# Partitioning of Poly(ethylene oxide), Poly(ethylene imide), and Bovine Serum Albumin in Isobutyric Acid + Water

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ABSTRACT: We present new measurements of the partitioning of the polymers (poly(ethylene glycol) (PEG) and poly(ethylene imine) (PEI)) and the protein bovine serum albumin (BSA) between coexisting liquid phases of isobutyric acid + water. We show that all partition unevenly between the liquid phases: In all cases, there is substantially more polymer or protein in the upper, isobutyric acid-rich phase. At molecular masses above about 10 kg/mol, PEG and PEI fractionate between the liquid phases, with a higher average molecular mass in the lower, water-rich phase. The fractionation and partitioning are amplified both when the molecular mass of the polymer is increased, and when $D_2O$ + isobutyric acid is used instead of $H_2O$ + isobutyric acid. The dependence of the distribution coefficient on molecular mass disagrees with the predictions of Flory−Huggins mean field theory for all PEG and PEI systems studied. At low molecular weight, the PEG fractionations are consistent with theoretical predictions that the molecular mass distributions of the daughter phases will have the same mathematical forms as the parent phase. However, as the average molecular mass increases, the PEG molecular mass distributions of the daughter phases differ from those of the parent phases. The critical temperature, $T_c$, of PEG + isobutyric acid + $H_2O$ increases approximately linearly with PEG concentration, increases nonlinearly with PEG average molecular mass, and is independent of the terminating group.

## Introduction

Consider the addition of a polydisperse polymer to two coexisting liquid phases. If the monomer constituting the polymer is more attracted to one liquid phase than to the other, then that attraction will be amplified when the monomers combine to form a polymer, and the polymer will be drawn more to that phase at larger molecular mass. Therefore, the coexisting liquid phases will contain polymer molecules with different molecular mass distributions (MMD's), different average molecular masses, and different total masses of polymer. Such polymer fractionations, where the distribution/partition coefficient of the polymer is a function of the molecular mass, are well-known, but they are usually not very dramatic, with differences of average molecular mass between the two phases being less than 10%.[1,2]

Looking for a solvent system that might interact strongly with a polymer, we thought of earlier work with isobutyric acid + water.[3] We then found a very dramatic fractionation of poly(ethylene glycol) (PEG) in coexisting phases of this mixture: The average molecular mass of the PEG in the lower, water-rich phase is about twice that in the upper isobutyric acid-rich phase, for a polymer molecular mass of about 20 kg/mol.[4] In addition, the majority of the PEG molecules partition into the upper isobutyric acid-rich phase, and there is a considerable reduction in the polydispersity of the PEG chains in the lower phase. We have since shown, using small-angle neutron scattering and polarimetry with a chiral dopant, that the PEG has a coil configuration in water, but a helical configuration in isobutyric acid and its mixtures with water.[5,6] We hypothesize that the driving force for the partitioning and fractionation of PEG in isobutyric acid + water is related to the conformational change of the PEG molecules from coils in the lower water-rich phase to helices in the upper isobutyric acid-rich phase, and that the conformational change is induced by a hydration layer of water that is present on the PEG, even in the acid-rich phase.[6]

This case of significant fractionation offers an opportunity to study the factors affecting fractionation and to test theories of polymer fractionation. Here we address the effects of polymer average molecular mass and terminating group (−OH vs $-OCH_3$) on PEG fractionation in isobutyric acid + $H_2O$ (upper critical solution temperature, UCST, at about 26 °C[3,7]), and in isobutyric acid + $D_2O$ (UCST at about 45 °C[8,9]). We first measured the critical temperature, $T_c$, of PEG + isobutyric acid + $H_2O$ as a function of polymer concentration, molecular mass, and terminating group. We find that $T_c$ increases approximately linearly with PEG concentration, but increases nonlinearly with PEG molecular mass and is independent of the terminating group.

We also examine a polymer with a different but related backbone structure: poly(ethylene imine) (PEI). For PEI, the −O− atoms in the PEG backbone are replaced by −NH− groups. In PEG, the carbon atoms are hydrophobic and the oxygen atoms are hydrophilic, because they accept hydrogen bonds. In PEI, the −NH− groups can accept and donate hydrogen bonds and are very hydrophilic, which makes this polymer, like PEG, very hygroscopic.[10−12] Like PEG, PEI molecules form coils in water and helices in isobutyric acid.[6]

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In all cases discussed below, the majority of the PEG or the PEI migrates into the upper isobutyric acid-rich phase, even though the polymers are more soluble in water than in isobutyric acid. As the molecular mass of the polymer increases, the degree of fractionation is enhanced. The dependence of the distribution coefficient on molecular mass disagrees with the predictions of Flory−Huggins mean field theory for all PEG and PEI systems studied. For low average molecular masses of PEG, the mathematical forms of the MMD's of the polymers in the coexisting daughter phases are, within the experimental uncertainty, the same as the forms of the MMD's of the starting parent polymers. As the average molecular mass of PEG is increased, the functional form of the MMD of the PEG in the upper, isobutyric acid phase remains the same as that of the parent PEG, but the mathematical form of the MMD of the PEG in the lower, water-rich phase differs from that of the parent PEG. The effect of using $D_2O$ in the solvent mixture is to increase both partitioning and fractionation, as might be expected because $D_2O$ forms stronger hydrogen bonds and thus a more tightly bound hydration layer.

PEG and PEI molecules contain both hydrophilic and hydrophobic groups, and therefore their behavior in solution may be similar to the behavior of biomacromolecules, such as proteins. We include here the partitioning of bovine serum albumin (BSA) in isobutyric acid + $H_2O$ and show that BSA also partitions mostly into the upper isobutyric acid-rich phase, even though it is more soluble in water. We propose that here, too, a hydration layer supports the solubility.

## Theory and Prior Experiments

**A. How does the Addition of a Polymer Affect the $T_c$ of the Binary Liquid Mixture?** When a low concentration of a third component (an "impurity") is added to a binary liquid mixture with a consolute point, the critical temperature, $T_c$, and the critical composition, $x_c$, can change.[13] When the third component is a polymer, then the shifts in $x_c$ and $T_c$ depend on the average polymer molecular mass, as well as on the polymer concentration.[14] Studies in which the third component is a polymer[14−21] show that $T_c$ can increase or decrease on addition of polymer, and that $T_c$ is much more sensitive to the presence of polymer than is $x_c$.

The effect of the third component can be understood both in an intuitive manner[22] and in the context of a simple theory such as the regular solution theory[23] and also from thermodynamic arguments based on the Gibbs−Duhem equation.[24] If the third component is equally soluble in both liquids of the binary mixture, then we expect that third component to increase the mutual solubility of the two components and thus decrease a UCST and increase a lower critical solution temperature (LCST). If the third component is more soluble in one liquid component than in the other, then we expect that the mutual solubility will be decreased, thus increasing a UCST and decreasing an LCST. These generalizations, based in thermodynamics, are sometimes called the Timmermans rules.[25] On the other hand, the Timmermans rules have been found to be violated in some cases of binary fluid mixtures where one component is water.[25,26] A theoretical analysis based on lattice cluster theory for the addition of a block copolymer as an impurity to a binary polymer blend supports the Timmermans rules, except in the case of an LCST.[27]

PEG is much more soluble in pure water than in pure isobutyric acid, but when the two fluids are mixed and form two phases, the PEG distributes mostly into the isobutyric acid-rich upper phase.[4] The Timmermans rules would predict that PEG will increase the $T_c$ of isobutyric acid + water, but as noted above, aqueous systems often violate the Timmermans rules.

We are aware of no theoretical predictions of the functional dependence of $T_c$ and $x_c$ on the concentration of the third component. However, previous experiments all find that the shift in $T_c$ is linear in the concentration of the third component.[14,18,25,26,28−33] There is also evidence that the shift in $T_c$ is linear in the shift in $x_c$.[13]

**B. Can We Use the Mass Balance Equations To Describe the Polymer Partitioning?** It is useful to know the total partitioning of the polymer: the total mass of polymer, summed over all molecular masses, in each of the coexisting liquid phases. These values can be determined experimentally, and can be also calculated from the molecular mass distributions (see Appendix A). The total mass of polymer in each phase is given by equations developed by Smith:

$$W_A^T = \frac{w_A(M) - w_B(M)}{w(M) - w_B(M)} W^T \quad (1)$$

$$W_B^T = \frac{w_B(M) - w_A(M)}{w(M) - w_A(M)} W^T \quad (2)$$

where $W^T$ is the total polymer mass in both phases, $W_A^T$ and $W_B^T$ are the masses of polymer in each phase A and B, $w(M)$ is the *normalized* mass fraction of molecular mass $M$ in the one-phase region above $T_c$ (the "parent" phase), and $w_A(M)$ and $w_B(M)$ represent the mass fractions of molecular mass $M$ in phases A and B (the "daughter" phases). The values of $W_A^T$ and $W_B^T$ obtained from eqs 1 and 2 should be the same for any choice of $M$.

**C. Do Any of the Available Theories Describe the Partitioning and Fractionation of This Polymer + Binary Solvent?** Polymers in single solvents can separate into two phases, e.g., poly(styrene) in methylcyclohexane.[4,34] The distribution of the polymer components between the two phases in unary solvents has been addressed by Brönsted,[35] Schultz,[36,37] Flory,[38] and Scott.[39] Recently, Pagonabarraga and Cates proposed a density functional approach to Flory−Huggins (FH) theory.[40] The FH mean field theory leads to[41,42]

$$\ln[w_i^u/w_i^l] = \ln[r] - ai \quad (3)$$

where $w_i^u$ and $w_i^l$ are the masses of component of degree of polymerization (molecular mass divided by the mass of one monomer), $i$, in the upper and lower phases respectively, and $r$ is the ratio of the volumes of the two phases. The parameter $a$ is related to the interactions between the polymer and the solvent, and in the simplest FH theory, $a$ is taken to be a function of temperature and not a function of concentration. This original FH theory is only applicable to polymer + solvent systems with a UCST, such as poly(styrene) in methylcyclohexane. The FH theory predicts a straight line for a plot of $\ln[w_i^u/w_i^l]$ vs $i$, with a negative slope (indicative of larger polymers equilibrating to the lower, denser phase) and with an intercept of zero only when the composition is at the critical volume fraction, $\phi_c$.

