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Phase equilibria, solvent properties, and protein partitioning in aqueous polyethylene glycol-600-trimethylamine N-oxide and polyethylene glycol-600-choline chloride two-phase systems

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ABSTRACT

The phase diagram of a new aqueous two-phase system (ATPS) formed by polyethylene glycol with molecular weight 600 (PEG-600) and trimethylamine N-oxide (TMAO) in 0.01 M sodium phosphate buffer (NaPB), pH 7.4, is determined and hydrophobic, electrostatic and other solvent properties of the phases are characterized. The same properties are determined for the ATPS formed by PEG-600 and choline chloride in 0.01 M sodium phosphate buffer (NaPB), pH 7.4. Solvent properties of water (dipolarity/polarizability, hydrogen bond donor acidity, and hydrogen bond acceptor basicity) in aqueous solutions of polypropylene glycol-400 (PPG-400), polyethylene glycol dimethyl ether -250 (PEGDME-250), and choline chloride are determined at different concentrations. The concentrations of the aforementioned polymers, as well as PEG-600 and PEG-1000 required for phase separation in mixtures with choline chloride reported in the literature are analyzed. It is found that the concentrations of polymers needed for phase separation in mixtures with 35%wt. choline chloride are linearly related with water hydrogen bond donor acidity or hydrogen bond acceptor basicity in the individual polymer solutions at given concentrations. Partition behavior of nine proteins was examined in both systems. The partition coefficients of proteins in PEG-600-choline chloride ATPS exceeded those observed in PEG-600-TMAO ATPS from ca. 2 to ca. 75-fold possibly due to the larger difference between the composition of the coexisting phases in the former ATPS. Analysis of partition coefficients in the two ATPS were compared to those reported in Dextran-PEG ATPS, and proteins likely engaged in direct interactions with choline chloride were identified.

1. Introduction

Aqueous two-phase systems (ATPSs) commonly used in separation of biological products [1] and in analytical applications [2] are typically formed in aqueous mixtures of two polymers, a single polymer and salt [3,4], or two salts [5]. The ATPS formed by a single polymer and organic compound, such as a carbohydrate [6] or surfactant [7,8], have also been reported, but not well studied. Although properties of ATPSs formed by two polymers or by a single polymer and salt have been examined relatively well [2,4], those of ATPSs formed by a single polymer and an organic compound are not characterized in any detail.

It has been reported [9–14] previously that partition behavior of any solute from small organic compounds to proteins in aqueous two-phase systems formed by two polymers or a single polymer and inorganic salt is driven by differences between solute-solvent interactions in the two coexisting phases. The solvent properties of aqueous media governing partition in ATPS include solvent dipolarity/polarizability, π^*, representing ability of water to participate in dipole-dipole and dipole-induced dipole interactions, solvent hydrogen bond donor acidity, α, hydrogen bond acceptor basicity, β, and electrostatic properties including ion-ion and ion-dipole interactions. These properties of aqueous media in the phases of ATPS are altered relative to those in water as the result of effects of phase forming polymers and salts present in the phases. The partition coefficient of a solute (including proteins) may be described as [9–13]:

$$logK_i = S_s\Delta\pi*_i + B_s\Delta\alpha_i + A_s\Delta\beta_i + C_sc_i \quad (1)$$

where K is the solute partition coefficient; $\Delta\pi^*$ is the difference between the solvent dipolarity/polarizability of the two phases, $\Delta\alpha$ is the difference between the solvent HBD acidity of the two phases,

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$\Delta\beta$ is the difference between the solvent HBA basicity of the two phases; c is the difference between the electrostatic properties of the two phases; S_s, A_s, B_s, and C_s are constants (solute specific coefficients) quantifying the complementary interactions of the solute with the solvent media in the coexisting phases and representing the relative contributions of these interactions into partition coefficient of the solute; the subscript s designates the solute; the subscript i denotes the ATPS used; the difference for each solvent property is determined as the one between the upper and lower phases.

The solute specific coefficients may be determined for a given compound (including proteins) by the analysis of partition coefficients of this compound in multiple ATPSs with different polymer but same ionic composition with established solvent properties of the phases. Once $\Delta\pi^*$, $\Delta\alpha$, $\Delta\beta$, and c parameters in 5–10 different ATPSs are determined, the solute specific coefficients can be calculated by multiple linear regression analysis using Eq. (1). It was shown [13] also that the partition coefficient of a compound with pre-determined solute specific coefficients in a "new" ATPS with established solvent properties of the phases could be predicted with 90–95% accuracy.

We have reported [14] recently that protein partition behavior may be manipulated in polymer-polymer ATPS by addition of trimethylamine N-oxide (TMAO) at relatively high concentrations (up to ~2 M). It has been observed that TMAO at high concentrations (>ca.1.5 M) precipitates from aqueous solution of polyethylene glycol with an average molecular weight of 8000 (PEG-8000), while it is able to induce phase separation in aqueous mixtures with PEG of an average molecular weight 600 (PEG-600). Characterization of the PEG-600-TMAO ATPS, analysis of its properties and partition behavior of several proteins was the purpose of this study. The similar properties were also studied for the already described [15–21] system formed in aqueous mixtures of PEG-600 and choline chloride. In addition, we examined for the first time here how solvent properties of water in solutions of individual polymers relate to the polymers concentrations required for phase separation in aqueous mixtures with a fixed amount of choline chloride.

