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Phase diagrams of urethanized polyvinyl alcohol with a series of hydrophobically modified pH-responsive polymers containing amino acid residues

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Abstract−The composition and phase diagram of aqueous two-phase systems (ATPSs) of urethanized polyvinyl alcohol (UPVA) and a series of ionic co- and terpolymers (IPs) of pH-responsive hydrophilic diallylammonioethanoate, 0-3 mol% hydrophobic dodecyldiallylammonium chloride, and SO_2 have been studied for the first time. The presence of unquenched nitrogen valency in the repeat units of the IPs permitted the change of the charge types and their densities in the polymer chain. The effects of zwitterionic ($Z^{\pm}$) and anionic (A^-) charge densities on the polymer backbone, salt (NaCl) concentration, and hydrophobe content on the phase diagrams were investigated. The presence of the hydrophobe in the polymer shifted the binodal downward; magnitude of the shift increased with the increase in the hydrophobe content. The phase separation happened at relatively low total polymer concentrations (much below than 10%), which could be useful in separation and purification of biomaterials.

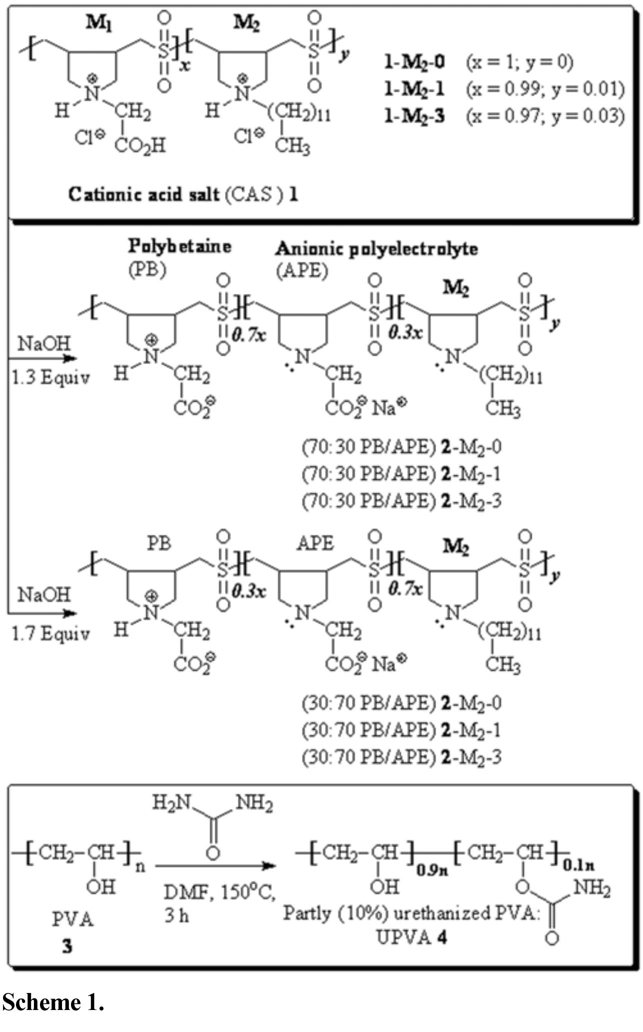
Key words: Aqueous Two-phase System, pH-responsive Polymers, Hydrophobicity, Urethanized Polyvinyl Alcohol, Phase Diagram

INTRODUCTION

Aqueous solutions of appropriate concentrations of two polymers, a polymer and salt or other alternative components, when combined above or below a certain critical temperature, lead to the construction of biphasic systems known as aqueous two-phase systems (ATPSs). ATPS, having the advantage to be readily scaled-up, are efficient, inexpensive, environment-friendly and biocompatible liquid-liquid extraction systems that have been widely used to separate and purify a variety of industrially important biomolecules [1-7]. Unequal distribution of biomolecules, such as amino acids and proteins in ATPSs, leads to the efficient separation and purification of the biomolecules in downstream processes of biotechnology [8,9].

The most commonly used ATPSs are based on poly(ethylene glycol) (PEG) and dextran [10-13]. In some cases, the polymers have been modified with hydrophobic groups and biospecific ligands for the affinity partitioning of biomolecules [14-18]. Two recent reviews [19,20] describe the ATPS based on ionic liquids as a novel green separation system. The efficient use of hydrophobically modified acrylamide-styrene copolymer-PEG aqueous two-phase system in the separation of bovine serum albumin has been demonstrated [21].

In an effort to develop suitable ATPSs, the effect of pH and salt concentration on the coexistence curves of a system containing pH responsive copolymer poly(diallylammonioethanoate-*alt*-sulfur dioxide) and polyethylene glycol has been reported [22]. The system has been successfully employed in the study of protein partitioning; extremely high partitioning levels (partition coefficient of 0.004)



Scheme 1.

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and very high separation ratios of bovine serum albumin and cytochrome c (upto 48) were recorded [23]. Hydrophobicity is a key feature that determines the partitioning of proteins in ATPSs [24]. Here in this work, the phase diagram data of a variety of new ATPSs involving pH-responsive hydrophobically modified cyclocopolymers of diallylammonioethanoate/sulphur dioxide **2** (obtained *via* neutralization of **1** with NaOH) having varying proportions of polybetaine (PB) and anionioic polyelectrolyte (APE) fractions and urethanized polyvinyl alcohol (UPVA) **4** is reported (Scheme 1). Cyclopolymer **2** is anticipated to be a promising candidate for the construction of recycling ATPS, since the pH-responsive polymer can be precipitated at lower pH values by its conversion to water-insoluble **1**. The amino acid residues in **2** mimic the biomolecules like proteins, and as such the ATPSs may be used in bioseparation involving monomeric amino acids or proteins. Polyvinyl alcohol (PVA), with free hydroxyl groups, offers considerable latitude for chemical transformations such as esterification, etherification and acetylization. It has been established [25] that partly urethanized PVA (UPVA) is more stable than PVA and readily soluble in solvents, such as water, DMF and DMSO. To our knowledge the UPVA has never been reported as a component for the construction of ATPS.

EXPERIMENTAL

1. Materials

The synthesis of polymers poly[(diallylammonioethanoate-*alt*-sulfur dioxide)-*ran*-(diallyldodecylammonium chloride-*alt*-sulfur dioxide)], also described as cationic acid salts (CAS) **1**-M_2-0, **1**-M_2-1, **1**-M_2-3, containing 0, 1 and 3 mol% of the hydrophobic monomer M_2 (diallyldodecylammonium chloride), respectively, has been reported elsewhere [26]. These polymers have similar weight average molar masses of around 1.86×10^5 g/mol and intrinsic viscosities [h] of ~1.0 dL/g for the anionic form of **2** having a polybetaine (PB)/anionic polyelectrolyte (APE) ratio of 0 : 100 (Scheme 1).

PVA **3** with a number average molecular weight of 72000, a degree of hydrolyzation of 97.5-99.5 mol% and a degree of polymerization of 1,600 was purchased from Fluka Chemie AG (Buchs, Switzerland). Partly urethanized PVA (UPVA) **4** was prepared [24] by adding urea to PVA in DMF at about 150 °C for 3 h (Scheme 1). The degree of urethanization and molar mass of the UPVA were estimated to be around 10 mol% of the repeating units and 68,000 g/mol, respectively [25]. The urethanization process is known to be accompanied by some chain cleavage.