This theory for a polymer + solvent was extended by ten Brinke and Szleifer[43] (TS theory) by the treating exactly the inclusion of the intramolecular interactions of each polymer chain, while keeping the mean field approximation for intermolecular interactions. This extended theory can treat systems with either a UCST or an LCST, but can only be used for polymers with degree of polymerization below 300 and is partly simulational. The TS theory predicts that the plot of $\ln[w_i^u/w_i^l]$

vs $i$ is a concave upward curve, and is not the straight line predicted by FH theory.

Wu and Prausnitz[44] used continuous thermodynamics to predict the distribution coefficient as a function of polymer molecular mass in *binary* solvents. Wu and Prausnitz predict that the distribution coefficient for a polymer in a binary solvent also follows an equation like eq 3, but with a parameter $a$ that is a complicated function of the binary interactions of the three components in the system. This theory also predicts a straight line for a plot of $\ln[w_i^u/w_i^l]$ vs $i$.

Experimentally, it has been shown that eq 3 does not describe the experimental data for poly(styrene) in dimethoxymethane + dimethylether,[45] for PEG in 2,6-lutidine + water,[46] or for PEG in isobutyric acid + water.[46] In fact, all the literature data, for single or binary solvents, indicate that the FH theory does not fully describe the distribution of polymer between coexisting phases, when a broad enough range of molecular mass is considered.[46] All the data seem to be qualitatively consistent with the ten Brinke and Szleifer theory in showing an overall negative slope and an upward concave curvature in plots of $\ln[w_i^u/w_i^l]$ vs $i$.

**D. What Are the Mathematical Forms of the Molecular Mass Distributions (MMD's) of the Polymer in the Parent and Daughter Phases, and How Do They Develop over Time?** The mathematical form of the MMD for the initial, parent polymer sample, determined by its synthesis, may not be the same as the MMD's of the daughter phases. The polymers of interest here, PEG and PEI, are synthesized by anionic polymerizations.[47] The time development of the MMD during an anionic polymerization has been reviewed and measured by Das et al.[48] At the beginning of the polymerization process, if the rate constant for propagation is much greater than the rate constant for dissociation, then the initial molecular mass distribution is a narrow, Poisson distribution.[49] As the polymerization proceeds, an intermediate Gaussian distribution is observed, and this evolves toward a final Flory−Schulz distribution (equivalent to an exponential distribution for large polymers), as expected from theoretical considerations.[1,50−53] The MMD of a particular initial, parent polymer sample will thus depend on the time at which the polymerization process was terminated.

The Gaussian distribution is used as an empirical description of the MMD's for polymers with narrow MMD's,[49,54] but cannot be used to describe polymers with wide distributions since the symmetry about the mean would require it to have chains of negative length. One way of solving this problem is to assume that the logarithm of the chain length has a Gaussian distribution; this distribution is termed the "log-normal distribution." The log-normal distribution has been used by Wesslau[55] and others,[56,57] but its use has been criticized as being "no more than curve fittings" and "lacking any real physical implications."[58]

Given the mathematical form of the MMD of the initial, parent polymer, what will be the forms of the MMD's of the polymers in each of the daughter phases? The moments of these MMD's have been considered by Evans and co-workers,[59−62] who predict (1) that the fractionation (difference in means or first moments) in the daughter phases increases when the polydispersity (second moment) of the parent distribution increases and (2) that the difference in polydispersities (second moments) of the daughter phases increases as the asymmetry (third moment) of the parent distribution increases. Xu and Baus have disputed these predictions for nearly symmetric distributions.[63] Measurements on colloidal particles[61,64,65] have supported the theory of Evans et al., albeit with large uncertainties in the experimental parameters.

For the full MMD's of the parent and daughter phases, Evans et al.[61] and Xu and Baus[63] predict that they will all have the same mathematical forms. In 2003, Kalyuzhnyi and Kahl[66] used a mean spherical approximation to consider the MMD's of a mixture of polydisperse particles interacting by hard-sphere Yukawa potentials: a system of just the particles and no solvent, so that the phase separation is a "liquid−gas" transition; they also predict that the mathematical forms of the MMD's in the daughter phases will be the same as that of the parent phase. Fasolo and Sollich presented a theory for polydisperse hard spheres that predicts phase separation into solid and liquid phases, with quite different MMD's in the daughter phases from the parent phase, and with the extent of the difference increasing as the polydispersity increases.[67]

Work in the 1970s on polymer fractionation had addressed this issue for a polymer + unary solvent system by using a FH theory with a polymer−solvent interaction parameter that is linearly dependent on concentration.[68] The calculations of Kamide et al.[68] show the following points: (1) an unsymmetric parent distribution results in daughter distributions that are more symmetric; (2) for the daughter phase with the lower average molecular mass, the polydispersity index increases with average molecular mass; (3) for the daughter phase with the higher average molecular mass, the polydispersity index decreases with average molecular mass. Our experimental approach corresponds to the first step of a "batch fractionation," but in a polymer + binary solvent system. Kamide et al. later applied FH theory to the polymer + binary solvent system and they found predictions 2 and 3 again for these systems, but they did not address the forms of the MMD's directly.[69−71]

The mathematical forms of the full MMD's of the polydisperse species in the parent and daughter phases have not previously been studied experimentally. Here we will address the mathematical forms of the MMD's, but we will not address the question of the effect of the polydispersity of the parent phase, because we did not have polymer samples with a large range of polydispersities. We will choose an empirical function to fit to the experimental parent MMD, and then test as to whether that same function will also fit to the daughter MMD's. The two functional forms that we will use are the Gaussian distribution and the log-normal distribution, given in eqs 4 and 5, respectively:

$$w(M_i) = \frac{A}{\sigma(2\pi)^{1/2}} \exp\left[-{}^1/_2\left(\frac{i-\bar{i}}{\sigma}\right)^2\right] \tag{4}$$

$$w(M_i) = \frac{A}{\sigma(2\pi)^{1/2}} \exp\left[-{}^1/_2\left\{\frac{\ln(i/\bar{i})}{\sigma}\right\}^2\right] \tag{5}$$

where $A$ is a scaling constant, $\sigma$ is the standard deviation, $i$ is the degree of polymerization, and $\bar{i}$ is the mean degree of polymerization (the degree of polymerization at the number-average molecular mass). From such fits, we aim to assess whether or not the daughter MMD's are different from the parent MMD.

The rates of development of the final MMD's in the two phases are also of interest. Warren[72] suggested that there may be a fast enthalpic relaxation to the equilibrium $M_n$ values, followed by a slow entropic relaxation to the equilibrium $M_w$ values: $M_n$ in each phase would relax more quickly than $M_w$. Our earlier experiments[4] did not show such a difference in the relaxation of the moments of the MMD's.

**Table 1. Poly(ethylene glycol) (PEG), Poly(ethylene imine) (PEI), and Bovine Serum Albumin (BSA) Samples**

| polymer | source | lot no. | $M_w \times 10^3$ (g/mol) | $M_n \times 10^3$ (g/mol) | $M_w/M_n$ |
|---|---|---|---|---|---|
| PEG-2kOH | Polymer Source | PEG2OH-2k | 1.91 | 1.70 | 1.14 |
| PEG-2k$OCH_3$ | Polymer Source | PEG2$OCH_3$-2k | 1.73 | 1.56 | 1.11 |
| PEG-10kOH | Polymer Source | PEG2OH-10k | 13.4 | 11.9 | 1.13 |
| PEG-10k$OCH_3$ | Polymer Source | P2963-2$OCH_3$ | 12.9 | 5.84 | 2.21 |
| PEG-20kOH | Fluka | 425182/1 | 23.8 | 18.5 | 1.29 |
| PEG-200k$OCH_3$ | Aldrich | 06275JO | 151 | 72.9 | 2.08 |
| PEG-252k $OCH(CH_3)CH_2OCH_3$ | Polymer Source | P3624-E$OOCH_3$ | 287 | 248 | 1.16 |
| PEG-1000kOH | Polymer Source | PEG-1000kOH | 933 | 457 | 2.04 |
| PEI-25k | Alfa Aesar | H08Q19 | 30.4 | 21.8 | 1.39 |
| BSA | Sigma | A6003 | 66.3 | 66.3 | 1.00 |

**E. What Is the Effect on Partitioning and Fractionation of Increasing the Strength of Hydrogen Bonds in the System?** Deuterium bonds are stronger than hydrogen bonds.[73] If the strong partitioning and fractionation seen in these systems are related to the hydrogen bonding of the hydration layer on the polymer,[6] then a change from a $H_2O$ in the solvent mixture to $D_2O$ should further increase partitioning and fractionation.

## Experimental Methods

**A. Materials.** All the polymer samples used are given in Table 1 with their corresponding weight ($M_w$) and number ($M_n$) average molecular masses and polydispersities ($M_w/M_n$), as determined in our laboratory by size exclusion chromatography (SEC) (see below). Bovine serum albumin (BSA) was purchased from Sigma (96% albumin). The solvents used were isobutyric acid (IBA, Aldrich, 99% purity), $D_2O$ (Aldrich, 99% D), and freshly distilled deionized water (Barnstead system, 18.2 MΩ cm). Acetic acid (AcOH, Fisher Scientific, 99.9% purity), sodium acetate (NaOAc, Fisher Scientific, 100% purity), and sodium hydroxide pellets (NaOH, Fisher Scientific, 87.6% purity) were used to prepare a buffer for the mobile phase when analyzing PEI by SEC.

The PEG samples were used as received without further purification, except for one sample. The 20kOH sample required further purification since the presence of impurities was leading to questionable results.[4] The polymer sample was dissolved in the minimum amount of warm methanol while stirring. The resulting solution was then cooled in an ice bath to precipitate the PEG; the crystalline PEG was removed by filtering and dried in a vacuum oven at ~50 °C overnight.