2. Experimental

2.1. Materials

2.1.1. Polymers, organic compounds, and other chemicals

Polyethylene glycol PEG-600 (Lot 47171728) with an average molecular weight (M_n) of 600 was purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA). Choline chloride (99% purity) and polyethylene glycol dimethyl ether (PEGDME) with molecular weight of ca. 250 was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Polypropylene glycol (PPG) with molecular weight 400, trimethylamine N-oxide (TMAO) and o-phthaldehyde (OPA) reagent solution (complete) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dinitrophenylated (DNP-) amino acids – DNP-glycine, 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 and were used without further purification.

2.1.2. Proteins

α-Chymotrypsin, α-chymotrypsinogen A, ribonuclease A, ribonuclease B, and trypsinogen from bovine pancreas, concanavalin A from *Canavalia ensiformis* (jack beans), β-lactoglobulin A from bovine milk (>90%), β-lactoglobulin B from bovine milk (>90%) were purchased from Sigma–Aldrich. Lysozyme (salt free) from chicken egg white was obtained from Worthington Biochemical Corp. (Lakewood, NJ, USA). Purity of all proteins was verified by electrophoresis.

2.2. Methods

2.2.1. Phase diagrams

Binodals of the PEG-600-TMAO-0.01 M sodium phosphate buffer (NaPB), pH 7.4 and PEG-600-choline chloride-0.01 M NaPB, pH 7.4 were determined by turbidimetric titration using 90%wt. stock solution of PEG-600, 37.24%wt. stock solution of TMAO, 70.0%wt. stock solution of choline chloride, and 0.5 M stock solution of sodium phosphate buffer (NaPB), pH 7.4. Stock sodium phosphate buffer (NaPB; 0.5 M, pH 7.4) was prepared by mixing appropriate amounts of Na_2HPO_4 and NaH_2PO_4. All the stock solutions were prepared in deionized (DI) water. The PEG-600-TMAO and PEG-600-choline chloride two-phase systems with varied ratio of polymer and TMAO or choline chloride were prepared with 0.01 M NaPB, pH 7.4 and titrated dropwise, with 0.01 M NaPB, pH 7.4 until one-phase homogeneous systems were formed.

2.2.2. Aqueous two-phase systems (ATPSs)

ATPSs were prepared as described elsewhere [14]. Appropriate amounts of polymer, TMAO or choline chloride and NaPB, pH 7.4, stock solutions were added to the systems. Each ATPS had total weight of 0.5 g (total volume 472 ± 2 μL for PEG-600-TMAO ATPS and 453 ± 2 μL for PEG-600-choline chloride ATPS). The PEG-TMAO two phase systems used had the same compositions of 37.8 wt.% PEG-600, 18.6 wt.% TMAO and same ionic composition of 0.01 M NaPB, pH 7.4. The PEG-600-choline chloride ATPSs used had the same composition of 47.0 wt.% PEG-600 and 30.0 wt.% choline chloride and the same ionic composition of 0.01 M NaPB, pH 7.4.

The systems of the above compositions but of 4.0 g total weight were prepared separately in order to estimate the volumes and the densities of the phases.

2.2.3. Partitioning

Partitioning experiments in the aqueous two-phase systems explored were performed as described in detail in Supplementary Information (S1).

The partition coefficient (K) value for each solute was determined as the slope of the concentration (fluorescence intensity for proteins or UV absorbance for dinitrophenylated amino acids) in the top phase plotted as a function of the concentration in the bottom phase averaged over the results obtained from two to four partition experiments carried out at the specified composition of the system. The deviation from the average K value was always less than 3% and in most cases lower than 1%.

2.2.4. Solvatochromic studies

The solvatochromic probes 4-nitroanisole, 4-nitrophenol, and Reichardt's carboxylated betaine dye were used to determine the solvent dipolarity/polarizability π^*, H-bond acceptor (HBA) basicity β, and H-bond donor (HBD) acidity α of the media in the separated phases of the ATPS, and individual solutions of choline chloride, polyethylene glycol dimethyl ether, and polypropylene glycol in water as described previously [22]. The detailed description of the solvatochromic analysis is presented in Supplementary Information (S2).

3. Results and discussion

3.1. Phase diagrams

The phase diagrams determined for the two ATPS examined are presented in Fig. 1a. It should be noted in particular that the PEG-

