, methyl, and $\alpha$ protons with increasing concentrations of NaI and NaSCN, respectively. In contrast with the data above, only the $N$-$CH_2$ and methyl protons shift linearly, while the $\alpha$ protons (red) display distinct nonlinearity. Such nonlinearity in the chemical shift is consistent with a binding interaction between these ions and the polymer at the site of the $\alpha$ protons and the corresponding $\alpha$ carbon. This result is analogous to previous reports, where $SCN^-$ and $I^-$ led to nonlinear NMR shifts of $\alpha$ protons in the backbones of elastin-like polypeptides.[26] The difference here, of course, is that such shifts are occurring on a polymer that contains no NH moiety within the amide group. In the present case, it would appear that the backbone is the key interaction site of the weakly hydrated anions with the polymer. The chemical shift changes of $\alpha$ protons of PDEA as a function of NaSCN and NaI can be fit to eq 2.

$$\Delta\delta \text{ (ppm)} = c[M] + \frac{\delta_{max}[M]}{K_d + [M]} \quad (2)$$

It should be noted that eq 2 is analogous to eq 1 and has a linear and nonlinear term, where now the coefficient $c$ has units of chemical shift per molarity of salt added. The nonlinear term represents direct anion binding with the coefficient $\delta_{max}$ representing the maximum change in the chemical shift upon saturation ion binding, and $K_d$ is the apparent equilibrium dissociation constant. After subtraction of the linear portion of the data for the $\alpha$ protons, the residual changes in the chemical shift follow a binding curve for both NaSCN and NaI (green and black data points in Figure 4, respectively). The residual nonlinear portions of the LCST data from Figure 2B for NaSCN and NaI are also shown. As seen, NaSCN binds slightly tighter to the polymer than NaI for both the NMR and LCST data, which is consistent with previous results.[26] The abstracted apparent $K_d$ values from both fitted data sets are shown in Table 1. Overall, the apparent dissociation constants from the LCST measurements were weaker than those of the corresponding proton NMR measurements. This is consistent with the notion that the $K_d$ values abstracted from the LCST measurements represent averages of interactions with ions over



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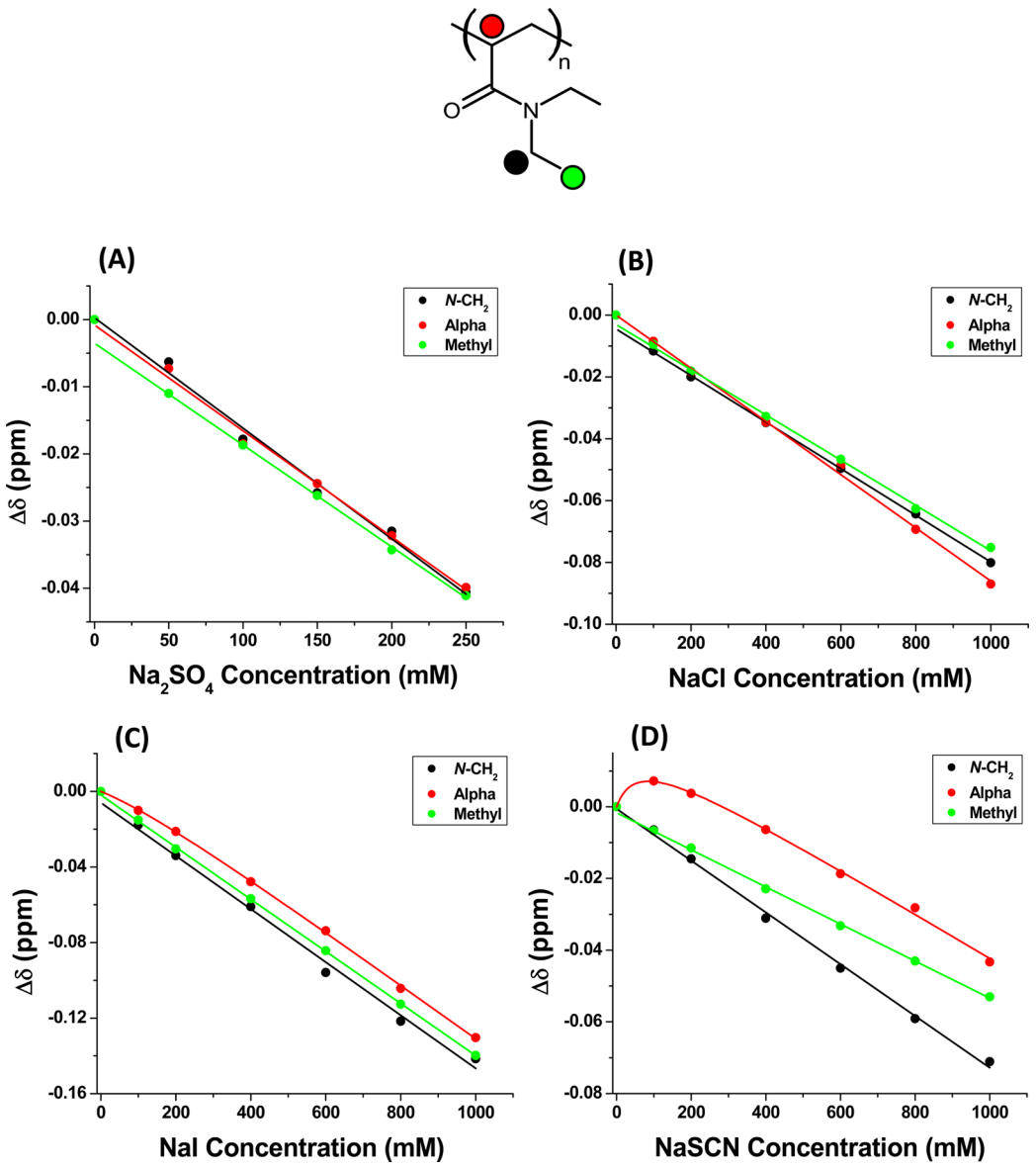


