Origin of salt additive effect on solute partitioning in aqueous polyethylene glycol-8000–sodium sulfate two-phase system

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ARTICLE INFO

Article history:

Received 6 November 2013

Received in revised form 31 January 2014

Accepted 3 February 2014

Available online 18 February 2014

Keywords:

PEG–sulfate aqueous two-phase systems

Salt additives

Ionic and non-ionic solutes partitioning

Hofmeister series

Relative hydrophobicity

Electrostatic properties

ABSTRACT

Partitioning of a homologous series of dinitrophenylated (DNP-) amino acids with aliphatic side chains was examined in aqueous polyethylene glycol (PEG)-8000–sodium sulfate two-phase systems (ATPS) with the additives NaSCN, NaClO₄, and NaH₂PO₄ at concentrations varied from 0.025 M up to 0.54 M. The differences between the relative hydrophobicities and electrostatic properties of the two phases in all ATPS were estimated. Partitioning of adenine, adenosine mono-, di- and tri-phosphates was also examined in all ATPSs, including those with NaCl additive. Partition coefficients for these compounds and for nonionic organic compounds previously reported [L.A. Ferreira, P. Parpot, J.A. Teixeira, L.M. Mikheeva, B.Y. Zaslavsky, J. Chromatogr. A 1220 (2012) 14.] were analyzed in terms of linear solvent regression relationship. The results obtained suggest that the effects of the salts additives are related to their influence on the water structure.

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1. Introduction

Aqueous two-phase systems (ATPSs) are formed in mixtures of two (or more) water-soluble polymers or a single polymer and specific salt in an aqueous solution above certain critical concentrations or temperature. Two immiscible aqueous phases are formed in such mixtures. The uniqueness of these systems is that each of the phases contains over 80% water on a molal basis but the phases are immiscible and differ in their solvent properties [1–4]. Therefore they can be used for the discriminating distribution of added solutes. ATPSs have been used for separation of biological macromolecules and particles for over 50 years [5–7] and recently found use in a variety of analytical applications as well [8,9].

The most studied polymer–salt ATPSs are formed by polyethylene glycol (PEG) and inorganic salts, such as sodium sulfate, phosphate, carbonate or citrate. Salt effects on polymers and biopolymers, such as proteins, in water generally follow the well known Hofmeister series [10] with qualitative order of the anions being citrate^{3−} > CO₃^{2−} > SO₄^{2−} > H₂PO₄[−] > F[−] > OH[−] > Cl[−] > NO₃[−] >

Br[−] > I[−] > ClO₄[−] > SCN[−]. Anions to the left of chloride are often called kosmotropes and they typically stabilize proteins and salt them out of solution. Anions to the right of chloride are called chaotropes and they commonly destabilize proteins and salt them into solution. The positions of some anions in the series, such as ClO₄[−] and SCN[−] or SO₄^{2−} and H₂PO₄[−] may interchange depending on the particular effect in question and the nature of cation. The Hofmeister effect is commonly explained by the ability of various ions to “make” or “break” water structure in aqueous solution [11,12]. This explanation was put in doubt by many recent experimental and theoretical [13–16] studies showing that the properties of bulk water are not noticeably perturbed by ions in solution. It should be mentioned, however, that some recent experimental data [11,12,17] contradict this conclusion. The mechanism of the ions effects on proteins in aqueous solutions remains debatable.

The anions of sodium salts capable of forming ATPS with PEG (citrate, carbonate, sulfate, phosphate, fluoride, and hydroxide) all belong to the kosmotropes. Attempts to investigate mechanism of phase separation in aqueous polymer–salt mixtures based on thermodynamic analysis of experimental data [18,19] can hardly be viewed as successful and any attempt at considering phase separation in aqueous solution based on deviation from ideality is doomed. This issue, however, is beyond the scope of the present work.