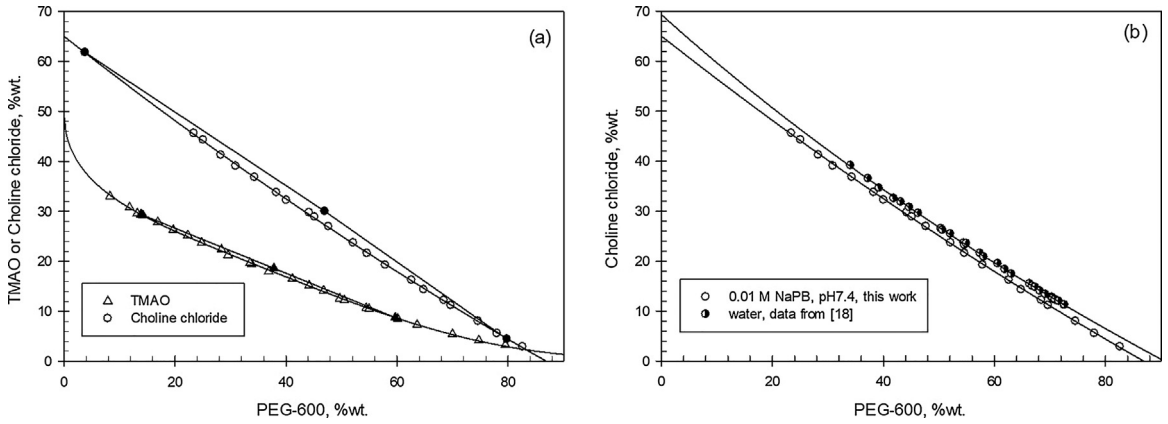

Fig. 1. **(a)** Phase diagrams of PEG-600-TMAO and PEG-600-Choline chloride aqueous two-phase systems both containing 0.01 M sodium phosphate buffer, pH 7.4. **(b)** Phase diagrams of PEG-600-Choline chloride aqueous two-phase systems in water [18] and in 0.01 M sodium phosphate buffer, pH 7.4.

Table 1
Differences between the hydrophobic and electrostatic properties of the phases in PEG-600 (37.8%wt.)- TMAO (18.6%wt.)-0.01 M NaPB, pH 7.4 and PEG-600 (47.0%wt.) – choline chloride (30.0%wt.) – 0.01 M NaPB, pH 7.4 ATPS.[a]

	PEG-TMAO	PEG-choline chloride
[PEG] in top phase	13.9%wt.	3.85%wt.
[PEG] in bottom phase	59.69%wt.	79.82%wt.
TMAO/choline chloride in top phase	29.37%wt.	61.78%wt.
TMAO/choline chloride in bottom phase	8.74%wt.	4.43%wt.
E[b]	-0.088 ± 0.0035	-0.177 ± 0.0008
$\Delta G(CH_2)$[b], cal/mole	-120 ± 4.7	-240 ± 1.2
C[c]	-0.568 ± 0.012	-0.678 ± 0.003
$\Delta\pi^*$[d]	0.022 ± 0.001	0.100 ± 0.002
$\Delta\beta$[d]	Nd[e]	-0.071 ± 0.002

[a] NaPB – 0.01 M sodium phosphate buffer, pH 7.4.
[b] Parameters E and $\Delta G(CH_2)$ values characterize the difference between the relative hydrophobicities of the coexisting phases of a given ATPS.
[c] parameter C value characterizes the difference between the electrostatic properties of the phases (for explanation see text).
[d] $\Delta\pi^*$ – difference between the solvent dipolarity/polarizability of aqueous media in the coexisting phases; $\Delta\beta$ – difference between the solvent hydrogen bonding acceptor basicity of aqueous media in the coexisting phases.
[e] Nd – not determined (see in the text).

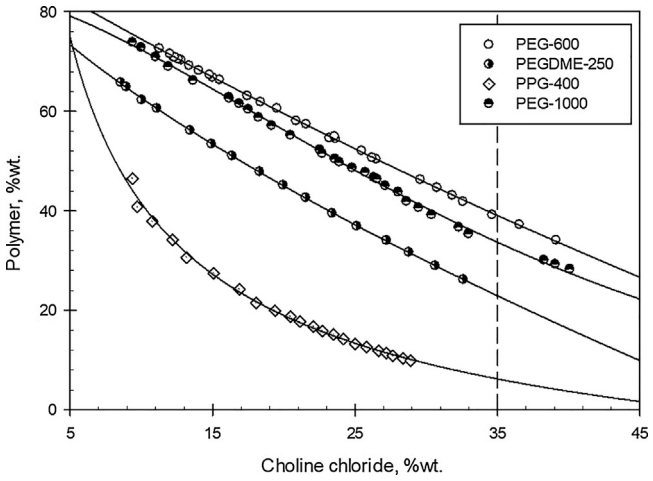

Fig. 2. Binodal lines of PEG-600-Choline chloride (data from [18]), polyethylene glycol dimethyl ether 250 (PEGDME)-Choline chloride (data from [21]), and polypropylene glycol-400 (PPG-400)-Choline chloride (data from [17]) aqueous two-phase systems.

600-choline chloride-0.01 M NaPB ATPS requires concentrations of the polymer and salt for phase separation significantly higher than in the PEG-600-TMAO-0.01 M NaPB ATPS. It should be noted that the compositions of the phases were estimated according to the approach used in [23,24]. It should be mentioned that in contrast to the phase diagram for PEG-600-choline chloride the binodal line for the PEG-TMAO ATPS could not be described by the empirical equation derived by Merchuk et al. [25] likely in view of the flatness of the line and was described by exponential expression. The tie-line slope in the formed ATPS (−0.75) is also exceeds that in the PEG-TMAO system (−0.45) indicating that the difference between the compositions of the coexisting phases in the PEG-600-choline chloride-NaPB ATPS exceeds that in the PEG-600-TMAO-NaPB ATPS (see below in Table 1). The ratios of the volumes of the upper to lower coexisting phases in the ATPS explored here are: ca. 0.95 in the PEG-TMAO ATPS and ca. 0.77 in the PEG-choline chloride ATPS.

There are several phase diagrams reported for polymer-choline chloride ATPSs formed by PEG-600 and PEG-1000 [18], polypropylene glycol (PPG) 400 [17], and polyethylene glycol dimethyl ether-250 [21]. Analysis of the data reported [17,18,21] shows that the addition of 0.01 M sodium phosphate buffer, pH 7.4 results in a slight but noticeable downward shift of the binodal in the PEG-600-choline chloride ATPS [18] as shown in Fig. 1b, while an increase in the relative hydrophobicity of the polymer (from PEG-600 to polyethylene glycol dimethyl ether-250 [21] to PPG-400 [17]) leads to significant decrease of the concentrations of the polymer and choline chloride in their aqueous mixtures necessary for phase separation (see in Fig. 2).