Fresh water was used to prepare each solution. Water which had been stored in polycarbonate bottles for even a few hours led to variations in $T_c$, probably because of the leaching of plastic out of the walls of the container.[4]

**B. The Effect of Polymer Concentration and Molecular Mass on $T_c$ for Poly(ethylene glycol) in Isobutyric Acid + Water.** To study the effect of polymer molecular mass on $T_c$, samples of PEG of various molecular masses (see Table 1) were each dissolved to a concentration of 2.20 ± 0.01 mg/mL in a mixture of isobutyric acid + water at the critical concentration (39.0% isobutyric acid by mass).[7] To study the effect of PEG concentration on $T_c$, solutions of 2kOH PEG at several concentrations were prepared in the isobutyric acid + $H_2O$ mixture at the critical composition. Since the critical composition is not affected by the small amount of PEG (see Theory and Experiments, part A), the ratio of the volumes of the two phases is unity for all the experiments. For each sample, the temperature was first set above $T_c$ and the sample stirred for at least 24 h to ensure homogeneity; then the temperature was decreased stepwise until a meniscus formed.

**C. Partitioning and Fractionation between Liquid Phases.** For each polymer/solvent system, about 20 mL of solvent mixture was prepared at its critical composition and the polymer added to a concentration of 2.0 ± 0.1 mg/mL, in a glass vial with a magnetic stirrer bar. This vial was then sealed with a septum cap and clamped into a temperature-controlled water bath (see section D below), where it rested on a VWR 230 submersible stirrer. The stirrer allowed mixing of the solutions in the one-phase region, but the two-phase solutions were not stirred. Each experiment began with the solution stirred at a temperature in the one-phase region for several days, after which a 1.0 mL sample was extracted. The temperature of the bath was then decreased, and at each temperature in the two-phase region, and 1.0 mL samples of each liquid phase (upper and lower) were extracted each day over a period of about a week. Extractions were made using a hypodermic syringe needle through the septum cap. For each extracted sample, the solvent was evaporated in a vacuum oven overnight, water was added, and the aqueous polymer solution was analyzed by SEC. In our prior study[4] we made extractions at different ($T - T_c$) values, but since there was no effect of the temperature on the MMD's, we have not varied ($T - T_c$) in the present studies. The temperatures at which all extractions were made are given in Table 3.

**Table 2. The Observed Upper Critical Solution Temperatures, $T_c$, as a Function of Polymer Concentration (for 2kOH PEG) and Polymer Molecular Mass $M_n$ (for 2.20 mg/mL)**

| polymer concn (mg/mL) (± 0.01) | $T_c$ (°C) (± 0.1 °C) | $M_n \times 10^3$ (g /mol) | $T_c$ (°C) (± 0.1 °C) |
|---|---|---|---|
| 0 | 26.0 | 0 | 26.0 |
| 1.00 | 27.9 | 1.70 | 29.4 |
| 2.00 | 29.1 | 5.84[b] | 40.7 |
| 4.00 | 30.8 | 11.9 | 44.7 |
| 6.00 | 32.6 (30.6[a]) | 18.5 | 53.0 |
| 8.00 | 35.3 | 248 | >90.0 |
| 10.00 | 37.0 | 457 | >90.0 |
| 12.00 | 39.0 | | |

[a] $T_c$ as determined by Castellanos et al.[77] [b] Polymer terminated by $-OCH_3$.

At the end of an experiment, the temperature was again raised to ($T - T_c$) > 0. The sample was stirred and another one-phase extraction made. This allowed us to assess how much the overall composition and MMD of the sample changed as a result of the extractions. In all cases, the MMD in the parent phase remained the same before and after the temperature cycling and the extractions.

Partitioning/fractionation studies were made on the following systems: (1) PEG in isobutyric acid + $H_2O$ at different average polymer molecular masses and with different polymer terminating groups; (2) PEG in isobutyric acid + $D_2O$ at different polymer molecular masses; (3) PEI in isobutyric acid + $H_2O$ at one average molecular mass; (4) BSA in isobutyric acid + $H_2O$.

For BSA, we first tested the solubility in both water and isobutyric acid. The solubility was determined by UV spectroscopy (Milton Ray Spectronic 3000, $\lambda = 278$ nm). We found that BSA is readily soluble in water, but insoluble in pure isobutyric acid, even at elevated temperatures. However, BSA is soluble in the critical mixture of isobutyric acid + $H_2O$, and its UV absorption was used to determine the concentrations of BSA in the coexisting phases. The pH of the isobutyric acid + $H_2O$ mixture at the critical composition was 2.0 ± 0.3.

$T_c$ was measured for BSA + isobutyric acid + $H_2O$ at two different concentrations of BSA, 1.00 and 5.00 mg/mL. $T_c$ was 26.3 ± 0.3 °C at [BSA] = 1.00 mg/mL and 26.9 ± 0.3 °C at [BSA] = 5.00 mg/mL, as compared to 26.0 ± 0.1 °C with no BSA, so BSA increases $T_c$, but not to the same extent as does PEG (see below).

Extractions were taken on a mixture of 5.00 mg/mL BSA in a critical mixture of isobutyric acid + $H_2O$. The isobutyric acid was

**Table 3. Mass and Number-Average Molecular Mass ($M_w$ and $M_n$), Polydispersity ($M_w/M_n$), Mass Fraction of Polymer ($W$), and the Mathematical Form of the Molecular Mass Distributions (MMD) for the Parent and Daughter Distributions**

| polymer system[a,b] | phase/temp (°C)[c] | $M_w \times 10^3$ (g/mol) | $M_n \times 10^3$ (g/mol) | $M_w/M_n$ | $W_{\text{area under MMD method}}$ | $w_{\text{eqs 1 and 2}}$ | MMD form |
|---|---|---|---|---|---|---|---|
| 2kOH PEG + IBA + $H_2O$ | parent/38.8 | 1.91 | 1.68 | 1.14 | | | Gaussian |
| | upper/37.3 | 1.85 | 1.53 | 1.21 | 68 ± 4% | 59 ± 4% | Gaussian |
| | lower/37.3 | 1.93 | 1.71 | 1.13 | 32 ± 4% | 41 ± 4% | Gaussian |
| 2k$OCH_3$ PEG + IBA + $H_2O$ | parent/37.1 | 1.97 | 1.73 | 1.14 | | | log normal |
| | upper/25.7 | 1.95 | 1.73 | 1.13 | 73 ± 5% | 79 ± 5% | log normal |
| | lower/25.7 | 1.92 | 1.73 | 1.11 | 27 ± 5% | 21 ± 5% | log normal |
| 10kOH PEG + IBA + $H_2O$ | parent/60.0 | 13.2 | 11.9 | 1.11 | | | Gaussian |
| | upper/25.0 | 10.3 | 7.6 | 1.35 | 99.4 ± 0.5% | 99.6 ± 0.5% | Gaussian |
| | lower/25.0 | 34.8 | 32.5 | 1.07 | 0.6 ± 0.5% | 0.4 ± 0.5% | log normal |
| 20kOH PEG + IBA + $H_2O$ | parent/28.9 | 23.5 | 18.0 | 1.31 | | | Gaussian |
| | upper/24.6 | 19.0 | 10.0 | 1.90 | 88 ± 5% | 86 ±± 15% | Gaussian |
| | lower/24.6 | 38.2 | 37.0 | 1.03 | 12 ± 5% | 14 ± 15% | log normal |
| 2kOH PEG + IBA + $D_2O$ | parent/47.7 | 1.99 | 1.83 | 1.09 | | | Gaussian |
| | upper/45.2 | 2.00 | 1.82 | 1.10 | 90 ± 2% | 86 ± 3% | Gaussian |
| | lower/45.2 | 2.04 | 1.90 | 1.07 | 10 ± 2% | 14 ± 3% | Gaussian |
| 10kOH PEG + IBA + $D_2O$ | parent/60.0 | 13.4 | 12.2 | 1.10 | | | Gaussian |
| | upper/25.0 | 11.3 | 8.4 | 1.34 | 99.7 ± 0.5% | 99.5 ± 3% | Gaussian |
| | lower/25.0 | 30.0 | 28.9 | 1.03 | 0.3 ± 0.5% | 0.5 ± 3% | log normal |
| 20kOH PEG + IBA + $D_2O$ | parent/60.0 | 19.1 | 14.2 | 1.35 | | | Gaussian |
| | upper/45.2 day 1 | 8.73 | 4.70 | 2.04 | 99.7 ± 0.5% | 99.7 ± 3% | log normal (day 1); Gaussian (day 7) |
| | lower/45.2 day 1 | 44.4 | 44.3 | 1.00 | 0.3 ± 0.5% | 0.3 ± 3% | log normal (day 1); no polymer (day 7) |
| 25k PEI + IBA + $H_2O$ | parent/33.9 | 29.1 | 18.7 | 1.56 | | | log normal |
| | upper/24.8 | 17.2 | 10.6 | 1.62 | 99 ± 5% | 96 ± 12% | log normal |
| | lower/24.8 | 29.3 | 23.6 | 1.24 | 1 ± 5% | 4 ± 12% | log normal |

[a] IBA refers to isobutyric acid [b] Polymer concentrations were 2.0 mg/mL, except for 20kOH PEG + IBA + $H_2O$, where the concentration was 1.0 mg/mL in order to avoid shifting $T_c$ to an inconveniently high value. [c] Temperature at which extractions made, ±0.1 °C.

then removed from the extracted aliquots by dialysis, because the isobutyric acid absorbs UV in the same range of wavelengths as does BSA. For each extraction, 1.0 mL of sample was transferred to membrane dialysis tubing (Fisher Scientific, molecular mass cut off = 12 −14 kDa). The samples were dialyzed in 2 L of distilled, deionized water, continuously stirred and changed every 12 h for 5 days. The dialysis was complete when the pH of the dialysis water was no longer changing, which happened at pH between 4.5 and 5.0. The BSA concentrations in the dialyzed extractions were determined by UV spectroscopy. Circular dichroism (CD) (Jasco J-810 Spectropolarimeter) was used to analyze the α-helical content in the samples.

A study of the BSA content in each phase over time was made using the methods described above. In this case, the initial concentrations of BSA were 0.25 mg/mL and 1.00 mg/mL.