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One of the most interesting aspects of solutes behavior in PEG–salt ATPS is that relatively small amounts of salt additives may significantly affect the solute partitioning [20–22]. The most widely used salt additive in this ATPS is NaCl [20–22]. The data obtained in the studies of protein partition behavior, however, typically leave too much room for interpretation being assigned to the protein–ion specific interactions, protein conformational changes and other often hard to prove (and disprove) possibilities. The data obtained for structurally simple organic compounds generally cannot be interpreted as ambiguously and hence may provide better insight into mechanism of the effect under study.

Recently, effects of different salt additives (NaCl, NaSCN, NaClO₄, NaH₂PO₄) over a wide concentration range up to ca. 2 M on partition behavior of small organic compounds in PEG-8000–Na₂SO₄ were reported [23]. Later we examined the effect of NaCl in the concentration range of 0–1.9 M on the phase diagram of this ATPS and characterized solvent properties of the phases [24].

We extended this study here and examined the differences between the relative hydrophobicities and electrostatic properties of the phases in PEG-8000–Na₂SO₄ ATPS with additives of NaSCN, NaClO₄, and NaH₂PO₄. As reported previously [23], adenosine and guanosine displayed complicated partition behavior in PEG-8000–Na₂SO₄ ATPS with increasing concentration of NaSCN, NaClO₄, and NaH₂PO₄ additives. Their partition coefficients increased with salt additive concentration increasing up to ~0.2 M and decreased with further increasing salt concentration. Therefore we also examined here partitioning of adenine and adenosine mono-, di-, and tri-phosphates in all the ATPSs, including those with NaCl additive. We attempted to answer the question of how relatively small amounts of the above salt additives may affect solute partitioning in ATPS formed by exceeding amount of sodium sulfate and PEG.

2. Experimental

2.1. Materials

Polyethylene glycol-8000 (Lot 048K00241) with an average molecular weight (M_w) of 8000 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Adenine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) were purchased from Sigma-Aldrich. Dinitrophenylated (DNP) amino acids—DNP-alanine, DNP-norvaline, DNP-norleucine, and DNP- α -amino-*n*-octanoic acid, were purchased from Sigma-Aldrich. The sodium salts of the DNP-amino acids were prepared by titration.

All salts and other chemicals used were of analytical-reagent grade.

2.2. Methods

2.2.1. Aqueous two-phase systems

Stock solutions of PEG 8000 (50 wt%) and Na₂SO₄ (20.3 wt%) were prepared in water. Sodium phosphate buffer (NaPB; 0.5 M, pH 6.8) was prepared by mixing 3.45 g of NaH₂PO₄·H₂O and 6.70 g Na₂HPO₄·7H₂O in 100 ml aqueous solution. A mixture of PEG and salts was prepared by dispensing appropriate amounts of the aqueous stock PEG-8000, Na₂SO₄ and NaPB solutions into a 1.2 ml microtube using a Hamilton (Reno, NV, USA) ML-4000 four-probe liquid handling workstation. Appropriate amounts of water and/or stock salt additives solutions were added to give the required ionic and polymer composition of the final system with total weight of 0.5 g (after addition of the solute sample, see below). All aqueous two-phase systems had a fixed composition of 11.10 wt% PEG-8000, 6.33 wt% Na₂SO₄ and 0.01 M NaPB, pH 6.8, with different salt

additive concentrations as indicated below. Stock solutions of each salt additive (NaCl, NaClO₄, NaSCN or NaH₂PO₄) of 0.5 or 5.0 M concentration were prepared and appropriated amounts were added to the two-phase systems to provide the required concentrations from 0.027 M up to ca. 0.5 M.