2. Phase Compositions and Phase Diagram of UPVA 4-[CAS 1+1.3 or 1.7 Equiv NaOH] -H_2O (0.1 or 0.4 N NaCl) System

2-1. The Tie Lines by NMR Method

A stock solution of 20%w/w of UPVA **4** (molar mass of the repeating unit 48.35) was prepared in salt-free water. The stock solutions of (70 : 30 PB/APE) **2** and (30 : 70 PB/APE) **2** were made by treating CAS.H_2O **1** (11-20%w/w) (molar mass of repeating unit 273.73) or **1**-M_2-1 (molar mass 274.65), or **1**-M_2-3 (molar mass 276.50) with 1.3 and 1.7 equivalents of NaOH, respectively. One equivalent of NaOH would be required to neutralize a CAS **1** sample to give PB/APE **2** with a PB/APE ratio of ~100 : 0 which in turn also generates 1 equivalent of NaCl (Scheme 1). Further addition of NaOH (0.3 and 0.7 equivalent) would change the (100 : 0 PB/APE) **2** to polymers (70 : 30 PB/APE) **2** and (30 : 70 PB/APE) **2** containing a mixture of zwitterionic and anionic units in a respective ratio of 70 : 30 and 30 : 70 (Scheme 1). A set of total systems of a volume of ~7 cm^3 was prepared by using the stock solutions of UPVA **4** and PB/APE **2** (i.e., 1.3 or 1.7 equiv NaOH-treated CAS-**1**). The salt concentration in the total systems (Tables 1-8) (*see below*) was kept 0.1 N or 0.4 N NaCl by adding additional solid salt (NaCl) when required. The determination of the final salt (NaCl) concentration took into account the NaCl produced from the neutralization reaction.

The mixtures of the total systems after thorough shaking were centrifuged for 10 min to ensure a complete phase separation and kept at 23 °C for 24 h. The volume and the density of the top layers (~1.03 g/cm^3) and bottom layers (~1.07 g/cm^3) were measured. The ^{1}H NMR spectra of the top and bottom layers were measured after exchanging H_2O with D_2O. The ^{1}H NMR spectra of (a) PVA **3**, (b) UPVA **4**, (c) sample from bottom phase of system 1 of Fig. 2(a) (Table 1), and (d) sample from top phase of system 4 of Fig. 2(c)

Table 1. Phase composition of the UPVA 4 - [CAS 1-M_2-0+1.3 equiv NaOH] -H_2O (0.4 N NaCl) system at 23 °C (shown in Fig. 2(a))

NMR method

System	Total system		Top phase		Bottom phase		Volume ratio[a]
	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	
1	5.591	10.02	0.224	16.5	13.2	0.266	1.48
2	4.015	6.838	0.380	11.3	9.67	0.305	1.54
3	2.519	4.449	0.557	7.06	5.41	0.678	1.48
4	3.513	4.245	0.538	8.17	6.16	0.643	0.926

Dilution method

System	Binodal data		System	Binodal data	
	CAS %w/w	UPVA %w/w		CAS %w/w	UPVA %w/w
a	1.35	5.65	d	2.45	3.01
b	1.75	4.45	e	3.15	2.35
c	2.11	3.7	f	4.03	1.77

[a]Volume ratio of top and bottom phase

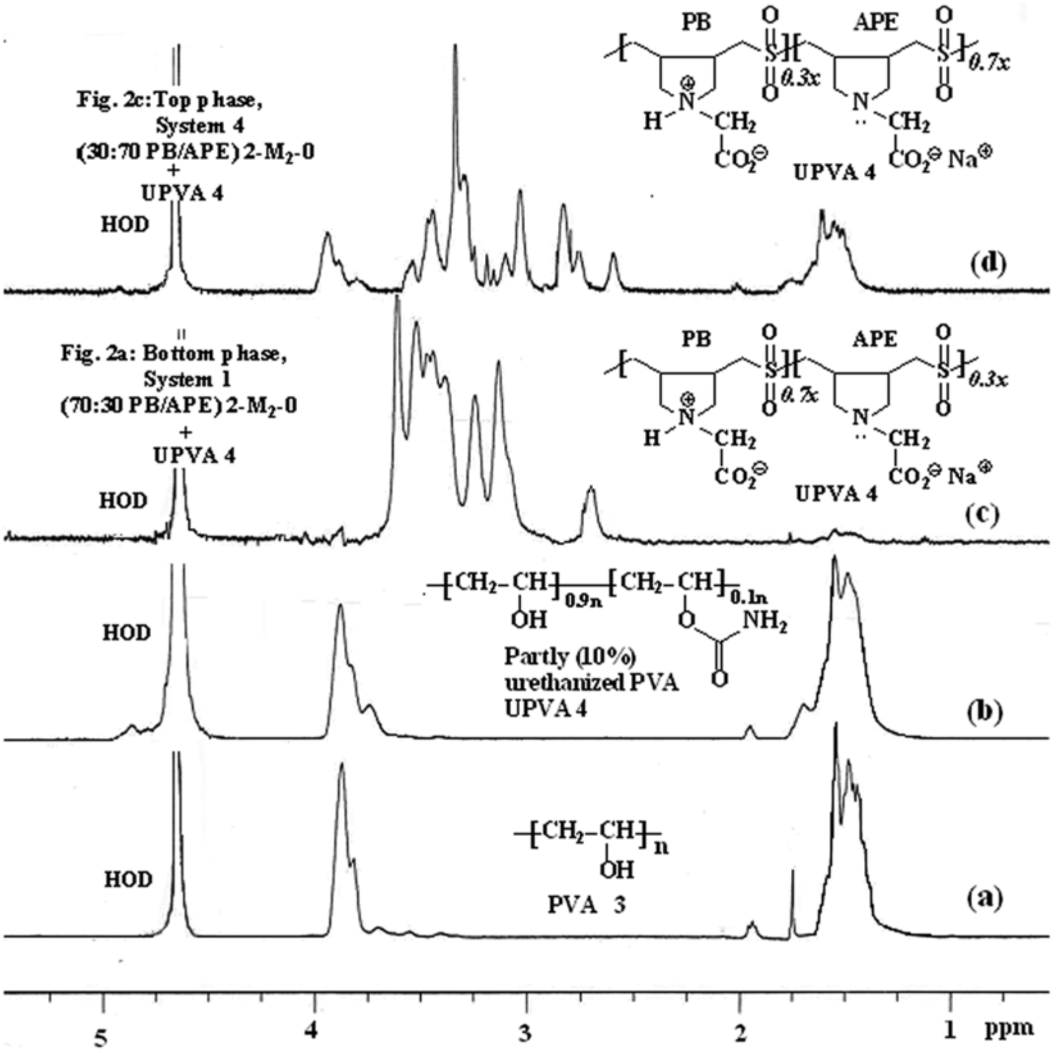


Fig. 1. ^{1}H NMR spectra of (a) PVA; (b) UPVA; (c) Fig. 2(a): Bottom phase, System 1: UPVA 4-[(70 : 30) PB/APE 2 M_2-0]; (d) Fig. 2(c): Top phase, System 4: UPVA 4-[(30 : 70) PB/APE 2 M_2-0].

(Table 1), are displayed in Fig. 1.