3.2. Solvent properties of water in aqueous solutions of choline chloride and polymers

Solvent features of aqueous solutions of choline chloride and several different polymers capable to induce phase separation (the solvent dipolarity/polarizability, π^*, hydrogen bond donor acidity, α, and hydrogen bond acceptor basicity, β) examined with solvatochromic dyes, as described above, are listed in Table 2. It should be mentioned that in the previously examined (see in [22]) aqueous solutions of various nonionic polymers, organic compounds and salts, we typically observed the different solvent features gradually changing with the increasing compound concentration. In solutions of polypropylene glycol (PPG) and polyethylene glycol dimethyl ether (PEGDME) intermittent changes in the solvent dipolarity/polarizability of water with increasing polymer concentration were observed. We currently cannot explain the reasons for these effects.

Binodals presented in Fig. 2 show that high polymer concentrations are necessary for phase separation in mixtures with choline chloride. In order to quantify concentrations of different polymers

Table 2
Solvent features[a] of water in aqueous solutions of choline chloride and different polymers.

Compound	Concentration	π^*	α	β
None		1·096 ± 0·003	1·237 ± 0·003	0·596 ± 0·001
Choline chloride	0.5 M (ca 6.4%wt.)	1.107 ± 0.001	1.204 ± 0.002	0.592 ± 0.001
	1.0 M (ca 12.6%wt.)	1.113 ± 0.001	1.185 ± 0.001	0.589 ± 0.002
	1.5 M (ca. 18.8%wt.)	1.113 ± 0.001	1.169 ± 0.001	0.589 ± 0.002
	2.0 M (ca. 24.8%wt.)	1.116 ± 0.001	1.152 ± 0.002	0.588 ± 0.001
	3.0 M (ca. 36.7%wt.)	1.111 ± 0.001	1.124 ± 0.001	0.591 ± 0.001
PPG−400[b]	10%wt.	1.118 ± 0.001	1.052 ± 0.001	0.623 ± 0.001
	20%wt.	1.117 ± 0.001	0.934 ± 0.001	0.658 ± 0.001
	30%wt.	1.089 ± 0.001	0.916 ± 0.001	0.688 ± 0.001
	40%wt.	1.029 ± 0.001	Nd	0.718 ± 0.001
PEGDME−250[c]	10%wt.	1.101 ± 0.001	1.105 ± 0.001	0.610 ± 0.002
	20%wt.	1.104 ± 0.001	0.992 ± 0.001	0.628 ± 0.001
	30%wt.	1.100 ± 0.002	0.885 ± 0.002	0.653 ± 0.002
	40%wt.	1.093 ± 0.001	0.780 ± 0.001	0.680 ± 0.001
PEG−1000[d]	10%wt.	1.107 ± 0.002	1.085 ± 0.003	0.611 ± 0.002
	20%wt.	1.110 ± 0.002	0.987 ± 0.003	0.627 ± 0.002
	30%wt.	1.116 ± 0.002	0.889 ± 0.002	0.646 ± 0.002
	40%wt.	1.121 ± 0.003	0.789 ± 0.003	0.669 ± 0.003
PEG−600[d]	10%wt.	1.106 ± 0.001	1.113 ± 0.002	0.611 ± 0.002
	20%wt.	1.107 ± 0.001	1.009 ± 0.003	0.626 ± 0.002
	30%wt.	1.109 ± 0.002	0.899 ± 0.002	0.644 ± 0.001
	40%wt.	1.110 ± 0.002	0.790 ± 0.001	0.669 ± 0.001

[a] π^* – solvent dipolarity/polarizability; α – solvent H-bond donor acidity; β – solvent H-bond acceptor basicity.
[b] PPG-400 – polypropylene glycol with molecular weight 400.
[c] PEGDME-250 – polyethylene glycol dimethyl ether with molecular weight 250.
[d] PEG-1000 – polyethylene glycol with molecular weight 1000 and PEG-600 – polyethylene glycol with molecular weight 600 (data from [22]).

Table 3
Concentrations of polymers inducing phase separation in aqueous mixtures with 35.0%wt. choline chloride and solvent features[a] of water in their aqueous solutions.

Polymer	Concentration	π^*	α	β
PPG−400[b]	6.22%wt.	1.113 ± 0.002	1.100 ± 0.002	0.614 ± 0.002
PEGDME−250[b]	22.93%wt.	1.104 ± 0.002	0.958 ± 0.002	0.636 ± 0.002
PEG−1000[b]	33.69%wt.	1.118 ± 0.003	0.845 ± 0.003	0.655 ± 0.002
PEG−600[b]	39.09%wt.	1.110 ± 0.002	0.801 ± 0.002	0.666 ± 0.002

[a] π^* – solvent dipolarity/polarizability; α – solvent H-bond donor acidity; β – solvent H-bond acceptor basicity.
[b] PPG-400 – polypropylene glycol with molecular weight 400; PEGDME-250 – polyethylene glycol dimethyl ether with molecular weight 250; PEG-1000 – polyethylene glycol with molecular weight 1000 and PEG-600 – polyethylene glycol with molecular weight 600.

inducing phase separation at the fixed concentration of choline chloride, we selected the latter at 35.0%wt. At this concentration, the binodals for mixtures of choline chloride with PPG-400 and PEGDME-250 had to be extrapolated but the gradual changes in the binodals are well established, and at this concentration of choline chloride, all the polymers concentrations were found to be within the range of up to ~40%wt.; i.e. in the range of the experimentally determined solvent features of polymers solutions (see in Table 2). The identified concentrations of polymers inducing phase separation in mixtures with 35.0%wt. choline chloride are listed in Table 3. The values of the solvent features for the aqueous solutions of individual polymers were determined by interpolation of these features from the corresponding concentration dependences and are presented in Table 3.