2.2.2. Partitioning experiments

The aqueous two-phase partitioning experiments were performed using an automated instrument, Automated Signature Workstation, ASW (Analiza, Cleveland, OH, USA). The ASW system is based on the ML-4000 liquid-handling workstation (Hamilton Company) integrated with a UV–VIS microplate spectrophotometer (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA, USA). Solutions of all compounds were prepared in water at concentrations of 2–100 mM, depending on the compound solubility. Varied amounts (0, 20, 40, 60, 80, and 100 μ l) of a given compound solution and the corresponding amounts (100, 80, 60, 40, 20, and 0 μ l) of water were added to a set of the same polymer/salts mixtures. The systems were vortexed in a multi-pulse vortexer and centrifuged for 60 min at 3000 $\times$ g at 23 °C temperature in a refrigerated centrifuge (Jouan, BR4i) to accelerate phase settling. The upper phase in each system was partially removed, the interface discarded, and aliquots of 15–75 μ l from the upper and lower phases were withdrawn in duplicate for analysis. The aliquots were transferred into microplate wells and diluted up to 300 μ l. In the cases of considerable difference between the concentrations of a given compound in one phase relative to the other phase, different dilution factors were used for the upper and lower phases. Water was used as a diluent for all compounds. The microplate was sealed, and following moderate shaking for 30 min in an incubator (Vortemp 56EVC, Labnet International, Edison, NJ, USA) at room temperature and short centrifugation (3 min at 1500 rpm), optical absorbance was measured at the wavelength of maximum absorption with the UV–VIS plate reader. The maximum absorption wavelength for each compound was determined in separate experiments by analysis of the absorption spectrum over the 250–500 nm range. In all measurements the correspondingly diluted pure phases were used as blank solutions.

The partition coefficient, K , defined as the ratio of the sample concentration in the upper phase to the sample concentration in the lower phase was determined as the slope of the compound concentration in the upper phase plotted as a function of the concentration in the lower phase averaged over the results obtained from two to four partition experiments carried out at the specified polymer and salt composition of the system, taking into consideration the corresponding dilution factors used in the experiment. Deviation from the average K -value was consistently below 3% and in most cases lower than 2%.

3. Results and discussion

In order to characterize the differences between the relative hydrophobicities and electrostatic properties of the phases in the ATPSs with varied concentrations of salt additives, the partition coefficients of the homologous series of sodium salts of DNP-amino acids with aliphatic side-chains (alanine, norvaline, norleucine, and α -amino-*n*-caprylic acid) in each ATPS were analyzed as follows. Typical experimental data obtained for sodium salts of the DNP-amino acids are plotted in Fig. 1, and the linear curves observed may be described as:

$$\ln K_{\text{DNP-AA}}^{(i)} = C^{(i)} + E^{(i)}N_C \quad (1)$$

where $K_{\text{DNP-AA}}$ is the partition coefficient of a DNP-amino acid with aliphatic side-chain; superscript (i) denotes the particular i -th ATPS used for the partition experiments; N_C is equivalent number of CH₂ groups in the aliphatic side-chain of a given DNP-amino acid; E is

Fig. 1. Partition coefficients of sodium salts of DNP-amino acids with aliphatic side-chains as functions of side-chain lengths in aqueous PEG-8000–Na₂SO₄ two-phase system with indicated concentration of salt additive (pH 6.8; temperature 23 °C).

an average $\ln K$ increment per CH₂ group; C represents the total contribution of the non-alkyl part of the structure of a DNP-amino acid into $\ln K_{\text{DNP-AA}}$.

The coefficients $E^{(i)}$ and $C^{(i)}$ values determined for all the ATPSs examined are listed in Table 1. As the standard free energy of transfer of a solute between the coexisting phases is described as:

$$\Delta G^0 = -RT \ln K \quad (2)$$

where R is the universal gas constant and T is the absolute temperature in Kelvin, it follows that

$$\Delta G^0(\text{CH}_2) = -RTE \quad (3)$$

where $\Delta G^0(\text{CH}_2)$ is the standard free energy of transfer of a methylene group from one phase to another. The $\Delta G^0(\text{CH}_2)$ values calculated from the experimental data with Eqs. (1)–(3) are listed in Table 1.

Examination of the parameter E or $\Delta G^0(\text{CH}_2)$ values listed in Table 1, and plotted in Fig. 2, indicates that the difference between the relative hydrophobicity of the phases increases with increasing concentration of each salt additive. The difference between the electrostatic properties of the phases represented by parameter C value in Eq. (1) (see in Table 1) also changes with increasing

Fig. 2. The standard free energy of transfer of a CH₂ group between the coexisting phases, $\Delta G^0(\text{CH}_2)$, as function of the salt additive concentration. Lines are added for eye-guidance only.

