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Journal:	*Physical Chemistry Chemical Physics*
Manuscript ID	CP-ART-10-2019-005810.R1
Article Type:	Paper
Date Submitted by the Author:	21-Jan-2020
Complete List of Authors:	Titus, Amber; Department of Biological Sciences, Kent State University Ferreira, Luisa; Cleveland Diagnostics, Inc. Belgovskiy, Alexander; Cleveland Diagnostics, Inc. Kooijman, Edgar; Kent State University, Biological Sciences Mann, Elizabeth; Kent State University, Physics Mann Jr. , J. Adin; Department of Chemical and Biomolecular Engineering, Case Western Reserve University Meyer, William; Scattering Solutions, Inc. Smart , Anthony; Scattering Solutions, Inc. Uversky, Vladimir; University of South Florida, Department of Molecular Medicine Zaslavsky, Boris; Cleveland Diagnostics, Inc.

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Interfacial Tension and Mechanism of Liquid-Liquid Phase Separation in Aqueous Media

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Amber R. Titus,[a] Luisa A. Ferreira,[b] Alexander I. Belgovsky,[b] Edgar E. Kooijman,[a] Elizabeth K. Mann,[c] J. Adin Mann Jr.,[d] William V. Meyer,[e] Anthony E. Smart,[f] Vladimir N. Uversky,[g,h] and Boris Y. Zaslavsky[b*]

The organization of multiple subcellular compartments is controlled by liquid-liquid phase separation. Phase separation of this type occurs with the emergence of interfacial tension. Aqueous two-phase systems formed by two non-ionic polymers can be used to separate and analyze biological macromolecules, cells and viruses. Phase separation in these systems may serve as the simple model of phase separation in cells also occurring in aqueous media. To better understand liquid-liquid phase separation mechanisms, interfacial tension was measured in aqueous two-phase systems formed by dextran and polyethylene glycol and by polyethylene glycol and sodium sulfate in the presence of different additives. Interfacial tension values depend on differences between the solvent properties of the coexisting phases, estimated experimentally by parameters representing dipole-dipole, ion-dipole, ion-ion, and hydrogen bonding interactions. Based on both current and literature data, we propose a mechanism for phase separation in aqueous two-phase systems. This mechanism is based on the fundamental role of intermolecular forces. Although it remains to be confirmed, it is possible that these may underlie all liquid-liquid phase separation processes in biology.

Introduction

The importance of liquid-liquid phase separation (LLPS) in the organization and function of cells is increasingly recognized. LLPS controls the formation of multiple cellular membrane-less organelles such as stress granules, centrosomes, P-bodies, and Cajal bodies, which are observed in cytoplasm and nucleoplasm as liquid drops capable of forming and dissipating in response to various external stimuli.[1-9] The primary mechanism of LLPS is not currently known. An understanding of the molecular mechanism behind LLPS is not only important from the theoretical point of view; in practice it may lead to the development of new types of drugs for regulation of these and related processes involved in multiple diseases.[3, 10]

A distinctive feature of LLPS in cells is that it occurs in aqueous media and typically depends on the presence of compounds such as proteins, nucleic acids and metabolites.[1] The LLPS in aqueous mixtures of three to six non-ionic or ionized polymers may lead to separation of such mixtures into three to six[11] or even 18 phases.[12] Because aqueous two-phase systems (ATPS) formed by two non-ionic polymers are much better characterized and understood than multi-phase systems, the phase separation in ATPS was suggested[13, 14] to be the simplest model of LLPS in a cell.

ATPS arise in water when the concentrations of two different specific polymers, such as dextran and polyethylene glycol (PEG) or Ficoll™ exceed a certain threshold. ATPS also arise in mixtures of a polymer of low molecular weight, such as PEG-600, whose molecular weight is 600, with small organic compounds such as trimethylamine N-oxide (TMAO)[15] or small ionized compounds such as choline chloride.[16]

ATPS formed by two non-ionic polymers can be used to separate and analyze biological macromolecules, cells, viruses, etc. Proteins added to ATPS distribute between the two phases, with a partition coefficient defined as the ratio of the protein concentrations in each of the two phases. The partition coefficient value depends on the nature and spatial arrangement of groups exposed to the solvent and the solvent properties of aqueous media in the two phases.[17] Since proteins typically do not interact with phase-forming polymers,[18] the high sensitivity of analysis of protein partitioning to spatial arrangement of protein groups exposed to the solvent enables detection of protein misfolding, aggregation, and single-point mutations. Extreme sensitivity to changes in the nature of the solvent exposed groups makes it possible to detect changes in protein-protein interactions,[19] and post-translational modifications.[20] The protein partition analysis may be used for