The data presented in Table 3 show that there is a linear relationship between the solvent hydrogen bond donor acidity, α, and hydrogen bond acceptor basicity, β, of water in aqueous solutions of different polymers at the concentrations necessary for phase separation with 35.0%wt. choline chloride. This relationship is illustrated graphically in Fig. 3a, and it may be described as:

$$\beta_i = 0.801_{\pm 0.008} - 0.170_{\pm 0.009}\alpha_i \quad (2)$$

$$N = 4; r^2 = 0.9944; SD = 0.002; F = 357$$

where subscript "i" denotes the i^{th} polymer, N – number of polymers, r – correlation coefficient; SD – standard deviation, and F – ratio of variation.

Further analysis of the data in Table 3 shows that the polymer concentration required for phase separation in aqueous mixture with 35.0%wt. choline chloride is linearly related with the solvent hydrogen bond donor acidity, α, in the aqueous solution of the polymer. This relationship is graphically illustrated in Fig. 3b, and it may be described as:

$$[polymer_i] = 126_{\pm 2.9} - 109_{\pm 3.1}\alpha_i \quad (3)$$

$$N = 4; r^2 = 0.9984; SD = 0.72; F = 1215$$

where $[polymer_i]$ is the concentration of the i^{th} polymer (in%wt.) necessary for phase separation in aqueous mixture with 35%wt. choline chloride, all the other parameters are as defined above. It should be mentioned that in view of the linear relationship between the solvent features α_i and β_i described by Eq. (2), the linear dependence of the polymer concentration $[polymer_i]$ on the solvent hydrogen bond acceptor basicity of water, β_i, similar to the one described by Eq. (3) is observed.

The established dependence of the concentrations of different polymers needed for phase separation with choline chloride at the fixed concentration of 35.0%wt. upon the properties of water to donate or accept hydrogen bond in solutions of these polymers indicates that phase separation is caused or related to particular changes in the solvent properties of water in all the mixtures. The occurrence of such changes, on one hand, agrees with the multiple previous observations of the differences between solvent properties of water in the coexisting phases of ATPS, and, on the other hand, with the supposition [22] that changes in the solvent properties of water are related to those in water structure rearrangement.

3.3. Differences between the properties of the coexisting phases

Partition coefficients of Na-salts of DNP-amino acids in the ATPS under study are listed in Table 4 and shown graphically as functions of the equivalent number of methylene groups representing the

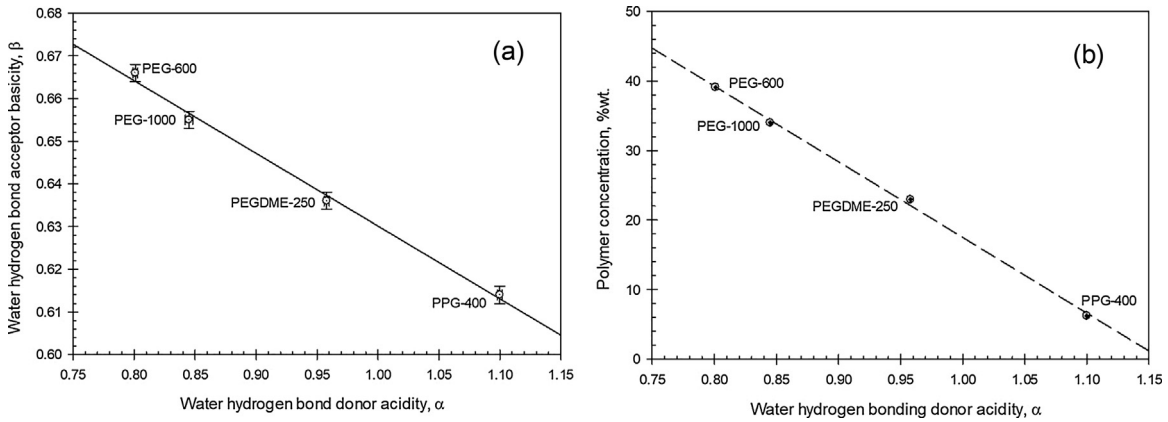

Fig. 3. **(a)** Relationship between water hydrogen bond donor acidity, α, and hydrogen bond donor basicity, β, in aqueous solutions of various polymers at concentrations required for phase separation in aqueous mixtures with 35.0%wt. choline chloride. **(b)** Relationship between water hydrogen bond donor acidity, α, in aqueous solutions of various polymers and their concentration required for phase separation in aqueous mixtures with 35.0%wt. choline chloride.

Table 4
Partition coefficients for sodium salt DNP-amino acids and proteins in PEG-600 (37.8%wt.)- TMAO (18.6%wt.)-0.01 M NaPB, pH 7.4 and PEG-600 (47.0%wt.) – choline chloride (30.0%wt.) – 0.01 M NaPB, pH 7.4 ATPS[a].