**D. Temperature Control and Measurement.** The samples were all held in a water bath with the temperature controlled to within a few mK.[74] The temperature was measured using a Guildline (Orlando, FL) platinum resistance thermometer with an accuracy of 0.01 K and a precision of 0.001 K.

**E. Size Exclusion Chromatography (SEC).** After each polymer solution was extracted and dried under vacuum overnight, 1.0 mL of water was added. These samples were allowed to fully solvate overnight, filtered, and analyzed by SEC. The SEC system and its calibration have been described previously.[5] For the PEG samples, a mobile phase of freshly distilled, deionized $H_2O$ was used. For the PEI samples, a buffer solution of 0.5 M sodium acetate + 0.5 M acetic acid (pH = 7.0) was used. For each sample, 100 μL were injected for a run time of 60 min at a flow rate of 0.6 mL/min.

The chromatograms were analyzed and converted into full molecular mass distributions using the Waters software package Breeze (Version 3.30). This software (like many other available SEC analysis programs) does not correct for the column broadening effect,[75] which may lead to an overestimate of $M_w/M_n$. However, analysis software that does correct for column broadening (GPC for Windows, Version 1.0[76]) was used for one sample (20kOH PEG) and there was no significant difference between the SEC traces or $M_w/M_n$ between the two programs. We used the Breeze software for all analyses because GPC for Windows does not run on contemporary computer platforms.

## Results and Discussion

**A. The Effect of Polymer Concentration and Molecular Mass on $T_c$ for Poly(ethylene glycol) in Isobutyric Acid + Water.** We have measured the change of $T_c$ of isobutyric acid + $H_2O$ when PEG is added of various molecular masses and at various concentrations. We have not measured the effect of PEG on $x_c$ because that effect is very small, as indicated by the appearance of the liquid−liquid menisci in the middle of the samples (with equal volumes of the two liquid phases) for all the samples studied. Table 2 lists the resulting values of $T_c$.

Figure 1a shows the effect of the change of concentration of 2kOH PEG on $T_c$. At zero PEG concentration, a critical mixture of isobutyric acid + $H_2O$ (39% mass fraction isobutyric acid) has a $T_c = 26.0 \pm 0.1$ °C, in agreement with previous studies.[3,7] Figure 1a shows that as the PEG concentration increases, $T_c$ *increases* linearly. Also shown is a point from Castellanos et al.[77] (30.6 ± 0.1 °C for the addition of 6 mg/mL 2kOH PEG to the isobutyric acid + $H_2O$ mixture; it is lower than the value determined in the present work, but does support an increase in $T_c$; differences in sample preparation technique could account for the difference in values. We conclude that on the addition of PEG, a polymer that is more soluble in $H_2O$ than in isobutyric acid, the two solvents become mutually less soluble and this raises $T_c$.

Figure 1b shows how $T_c$ changes as a function of PEG molecular mass and terminating group at polymer concentrations of 2.20 ± 0.01 mg/mL. There is a marked increase in $T_c$ as the PEG molecular mass increases. Data for 252k PEG and 1000k PEG (Table 1) are not shown in Figure 1b because, at this concentration, the $T_c$ values at these high average molecular masses are very high and inaccessible using our water baths. These high molecular mass samples were heated on a stirrer hot plate and were still in the two-phase region at 90 °C, even after a few hours. Figure 1b also shows that there is little change in $T_c$ as the terminating group is changed from −OH to −$OCH_3$.

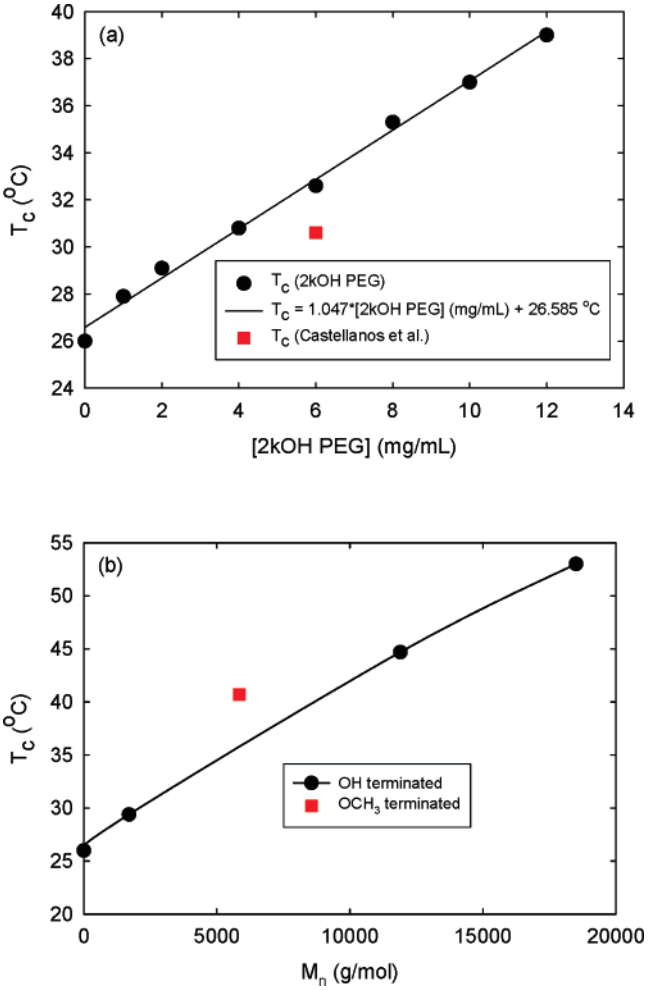


**Figure 1.** Critical temperature, $T_c$, for poly(ethylene glycol) (PEG) + isobutyric acid + $H_2O$ as a function of (a) 2kOH PEG concentration, and (b) PEG molecular mass and terminating group at 2.20 ± 0.01 mg/mL. Error bars for $T_c$ (±0.1 °C) are smaller than the size of the symbols. The solid line is a linear fit to the new data. The red point is from an earlier study.[77] Table 1 gives the characterizations of the PEG samples.

Our results for the polymer concentration dependence of $T_c$ in Figure 1a are in disagreement with those of Venkatesu,[18] who put PEG of molecular mass $9 \times 10^5$ g/mol and at concentrations 0 to 1.6 mg/mL into isobutyric acid + $H_2O$ and found that $T_c$ *decreased* as polymer concentration increased. The molecular mass for the polymer used in our Figure 1a is 4 orders of magnitude smaller than that used by Venkatesu. The concentration range in Figure 1a is 0 to 13 mg/mL, overlapping the range of Venkatesu and extending further. Venkatesu did not characterize his polymer sample except by citing the supplier's (Aldrich) nominal "MW." Recall from above that our own attempts to examine the $T_c$ shift for a polymer of high molecular mass did not succeed due to long equilibration times. However, it is possible that PEG of a much larger molecular mass affects $T_c$ differently than that of lower molecular mass—i.e., that there is somehow a peak in $T_c$ at higher mass in Figure 1b.

**B. Partitioning and Fractionation between Liquid Phases. (1) Poly(ethylene glycol) in Isobutyric Acid + $H_2O$. 2kOH PEG + Isobutyric Acid + $H_2O$.** Figure 2 shows the partitioning of 2kOH PEG between isobutyric acid-rich and $H_2O$-rich phases. These data are shown in three ways: (a) the change of $M_n$, $M_w$, and $M_w/M_n$, with time; (b) The normalized area under the SEC trace as a function of molecular mass, giving the degree of partitioning of the total polymer mass between the upper and lower phases, at equilibrium; (c) MMD's in the parent, upper, and lower phases, at equilibrium. The normalized area under the SEC trace is the area under the "slice" of the trace at a particular molecular mass.

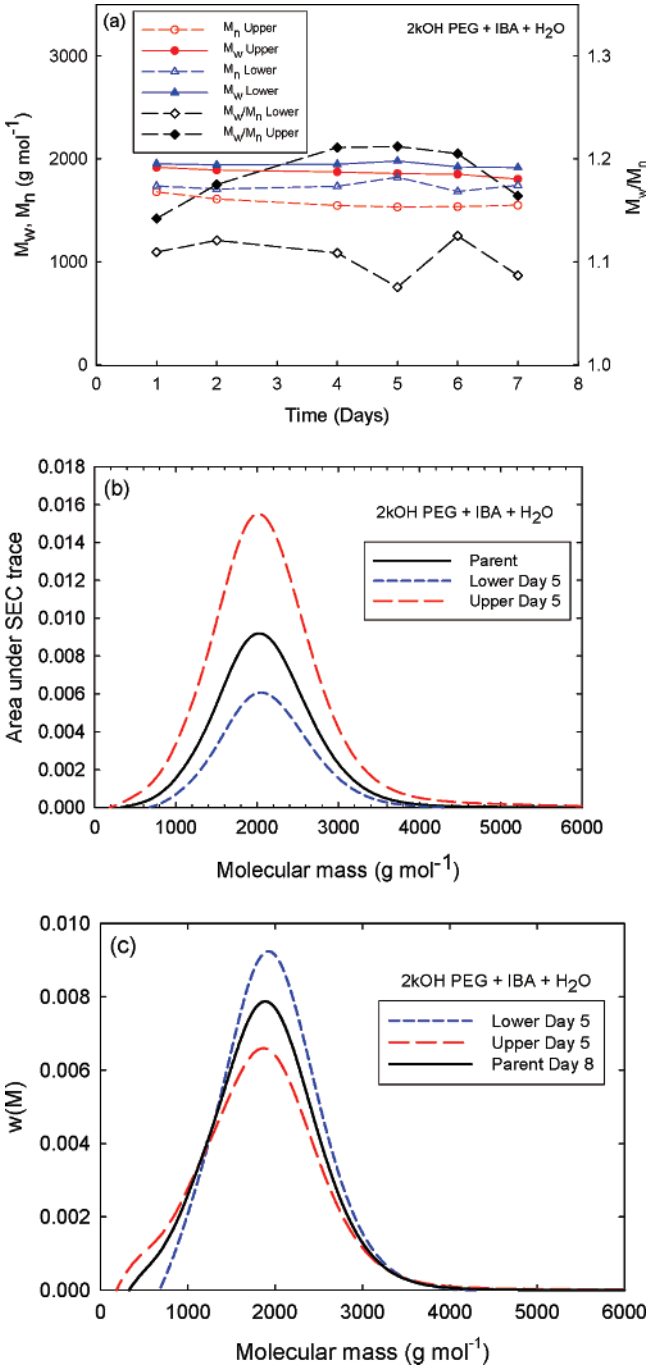


**Figure 2.** Partitioning of 2kOH PEG in isobutyric acid (IBA) + $H_2O$: (a) $M_w$, $M_n$, and $M_w/M_n$ for the upper and lower daughter phases as functions of time; (b) area under SEC trace as a function of molecular mass for parent and daughter phases at equilibrium; (c) mass fraction molecular mass distribution, $w(M)$, for parent and daughter phases at equilibrium. Error bars on $M_n$, $M_w$, and $M_w/M_n$ (not shown) are about 10% of the values.