**Figure 3.** Relative chemical shift changes ($\Delta\delta$) of $N$-$CH_2$, $\alpha$, and methyl protons of PDEA as a function of the salt concentration for (A) $Na_2SO_4$, (B) NaCl, (C) NaI, and (D) NaSCN. The solid lines represent best fits of the data points by eq 2. The structure of PDEA is also provided with the corresponding positions of the $N$-$CH_2$ (black dots), $\alpha$ (red dots), and methyl (green dots) proton sites.

the entire polymer surface, while the $K_d$ values from the NMR data represent a single site of interaction, i.e., the $\alpha$ proton. Moreover, as noted above, the NMR data were measured isothermally, while the LCST measurements were not. Nevertheless, the spectroscopic dissociation constants are only about a factor of ~2.5 tighter for $SCN^-$ and ~3.6 tighter for $I^-$ compared to the thermodynamic measurements, which is fairly close for values obtained by very different methods.

It should be noted that Figure 3 only plots data from the $N$-$CH_2$ protons, methyl protons, and backbone $\alpha$ protons but not from the methylene groups on the backbone. Unfortunately, the peaks associated with this site were too broad to abstract the corresponding peak shifts. This was true for all of the salts that were tested.

**FTIR of PDEA versus Sodium Salts.** In a final set of experiments, FTIR measurements were made to probe the interactions of various sodium salts with the carbonyl groups of PDEA. Figure 5 shows the spectrum of the amide I region of PDEA in $D_2O$. It should be noted that the amide I band of PDEA arises mostly from the carbonyl stretch in $D_2O$,[37] yet the peak shape is somewhat different compared to PNIPAM.[38] Specifically, PDEA gave rise to two absorption peaks centered at 1619 and 1599 $cm^{-1}$ below its LCST,[39] while PNIPAM only has one amide band. Figure 6 shows the carbonyl peak position of both the 1619 $cm^{-1}$ peak and the 1599 $cm^{-1}$ peak of PDEA as a function of salt concentrations for four different anions. As seen, both peak positions remained unchanged within the experimental error. Such FTIR data suggest that there is little, if any, direct interactions between the sodium salts and the carbonyl groups. Moreover, the results are consistent with previous NMR, FTIR, and MD simulation data for amides, where such interactions were found to be unfavorable.[22,24,25,40,41]



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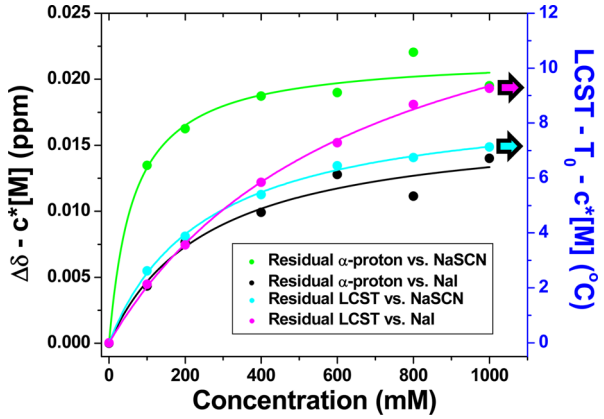


**Figure 4.** Residual change in the chemical shift and LCST data after subtraction of the linear portions of the data. The chemical shift residuals are plotted on the left axis, while the LCST residuals are plotted on the right axis.

