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Liquid−Liquid Equilibria of Aqueous Two-Phase Systems Containing Poly(ethylene glycols) of Different Molecular Weight and Sodium Citrate

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The phase diagrams corresponding to systems of sodium citrate and poly(ethylene glycol) of different molecular weights (600, 1000, 1450, 3350, and 8000) were determined at (22, 37 and 50) °C. Moreover, the medium pH (5.2, 8.2, and 9.2) effect was also studied. The binodal curves were described using a four-parameter sigmoidal equation, and the tie-lines were satisfactorily described using the Othmer−Tobias and Bancroft equations. An increase in temperature and pH caused the expansion of the two-phase area. The binodal curves became more asymmetric and close to the origin with an increase in poly(ethylene glycol) molecular weight.

Introduction

Aqueous two-phase systems (ATPSs) can be formed by combining either two incompatible polymers or a polymer and a salt in water above a certain critical concentration.[1] In laboratory-scale separations, the most commonly used systems are comprised of the polymers poly(ethylene glycol) (PEG) and dextran, while for large-scale enzyme extraction, PEG + salt systems are used. These systems are attractive because of their low-cost, rapid phase disengagement, and availability of commercial separators, which allow a faster and continuous protein separation. The most commonly inorganic salt employed is potassium phosphate, but the high salt concentration required represent a waste disposal problem, which leads to environmental concerns. Other authors[2] have investigated citrate as a substitute for inorganic salts and found that citrates form aqueous two-phase systems with PEG. Citrates are biodegradable and non-toxic and can be discharged into biological wastewater treatment plants. To aid the design and process optimization of the aqueous two-phase extraction technique, more detailed information on the phase composition and the physicochemical properties of these systems is desirable. The dependence of phase composition on the polymer concentrations at a given temperature, pressure, and molecular weight of the polymer is often graphically displayed in a phase diagram. Present on the diagram is the binodal curve, which delineates the potential working area for a particular two-phase system and is a fingerprint unique to that system under set conditions of, for example, pH and temperature.[3] Zafarani-Moattar et al.[4] determined the liquid−liquid equilibria for the PEG6000 + sodium citrate at different temperatures and used the osmotic virial and the UNIQUAC equations to correlate the phase behavior of this system. Recently, Murugesan and Perumalsamy[5] obtained the binodal data for PEG 2000 + sodium citrate at five different temperatures and correlated them with model equations. However, the available experimental data for PEG + sodium citrate + water ATPSs are limited, and data for the binodal and the phase diagram of PEG of other molecular masses (600, 1000, 1450, 3350, and 8000) at different pH values are not available. The goal of this work is to obtain a complete set of measurements on the phase equilibrium for PEGs of increasing molecular weights and sodium citrate at different pH values and temperatures. In addition to the equilibrium measurements, the empirical description of the phase-equilibrium data with a four-parameter sigmoidal equation was performed in order to provide the basis for interpolating and extrapolating experimental data and predicting phase compositions when such data are not available.

Experimental Section

Materials. Poly(ethylene glycols) of the following average molecular masses: 600 (PEG600), 1000 (PEG1000), 1450 (PEG1450), 3350 (PEG3350), and 8000 were purchased from Sigma Chemical Co. and used without further purification. All the other reagents were of analytical grade with a minimum purity of 99%.

Experimental Procedure. The determination of the binodal curve was carried out by a turbidometric titration method.[6] Stock aqueous solutions of PEG of different molecular weight with mass fractions of (40 to 50) % and mass fractions of citric acid of 25 % were prepared by weighing known quantities of the polymer and acid, respectively. The pH of the citric stock solutions was adjusted to 5.20, 8.20, and 9.20, respectively, by the addition of the appropriate quantities of sodium hydroxide. Small aliquots, approximately (0.01 to 0.05) g, of the polymer stock solution were added to 1 g of the sodium citrate stock solution and placed in a glass tube. After each aliquot addition, the system was thoroughly mixed. The first appearance of turbidity (the cloud point) indicated that the system was about to enter to the two-phase area. With knowledge of the composition of the starting polymer and salt solutions and of the added amounts (grams), the total system composition, just prior to the two-phase formation, was calculated and provided a point on the binodal curve. The starting and added solution masses were measured on an analytical balance with a precision of $\pm 1.\ 10^{-4}$ g. Additional binodal points were obtained by adding a small amount of water to clear the system and then enough drops of the PEG stock solution to produce turbidity again. To obtain the binodal points corresponding to higher polymer concentration, the above-mentioned procedure was inverted, thus titrating