Figure 2a shows that the values of $M_n$ and $M_w$ for 2kOH PEG over time show no dramatic change in $M_n$, $M_w$, or $M_w/M_n$ after just 1 day (within the 10% uncertainty), whereas Shresth et al. found that 20kOH PEG required 2−3 days to reach equilibrium.[46] It is reasonable that the smaller molecular mass

2kOH PEG would equilibrate faster than the 20kOH PEG. $M_n$ and $M_w$ do not show different relaxation rates.

Figure 2b and Table 3 show that the 2kOH PEG *partitions* or *distributes* unevenly between the upper and lower phases: Most of the PEG is found in the upper, isobutyric acid-rich phase, as was seen for 20kOH PEG.[46] We calculate the degree of total partitioning of the PEG between the two phases in two ways: (i) as the ratio of the areas under the MMD's of the two phases and (ii) from the full MMD's, as given by eqs 1 and 2. From the first method, the total mass of polymer in the lower phase is $W^T_{lower} = 32 \pm 4\%$ and a total mass fraction in the upper phase, $W^T_{upper} = 68 \pm 4\%$. From eqs 1 and 2, $W^T_{lower} = 41 \pm 4\%$ and $W^T_{upper} = 59 \pm 4\%$ (see also Table 3). The uncertainties are given as one standard deviation. Within experimental uncertainty (3 standard deviations), the results are in agreement. In using eqs 1 and 2, care must be taken in choosing limits: The limits of the molecular masses used in the calculation must be within the limits of the molecular mass distributions of the upper, the lower, and parent phases.

However, Figure 2c shows that there is no significant *fractionation* of the polymer between the two phases for 2kOH PEG: The MMD's in the upper and lower phases are the same within their 10% uncertainty, in contrast to 20kOH PEG in isobutyric acid + $H_2O$, where $M_w$(lower) is about twice $M_w$-(upper).[4] On close inspection of Figure 2c, a broad shoulder in $w(M)$(upper) and $w(M)$ (parent) is evident at ~500 g/mol. This feature is not seen in $w(M)$(lower) and is amplified somewhat in the upper phase as compared to the parent; this is evidence of some slight fractionation. Figure 2, parts a and c, and Table 3 show that $w(M)$ is only slightly less polydisperse in the lower phase than in the upper phase, whereas there is a 23% reduction in polydispersity in the lower phase for 20kOH PEG.[4]

Figure 3 shows fits of the scaled Gaussian function (eq 4) to $w(M)$ for the parent phase, the lower phase, and the upper phase. The insets to Figure 3 show that the residuals are within three standard deviations of the mean, and thus are not significant except at the very highest degrees of polymerization. Niamke[78] also found that this function gave relatively random residual plots when fitted to the data of Shresth et al. for 20kOH PEG.[4] The fitted parameters are tabulated in Table 4. The conclusion is that the partitioning of the 2kOH PEG between the two liquid phases of isobutyric acid + $H_2O$ does not affect the mathematical form of the MMD's: The data from the parent and both daughter phases can all be described by Gaussian functions.

Figure 4 shows the MMD for 2kOH PEG in isobutyric acid + $H_2O$ data, plotted as suggested by eq 3. We plot molecular mass on the abscissa (as opposed to the degree of polymerization, $i$) and the logarithm of the ratio of $w(M)$ in the two phases on the ordinate (as opposed to the mass fraction in each phase). The plotted parameters are proportional to those in eq 3 and only differ by constants (monomer molecular mass and total mass of polymer, respectively). Our data show an overall negative slope, but are concave upward, as predicted by ten Brinke and Szleifer.[43]

Indeed, for all the samples in Table 3, plots of $\ln[w(M)^{upper}/w(M)^{lower}]$ vs molecular mass are similar to those shown in Figure 4 and thus are not consistent with Flory−Huggins mean field theory. We do not show the other analogous plots.

**2kOCH$_3$ PEG + Isobutyric Acid + H$_2$O.** Here we study the effect of changing the terminating group on the PEG from −OH to −OCH$_3$: Samples 2kOH PEG and 2kOCH$_3$ PEG have essentially the same molecular mass, but differ in the terminating group. The results are listed in Table 3, but are not plotted. $M_n$ and $M_w$ reached equilibrium in less than 1 day, like sample 2kOH PEG (see Figure 2a).

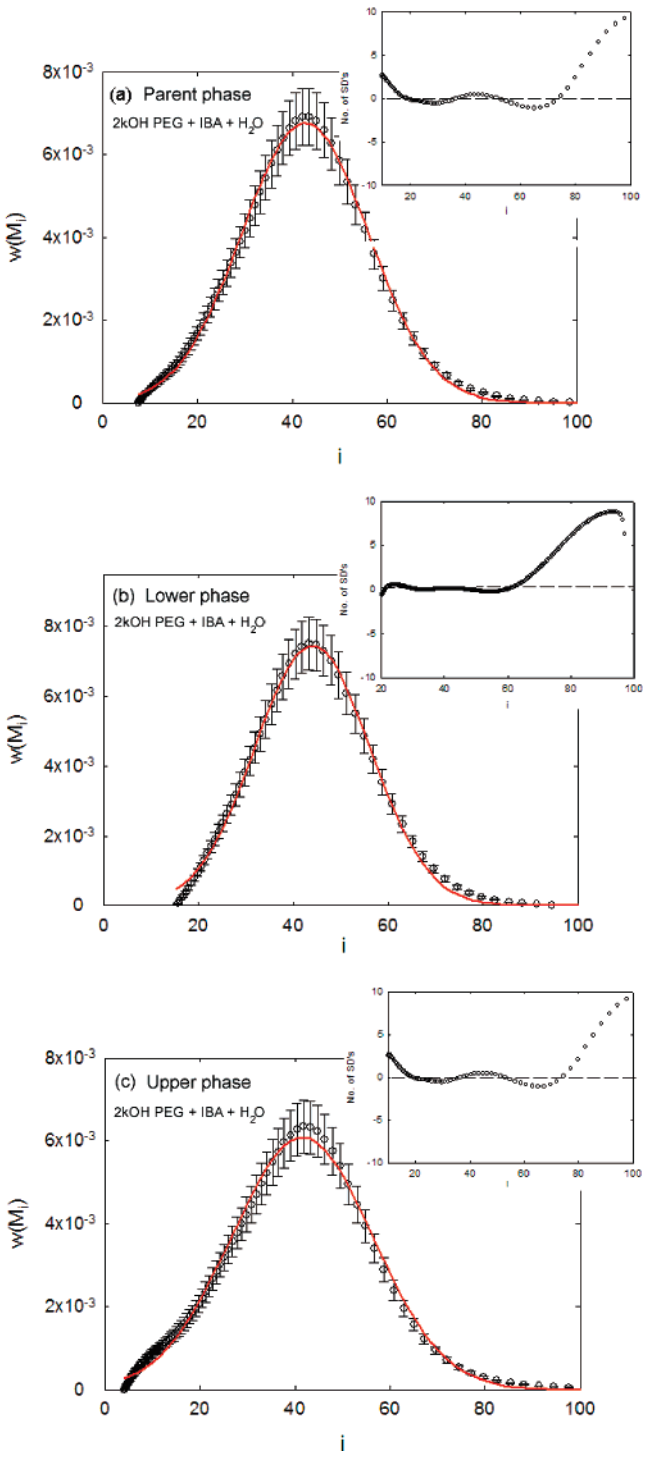


**Figure 3.** Molecular mass distribution $w(M)$ vs degree of polymerization i, for 2kOH PEG + isobutyric acid + $H_2O$ in (a) the parent phase, (b) the lower phase, and (c) the upper phase. Experimental data are open circles. Solid lines are Gaussian functions that fit the experimental data. The insets show the residual plots of the data from the fits. For all experimental data and residual plots, only every fifth data point has been plotted for clarity. The error bars for $w(M)$ are about 10%.