	Partition coefficient, K	
Compound	PEG-TMAO	PEG-choline chloride
DNP-Gly	0.267 ± 0.001	0.205 ± 0.001
DNP-Ala	0.209 ± 0.002	0.122 ± 0.0008
DNP-NVal	0.151 ± 0.0008	0.0715 ± 0.0006
DNP-NLeu	0.1325 ± 0.0005	0.046 ± 0.0002
DNP-AO[b]	0.0744 ± 0.0004	0.016 ± 0.001
Chymotrypsin	2.622 ± 0.055	77.2 ± 0.54
α-Chymotrypsinogen	1.393 ± 0.009	109 ± 2.9
Concanavalin A	5.099 ± 0.025	9.75 ± 0.062
β-Lactoglobulin A	0.078 ± 0.004	5.09 ± 0.064
β-Lactoglobulin B	0.044 ± 0.0008	36.2 ± 0.29
Lysozyme	3.365 ± 0.019	103.7 ± 1.13
Ribonuclease A	3.440 ± 0.033	34.1 ± 0.29
Ribonuclease B	12.16 ± 0.13	36.0 ± 0.25
Trypsinogen	3.510 ± 0.029	59.4 ± 0.48

[a] NaPB – 0.01 M sodium phosphate buffer, pH 7.4.
[b] Na-salt DNP-amino-n-octanoic acid.

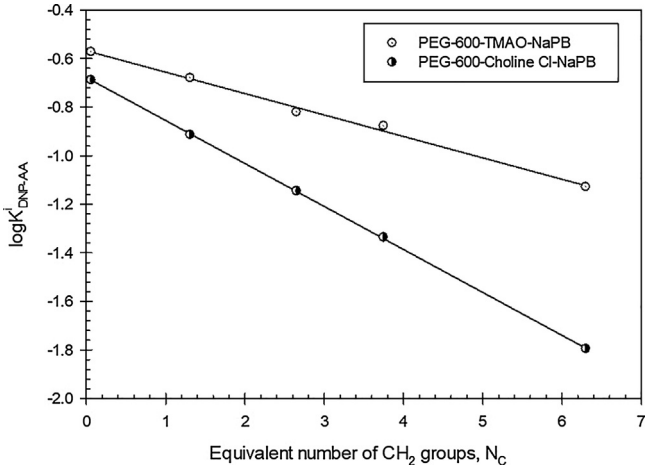

Fig. 4. Logarithm of the partition coefficient value, $\log K_{DNP\text{-}AA}$, for sodium salts of DNP-amino acids with aliphatic side-chains in aqueous PEG-600-TMAO and PEG-600-Choline chloride aqueous two-phase systems both containing 0.01 M sodium phosphate buffer, pH 7.4 as a function of equivalent length of the side-chain, N_C, expressed in terms of equivalent number of CH_2 units.

length of the alkyl side-chain in Fig. 4. The data in Fig. 4 may be described as:

$$logK^{(i)}_{DNP\text{-}AA} = C^{(i)} + E^{(i)}N_C \quad (4)$$

where $K_{DNP\text{-}AA}$ is the partition coefficient of a sodium salt of DNP-amino acid with aliphatic side-chain; superscript *(i)* denotes the particular i^{th} ATPSs used for the partition experiments; N_C is equivalent number of CH_2 groups in the aliphatic side-chain of a given DNP-amino acid; *E* is an average *logK* increment per CH_2 group; *C* represents the total contribution of the non-alkyl part of the structure of a DNP-amino acid into $logK_{DNP\text{-}AA}$ and used to characterize the difference between the electrostatic properties of the coexisting phases as described previously [4,14].

It is interesting that while the difference between the relative hydrophobic character of the phases in the PEG-TMAO and PEG-choline chloride is very substantial (2-fold), the difference between the electrostatic properties of the phases is relatively small (ca. 1.2-fold). Commonly, the differences between electrostatic properties of the phases in polymer-salt ATPS are larger than those in two polymer ATPS, because the salt distribution between the phases is believed to be the main reason for the difference between electrostatic properties of the phases. The small differences between electrostatic properties of the two ATPSs under discussion is therefore rather surprising, as the electrostatic properties in the phases of PEG-TMAO ATPS are likely to be determined by distribution of the sodium phosphate buffer between the phases, while in the PEG-choline chloride, it may be expected to be determined by both – choline chloride and phosphate buffer.

Investigation of the solvent properties of aqueous phases measured with the aforementioned solvatochromic probes showed that only some of these properties could be estimated in the two ATPSs examined because of the deterioration of the dyes spectra in the presence of high TMAO, PEG-600, and choline chloride concentrations. Only the solvent dipolarity/polarizability, π^*, could be determined in the phases of the PEG-TMAO ATPS and the dipolarity/polarizability, π^*, and hydrogen bonding acceptor basicity, β, could be estimated in the phases of PEG-600-choline chloride ATPS. The differences between these solvent properties, $\Delta\pi^*$ and $\Delta\beta$, are listed in Table 1, and their values are surprisingly large for the latter ATPS. Previously, the largest $\Delta\pi^*$ value of 0.140 was observed in the ATPS formed by 15.0%wt. of PEG-8000 and 30.0%wt. Ucon (copolymer of ethylene glycol and propylene glycol) in 0.15 M NaCl in 0.01 M NaPB, pH 7.4 [21]. In the PEG-8000-Na_2SO_4-0.5 M sucrose-0.01 M NaPB, pH 6.8 ATPS, the same difference amounts to −0.077 [12]. The largest difference between the solvent hydrogen bonding acceptor basicity of the aqueous media in the coexisting