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Table 1. Phase Compositions for the Water (1) + PEG (2) + Citrate (3) Systems at 22 °C

tie line	total system		bottom phase		top phase			tie line	total system		bottom phase		top phase		
	100 w_2	100 w_3	100 w_2	100 w_3	100 w_2	100 w_3	STL[a]		100 w_2	100 w_3	100 w_2	100 w_3	100 w_2	100 w_3	STL[a]
PEG600 + Sodium Citrate at pH 5.20															
1	17.14	14.11	10.08	18.13	24.31	10.02	−1.75	3	18.55	15.26	5.21	22.97	33.17	6.81	−1.73
2	17.83	14.67	7.31	20.64	29.52	8.03	−1.76								
PEG1000 + Sodium Citrate at pH 5.20															
1	15.29	13.42	8.44	16.62	24.20	9.26	−2.14	3	15.92	13.97	5.37	18.94	29.21	7.70	−2.12
2	15.53	13.63	7.19	17.50	26.63	8.48	−2.15								
PEG1450 + Sodium Citrate at pH 5.20															
1	15.01	11.15	8.16	14.53	19.73	8.82	−2.03	3	15.75	11.70	5.02	17.01	25.30	6.97	−2.02
2	15.48	11.50	5.88	16.26	23.45	7.55	−2.02	4	16.27	12.09	3.68	18.34	28.36	6.09	−2.01
PEG3350 + Sodium Citrate at pH 5.20															
1	10.12	10.53	5.52	12.70	15.96	7.78	−2.12	3	10.78	11.22	3.03	14.86	21.56	6.16	−2.13
2	10.45	10.87	3.96	13.96	18.76	6.92	−2.10	4	11.14	11.59	2.26	15.72	24.19	5.51	−2.15
PEG8000 + Sodium Citrate at pH 5.20															
1	4.26	11.83	2.68	12.29	13.92	6.89	−2.08	4	4.79	12.95	1.08	14.40	27.56	4.03	−2.55
2	4.39	11.88	2.10	12.92	17.16	6.07	−2.19	5	4.94	13.38	0.75	15.03	29.56	3.70	−2.54
3	4.65	12.57	1.30	14.02	22.08	5.02	−2.31								
PEG600 + Sodium Citrate at pH 8.20															
1	16.13	10.84	7.73	14.41	26.20	6.56	−2.35	3	17.95	12.06	2.87	18.65	36.04	4.15	−2.29
2	16.98	11.41	4.86	16.66	31.16	5.27	−2.31								
PEG1000 + Sodium Citrate at pH 8.20															
1	9.88	12.36	7.51	13.29	25.85	6.11	−2.55	4	10.84	13.56	2.89	16.84	33.01	4.40	−2.42
2	10.18	12.73	5.52	14.64	27.29	5.74	−2.45	5	11.20	14.01	2.12	17.63	37.72	3.44	−2.50
3	10.50	13.13	4.08	15.77	30.20	5.03	−2.43								
PEG1450 + Sodium Citrate at pH 8.20															
1	9.34	10.40	5.45	12.08	20.26	5.69	−2.32	3	10.37	11.56	2.57	14.81	29.41	3.62	−2.40
2	9.75	10.87	3.97	13.32	24.29	4.69	−2.35	4	10.71	11.93	1.99	15.55	31.81	3.18	−2.41
PEG3350 + Sodium Citrate at pH 8.20															
1	8.89	8.09	4.91	9.55	16.64	5.24	−2.72	3	9.70	8.82	2.35	11.49	23.51	3.79	−2.75
2	9.28	8.44	3.40	10.59	20.21	4.44	−2.73	4	10.16	9.24	1.55	12.34	26.95	3.19	−2.77
PEG8000 + Sodium Citrate at pH 8.20															
1	5.86	7.97	3.33	8.61	13.05	6.15	−3.95	3	6.24	8.48	1.79	9.54	20.09	5.18	−4.20
2	6.04	8.21	2.48	9.07	16.98	5.58	−4.16	4	6.46	8.78	1.29	9.97	24.03	4.73	−4.33
PEG600 + Sodium Citrate at pH 9.20															
1	17.42	11.15	7.93	15.98	23.87	7.87	−1.97	3	18.20	11.64	6.32	17.35	30.08	5.93	−2.08
2	17.89	11.40	7.31	16.48	28.04	6.52	−2.08	4	18.71	11.97	5.15	18.50	32.82	5.18	−2.07
PEG1000 + Sodium Citrate at pH 9.20															
1	14.86	10.23	7.00	13.27	24.61	6.46	−2.58	3	15.83	10.90	3.69	15.64	31.12	4.92	−2.56
2	15.36	10.57	4.93	14.67	27.88	5.65	−2.54	4	16.23	11.17	2.76	16.47	33.21	4.49	−2.54
PEG1450 + Sodium Citrate at pH 9.20															
1	18.97	7.17	4.71	13.21	22.82	5.54	−2.36	3	20.83	7.87	2.14	15.66	30.55	3.82	−2.40
2	20.17	7.62	2.75	15.00	27.84	4.37	−2.36								
PEG3350 + Sodium Citrate at pH 9.20															
1	8.18	8.96	5.36	10.08	14.41	6.49	−2.53	3	8.72	9.54	3.21	11.63	21.00	4.88	−2.63
2	8.44	9.23	4.12	10.90	17.78	5.62	−2.58	4	9.00	9.85	2.39	12.43	22.61	4.54	−2.56
PEG8000 + Sodium Citrate at pH 9.20															
1	5.86	8.87	1.49	10.35	18.70	4.51	−2.95	3	6.55	9.91	0.34	11.88	28.27	3.01	−3.15
2	6.24	9.45	0.78	11.20	24.71	3.54	−3.13								