The partitioning between the two phases was calculated by the two methods of analysis and, like 2kOH PEG, 2kOCH$_3$ PEG partitions more into the upper phase. $M_n$ and $M_w$ are, within the experimental uncertainty, the same in both phases, indicating that for 2kOCH$_3$ PEG no significant fractionation occurs. The function that best fits the parent $w(M)$ (see Table 4) differs for

**Table 4. Parameters Obtained from Fits of Experimental Molecular Mass Distribution (MMD) Data by Mathematical Forms (Gaussian and Log Normal) for the Parent and Daughter Phases of Polymers in Isobutyric Acid (IBA) + $H_2O$ or $D_2O$ Where the Uncertainties Are to Three Standard Deviations and Where $\chi^2_{reduced}$* Is the Reduced $\chi^2$ Squared Value for the Poorer of the Two Fits, Gaussian (G) or Log Normal (LN)**

| sample | phase | function | $A$ | $\sigma$ | $\bar{i}$ | $\chi^2_{reduced}$ | $\chi^2_{reduced}$* |
|---|---|---|---|---|---|---|---|
| 2kOH PEG + IBA+ $H_2O$ | parent | Gaussian | $0.032 \pm 0.001$ | $19.6 \pm 0.2$ | $43.3 \pm 0.2$ | 1.12 | 19.1 (LN) |
| | upper | Gaussian | $0.0306 \pm 0.0009$ | $21.7 \pm 0.2$ | $41.0 \pm 0.2$ | 0.54 | 15.4 (LN) |
| | lower | Gaussian | $0.032 \pm 0.001$ | $17.7 \pm 0.2$ | $45.0 \pm 0.2$ | 1.23 | 4.98 (LN) |
| 2k$OCH_3$ PEG + IBA+ $H_2O$ | parent | log normal | $2 \times 10^{-4} \pm 6 \times 10^{-6}$ | $0.012 \pm 0.0006$ | $36.8 \pm 0.2$ | 1.05 | 2.40 (G) |
| | upper | log normal | $2 \times 10^{-4} \pm 7 \times 10^{-6}$ | $0.012 \pm 0.0002$ | $37.9 \pm 0.2$ | 1.33 | 2.56 (G) |
| | lower | log normal | $2 \times 10^{-4} \pm 6 \times 10^{-6}$ | $0.010 \pm 0.0001$ | $36.9 \pm 0.2$ | 2.14 | 6.09 (G) |
| 10kOH PEG + IBA + $H_2O$ | parent | Gaussian | $0.030 \pm 0.0009$ | $120 \pm 2$ | $294 \pm 1$ | 1.39 | 7.49 (LN) |
| | upper | Gaussian | $0.033 \pm 0.0009$ | $166 \pm 3$ | $212 \pm 3$ | 0.91 | 6.10 (LN) |
| | lower | log normal | $1.7 \times 10^{-6} \pm 6 \times 10^{-7}$ | $0.014 \pm 0.0009$ | $650 \pm 27$ | 1.50 | 1.81 (G) |
| 20kOH PEG + IBA+ $H_2O$ | parent | Gaussian | $0.486 \pm 0.01$ | $279 \pm 3$ | $560 \pm 2$ | 1.27 | 10.8 (LN) |
| | upper | Gaussian | $0.517 \pm 0.02$ | $312 \pm 6$ | $537 \pm 5$ | 0.58 | 5.67 (LN) |
| | lower | log normal | $6 \times 10^{-5} \pm 1 \times 10^{-6}$ | $0.0064 \pm 0.0003$ | $817 \pm 6$ | 1.84 | 3.32 (G) |
| 2kOH PEG + IBA+ $D_2O$ | parent | Gaussian | $0.039 \pm 0.001$ | $17.6 \pm 0.3$ | $45.1 \pm 0.2$ | 0.67 | 7.61 (LN) |
| | upper | Gaussian | $0.039 \pm 0.001$ | $16.4 \pm 0.2$ | $44.8 \pm 0.2$ | 0.89 | 4.29 (LN) |
| | lower | Gaussian | $0.037 \pm 0.002$ | $16.7 \pm 0.4$ | $44.5 \pm 0.2$ | 0.15 | 6.73 (LN) |
| 10kOH PEG + IBA + $D_2O$ | parent | Gaussian | $0.0031 \pm 0.0009$ | $122 \pm 1$ | $300 \pm 1$ | 1.01 | 6.68 (LN) |
| | upper | Gaussian | $0.0031 \pm 0.0009$ | $175 \pm 3$ | $231 \pm 2$ | 1.25 | 4.83 (LN) |
| | lower | log normal | $1.6 \times 10^{-6} \pm 1 \times 10^{-7}$ | $0.0041 \pm 0.0001$ | $513 \pm 1$ | 2.23 | 2.44 (G) |
| 20kOH PEG + IBA + $D_2O$ | parent | Gaussian | $0.031 \pm 0.0009$ | $265 \pm 4$ | $430 \pm 3$ | 0.60 | 3.28 (G) |
| | upper | log normal | $8 \times 10^{-6} \pm 2 \times 10^{-7}$ | $0.034 \pm 0.0006$ | $62 \pm 1$ | 1.52 | 0.97 (G)[a] |
| | lower | log normal | $1 \times 10^{-6} \pm 7 \times 10^{-8}$ | $0.0014 \pm 0.00005$ | $1006 \pm 2$ | 6.68 | 8.15 (G) |
| 25k PEI + IBA + $H_2O$ | parent | log normal | $3 \times 10^{-5} \pm 9 \times 10^{-7}$ | $0.0243 \pm 0.0007$ | $313 \pm 5$ | 0.61 | 5.89 (G) |
| | upper | log normal | $3 \times 10^{-5} \pm 1 \times 10^{-6}$ | $0.0250 \pm 0.0009$ | $126 \pm 4$ | 0.82 | 2.83 (G) |
| | lower | log normal | $3 \times 10^{-5} \pm 3 \times 10^{-6}$ | $0.023 \pm 0.002$ | $408 \pm 10$ | 1.75 | 2.83 (G) |

[a] This fit shows a lower $\chi^2_{reduced}$ value; however the uncertainties associated with the fit are larger than the parameters themselves and therefore this is not a reasonable fit to these data.

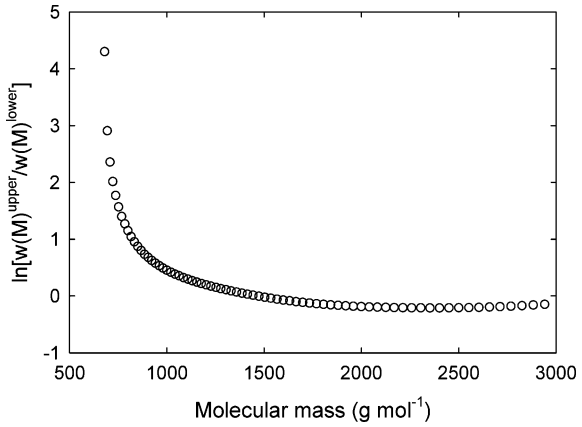


**Figure 4.** $\ln[w(M)^{upper}/w(M)^{lower}]$ vs molecular mass for 2kOH PEG + isobutyric acid + $H_2O$.

the two polymer samples—a modified Gaussian fits better for 2kOH PEG and a modified log-normal distribution fits better for 2k$OCH_3$ PEG, but this can be due to a difference in the original synthesis of each parent polymer. For 2k$OCH_3$ PEG, the same log-normal functional form (eq 5) that fits the MMD of the parent polymer also fits the MMD's of the polymers in both daughter phases.

**10kOH PEG + Isobutyric Acid + $H_2O$.** On increasing molecular mass by a factor of about 5, we find that the system requires about 2 days to reach equilibrium, somewhat longer than the systems with PEG of smaller molecular mass discussed above (see Figure 2a). $M_n$ and $M_w$ equilibrate at the same rate. The 10kOH polymer chains partition to a greater extent than does 2kOH PEG. Most importantly, they also fractionate: the lower water-rich phase contains polymer of higher average molecular mass than does the upper isobutyric acid-rich phase (see Table 3; data are not plotted).

Table 4 shows that the parent and upper phase MMD data are better described by Gaussian functions, but that the lower phase MMD is better described as a log-normal distribution.

**20kOH PEG + Isobutyric Acid + $H_2O$.** Like Shresth et al.,[4] we found that this higher average molecular mass takes still longer to equilibrate, 2−3 days, and that $M_n$ and $M_w$ equilibrate at the same rate. The new data (Table 3) replicate Shresth et al. in showing that the majority of the PEG partitions into the upper isobutyric acid-rich phase, that the difference in $M_n$ or $M_w$ between coexisting liquid phases is about a factor of 2 ($M_w$(upper) = 19.0 kg/mol and $M_w$(lower) = 38.2 kg/mol; cf. Shresth $M_w$(upper) = 18.0 kg/mol and $M_w$(lower) = 36.7 kg/mol) and that the polydispersity in the lower phase is reduced considerably. For this sample, too, we find that the mathematical forms of the MMD's of the parent and daughter phases differ—see Table 4 and Figure 5. The MMD's of the parent polymer and the upper-phase polymer can be reasonably well-described as Gaussian, but the MMD of the PEG in the lower phase cannot be described as Gaussian and is not even well-described as a log-normal distribution (see Figure 5b).

**200kOH PEG + Isobutyric Acid + $H_2O$.** This sample was stirred constantly at almost 70 °C for over a week before a clear one-phase region was evident. After 3 weeks at temperatures much lower than $T_c$, the sample still had not separated into two distinct phases. We abandoned the study of this sample because of the long equilibration times.

**(2) Poly(ethylene glycol) in Isobutyric Acid + $D_2O$.** Now we consider systems where the $H_2O$ in the solvent is replaced by $D_2O$, in order to increase the hydrogen bonding of the hydration layer on the polymer.[6]

**2kOH PEG + Isobutyric Acid + $D_2O$.** Unlike 2kOH PEG in isobutyric acid + $H_2O$ (Figure 2a), where equilibrium was reached quickly, 2kOH PEG in isobutyric acid + $D_2O$ shows changes in $M_n$, $M_w$, and $M_w/M_n$ over time, taking about 2 days to achieve steady values (Figure 6a), and with $M_n$ and $M_w$ relaxing at the same rate. Like 2kOH PEG in isobutyric acid + $H_2O$, the majority of the PEG partitions into the upper isobutyric acid-rich phase, and there is no significant fractionation (Table 3).