Fig. 3. The difference between the electrostatic properties of the phases, parameter C , as function of the salt additive concentration. Lines are added for eye-guidance only.

concentration of each salt additive in a salt-specific manner as shown in Fig. 3.

It was suggested by Zaslavsky [1], pp. 208–216, to use the contribution of an ionic group into the solute partition coefficient as an empirical measure of the difference in question. The experimental results here were obtained with sodium salts of *p*-dinitrophenyl-amino acids, i.e. compounds possessing a DNP–NH–CH–COO[−]–Na⁺ group. This moiety is bulky and contains a substituted aromatic ring. Use of this particular group as a probe for electrostatic ion–ion, ion–dipole and dipole–dipole interactions obviously has some limitations. Only to a first approximation the free energy of transfer of this group between the coexisting phases of an ATPS may be viewed as a measure of the ability of aqueous media to participate in a particular kind of intermolecular interactions. The difference between the electrostatic properties of the two phases characterized by parameter C value is governed mostly by the difference between the ionic composition of the phases.

As shown in Fig. 3, in the presence of NaCl additive the parameter C value decreases gradually with increasing NaCl concentration except at the NaCl concentration of ~0.1 M where parameter C -value increases. It might be attributed to the fact that the phase diagrams for the ATPSs with 0–0.05 M NaCl additive are extremely close [23], their phase compositions are very similar, and that the first noticeable shift of the phase diagram is observed at NaCl concentration of ~0.1 M. The data presented in Fig. 3 show that the difference between the electrostatic properties of the phases increases significantly with increasing concentration of NaH₂PO₄ additive likely because this salt is immiscible with PEG and in the PEG–sulfate ATPS it concentrates in the bottom sulfate-rich phase. NaSCN and NaClO₄ additives in contrast to NaH₂PO₄ are likely to distribute predominantly in the upper PEG-rich phase [1] and that is why the parameter C value gradually decreases with increasing NaSCN and NaClO₄ concentrations. It seems that the salt additive effect changes according to the anion position in Hofmeister series: H₂PO₄[−] > Cl[−] > ClO₄[−] ≅ SCN[−].

Partition coefficients for adenine, AMP, ADP, and ATP, in all ATPSs are listed in Table 2. It was reported recently [25] that partition coefficients of solutes in PEG–salt ATPSs with and without salt additives may be compared based on the so-called Collander linear solvent regression relationship [26–30]:

$$\ln K_{\text{PEG-Sulfate-Salt}}^i = a_{io} + b_{io} \ln K_{\text{PEG-Sulfate}}^i \quad (4)$$

where $K_{\text{PEG-Sulfate-Salt}}^i$ is the partition coefficient of the i -th compound in PEG–sulfate ATPS with a given salt additive; $K_{\text{PEG-Sulfate}}^i$ is

Table 1
Coefficients E and C (see Eq. (1) in text) and the free energy of transfer of a CH_2 group between the coexisting phases of PEG– Na_2SO_4 ATPS of a fixed composition with indicated concentration of salt additives.