[a] Slope of the tie line.

the stock polymer solution with the stock salt solution. The system temperature was maintained constant and controlled to within ± 0.05 °C by immersing the glass tube and the stock solutions in a thermostatic bath.

For the determination of the tie lines, a series of ATPSs of at least three different known total compositions were prepared in graduated glass tubes and placed in a thermostatic bath. After reaching the phase equilibrium, visual estimates of top and bottom volumes (V_T and V_B, respectively) were made. The phases were separated, and their densities were measured with an Anton Paar DMA 35 N (precision of $\pm 1{\cdot}10^{-4}$ g/cm^3) density meter. PEG and citrate equilibrium compositions in top and bottom phases were measured by an enzymatic and colorimetric assay, respectively. Citrate was converted to oxaloacetate and acetate in a reaction catalyzed by the enzyme citrate lyase (CL):[7]

$$\text{citrate} \xrightarrow{\text{CL}} \text{oxaloacetate} + \text{acetate}$$

In the presence of the enzymes L-malate and (L-MDH) and L-lactate dehydrogenase (L-LDH), oxalacetate and its decarboxylation product pyruvate were reduced to L-malate and L-lactate, respectively, by reduced nicotinamide-adenine dinucleotide (NADH):

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-MDH}} \text{L-malate} + \text{NAD}^+$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-MLDH}} \text{L-lactate} + \text{NAD}^+$$

The amount of NADH oxidized in the above reactions is stoichiometric to the amount of citrate. NADH was determined by means of its light absorbance at 340 nm. The uncertainty in the measurement of the mass fraction of the salt was estimated to be better than ± 0.001.

The PEG compositios in both phases were determined according to a colorimetric assay based on the partitioning of a chromophore present in ammonium ferrothiocyanate reagent from an aqueous to an organic (chloroform) phase in the presence of PEG.[8] A total of 2.5 mL of ammonium ferrothiocyanate 0.1 mol·L^{-1} and 2.5 mL of chloroform were taken in a centrifuge tube. Aliquots (50 μL) containing PEG (from

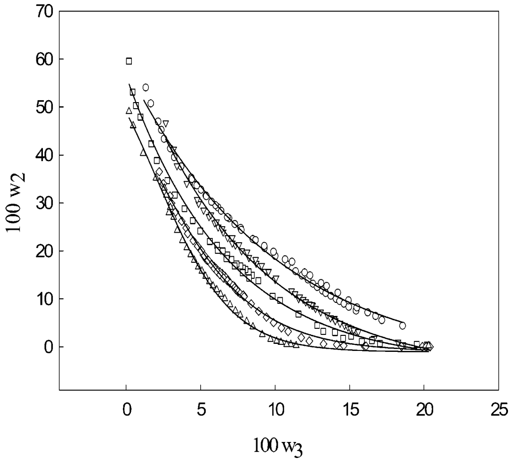

Figure 1. Binodal curves for water (1) + PEG of varying molecular weight (2) + citrate (3) at 22 °C and pH 9.20: ○, PEG600; ▽, PEG1000; □, PEG1450; ◇, PEG3350; △, PEG8000.

appropriate dilutions of top and bottom phases) were added to tubes containing the above biphasic system. After vigorous mixing for 30 min, the tubes were centrifuged at 3000*g* for 2 min. The lower chloroform layers were removed, and their absorbances were recordered at 510 nm. Aqueous PEG standards obtained by serial dilution of a stock solution with mass fraction of 30 % were used to prepare a calibration curve for each PEG of a given molecular weight. The uncertainty in the measurement of the mass fraction of the polymer was estimated to be ± 0.002.