However, the degree of partitioning of 2kOH PEG between the phases increases when $D_2O$ is substituted for $H_2O$ (Table

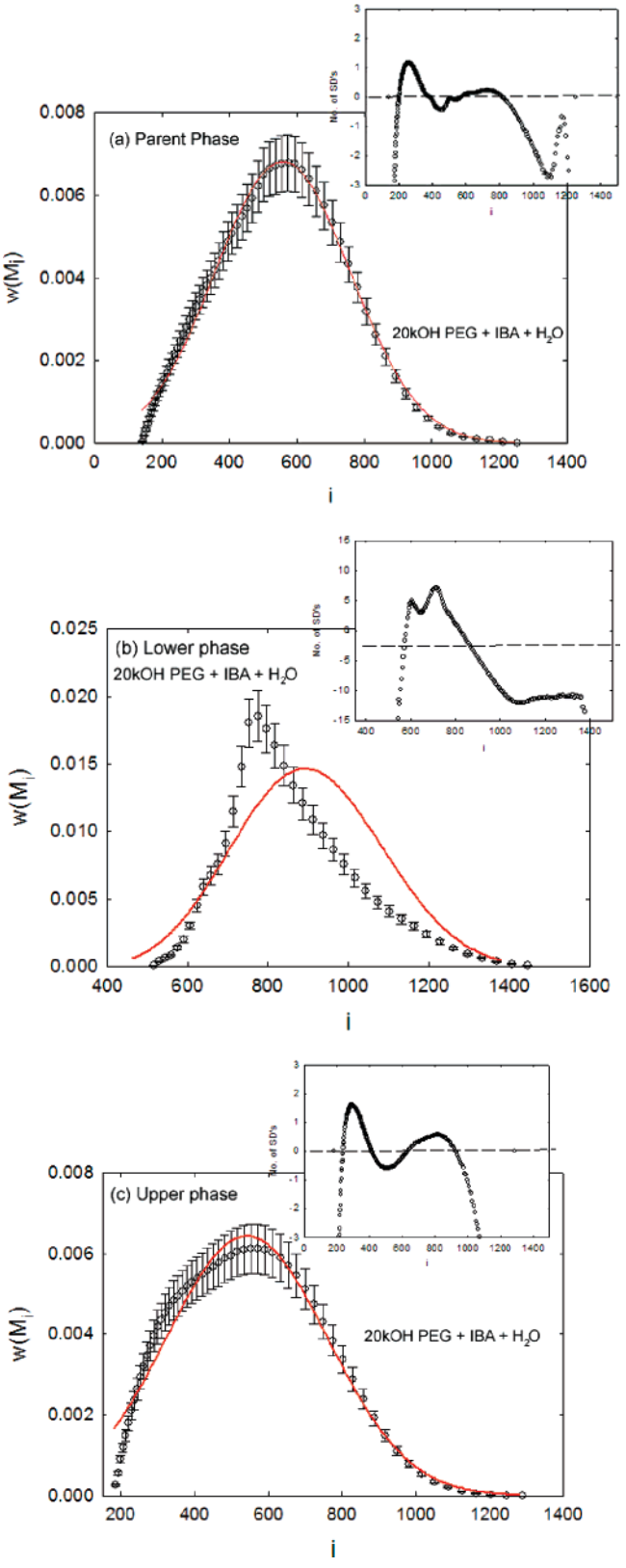


**Figure 5.** Molecular mass distribution $w(M)$ vs degree of polymerization i, for 20kOH PEG + isobutyric acid + $H_2O$ in (a) the parent phase, (b) the lower phase, and (c) the upper phase. Experimental data are open circles. Solid lines are functions fitted to the experimental data: Gaussian for the parent MMD and for the upper daughter phase MMD; log-normal for the lower daughter phase MMD. The insets show the residual plots of the data from the fits. For all experimental data and residual plots, only every fifth data point has been plotted for clarity. The error bars for $w(M)$ are about 10%.

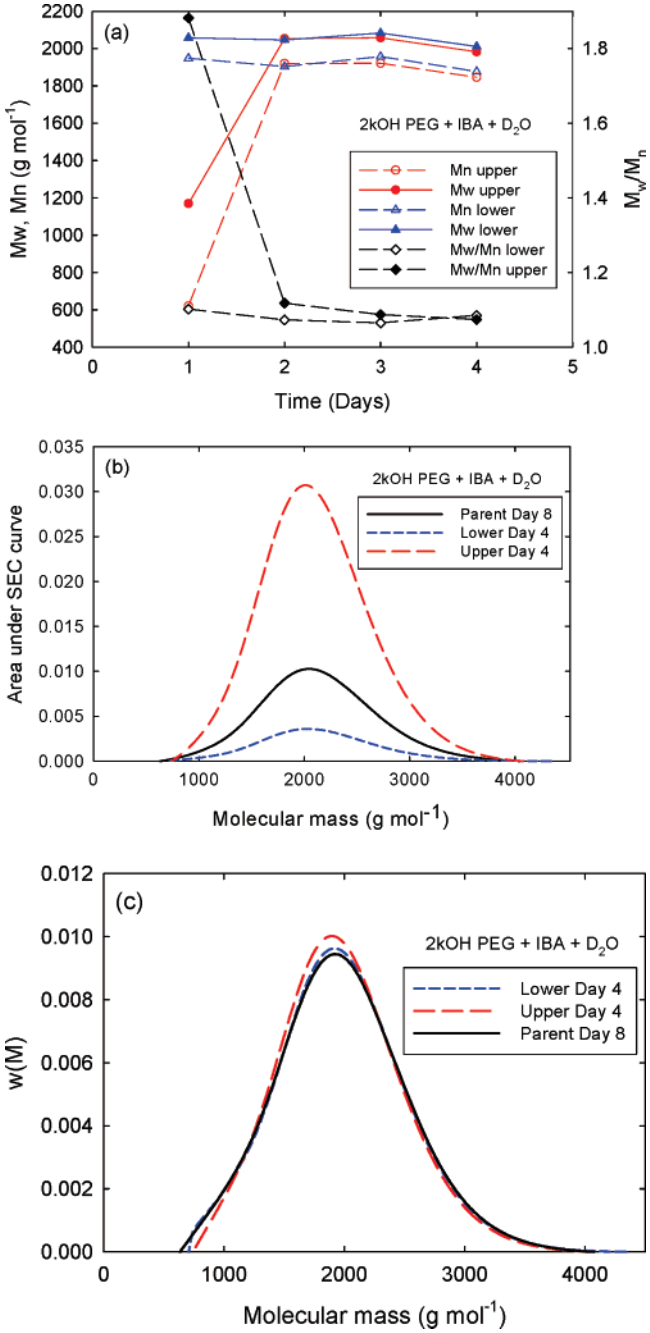


**Figure 6.** Partitioning of 2kOH PEG in isobutyric acid + $D_2O$: (a) $M_w$, $M_n$, and $M_w/M_n$ for the upper and lower daughter phases as functions of time; (b) area under SEC trace as a function of molecular mass for parent and daughter phases at equilibrium; (c) mass fraction molecular mass distribution, $w(M)$, for parent and daughter phases at equilibrium.

3), from 60 to 68% in the upper phase to about 90% (compare Figure 6b with Figure 2. The mathematical form of MMD is the same (Gaussian) for the parent and daughter phases, as for the 2kOH PEG + isobutyric acid + $H_2O$ system (see Table 4). The increase in equilibration time and the increase in degree of partitioning can be due to the stronger hydrogen bonding of the hydration layer to the polymer in the $D_2O$ system.

**10kOH PEG + Isobutyric Acid + $D_2O$.** On increasing the PEG molecular mass from 2 to 10 kg/mol in the PEG + isobutyric acid + $D_2O$ system, the polymer equilibrates in 2 days and not only partitions unevenly between the two daughter phases but also fractionates significantly and has a lower polydispersity in the lower phase (see Table 3). For the parent

and upper phases, a scaled Gaussian function fits the polymer MMD, whereas in the lower phase, a log-normal function better fits the MMD of the polymer (see Table 4).

**20kOH PEG + Isobutyric Acid + $D_2O$.** When the polymer molecular mass is increased still further for the PEG + isobutyric acid + $D_2O$ system (Table 3), the equilibration time becomes very long (7 days), the PEG chains still partition unevenly between the upper and lower phases, and the fractionation of the polymer between phases is slightly more pronounced, indicating an increase in fractionation as the molecular mass of the polymer increases.

After 24 h, both daughter phase MMD's are better described by log-normal distributions, while the parent phase MMD is better described by a Gaussian curve. After 7 days, however, the degree of partitioning is so great that no polymer chains are detected in the lower phase from SEC measurements, and the MMD of the polymer in the upper phase is better fitted by a Gaussian distribution (see Table 4).

**(3) Poly(ethylene imine) (PEI) in Isobutyric Acid + $H_2O$.** When the −O− heteroatom in the PEG chain is replaced by an −NH− group, we observe behavior to quite similar to that of the PEG systems (see Table 3): The 25k PEI chains partition dramatically between daughter phases, the PEI chains fractionate with the larger average polymer molecular mass in the lower $H_2O$-rich phase, and the polydispersity of the PEI chains is less in the lower phase than in the upper phase. Equilibrium for $M_w$ and for $M_n$ was achieved for 25k PEI after 5 days.

The MMD plots (not shown) for 25k PEI + isobutyric acid + $H_2O$ are highly skewed and thus modified log-normal function fit the MMD's better for the parent phase and for both daughter phases. Unlike the analogous PEG system (20k PEG + isobutyric acid + $H_2O$), the same mathematical function can be used to describe all three MMD's. However, the quality of the fit is lower for the MMD of PEG in the lower phase, indicating that perhaps the MMD is not quite the same as for the upper phase (see Table 4).

**(4) Bovine Serum Albumin (BSA) in Isobutyric Acid + Water.** Since BSA is insoluble in isobutyric acid (see Experimental Methods, section C), we would expect that the BSA would be found predominantly in the lower $H_2O$-rich phase. However, UV analysis indicated that the BSA from the parent phase partitions entirely into the upper isobutyric acid-rich phase. The lower $H_2O$-rich phase does not contain measurable amounts of BSA. This is analogous to the PEG and PEI cases discussed above, where the polymers partition mainly into the isobutyric acid-rich phase, even though they are more soluble in water.

The partitioning of BSA over time at two different initial concentrations in isobutyric acid + $H_2O$ at 25.6 °C is shown in Figure 7, parts a and b. The BSA content in the upper phase decreases with time. This may be due to more BSA partitioning into the lower phase. However, the amount of BSA in the lower phase is negligible within the experimental uncertainty. The reduction in BSA concentration in the upper phase is more likely due to adsorption of BSA onto the walls of the glass vials.[79] The CD analyses shown in Figure 7c show the presence of α-helices[80] in both the parent phase and the upper isobutyric acid -rich phase. No helical content was observed in the lower $H_2O$-rich phase. From these data we conclude that the majority of the BSA partitions into the upper isobutyric acid-rich phase and that the protein is not denatured by the acid.