NaCl^a (M)	E^a	C^a	$\pm\Delta G(\text{CH}_2)^a$ (cal mol $^{-1}$)	NaSCN (M)	E	C	$\pm\Delta G(\text{CH}_2)$ (cal mol $^{-1}$)
0	0.207 ± 0.005	1.12 ± 0.02	122 ± 2.9	0	0.207 ± 0.005	1.12 ± 0.02	122 ± 2.9
0.027	0.214 ± 0.006	1.04 ± 0.02	126 ± 3.5	0.025	0.209 ± 0.004	1.10 ± 0.02	123 ± 2.4
0.053	0.223 ± 0.005	1.02 ± 0.02	131 ± 2.9	0.050	0.23 ± 0.011	1.05 ± 0.04	135 ± 6.5
0.107	0.230 ± 0.002	1.086 ± 0.008	135 ± 1.2	0.100	0.246 ± 0.005	1.01 ± 0.02	145 ± 2.9
0.215	0.259 ± 0.001	1.002 ± 0.005	152 ± 0.6	0.215	0.26 ± 0.01	0.81 ± 0.04	153 ± 5.9
0.543	0.284 ± 0.002	0.983 ± 0.008	167 ± 1.2	0.543	0.263 ± 0.003	0.59 ± 0.01	155 ± 1.8
NaClO_4 (M)	E	C	$\pm\Delta G(\text{CH}_2)$ (cal mol $^{-1}$)	NaH_2PO_4 (M)	E	C	$\pm\Delta G(\text{CH}_2)$ (cal mol $^{-1}$)
0	0.207 ± 0.005	1.12 ± 0.02	122 ± 2.9	0	0.207 ± 0.005	1.12 ± 0.02	122 ± 2.9
0.027	0.219 ± 0.002	1.175 ± 0.007	129 ± 1.2	0.027	0.22 ± 0.01	1.06 ± 0.04	131 ± 5.9
0.054	0.228 ± 0.005	1.19 ± 0.02	134 ± 2.9	0.054	0.230 ± 0.008	1.17 ± 0.03	135 ± 4.7
0.108	0.250 ± 0.010	1.03 ± 0.03	147 ± 5.9	0.108	0.249 ± 0.005	1.28 ± 0.02	147 ± 2.9
0.216	0.263 ± 0.005	0.91 ± 0.02	155 ± 2.9	0.217	0.276 ± 0.002	1.562 ± 0.009	162 ± 1.2
0.549	0.300 ± 0.001	0.706 ± 0.005	177 ± 0.6	0.556	0.42 ± 0.01	1.93 ± 0.05	247 ± 5.9

^a Data for the NaCl additive were previously reported [24] and are presented here for comparison.

the partition coefficient of the same i -th compound in PEG–sulfate ATPS without salt additive; and a_{i0} and b_{i0} are constants depending on the ATPSs under comparison. The Collander equation [26–30] describes a linear correlation between distribution coefficients of solutes of the similar chemical nature in different organic solvent–water biphasic systems. The coefficients of the relationship (slope and intercept) depend on the particular systems under comparison as well as on the type of the solutes being examined. It was suggested that these coefficients represent the distinctive features of the interactions of the solute functional moieties with the solvents being compared [1]. It has been shown that the Collander relationship may be used for comparison of partition coefficients for solutes in PEG– Na_2SO_4 ATPSs with NaCl additives at different concentrations [24].

Typical examples of the observed relationships described by Eq. (4) are presented in Fig. 4. The a_{i0} and b_{i0} coefficients values and the corresponding correlation coefficients for compounds examined here and previously reported [23,24] are listed in Table 3. These data indicate that: (i) the relationships for nonionic and charged compounds are shifted relatively to each other but are essentially parallel (have the same b_{i0} coefficients), and (ii) the data scatter

Fig. 4. Logarithms of partition coefficients for ionic and nonionic compounds in aqueous PEG–8000– Na_2SO_4 two-phase system with 0.108 M NaSCN versus logarithms of partition coefficients for same compounds in aqueous PEG–8000– Na_2SO_4 two-phase system without salt additive (reference system).

Table 2
Partition coefficients of compounds in 11.10 wt% PEG–6.33 wt% Na_2SO_4 –0.01 M NaPB, pH 6.8 ATPS with indicated concentration of salt additives.