A mass balance check was made between the initial mass of each component and the amounts in the bottom and top phases on the basis of equilibrium compositions. The relative error in the mass balance was < 2 %. The tie line lengths (TLL) for the different compositions were calculated according to

$$\mathrm{TLL} = [(w_2^{\mathrm{T}} - w_2^{\mathrm{B}})^2 + (w_3^{\mathrm{T}} - s_3^{\mathrm{B}})^2]^{1/2} \tag{1}$$

where w_2^{T}, w_3^{T}, w_2^{B}, and w_3^{B} are the top (T) and bottom (B) equilibrium mass fractions of PEG (2) and citrate (3). The tie line lengths are expressed in mass fractions.

Results and Discussion

Effect of PEG Molecular Weight on Binodal Diagram. The phase compositions for the systems with varying PEG molecular weight at three different pH values are shown in Table 1. For most systems, the PEG concentration in the bottom phase is very small, and in some cases, PEG is almost excluded from this phase while the opposite behavior is observed in the top phase. A similar behavior was observed for the traditional PEG + phosphate ATPSs.[9] The binodal curves for ATPSs of sodium citrate and PEGs of different molecular weight at 22 °C, pH 9.20, are shown in Figure 1. It can be seen in this figure that binodal curves became more asymmetric and close to the origin with the increase in PEG molecular weight, thus requiring lower concentrations for phase separation. This may be caused by the increase in the incompatibility between the system components due to the more hydrophobical character of PEGs of higher molecular weight.[10] In fitting the binodal data for water (1) + PEG (2) + citrate (3) system to different expressions,[11,12] better results were obtained with the following empirical function:

$$w_2 = y_0 + \frac{i}{1 + e\,\dfrac{w_3 - x_0}{j}} \tag{2}$$

where w_2 and w_3 are the coordinates of the binodal points; y_0, x_0, i, and j are the fit parameters. The parameters of eq 2 along with the corresponding standard deviations (σ) for each PEG molecular weight are summarized in Table 2. The low values of σ indicate that the equation was suitable to fit the data. A similar behavior was observed for these ATPSs at other pH values and temperatures (data not shown).

Table 2. Values of the Fit Parameters y_0, x_0, i, and j of Equation 2 for Water (1) + PEG (2) + Citrate (3) Systems at 22 °C and pH 9.20

	parameters				
PEG	i	j	x_0	y_0	σ^a
600	1209.94	−9.39	−27.36	−3.98	0.90
1000	1307.15	−7.61	−22.08	−5.44	0.73
1450	1658.91	−6.57	−21.57	−3.59	1.16
3350	88.94	−3.60	0.89	−1.07	0.51
8000	71.78	−2.51	2.09	−1.07	0.71

[a] σ, standard deviation.

Table 3. Values of the Parameters of Othmer−Tobias and Bancroft Equations, k, n, k_1, and r for Water (1) + PEG (2) + Citrate (3) Systems at 22 °C and pH 9.20

PEG	k	n	σ^a	k_1	r	σ^a
600	2.990	0.545	0.0046	0.0998	2.072	0.015
1000	3.392	0.578	0.0034	0.0719	2.048	0.011
1450	3.583	0.499	0.0060	0.0552	2.207	0.022
3350	4.412	0.398	0.0065	0.0149	2.764	0.026
8000	5.855	0.273	0.0022	0.0010	3.932	0.011

[a] σ, standard deviation.

For most of the assayed systems, the total system composition has no significant effect upon the slope of the tie lines, which implies that they are parallel to each other, thus allowing us to know the coexisting phase compositions for any given total polymer phase-forming composition. A different behavior was observed for systems of PEG8000 whose tie lines became steeper for total compositions far away from the critical point.

Empirical equations have been proposed to ascertain the reliability of calculated tie line data in traditional liquid−liquid extraction, the most used being those of Othmer−Tobias[13] (eq 3) and Bancroft[11] (eq 4):

$$\left(\frac{1 - w_2^{\mathrm{T}}}{w_2^{\mathrm{T}}}\right) = k\left(\frac{1 - w_3^{\mathrm{B}}}{w_3^{\mathrm{B}}}\right)^n \tag{3}$$

$$\left(\frac{w_1^{\mathrm{B}}}{w_e^{\mathrm{B}}}\right) = k_1\left(\frac{w_1^{\mathrm{T}}}{w_2^{\mathrm{T}}}\right)^{\mathrm{r}} \tag{4}$$

where w_1^{T} and w_1^{B} are the mass fractions of water in the top (T) and bottom (B) phases; k, n, k_1, and r represent the parameters to be determined. Linearization of both equations produces acceptable consistency in the results. Values of the fit parameters and the corresponding standard deviations are given in Table 3.