The ^{1}H NMR signals for the polymers were well separated and the mole ratios of the repeating units of the polymers were easily calculated using the integration of the underlined two-proton multiplet ($\underline{\text{H}}_2$CC) at δ1.2-2.0 ppm for UPVA **4** (Fig. 1(b)) and the twelve-proton complex signals in the range δ2.5-3.7 for the polymers PB/APE **2** (i.e., CAS **1**+1.3 or 1.7 equivalent NaOH) (Fig. 1(c), (d)). For UPVA, the underlined protons C$\underline{\text{H}}$OH and C$\underline{\text{H}}$OCONH$_2$ appeared at δ3.60-3.95 and ≥4.65 ppm, respectively (Fig. 1(b)). The later signal was overlapped with the signal of HOD which also appeared at 4.65 ppm. For the area (A) under δ1.2-2.0 for CH$_2$ of UPVA, the area for a single H becomes (A/2). The PVA is urethanized to the extent of 10%, and as such the area of the non-urethanized, i.e., PVA portion of C$\underline{\text{H}}$OH (90%) at δ3.60-3.95 should translate into (A/2)×0.90. Since this proton (C$\underline{\text{H}}$OH) signal overlaps with the signals of the NaOH-treated CAS **1** (PB/APE **2**), the area (B) in the range δ2.5-4.0 ppm belongs to the 12 Hs of PB/APE **2** (CAS **1**+NaOH) repeat unit and C$\underline{\text{H}}$O protons of non-urethanized PVA. So, the area of a single H for CAS becomes [B−(A/2)×0.90]/12. The mole ratio of CAS/UPVA (i.e. the ratio of area of single H of CAS and UPVA) thus becomes:

$$\frac{\text{mol UPVA}}{\text{mol CAS}}=\frac{\text{Area of 1H of UPVA}}{\text{Area for 1H of CAS}}=\frac{\text{A}/2}{[\text{B}-(\text{A}/2)\times 0.9]/12} \qquad (1)$$

^{1}H NMR measurements were used to determine the mole ratios. The tie lines were constructed for the systems shown in Figs. 2, 3 and 4(a). The compositions of the systems shown in Fig. 2(a) are also listed in Table 1; however, the systems shown in the other figures are not tabulated in order to save space. The remaining Tables 2-8 representing the data for Figs. 2(b), 2(c), 3(a), 3(b), 3(c), 3(d) and 4(a) are included as supplementary information.

Weight percent of each polymer is determined by using the Eqs. (2) and (3) as described in our earlier work [27].

$$[\text{CAS}_b]=\frac{\text{UPVA}_0/48.35-(\text{CAS}_0/273.73)([\text{UPVA}]/[\text{CAS}])_t}{\text{V}_b\{([\text{UPVA}]/[\text{CAS}])_b-([\text{UPVA}]/[\text{CAS}])_t\}} \qquad (2)$$

Where, subscripts t and b represent top and bottom phase, respectively. [CAS] and [UPVA] represent the concentration of the repeating units in mmol of repeat unit cm^{-3}. CAS$_0$ and UPVA$_0$ represent total mass in mg of the polymers, and V represents the volume in

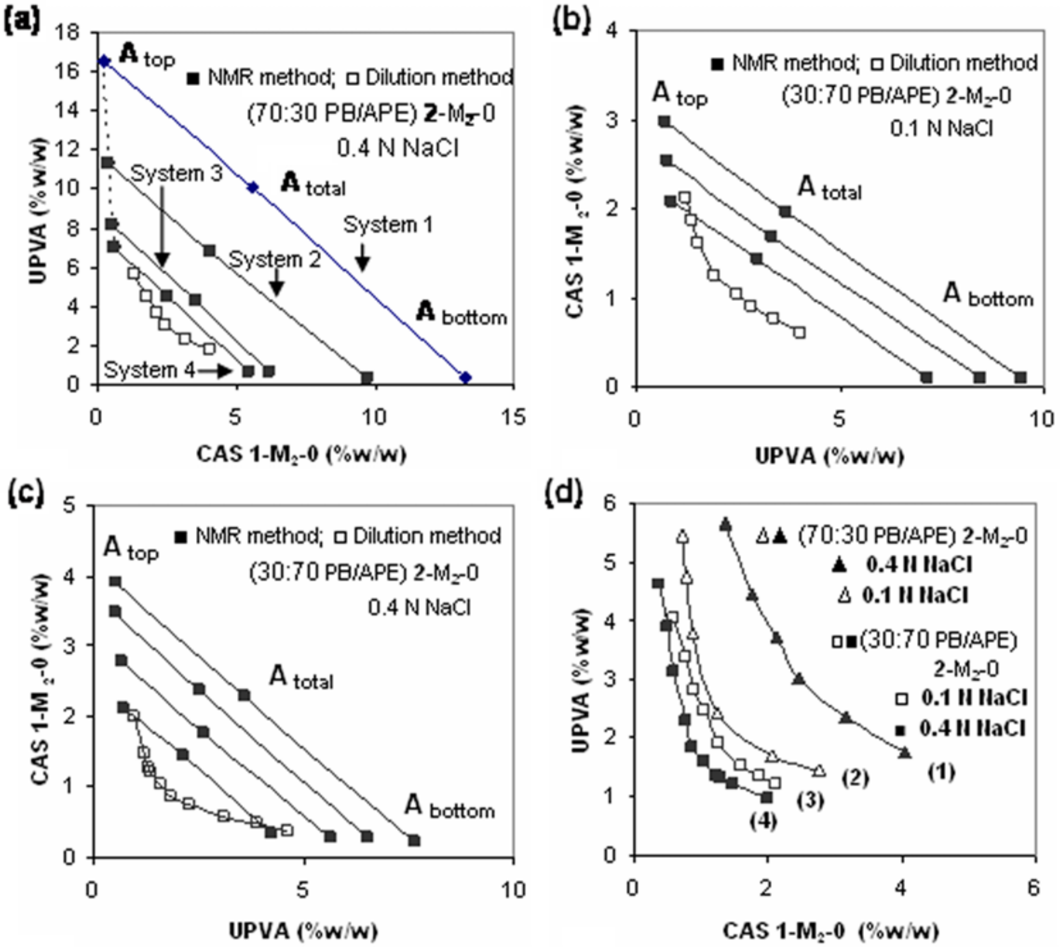


Fig. 2. Phase diagram at 23 °C of (a) UPVA4- [CAS 1-M_2-0+1.3 equiv NaOH]-water (0.4 N NaCl); (b) UPVA4- [CAS 1-M_2-0+1.7 equiv NaOH]-water (0.1 N NaCl); (c) UPVA4- [CAS 1-M_2-0+1.7 equiv NaOH]-water (0.4 N NaCl); (d) UPVA4- [CAS 1-M_2-0+1.3 or 1.7 equiv NaOH]-water (0.1 N and 0.4 N NaCl).

cm^3. Molar masses of the repeat units of the CAS.H_2O **1**-M_2-0 and UPVA were taken as 273.73 and 48.35, respectively. For CAS.H_2O **1**-M_2-1 and 1-M_2-3, the respective molar masses of 274.65 and 276.50 were used. [UPVA]/[CAS] represents the molar ratio of the polymers as determined by ^{1}H NMR integration. The mass of the polymer CAS in the bottom phase is then calculated as:

$$CAS_b=[CAS_b]V_b\times 273.73 \text{ mg} \tag{3}$$

Once one of the polymer concentrations in a phase is known, then the concentrations and weight percents of the polymers in the two phases are easily calculated from the known volume, density and mass of the two phases.