NaCl (M)	Adenine	AMP	ADP	ATP
0	3.4 ± 0.12	1.01 ± 0.05	0.75 ± 0.02	0.62 ± 0.04
0.027	3.2 ± 0.12	0.97 ± 0.06	0.70 ± 0.02	0.57 ± 0.03
0.053	2.95 ± 0.08	0.95 ± 0.06	0.57 ± 0.02	0.52 ± 0.02
0.107	2.8 ± 0.11	0.88 ± 0.04	0.60 ± 0.01	0.46 ± 0.02
0.215	3.7 ± 0.16	0.83 ± 0.04	0.54 ± 0.03	0.40 ± 0.02
0.543	4.1 ± 0.15	0.72 ± 0.02	0.43 ± 0.03	0.30 ± 0.02
NaClO_4 (M)				
0.027	3.29 ± 0.05	0.91 ± 0.01	0.63 ± 0.01	0.46 ± 0.01
0.054	3.57 ± 0.07	0.80 ± 0.03	0.53 ± 0.01	0.37 ± 0.02
0.108	3.78 ± 0.07	0.65 ± 0.02	0.40 ± 0.01	0.26 ± 0.02
0.216	4.1 ± 0.2	0.50 ± 0.01	0.296 ± 0.009	0.178 ± 0.007
0.549	4.1 ± 0.1	0.335 ± 0.008	0.19 ± 0.01	0.109 ± 0.009
NaSCN (M)				
0.025	3.27 ± 0.04	0.9 ± 0.01	0.637 ± 0.007	0.468 ± 0.008
0.050	3.52 ± 0.01	0.8 ± 0.01	0.540 ± 0.003	0.388 ± 0.005
0.100	3.7 ± 0.2	0.678 ± 0.009	0.431 ± 0.004	0.29 ± 0.02
0.215	3.75 ± 0.07	0.521 ± 0.005	0.316 ± 0.006	0.210 ± 0.008
0.543	3.51 ± 0.05	0.405 ± 0.008	0.253 ± 0.003	0.164 ± 0.007
NaH_2PO_4 (M)				
0.027	2.90 ± 0.02	1.28 ± 0.02	0.89 ± 0.02	0.640 ± 0.006
0.054	2.80 ± 0.03	1.38 ± 0.02	0.89 ± 0.01	0.63 ± 0.01
0.108	2.61 ± 0.04	1.41 ± 0.03	0.86 ± 0.01	0.570 ± 0.005
0.217	2.18 ± 0.09	1.26 ± 0.02	0.741 ± 0.006	0.410 ± 0.006
0.556	1.50 ± 0.04	0.88 ± 0.03	0.406 ± 0.008	0.180 ± 0.002

Table 3

Coefficients a_{io} and b_{io} in solvent regression equation (Eq. (4)) with PEG–sodium sulfate ATPS without salt additive used as a reference system.