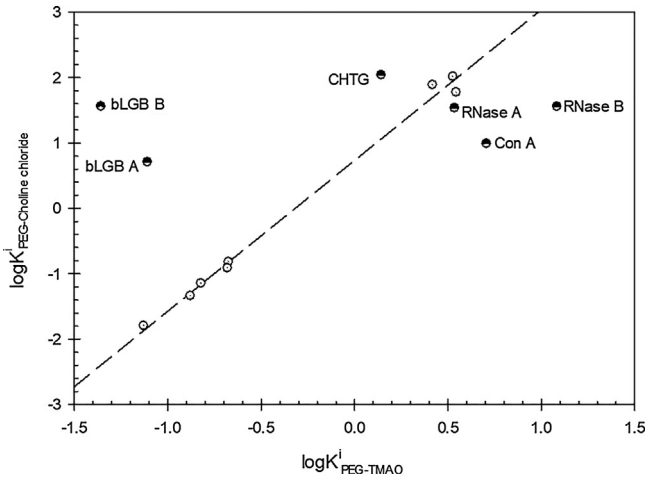

Fig. 5. Logarithm of the partition coefficient value, $logK^i$, for i-th compound in the PEG-600-Choline chloride aqueous two-phase system containing 0.01 M sodium phosphate buffer, pH 7.4 plotted against logarithm of the partition coefficient value for the same i-th compound in the PEG-600-TMAO aqueous two-phase system containing 0.01 M sodium phosphate buffer, pH 7.4. Half-filled circles represent the proteins not fitting the linear relationship: CHTG – α-chymotrypsinogen, Con A – concanavalin A, bLGB A – β-lactoglobulin A, bLGB B – β-lactoglobulin B, RNase A – ribonuclease A, RNase B – ribonuclease B.

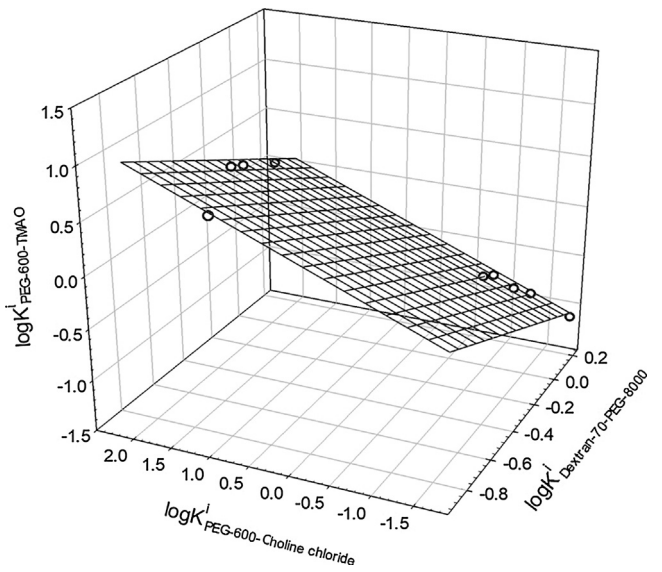

Fig. 6. Logarithm of the partition coefficient value, $logK^i_{PEG\text{-}600\text{-}TMAO}$, for i-th compound in the PEG-600-TMAO aqueous two-phase system as a function of the logarithms of the partition coefficients of the same compound in the Dextran-70-PEG-8000 aqueous two-phase system (data from [29]) and in the PEG-600-Choline chloride aqueous two-phase system (all systems contain 0.01 M sodium phosphate buffer, pH 7.4).

phases of 0.112 value was observed in the aforementioned PEG-Ucon ATPS [26], while in PEG-8000-Na_2SO_4 ATPS [12] it was varied between 0.013 and 0.028.

3.4. Partition behavior of proteins

Partition coefficients of nine proteins in the PEG-600-TMAO and PEG-choline chloride ATPSs are listed in Table 4. Analysis of the *K*-values for these proteins shows several surprising findings. First, partition coefficients in PEG-choline chloride ATPS hugely exceed those determined for the same proteins in PEG-TMAO ATPS from 1.9-fold for concanavalin A up to ~820 for β-lactoglobulin B. On the other hand, the partition coefficients of ribonuclease A and ribonuclease B differ 3.5-fold in the PEG-TMAO ATPS and less than 1.1-fold in the PEG-choline chloride ATPS. The partition coefficient of β-lactoglobulin A exceeds that for β-lactoglobulin B ca. 1.8-fold in the PEG-TMAO ATPS, while the partition coefficient of β-lactoglobulin B exceeds that for β-lactoglobulin A ~7-fold in the PEG-choline chloride ATPS. These data differ significantly from those reported [19] for clavulanic acid with partition coefficients varying in the PEG-600-choline chloride ATPS from 0.6 to 1.6 depending on the particular ATPS composition used. The possible reason may be because the ATPS used did not include buffer, and pH in the phases varied from 4.7 to 7.0 [19].