2-2. Binodals by the Turbidity Method

The turbidity method: About 1.5 g of a concentrated solution (~10% w/w) of CAS **1** (treated with 1.3 or 1.7 equivalents of NaOH) in 0.1 or 0.4 N NaCl and a known weight of a concentrated solution (~20%w/w) of the UPVA **4** (in 0.1 or 0.4 N NaCl) were taken in a flask and stirred with a magnetic stir bar until the transparent system turned turbid. Then a known weight of a 0.1 or 0.4 N NaCl solution was added dropwise until the system became transparent again. At this point, the final composition of the two polymers calculated corresponds to a point on the binodal curve. After the first point was obtained, a concentrated solution of UPVA was added again to obtain a turbid suspension, and dilution with water (0.1 N NaCl) was repeated to obtain a second point on the binodal. This procedure was continued until a sufficient number of points for the construction of the binodal curve were obtained. To obtain points on the other end of the bimodal curve, the concentrated solution of the UPVA was titrated with the CAS **1** (treated with 1.3 or 1.7 equiv NaOH) solution following the same procedure as described above. Points obtained by turbidity method are joined together in the figures. All experiments were carried out at 23 °C.

2-3. Binodals by the Dilution Method

Turbidity method is the easier method for constructing the binodals. However, when the turbidity method did not give a clear-cut visualization of a phase transition, the binodals were obtained by a dilution method. Systems with known total compositions were diluted with water (NaCl), a few drops at a time, and then allowed to separate into two phases. This process was continued until the two phases became one. The composition of the diluted system (before the last drop makes the system homogeneous) was determined, and these points along with the composition determined by ^{1}H NMR analyses as well as by turbidity methods were used to construct the phase diagrams.

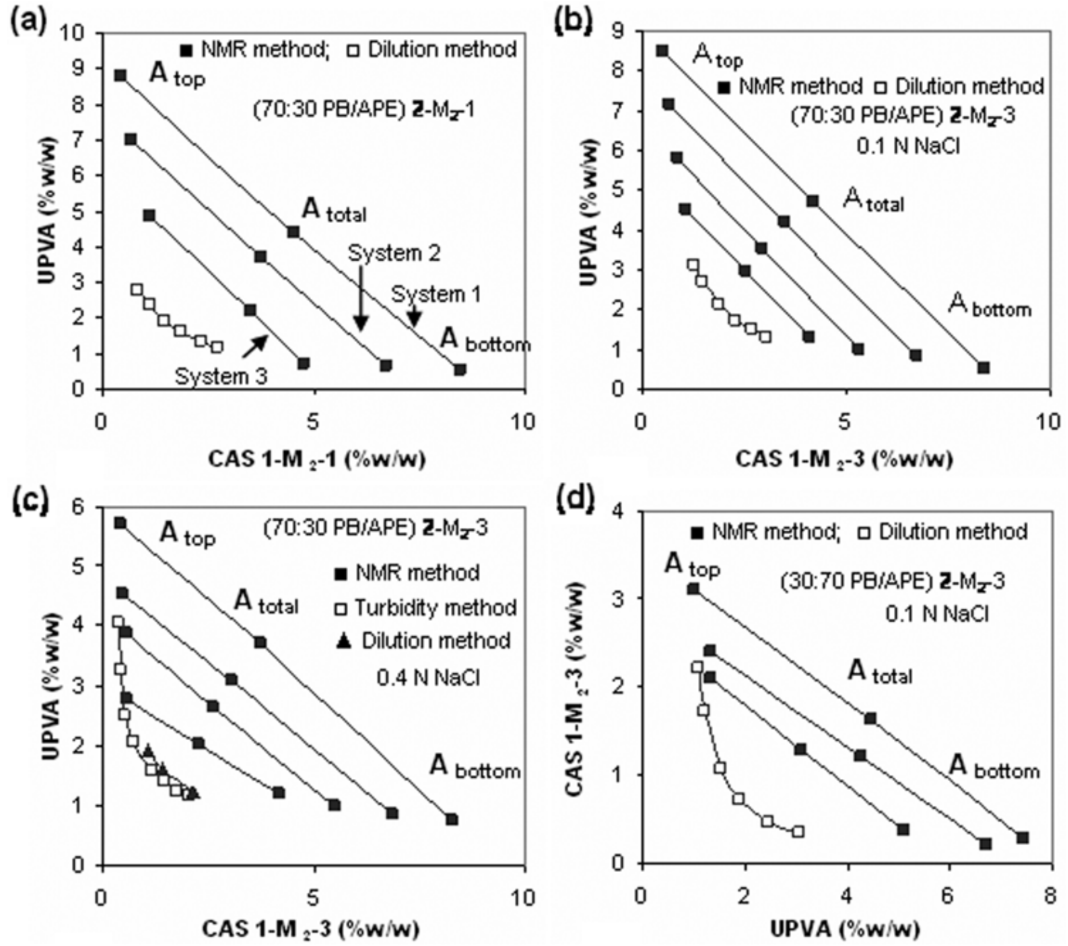


Fig. 3. Phase diagram at 23 °C of (a) UPVA4- [CAS 1-M_2-1+1.3 equiv NaOH]-water (0.4 N NaCl); (b) UPVA4- [CAS 1-M_2-3+1.3 equiv NaOH]-water (0.1 N NaCl); (c) UPVA4- [CAS 1-M_2-3+1.3 equiv NaOH]-water (0.4 N NaCl); (d) UPVA4- [CAS 1-M_2-3+1.7 equiv NaOH]-water (0.1 N NaCl).

RESULTS AND DISCUSSION

1. Phase Diagrams Using UPVA -[CAS 1-M_2-0+NaOH]-water (NaCl) Systems

Cationic acid salt (CAS) 1 is pH-responsive due to the presence of an amino acid residue having an unquenched nitrogen valency. On treatment with 1 and 2 equivalents of NaOH, CAS 1 can be converted into (PB/APE) 2 with a PB/APE ratio of ~100 : 0 and ~0 : 100, respectively (Scheme 1). Base treatment in the equivalent range 1< NaOH<2 is expected to generate polymers having varying proportions of PB and APE fractions in the polymer chain. While CAS 1 and (100 : 0 PB/APE) 2 were found to be almost insoluble in common solvents including water in the presence or absence of salt, the corresponding (0 : 100 PB/APE) 2 (i.e., APE 2) is water-soluble [28]. As the APE fraction in 2 crosses the 20% limit, it becomes water-soluble.

CAS 1-M_2-0 and 1-M_2-3, for instance, indicate the presence of 0 and 3 mol%, respectively, of the hydrophobic repeat units (M_2) having a C_{12} alkyl pendent as the hydrophobe (Scheme 1). Phase compositions of several UPVA 4-[CAS 1-M_2-0 or 1-M_2-1 or 1-M_2-3+ 1.3 or 1.7 equivalents of NaOH]-H_2O (0.1 or 0.4 N NaCl) systems were determined using three different techniques: the NMR, turbidity and dilution methods as described in the experimental section. Polymer concentrations of the two phases in equilibrium with each other, as determined by ^{1}H NMR, are connected by tie lines. Turbidity and/or dilution methods are used to obtain the binodal curves.