[Salt], M	Compounds	a_{io}	b_{io}	R^2
NaCl, 0.027	Nonionic	-0.02 ± 0.02	1.05 ± 0.01	0.9981
	Charged	-0.06 ± 0.01	1.04 ± 0.01	0.9991
NaCl, 0.053	Nonionic	-0.04 ± 0.01	1.09 ± 0.01	0.9989
	Charged	-0.15 ± 0.03	1.11 ± 0.02	0.9966
NaCl, 0.107	Nonionic	-0.07 ± 0.03	1.17 ± 0.03	0.9942
	Charged	-0.18 ± 0.02	1.17 ± 0.01	0.9991
NaCl, 0.215	Nonionic	-0.04 ± 0.02	1.25 ± 0.01	0.9987
	Charged	-0.27 ± 0.02	1.24 ± 0.02	0.9983
NaCl, 0.543	Nonionic	-0.03 ± 0.02	1.38 ± 0.02	0.9979
	Charged	-0.46 ± 0.03	1.38 ± 0.02	0.9982
NaClO ₄ , 0.027	Nonionic	-0.02 ± 0.02	1.14 ± 0.01	0.9984
	Charged	-0.15 ± 0.01	1.18 ± 0.01	0.9990
NaClO ₄ , 0.054	Nonionic	-0.04 ± 0.01	1.09 ± 0.01	0.9956
	Charged	-0.07 ± 0.03	1.16 ± 0.02	0.9984
NaClO ₄ , 0.108	Nonionic	-0.12 ± 0.05	1.41 ± 0.04	0.9888
	Charged	-0.55 ± 0.03	1.42 ± 0.02	0.9983
NaClO ₄ , 0.215	Nonionic	-0.14 ± 0.07	1.52 ± 0.06	0.9784
	Charged	-0.83 ± 0.04	1.50 ± 0.03	0.9968
NaClO ₄ , 0.549	Nonionic	-0.3 ± 0.1	1.73 ± 0.09	0.9609
	Charged	-1.23 ± 0.04	1.68 ± 0.03	0.9973
NaSCN, 0.025	Nonionic	-0.04 ± 0.02	1.14 ± 0.01	0.9984
	Charged	-0.16 ± 0.02	1.12 ± 0.01	0.9990
NaSCN, 0.050	Nonionic	-0.05 ± 0.03	1.22 ± 0.02	0.9968
	Charged	-0.29 ± 0.02	1.22 ± 0.02	0.9984
NaSCN, 0.100	Nonionic	-0.05 ± 0.04	1.30 ± 0.03	0.9926
	Charged	-0.50 ± 0.02	1.33 ± 0.02	0.9987
NaSCN, 0.215	Nonionic	-0.12 ± 0.05	1.43 ± 0.04	0.9991
	Charged	-0.77 ± 0.03	1.40 ± 0.03	0.9971
NaSCN, 0.543	Nonionic	-0.17 ± 0.08	1.50 ± 0.07	0.9698
	Charged	-1.01 ± 0.03	1.42 ± 0.02	0.9983
NaH ₂ PO ₄ , 0.027	Nonionic	-0.02 ± 0.03	1.07 ± 0.03	0.9938
	Charged	0.11 ± 0.04	0.97 ± 0.03	0.9941
NaH ₂ PO ₄ , 0.054	Nonionic	-0.04 ± 0.02	1.13 ± 0.02	0.9839
	Charged	-0.12 ± 0.02	1.17 ± 0.01	0.9952
NaH ₂ PO ₄ , 0.108	Nonionic	-0.04 ± 0.09	1.20 ± 0.07	0.9458
	Charged	0.07 ± 0.03	1.17 ± 0.02	0.9978
NaH ₂ PO ₄ , 0.217	Nonionic	0.06 ± 0.04	1.35 ± 0.03	0.9942
	Charged	0.01 ± 0.05	1.42 ± 0.04	0.9947
NaH ₂ PO ₄ , 0.556	Nonionic	-0.07 ± 0.05	2.00 ± 0.04	0.9920
	Charged	-0.16 ± 0.01	1.97 ± 0.01	0.9997

R^2 —Regression coefficient.

increases (correlation coefficients decrease) with increasing salt additive concentration.

The coefficients b_{io} obtained for salt additives studied are plotted in Fig. 5 as functions of the salt additives concentrations. It should be noted particularly that the NaH₂PO₄ additive affects the

Fig. 5. Coefficients b_{io} in solvent regression equation (Eq. (4), PEG–sodium sulfate ATPS without salt additive used as a reference system) as functions of the salt additive concentration. Lines are added for eye-guidance only.

Table 4

Coefficients α^{salt} and β^{salt} values (Eq. (5)) and the structural entropy of hydration [31] of anions of Na salts additives.

Anion	α^{salt}	β^{salt}	$\Delta_{\text{struc}}S$ (J K ⁻¹ mol ⁻¹)
ClO ₄ ⁻	0.260 ± 0.009	0.122 ± 0.009	107
SCN ⁻	0.225 ± 0.004	0.107 ± 0.003	83
Cl ⁻	0.163 ± 0.002	0.098 ± 0.002	58
H ₂ PO ₄ ⁻	0.159 ± 0.003	0.083 ± 0.002	-4

coefficient b_{io} much more significantly (and linearly) than the other salts additives examined. The reason seems to be that this salt is the only one among those studied that is immiscible with PEG and concentrates completely in the sulfate-rich phase. Other salts distribute predominantly into PEG-rich phase (NaSCN, NaClO₄) or in both phases (NaCl) depending on the salt additive concentration. The salt additives concentrations dependences of the b_{io} coefficients shown in Fig. 5 may be described as:

$$\log b_{io}^{\text{salt}} = \alpha^{\text{salt}} + \beta^{\text{salt}} \log[\text{salt}] \quad (5)$$