Comparison of the partition coefficients for the studied compounds (sodium-salts of DNP-amino acids and proteins) in the two ATPS under discussion is illustrated graphically in Fig. 5. The linear relationship observed in Fig. 5 may be described as:

$$logK^i_{PEG\text{-}600\text{-}CholineCl} = 0.73_{\pm 0.05} + 2.30_{\pm 0.07} logK^i_{PEG\text{-}600\text{-}TMAO} \quad (5)$$

$$N = 8; r^2 = 0.9946; SD = 0.130; F = 1096$$

where $K^i_{PEG\text{-}600\text{-}CholineCl}$ is the partition coefficient of the i^{th} compound in the PEG-600-choline chloride ATPS, $K^i_{PEG\text{-}600\text{-}TMAO}$ is the partition coefficient of the i^{th} compound in the PEG-600-TMAO ATPS, N – number of compounds, SD – standard deviation, and F – ratio of variation. It should be emphasized that only 3 proteins (chymotrypsin, lysozyme, and trypsinogen) fit the linear relationship together with Na-salts of DNP-amino acids.

Eq. (5) describes the typical linear solvent regression relationship previously thought [4] to verify the lack of proteins interactions with the phase-forming components of an ATPS. It has been established [27], however, that this thought is oversimplified. In reality, the partition coefficient of a given compound may be described as a linear combination of various types of interactions of the compound with water in the coexisting phases of a given ATPS [28]. The above linear solvent regression relationship may not hold for compounds with the contributions of different types of interactions with water in the phases of one of the ATPS under comparison very different from those in the other ATPS (see in [27]). The presence of specific interactions with one of the phase-forming components of ATPS is displayed more reliably if the comparison is performed between three different ATPS. The reasons for this phenomenon are considered in [29].

Analysis of the partition coefficients of proteins and Na-salts of DNP-amino acids listed in Table 4 together with those reported previously [30] in Dextran-70-PEG-8000-0.01 M NaPB, pH 7.4 is illustrated graphically in Fig. 6. The relationship observed may be described as:

$$logK^i_{PEG\text{-}600\text{-}TMAO} = -0.36_{\pm 0.01} - 0.89_{\pm 0.05} logK^i_{Dex\text{-}70\text{-}PEG-8000} + 0.30_{\pm 0.01} logK^i_{PEG\text{-}600\text{-}Cholin\ Cl} \quad (6)$$

$$N = 9; r^2 = 0.9979; SD = 0.038; F = 1395$$

where $K^i_{Dex\text{-}70\text{-}PEG\text{-}8000}$ is the partition coefficient of the i^{th} compound in the Dextran-70-PEG-8000-0.01 M NaPB, pH 7.4 ATPS, all the other parameters are as defined above. Five proteins do not fit the above relationship: β-lactoglobulin A, β-lactoglobulin B, lysozyme, ribonuclease A, and ribonuclease B. It has been shown previously [27,28] that these proteins do not interact directly with Dextran, PEG, and TMAO, therefore it is reasonable to suggest that they are engaged in interactions with choline chloride.

It was found that five out of nine studied proteins, β-lactoglobulin A, β-lactoglobulin B, lysozyme, ribonuclease A, and ribonuclease B, probably interact directly with choline chloride. It should be noted that concanavalin A and α-chymotrypsinogen do

not fit the above linear solvent regression relationship described by Eq. (5), and lysozyme does fit it, though according to the relationship described by Eq. (6) the latter is engaged in the direct interactions with choline chloride. For other proteins, chymotrypsin, α-chymotrypsinogen, concanavalin A, and trypsinogen, the ca. 2–78-fold difference between the partition coefficients in the PEG-choline chloride and PEG-TMAO ATPSs may be explained by the differences between the other solvent properties of the coexisting phases (solvent dipolarity/polarizability, solvent hydrogen bonding donor acidity, and hydrogen bonding acceptor basicity) which were unmeasurable in the PEG-600-TMAO ATPS and only partially measurable in PEG-600-choline chloride ATPS.

4. Conclusions

In this study, the phase diagram for new aqueous two-phase system formed by polyethylene glycol (PEG-600) with molecular weight 600 and trimethylamine N-oxide (TMAO) is determined. The differences between hydrophobic, electrostatic, and other solvent properties of the coexisting phases for the system are analyzed and compared with those of the ATPS formed by PEG-600 and choline chloride. The data obtained in this study show that ATPSs may be formed in a mixture of low molecular weight polymer and nonionic compound. Comparison of the properties of such system with the polymer-salt system showed that the difference between the electrostatic properties of the phases in the PEG-600-choline chloride ATPS exceeds that in the PEG-600-TMAO ATPS just by ∼20%, while the difference between the relative hydrophobicities of the phases of the same system is 2-fold higher than in the PEG-TMAO system possibly because of the larger difference between the compositions of the two phases in the former ATPS.

Analysis of the solvent features of water in aqueous solutions of various polymers showed that the concentrations of polymers necessary for phase separation in aqueous mixtures with choline chloride at the fixed concentration of 35%wt. are linearly dependent on the water ability to participate in hydrogen bonding in solutions of individual polymers. This finding agrees with the hypothesis that changes in the solvent features of water indicate the structural rearrangement of hydrogen bonds network in solution and implies that phase separation in aqueous mixtures may result from formation of two incompatible water hydrogen bonds networks.

The study of partition behavior of several proteins in both systems shows that the partitioning of proteins in the aqueous PEG-600-choline chloride two-phase system is much more extreme than in the PEG-600-TMAO system possibly due to larger difference between the compositions of the coexisting phases in the former system. Proteins engaged in direct interactions with choline chloride were identified.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2018.01.010.

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