In the presence of 1.3 and 1.7 equivalents of NaOH, CAS **1** is converted to polymer (70 : 30 PB/APE) **2** and (30 : 70 PB/APE) **2**, respectively, having PB and APE fractions in a respective ratio of 70 : 30 and 30 : 70 (Scheme 1). Phase compositions in the top and bottom phases and the ratio of the phase volumes of UPVA **4**- [CAS **1**-M_2-0+1.3 equiv NaOH] -H_2O (0.4 N NaCl) systems are described in Table 1. The corresponding phase diagram is shown in Fig. 2(a). In the current and subsequent phase diagrams, the %w/w of the polymer, rich in the top phase, is assigned the y-axis, while the polymer rich in the bottom phase is assigned the x-axis. The polymers displayed segregative phase separation behaviour (Fig. 2(a)). The binodal was found to be fairly symmetrical; thus the two polymers may have very similar hydrodynamic volumes [27]. The phase separation happened at relatively low total polymer concentrations, much below than 10%. Phase separation at a low concentration could be useful from an industrial point of view. Several tie lines (1-4) [Fig. 2(a)], which connect the phases in equilibrium, are constructed by NMR technique. The tie lines are helpful in constructing two-phases with suitable volume ratio of the top and bottom phases. For instance,

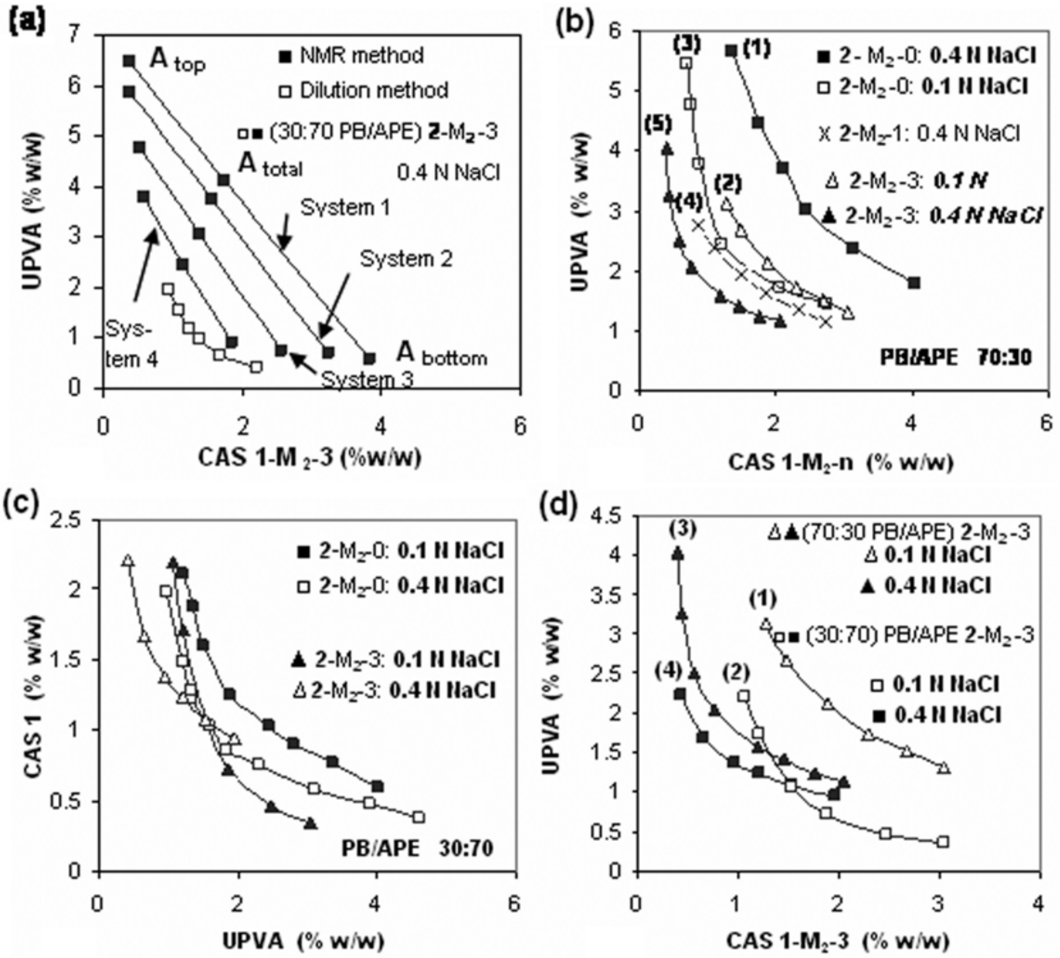


Fig. 4. Phase diagram at 23 °C of (a) UPVA4- [CAS 1-M$_2$-3+1.7 equiv NaOH]-water (0.4 N NaCl); (b) UPVA4- [CAS 1-M$_2$-n+1.3 equiv NaOH]-water (0.1 and 0.4 N NaCl); (c) UPVA4- [CAS 1-M$_2$-n+1.7 equiv NaOH]-water (0.1 and 0.4 N NaCl); (d) UPVA4- [CAS 1-M$_2$-3+1.3 or 1.7 equiv NaOH]-water (0.1 and 0.4 N NaCl).

Table 2. Phase composition of the UPVA 4-[CAS 1-M$_2$-0 1.7 equiv NaOH]-H$_2$O (0.1 N NaCl) system at 23 °C (shown in Fig. 2(b))

NMR method

System	Total system		Top phase		Bottom phase		Volume ratio[a]
	UPVA %w/w	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	CAS %w/w	
1	3.67	1.95	0.701	2.97	9.45	0.0871	1.93
2	3.33	1.64	0.772	2.52	8.43	0.0901	2.01
3	2.95	1.42	0.874	2.07	7.11	0.0937	2.05

Turbidity method

System	Binodal data		System	Binodal data	
	UPVA %w/w	CAS %w/w		UPVA %w/w	CAS %w/w
a	4.03	0.594	e	1.89	1.24
b	3.37	0.769	f	1.52	1.60
c	2.81	0.894	g	1.35	1.87
d	2.47	1.047	h	1.21	2.11

[a] Volume ratio of top and bottom phase

the total system represented by the point A_{total} will have the top and bottom phase compositions represented by A_{top} and A_{bottom}, respectively, but the volume or the mass ratio of the top and bottom phases will be determined by the ratio of the lengths of A-A_{bot} and A-A_{top}.