Effect of pH. The effect of pH on phase separation is viewed in Figure 2, which summarizes the phase diagrams corresponding to ATPSs of PEG3350 at different pH values. The binodal curves showed similar shape for the several pH values and tend to superimpose at high PEG or Cit concentrations, thus indicating that either the exclusion or the salting out effect respectively prevail in phase separation. When PEG and Cit concentrations adopt intermediate values, a smaller concentration of citrate is needed for two-phase formation at basic pH values with higher ratios between trivalent and divalent citrate ions.

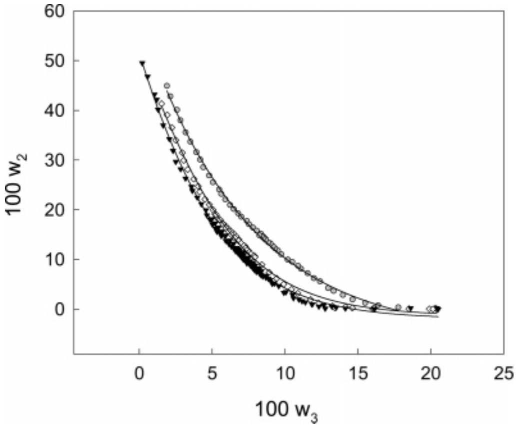

Figure 2. Effect of pH on binodal curve of ATPSs of water (1) + PEG3350 (2) + citrate (3) at 22 °C: ○, pH 5.20; ◇, pH 8.20; ▼, pH 9.20.

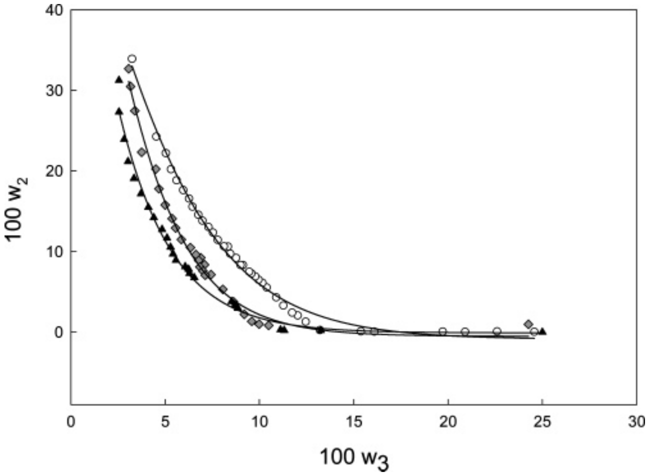

Figure 3. Effect of temperature on binodal curve corresponding to ATPS of water (1) + PEG8000 (2) + citrate (3) at pH 5.20: ○, 295 K; ◇, 310 K; ▲, 323 K.

This is in agreement with the well-known fact that trivalent ions are more efficient than divalent ions in promoting the phase separation.[14] This behavior was also observed for PEGs of the other molecular weight.

Effect of Temperature. Figure 3 shows that the increase in temperature causes an increase of the area of two-phase coexistence for all the assayed ATPSs. According to the model proposed by Cabezas,[15] the entropically unfavorable structuring of water produced by PEG at low temperatures is overcome due to the large decrease in enthalpy (due to the energetically favorable and highly directional interactions, such as hydrogen-bonding, between unlike molecules). At higher temperatures, provided that the structure of water in the PEG hydration shell does not break down too rapidly with increasing temperature, the unfavorable entropy contribution becomes preminent and the system phase separates itself. This trend also agrees with experimental results from the literature for other ATPSs.[16,17]

Conclusions

The aqueous two-phase partitioning method of liquid−liquid extraction is useful for separating material of biological origin. Water + PEG + NaCit systems are attractive for protein purification because of low cost, rapid phase separation, and the biodegradability of citrate. However, reliable and complete data on the composition and properties of these systems were not available at present. Since such information is necessary for the design of an extraction process, we analyzed the effect of different medium conditions such as temperature, pH, and PEG molecular weight on the liquid−liquid equilibria of these PEG + salt systems. All the binodal curves were satisfactory described using a four-parameter sigmoidal equation. An increase in temperature and PEG molecular weight caused the expansion of the two-phase area. The trivalent citrate showed to be more effective in phase separation since, at higher pH values, smaller concentrations of citrate were needed for two-phase formation.

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