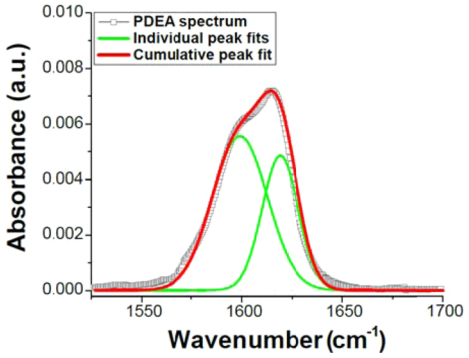


**Figure 5.** FTIR spectrum of PDEA in $D_2O$ at 5 °C, with individual Gaussian peaks fits (green curves) and a cumulative peak fit (red curve).

## CONCLUSION

The apparent equilibrium dissociation constants and salting-out coefficients for anions with PDEA found herein are quite close to the values for other poly(*N*-alkylacrylamide)-based polymers, including PNIPAM[2] and poly(vinylpyrrolidinone).[42] Moreover, these $K_d$ values are also relatively close to those found for elastin-like polypeptides (ELPs), where the amide moiety is in the backbone rather than the side chain.[3,26] Such findings suggests that the amide NH group is not decisive for the interactions between weakly hydrated anions and amide groups on polymers in aqueous solutions. Indeed, PNIPAM, ELPs, and PDEA all have similar $\alpha$ proton binding sites, which consist of a combination of an electron-withdrawing substituent directly adjacent to a CH or $CH_2$ moiety.[26] Unlike PNIPAM and ELPs, however, PDEA can only accept hydrogen bonds from water molecules rather than donate to them. In fact, although the interaction between an NH group and an anion may be favorable in the gas phase, there is an energetic penalty associated with displacing the hydrogen bond to the amide NH group from water upon anion binding in aqueous solutions. This probably leads to little net change in free energy upon anion binding at that site in solution. One might also wonder if the isopropyl group on PNIPAM sterically hinders the binding of the anion. However, weakly hydrated anions do not bind to polyacrylamide either;[26] therefore, it is not just steric hindrance that is preventing binding from occurring.

Curiously, the NMR data in Figure 4 reveal that only the backbone CH groups provide a binding site for the weakly hydrated anions, while the ethyl groups attached to the amide nitrogen do not (Figure 4). One might expect that the $CH_2$ groups directly adjacent to the amide nitrogen would be the most favorable site for the binding of weakly hydrated anions. However, these moieties may be sterically inaccessible. Moreover, the inductive effects from the adjacent amide may not be large enough to cause observable binding. In contrast, ELPs and other polypeptides have two amide dipoles withdrawing electron density from the adjacent aliphatic carbons.

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### Notes

The authors declare no competing financial interest.

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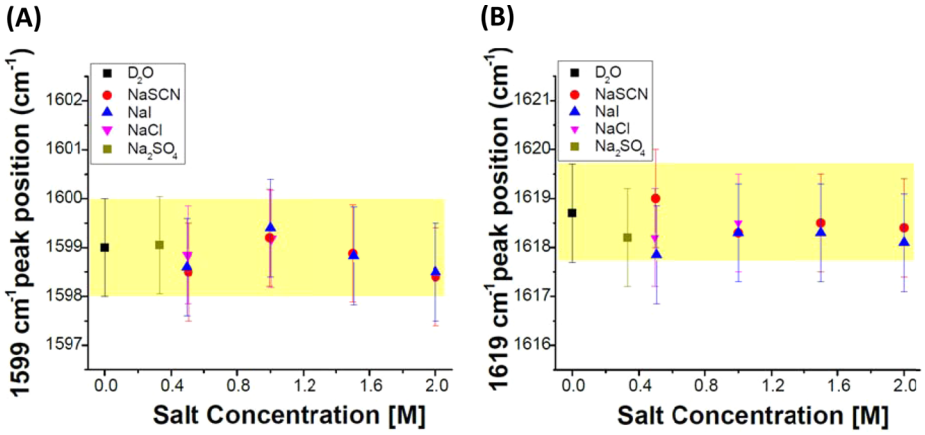


**Figure 6.** Individual amide I peak positions plotted as a function of the salt concentration for the (A) 1599 $cm^{-1}$ and (B) 1619 $cm^{-1}$ peak positions. The yellow highlighted regions represent the 2 $cm^{-1}$ resolution of the spectrometer centered at each of these two peaks in $D_2O$ in the absence of salt.



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