Phase compositions of UPVA **4**-[CAS **1**-M$_2$-0+1.7 equiv NaOH]-H$_2$O in the presence of 0.1 N NaCl and 0.4 N NaCl are given in

Table 3. Phase composition of the UPVA 4-[CAS 1-M$_2$-0+1.7 equiv NaOH] -H$_2$O (0.4 N NaCl) system at 23 °C (shown in Fig. 2(c))

NMR method

System	Total system		Top phase		Bottom phase		Volume ratio[a]
	UPVA %w/w	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	CAS %w/w	
1	3.57	2.30	0.525	3.90	7.63	0.238	1.33
2	2.54	2.36	0.549	3.47	6.52	0.294	2.01
3	2.62	1.76	0.671	2.78	5.63	0.292	1.51
4	2.11	1.44	0.721	2.12	4.24	0.342	1.60

Turbidity method

System	Binodal data		System	Binodal data	
	UPVA %w/w	CAS %w/w		UPVA %w/w	CAS %w/w
a	4.62	0.372	f	1.62	1.03
b	3.91	0.480	g	1.34	1.22
c	3.11	0.581	h	1.33	1.28
d	2.30	0.761	i	1.20	1.48
e	1.84	0.862	j	0.981	1.98

[a] Volume ratio of top and bottom phase

Table 4. Phase composition of the UPVA 4-[CAS 1-M$_2$-1+1.3 equiv. NaOH] -H$_2$O (0.4 N NaCl) system at 23 °C (shown in Fig. 3(a))

NMR method

System	Total system		Top phase		Bottom phase		Volume ratio[a]
	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	
1	4.54	4.38	0.421	8.76	8.50	0.513	0.944
2	3.75	3.71	0.680	7.01	6.71	0.62	0.955
3	3.52	2.19	1.13	4.85	4.79	0.713	0.526

Dilution method

System	Binodal data		System	Binodal data	
	CAS %w/w	UPVA %w/w		CAS %w/w	UPVA %w/w
a	2.71	1.13	d	1.48	1.91
b	2.32	1.34	e	1.1	2.35
c	1.85	1.61	f	0.85	2.75

[a] Volume ratio of top and bottom phase

Table 5. Phase composition of the [CAS 1-M$_2$-3+1.3 equiv. NaOH]-UPVA 4 -H$_2$O (0.1 N NaCl) system at 23 °C (shown in Fig. 3(b))

NMR method

System	Total system		Top phase		Bottom phase		Volume ratio[a]
	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	
1	4.20	4.71	0.503	8.52	8.41	0.532	1.12
2	3.51	4.20	0.651	7.15	6.72	0.82	1.16
3	2.92	3.51	0.850	5.82	5.31	1.01	1.15
4	2.52	2.96	1.07	4.50	4.08	1.28	1.11

Dilution method

System	Binodal data		System	Binodal data	
	CAS %w/w	UPVA %w/w		CAS %w/w	UPVA %w/w
a	3.05	1.31	d	1.88	2.13
b	2.67	1.52	e	1.47	2.67
c	2.29	1.73	f	1.27	3.12

[a] Volume ratio of top and bottom phase

Table 6. Phase composition of the [CAS 1-M_2-3+1.3 equiv. NaOH] -UPVA 4-H_2O (0.4 N NaCl) system at 23 ºC (shown in Fig. 3(c))

NMR method

System	Total system		Top phase		Bottom phase		Volume ratio[a]
	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	
1	3.75	3.71	0.45	5.7	8.3	0.75	1.43
2	3.05	3.07	0.51	4.51	6.89	0.87	1.50
3	2.65	2.65	0.594	3.87	5.53	0.99	1.39
4	2.29	2.01	0.568	2.76	4.18	1.18	1.12

Turbidity method

System	Binodal data		System	Binodal data	
	CAS %w/w	UPVA %w/w		CAS %w/w	UPVA %w/w
a	0.386	4.04	e	1.19	1.58
b	0.430	3.26	f	1.45	1.41
c	0.560	2.51	g	1.76	1.25
d	0.751	2.05	h	2.05	1.15

[a] Volume ratio of top and bottom phase

Table 7. Phase composition of the [CAS 1-M_2-3+1.7 equiv. NaOH] -UPVA 4 -H_2O (0.1 N NaCl) system at 23 ºC (shown in Fig. 3(d))

NMR method

System	Total system		Top phase		Bottom phase		Volume ratio[a]
	UPVA %w/w	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	CAS %w/w	
1	4.45	1.63	1.01	3.11	7.45	0.272	0.892
2	4.25	1.21	1.34	2.39	6.70	0.216	0.867
3	3.1	1.27	1.31	2.10	5.12	0.37	1.13

Dilution method

System	Binodal data		System	Binodal data	
	UPVA %w/w	CAS %w/w		UPVA %w/w	CAS %w/w
a	1.07	2.2	f	1.87	0.73
b	1.21	1.72	g	2.47	0.47
c	1.54	1.07	h	3.05	0.35

[a] Volume ratio of top and bottom phase

Table 8. Phase composition of the [CAS 1-M_2-3+1.7 equiv. NaOH]-UPVA 4 -H_2O (0.4 N NaCl) system at 23 ºC (shown in Fig. 4(a))

NMR method

System	Total system		Top phase		Bottom phase		Volume ratio[a]
	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	CAS %w/w	UPVA %w/w	
1	1.73	4.11	0.37	6.46	3.83	0.58	1.49
2	1.56	3.73	0.396	5.85	3.26	0.683	1.48
3	1.40	3.07	0.527	4.77	2.57	0.729	1.38
4	1.16	2.44	0.579	3.80	1.87	0.898	1.20

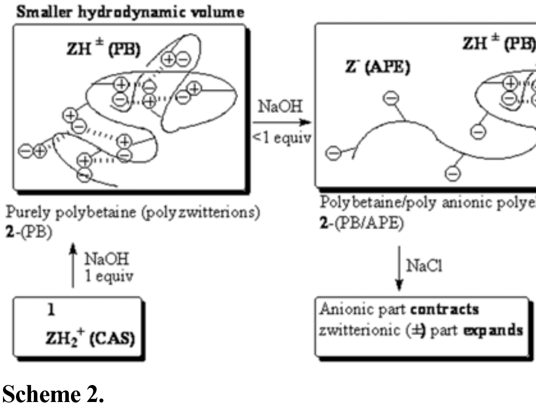
Turbidity method

System	Binodal data		System	Binodal data	
	CAS %w/w	UPVA %w/w		CAS %w/w	UPVA %w/w
a	0.95	1.95	d	1.38	0.962
b	1.08	1.54	e	1.67	0.651
c	1.24	1.20	f	2.22	0.422

[a] Volume ratio of top and bottom phase

Figs. 2(b) and (c), respectively. It is interesting that a phase reversion was observed with the zwitterionic (betainic)-poor (30 : 70 PB/APE) **2** [Figs. 2(b), (c)] contrary to the systems having a zwitterionic-rich (70 : 30 PB/APE) **2** with a PB/APE ratio of 70 : 30 [Fig. 2(a)]. In this case, the top and bottom layers were found to be rich in (30 : 70 PB/APE) **2** M_2-0 (i.e., [CAS **1**-M_2-0+1.7 equiv NaOH]) and UPVA **4**, respectively [Fig. 2(b) or (c)]. The rationale behind such phase reversion could be attributed to the differences in the solubility of the zwitterionic (PB) and anionic (APE) species' in water, while the former tends to be water-insoluble; the latter is very much water-soluble. The anionic rich (30 : 70 PB/APE) **2** is expected to attract much higher volume of water, and as such will have less density so as to occupy the top phase. The opposite is the case with zwitterionic-rich (70 : 30 PB/APE) **2**.