where [salt] is the salt additive concentration in PEG–sulfate ATPS, expressed in mole/kg; b_{io}^{salt} is the b_{io} coefficient for a given salt additive concentration; α^{salt} and β^{salt} are constants. The α^{salt} and β^{salt} coefficients values are listed in Table 4. The relationships under discussion are presented in Fig. 6. It should be mentioned that the concentration range for each salt additive used was up to ~0.5 M. For NaH₂PO₄ additive the b_{io} coefficients determined for nonionic solutes differ significantly from those for charged compounds, and only the former were used, as partition behavior of charged compounds in the ATPS PEG–Na₂SO₄–NaH₂PO₄ with both salts in the bottom phase only may have more complicated mechanism of partitioning. Averaged b_{io} coefficients determined for nonionic and charged compounds were used for ATPS with other salts additives.

It was suggested previously [1] that phase separation and partitioning of solutes in ATPS is governed by different effects of phase-forming components on the water structure. Hence, it seems reasonable to compare the effects of salts additives (or their anions) on the solute partitioning observed here and represented by the α^{salt} and β^{salt} coefficients with the effects of these anions on the water structure. If we define the structure of water according to Marcus [11], as the extent of hydrogen bonding, the effect of ions on the structure means the enhancement or diminution of this extent. These effects may be quantified by the structural entropies of hydration of ions as discussed by Marcus [11,31]. These entropies values, $\Delta_{\text{struc}}S$, taken from [31] are listed in Table 4 for the anions of the Na salts additives explored. The plots of α^{salt} and β^{salt} coefficients versus $\Delta_{\text{struc}}S$ are presented in Fig. 7a and b,

Fig. 6. Salt additive concentration dependence of the b_{io}^{salt} coefficients (Eq. (5)).

Fig. 7. Coefficients (a) α^{salt} and (b) β^{salt} values (Eq. (6)) as a function of the structural entropy of hydration [31] of anions of Na salts additives. In (a) line is for eye-guidance only.

respectively. The relationship observed for coefficient β^{salt} in Fig. 7b may be described as:

$$\beta^{\text{salt}} = -0.073 \pm 0.004 + 0.011 \pm 0.003 \times \exp\left(\frac{\Delta_{\text{struc}}S}{71 \pm 11}\right) \quad (6)$$

$$N = 4; \quad R^2 = 0.9988; \quad SE = 0.001; \quad F = 406$$

where β^{salt} and $\Delta_{\text{struc}}S$ are defined above; N —number of salt additives examined; R^2 is the correlation coefficient; SD is the standard deviation; and F is the ratio of variance. The relationship observed for α^{salt} coefficient may be partially fitted by power function as shown in Fig. 7a. The α^{salt} and β^{salt} coefficients in Eq. (5) characterize the effect of salt additive concentration on partition coefficients of different small organic compounds in the PEG–Na₂SO₄–salt additive ATPSs. The observed relationships for both α^{salt} and β^{salt} coefficients suggest that the effects of the salt additives on solute partitioning in the PEG–Na₂SO₄ ATPS originate from their effects on the water structure in the phases of ATPS. This finding agrees with the data reported previously [32] about effects of different salt additives on the solvent properties of aqueous media in different ATPSs formed by two nonionic polymers. It was shown in [32] that the difference between the electrostatic properties of the coexisting phases may be described only taking into account the effect

of a given salt additive on the hydrogen bonds in water. This effect was represented by a parameter (K_{b-1}), characterizing the equilibrium between populations of hydrogen bonds with a bent hydrogen bond conformation and with linear hydrogen bond conformation affected by a given salt additive influence on the hydrogen bonds network in water [17]. The fact that similar effects are displayed with the salt additive concentration of ca. 0.025–0.1 M on the background of ~ 1 M Na₂SO₄ in the lower phase is rather surprising. The limited number of salts additives examined here prevents any general conclusion. Further work is necessary and is currently in progress in our laboratories.

Acknowledgment

This work was financed by Fundação para a Ciência e a Tecnologia (FCT) of the Portuguese's Ministry for Science, Technology and Higher Education, in the framework of the Operational Programme COMPETE (PTDC/EQU-FTT/120332/2010), for which the authors are thankful. Nuno R. da Silva acknowledges the scholarship within this Project.

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