We have previously reported experimental evidence that PEG molecules take helical conformations in isobutyric acid, but they take coil conformations in water.[5] More recent experiments revealed that if trace amounts of water are removed from the

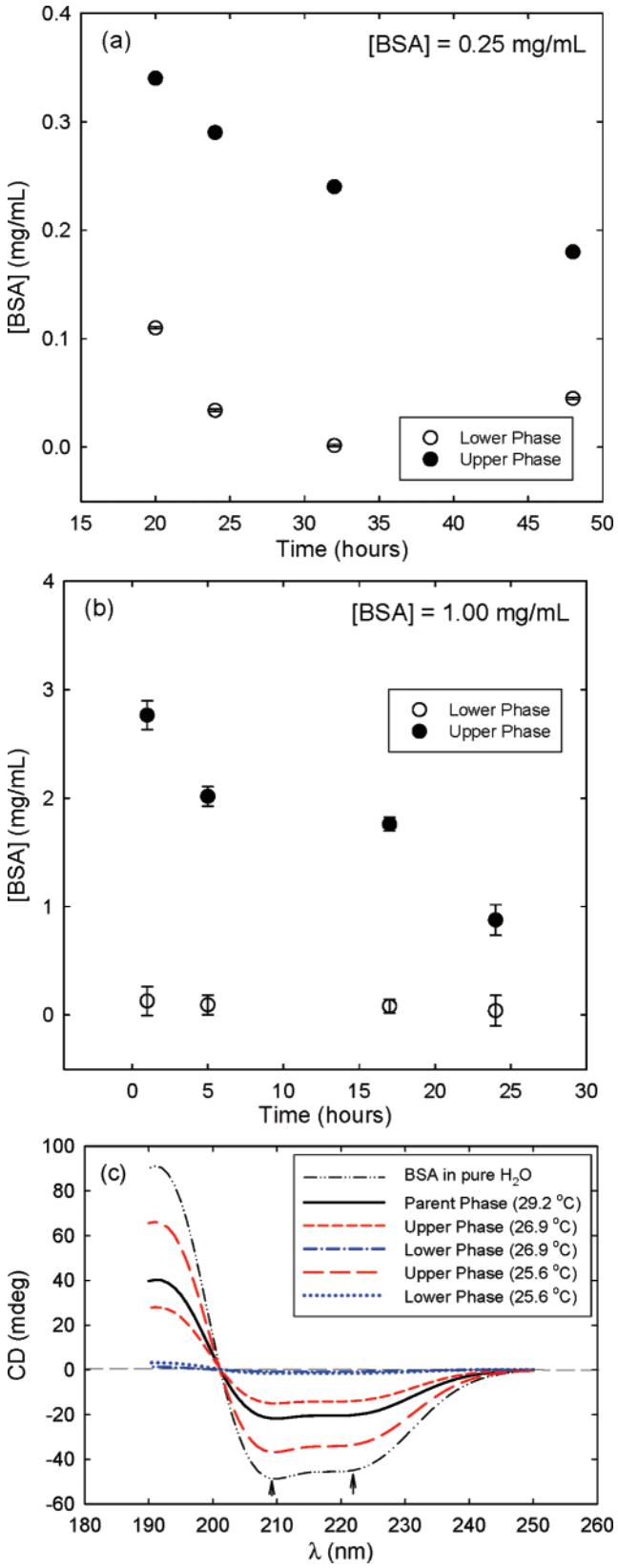


**Figure 7.** Partitioning of bovine serum albumin (BSA) in isobutyric acid + $H_2O$: (a) The concentration of BSA as a function of time for each daughter phase at $T < T_c$ for an initial concentration of 0.25 mg/mL; (b) the concentration of BSA as a function of time for each daughter phase at $T < T_c$ for an initial concentration of 1.00 mg/mL; (c) circular dichroism (CD) as a function of wavelength ($\lambda$) for BSA + isobutyric acid + $H_2O$ in the parent and daughter phases. The temperatures shown in the legend are the temperatures at which extractions were taken. The arrows indicate minima observed at 208 and 222 nm, indicative of α-helices.

isobutyric acid, the helices revert back to coils.[6] On rehydration, the coils then refold again into helices. Similarly, the trace water present in the upper phase of the BSA + isobutyric acid + $H_2O$ system could account for the fact that BSA is soluble in the upper isobutyric acid -rich phase. Perhaps a hydration layer acts as a "shield" between the protein and the isobutyric acid.

## Conclusions

In the Introduction, we raised several questions about polymer fractionation in binary solvents. We now address each of these questions.

**A. How Does the Addition of a Polymer Affect the $T_c$ of the Binary Liquid Mixture?** For PEG of molecular mass about 2 kg/mol, the $T_c$ of isobutyric acid + $H_2O$ increases linearly as the concentration of PEG increases. This is consistent with the "Timmermans rule"[22] that the addition of an impurity that is soluble in only one of the solvents of a binary solvent will decrease the solvent−solvent solubility and increase an upper critical solution temperature. The $T_c$ of isobutyric acid + $H_2O$ also increases, but nonlinearly, as the molecular mass of the added PEG increases. No study of $T_c$ was carried out for PEI. BSA increases $T_c$, but not to the same extent as does PEG.

**B. Can We Use the Mass Balance Equations To Describe the Polymer Partitioning?** Two methods were used to calculate the amount of polymer present in each phase: (i) the ratio of the areas under the MMD traces and (ii) the mass balance equations, eqs 1 and 2. The two methods agree within the experimental uncertainty for the data for the seven polymer−binary solvent systems studied. The mass balance equations can be used to calculate partitioning.

**C. Do Any of the Available Theories Decribe the Partitioning and Fractionation of This Polymer + Binary Solvent System?** We do not find simple Flory−Huggins behavior for the fractionation experiments of the eight polymer - binary solvent systems. We observe a nonlinearities in the ratio of $\ln[w(M)_{upper}/w(M)_{lower}]$ as a function of molecular mass that are consistent with qualitative predictions made by ten Brinke and Szleifer.[43] However, ten Brinke and Szleifer do not provide an analytical expression and we have not compared their theory directly to our experimental data.

**D. What Are the Mathematical Forms of the MMD's of the Polymer in the Parent and Daughter Phases and How Do They Develop over Time?** The MMD of the parent polymer is set by the conditions of the polymer synthesis, and there exists no theoretical guidance as to the expected MMD's of the polymer in the daughter phases. We have, therefore, used empirical fits to assess whether the mathematical forms of the polymer in the parent and daughter phases are the same. We find that at low average molecular masses (<10 kg/mol), the molecular mass distributions of PEG have the same mathematical forms in the parent and daughter phases. However, for 10 and 20 kg/mol PEG, we find changes in the MMD's upon fractionation. The MMD of the polymer in the upper polymer-rich daughter phase retains the mathematical form of the parent polymer, but the MMD of the polymer in the lower, polymer-lean phase differs from that of the parent polymer. To our knowledge, this is the first time that such an experimental observation has been reported. The sample of 25 kg/mol PEI did not show a significant difference in the forms of the MMD's in the coexisting phases. The rates of development of the MMD's do not agree with predictions:[72] $M_n$ does not relax more quickly than $M_w$, as was also found by Shresth et al.[4]

As discussed in the Introduction, some theories predict a change in the polymer MMD between parent and daughter phases, and some do not. We see a change as the PEG MMD's at higher polymer molecular masses. We do not see the more symmetric daughter MMD's predicted by Kamide and co-workers[68] for the polymer + unary solvent system. The explanation of the change in MMD upon fractionation between phases requires further exploration, by simulation and by theory.

**E. What Is the Effect on Partitioning and Fractionation of Increasing the Strength of Hydrogen Bonds in the System?** For the PEG samples studies, the degree of partitioning and the degree of fractionation were amplified when $H_2O$ was replaced by $D_2O$ in the solvent mixture. The partitioning and fractionation of PEG are driven by its ability to form a helix in isobutyric acid, and these helices are stabilized by a hydration layer.[6] When $D_2O$ is present, the hydration of the PEG helix in isobutyric acid will involve $D_2O$ molecules (there will be $D_2O$ present in the "isobutyric acid-rich" phase), which form stronger hydrogen bonds to the PEG than do $H_2O$ molecules. Hence the helix is even more favored and the driving force for partitioning and fractionation is increased.

**F. Other Observations.** These partitioning studies on synthetic polymers led to us to ask whether proteins would also partition in these solvent systems. We showed that BSA does indeed partition strongly into the upper isobutyric acid-rich phase. This result was unexpected since BSA is insoluble in pure isobutyric acid. Our other work details an hypothesis in that trace water acts as a stabilizing hydration layer on the polymer or protein.[6]

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## Appendix

**A. Mass Fraction of Polymer in Coexisting Phases.** Consider a polymer of mass $W^T$ with a molecular mass distribution (normalized) of $w(M)$. This sample is partitioned between phases A and B. The total mass of polymer in phase $A$ is $W_A^T$ with distribution $w_A(M)$. Similarly for phase B, we have $W_B^T$ and $w_B(M)$. The mass of each molecular mass is conserved,

$$W^T w(M) = W_A^T w_A(M) + W_B^T w_B(M) \quad \text{(i)}$$

as is the polymer mass:

$$W^T = W_A^T + W_B^T \quad \text{(ii)}$$

Solving for $W_A^T$:

$$W_A^T w_A(M) = W^T w(M) - W_B^T(M) w_B(M) \quad \text{(iii)}$$

$$W_A^T w_A(M) = W^T w(M) - [W^T - W_A^T] w_B(M) \quad \text{(iv)}$$

$$W_A^T w_A(M) = W^T w(M) - W^T w_B(M) + W_A^T w_B(M) \quad \text{(v)}$$

$$W_A^T [w_A(M) - w_B(M)] = W^T [w(M) - w_B(M)] \quad \text{(vi)}$$

$$\frac{W_A^T}{W^T} = \frac{w_A(M) - w_B(M)}{w(M) - w_B(M)} \quad \text{(vii)}$$

We refer to (vii) as the mass balance equation.

**Supporting Information Available:** A table giving MMD data for polymer in coexisting phases for 20kOH PEG in isobutyric acid + $H_2O$. This material is available free of charge via the Internet at http://pubs.acs.org.

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