The effects of NaCl concentration and the PB/APE ratio of polymer **2** on the binodal curves (obtained by dilution/turbidity methods) are displayed in Fig. 2(d). For (70 : 30 PB/APE) **2**-M_2-0, the binodal curve shifts downward as the concentration of NaCl becomes lower [*cf.* plot (1) vs. plot (2)], indicating that lower concentrations of the polymers are required to form the ATPS in 0.1 N NaCl than in 0.4 N NaCl. The 70% zwitterionic (betainic) and 30% anionic fractions in (70 : 30 PB/APE) **2**-M_2-0 are expected to contribute to 'antipolyelectrolyte' and 'polyelectrolyte' behavior, respectively (Scheme 1). According to Munk [29], the addition of small amounts of electrolytes (NaCl etc.) to the solution of simple linear polyelectrolytes leads to Debye-Hückel shielding effect that permits the polymer to adopt a more entropically favored compact conformation. The opposite behavior is typical for aqueous solutions of polyampholytes/polybetaines. The opposite charges of the zwitterionic species along the backbone tend to self neutralize the chain and cause it to collapse under attractions between the opposite charges (Scheme 2). Addition of a small electrolyte (like NaCl) tends to disrupt the zwitterionic interaction, thus resultiing in the increase of hydrodynamic volume (anti-polyelectrolyte effect). Since the polymer (70 : 30 PB/APE) **2**-M_2-0 has a higher degree of zwitterionic (70%) than anionic character (30%), it is expected to demonstrate overall antipolyelectrolyte behavior upon addition of NaCl. As the concentration of the added salt increases, the chain expansion increases. Thus an increased hydrodynamic volume of the polymer (70 : 30 PB/APE) **2**-M_2-0 in 0.4 N NaCl, makes it more compatible with the UPVA **4**, and therefore higher amounts of polymers are required for phase separation to occur. The above rationale gets credence from the observed intrinsic viscosity values. The intrinsic viscosity [h] of UPVA **4** at 30 °C in water was determined as 0.760 dL/g, whereas for polymer **2** having PB/APE ratios of 70 : 30, 30 : 70 and 0 : 100 at 30 °C in 0.1 N NaCl, the [h] values were found to be 0.175, 0.603 and 0.967 dL/g, respectively. Since it is well known that hydrodynamic volume of polymers in solution is directly proportional to their [h]s, it can be deduced that the dominance of the zwitterionic fragments PB causes the polymer chains to assume a more compact structure for a PB/APE ratio of 70 : 30. UPVA **4** is thus expected to have a much higher hydrodynamic volume than (70 : 30 PB/APE) **2**-M_2-0. The degree of the size asymmetry is expected to diminish in 0.4 N NaCl as a result of the coil expansion of the ionic polymer, thereby making the polymer pair more compatible (Scheme 2).



Scheme 2.

As the PB/APE ratio in **2**-M_2-0 was changed from 70 : 30 to 30 : 70, the binodals moved downwards both in 0.1 and 0.4 N NaCl [Fig. 2(d): plot (3) and (4)]. This change of ratio in favor of APE fraction is associated with an increase of the intrinsic viscosity (*see above*). Polymer (30 : 70 PB/APE) **2**-M_2-0 is thus expected to have a much higher hydrodynamic volume as well as net negative charges than a sample of (70 : 30 PB/APE) **2**. The higher net negative charges are responsible for moving the binodals downward, thus making it less compatible with the neural polymer UPVA **4**. Increasing the salt concentration to 0.4 N causes a contraction of the polymer chain since it is dominated by anionic motifs. Lower hydrodynamic volume of polymer (30 : 70 PB/APE) **2** in 0.4 N than in 0.1 N NaCl makes the polymer less compatible with the UPVA and therefore it requires less amounts of polymers for phase separation to occur.

2. Phase Diagrams Using UPVA-[CAS 1-M_2-(1 or 3)+NaOH]-water (NaCl) Systems

The effects of the presence of hydrophobe and its proportion on the binodals are demonstrated in Fig. 3(a)-(d) and Fig. 4(a) using UPVA **4**-[PB/APE **2**-M_2-1]- and UPVA **4**-[PB/APE **2**-M_2-3]-H_2O (0.1 and 0.4 N NaCl). As anticipated, the top and bottom layers were found to be overwhelmingly rich in UPVA **4** and (70 : 30 PB/APE) **2**-M_2-1 or -M_2-3, respectively, [Fig. 3(a)-(c)]. In the presence of zwitterionic-poor (30 : 70 PB/APE) **2**, a phase reversion was observed. For the UPVA **4**-[30 : 70 PB/APE **2**]-M_2-3 system**,** while the top layer was rich in (PB/APE **2**-M_2-3) in 0.1 N NaCl [Fig. 3(d)]**,** UPVA **4** preferred the top phase in 0.4 N NaCl [Fig. 4(a)].

The effects of NaCl concentration, the PB/APE ratio and hydrophobe content in polymer PB/APE **2** (obtained from CAS **1** treated with NaOH) on the binodal curves (obtained by dilution/turbidity methods) are displayed in Figs. 4(b)-(d). As discussed before, for (70 : 30 PB/APE) **2**-M_2-0 (containing 0 mol% hydrophobe), the binodal curve shifted downward as the concentration of NaCl became lower [Fig. 4(b): plot (1) vs. (3)]. The opposite trend was observed for (70 : 30 PB/APE) **2**-M_2-3 (containing 3 mol% hydrophobe) - the binodal shifted downward as the concentration of NaCl became higher [Fig. 4(b): plot (2) vs. (5)]. The presence of the hydrophobe also shifted the binodal downward; for the system containing the highest proportion of the hydrophobe, i.e., **2**-M_2-3, the binodal was shifted the most [Fig. 4(b): *cf.* plots (1), (4), and (5)]. For CAS **1** systems treated with 1.7 equivalents of NaOH, the presence of hydrophobe was also found to shift the binodals downward [Fig. 4(c)]. For the systems containing (30 : 70 PB/APE) **2**-M_2-3, the binodals [Fig. 4(d): plots (2) and (4)] were shifted downward compared to the systems having a PB/APE ratio of 70 : 30 [Fig. 4(d): plots (1) and (3)] both in 0.1 and 0.4 N NaCl. The presence of the hydrophobe and higher amount of APE fractions thus make it less compatible with the UPVA, hence requiring lesser amount of polymers for phase separation to occur. The well known effects of NaCl on hydrophobic association [30] dictate that the presence of added salt makes the aqueous system more hostile to the hydrophobes, thereby forcing them to associate intermolecularly. The hydrophobic moieties in the polymer molecules can undergo interchain associations

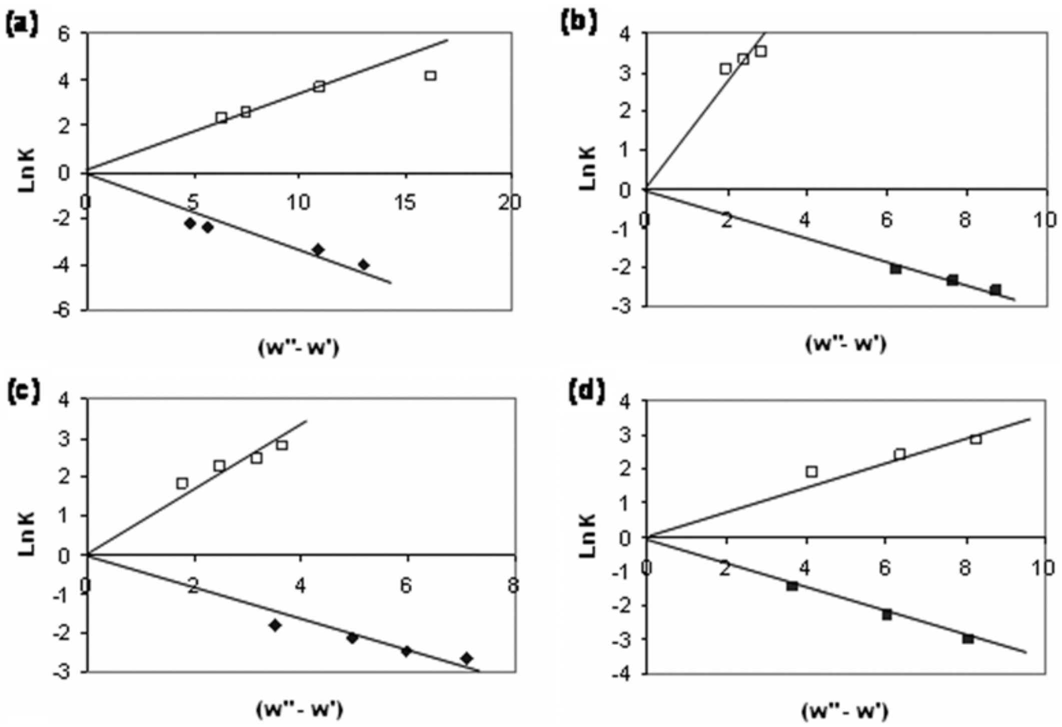


Fig. 5. Correlation of the phase diagrams of CAS 1-UPVA4-water (NaCl) systems using the method of Diamond and Hsu: Using Tie-line data from (a) (Fig. 2(a)); (b) (Fig. 2(b)); (c) (Fig. 2(c)); (d) (Fig. 3(a)).

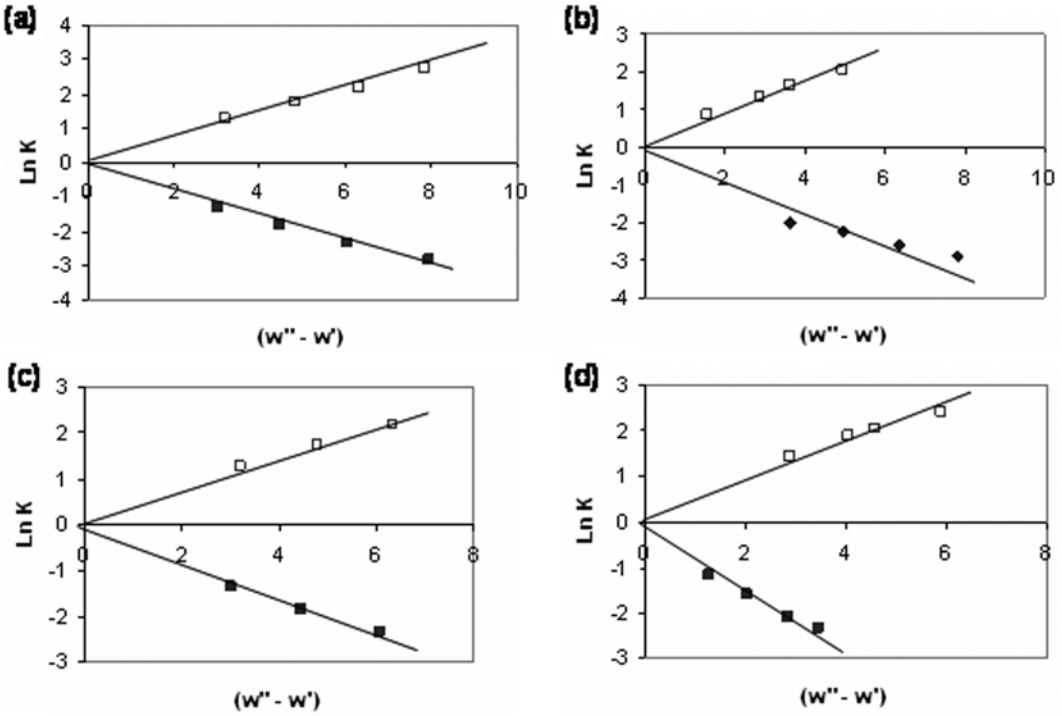


Fig. 6. Correlation of the phase diagrams of CAS 1-UPVA4-water (NaCl) systems using the method of Diamond and Hsu: Using Tie-line data from (a) (Fig. 3(b)); (b) (Fig. 3(c)); (c) (Fig. 3(d)); (d) (Fig. 4(a)).

to form an extended structure with a large molecular size. The formation of the physical network facilitates the incompatibility of the hydrophobically modified polymer (PB/APE) **2**-M_2-3 with UPVA **4**, and hence phase separation occurs at lower concentrations of the polymers.

3. The Correlation of the Phase Diagrams Using the Method of Diamond and Su

The consistency of the tie-lines of the [NaOH-treated CAS **1**]-UPVA **4**-water (NaCl) systems was checked using the following correlation developed by Diamond and Hsu [31] based on Flory-Huggins theory:

$$\ln K_1 = A_1 (w''_1 - w'_1)$$

and

$$\ln K_2 = A_2 (w''_2 - w'_2)$$

where w'' and w' are the polymer weight percent in the top and bottom phase, respectively, the slopes A_1 and A_2 are functions of the polymer molecular weight and the interactions between the polymers and water, K_1 and K_2 represent the partition coefficient (C_t/C_b) of the polymer between the top and bottom phase, and the subscripts 1 and 2 represent polymer 1 (PB/APE **2**) and polymer 2 (UPVA **4**). The correlation results are shown in Figs. 5 and 6. Straight lines were obtained indicating satisfactory representation of the phase behaviour by this model.

CONCLUSIONS

Urethanized polyvinyl alcohol (UPVA) has been introduced as a phase forming polymer for the first time. Cationic acid salt (CAS) polymers **1** with and without hydrophobic modification were used to construct ATPS, and to study the effects of hydrophobic modification on the phase behaviors. One of the most gratifying aspects of CAS **1** is that it has almost zero solubility in water below pH~7 in the presence or absence of added salt, thus making it a suitable candidate for industrial applications since the solubility behavior will permit its effective removal from solution by precipitation. The hydrophobicity, hydrophilicity and the hydrodynamic volume of the polymer **2** can be controlled by the degree of protonation of the pH-responsive amine functionality and by hydrophobic modification. The presence of hydrophobe as well as the increasing amount of anionic fractions in the polymer **2** chain was found to make the polymer less compatible with UPVA **4**, thereby requiring lesser amount of polymers for phase separation to occur. Hydrophobicity is a key factor that determines the partitioning of proteins in ATPSs. Further studies on the effect of the presence of hydrophobes and the charge density on the polymer chain in protein separation are currently under investigation in our laboratory. The phase separation takes place at relatively low total polymer concentrations, much below than 10%, which could be useful from an industrial point of view.

SUPPLEMENTARY INFORMATION

Tables 2, 3, 4, 5, 6, 7 and 8 representing the data for Figs. 2(b), 2(c), 3(a), 3(b), 3(c), 3(d) and 4(a) are included as supplementary information.

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