[a] Department of Biological Sciences, Kent State University, Kent, OH, USA.
[b] Cleveland Diagnostics, Cleveland, OH, USA.
[c] Department of Physics, Kent State University, Kent, OH, USA.
[d] Department of Chemical and Biomolecular Engineering, Case Western Reserve University, Cleveland, OH, USA.
[e] Scattering Solutions, Inc., Cleveland, OH, USA.
[f] Scattering Solutions, Inc., Costa Mesa, CA, USA.
[g] Department of Molecular Medicine and Byrd Alzheimer's Research Institute, Morsani College of Medicine, University of South Florida, Tampa, FL, USA
[h] Institute for Biological Instrumentation of the Russian Academy of Sciences, Federal Research Center "Pushchino Scientific Center for Biological Research of the Russian Academy of Sciences", Pushchino, Moscow region, Russia
* Correspondence to: Boris.Zaslavsky@cleveland-diagnostics.com.
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

quality control of biologics,[20] and it has been suggested[20] to be used for analysis of protein biomarkers based on their structural changes in contrast to their concentration changes. The new test based on this approach has been recently validated for clinical diagnostics of prostate cancer.[21, 22]

The solute-solvent interactions are of multiple types, such as ion-dipole, ion-ion, dipole-dipole, and hydrogen bonding. The relative ability of a solvent to participate in these interactions can be characterized with the Kamlet-Taft solvatochromic comparison method.[23, 24] This method uses three different solvatochromic dyes whose wavelengths of maximum absorbance depend on the ability of the solvent to participate in dipole-dipole and dipole-induced dipole interactions (solvent dipolarity/polarizability, π^*), the solvent ability to donate a hydrogen bond (solvent hydrogen bond donor acidity, or HBD acidity, α), and the solvent ability to accept a hydrogen bond (solvent hydrogen bond acceptor basicity, or HBA basicity, β). The difference between the electrostatic properties (ion-dipole and ion-ion interactions) of the two phases, characterized by c_i, may be estimated by analyzing the contribution of the ionic group to the logarithms of partition coefficients of a homologous series of aliphatic compounds (see Supplementary Material).

The differences between the above features of aqueous media in the two phases are the most important characteristics of ATPS. In the last decade, we established[18, 25] that the logarithm of partition coefficient of any solute (including proteins) may be described as a sum of the terms representing different solute-solvent interactions in the two phases. Each term includes the difference between one of the above solvent features of water in the two phases with a solute-specific coefficient representing the relative contribution of a given type of solute-solvent interactions to the solute partition coefficient. The solute-specific coefficients are determined by analysis of partition coefficients of the protein in five or more ATPSs of the same ionic composition but formed by different pairs of various non-ionic polymers. Once these coefficients are determined for a given protein the partition coefficient of that protein in any new ATPS of the same ionic composition with known solvent properties of the phases may be predicted with over 95% certainty.[18]

The emergence of an interfacial tension is the necessary condition for phase separation. The interfacial tension in ATPS has been reported[26-31] to vary from ~0.08 μN/m to over 10 μN/m depending on the ATPS composition, and is therefore of the same order of magnitude as the 0.4 to 3.0 μN/m estimated for membrane-less organelles.[32, 33]

Among various theoretical models of phase separation in ATPS the most successful in regard to quantitative description of phase diagrams is the binodal model pioneered by Guan *et al*.[34] This semi-empirical model is based on the assumption that each point on the binodal line may be viewed as a saturated solution of the phase-forming compound-1 in the solution of the phase-forming compound-2. It was suggested further[35] that the solubility of the compound-1 in solutions of compound-2 depends on the solvent properties of water in solutions of compound-2. The binodal model described in these terms was successfully applied[35] to the phase diagrams of ATPSs formed by pairs of various polymers (dextran, Ficoll, PEG-8000, and Ucon), ATPS formed by TMAO and polypropylene glycol-400 and TMAO and PEG-600, as well as ATPS formed by single polymer and salt and polymer and ionic liquids. Hence, we hypothesized that the differences between the solvent features of aqueous media in the coexisting phases of ATPS may describe the interfacial tension values in various ATPSs.

In this study we test the hypothesis that the interfacial tension in aqueous two-phase systems is determined by the same four characteristic parameters that were previously shown to determine solute partitioning:

$$\log_{10}(1+\gamma_i/\gamma_0) = k_\pi \Delta\pi^*_i + k_\alpha \Delta\alpha_i + k_\beta \Delta\beta_i + k_c c_i, \quad (1)$$

where γ_i is the interfacial tension in the ATPS with the i-th polymer composition; the ratio γ_i/γ_0 is used to convert dimensioned quantity into dimensionless argument of the logarithm; $\Delta\pi^*_i$, $\Delta\alpha_i$, $\Delta\beta_i$ and c_i are the differences in the solvent properties defined above for the i-th polymer composition, and k_π, k_α, k_β, and k_c are solvent-specific constants quantifying the relative contribution of the complementary interactions to the interfacial tension value. The form of the equation was chosen to be consistent with the relation already found to hold for the partition coefficients. The argument of the logarithm ensures that the limit as the solvent differences vanish corresponds to vanishing interfacial tension. For $\gamma_i \gg \gamma_0$, the value $\log_{10}(\gamma_0)$ can be considered as the zero-order term in a linear expansion of $\log_{10}(\gamma_i)$ in the parameters describing the solvent properties. Thus, γ_0 must be determined experimentally.

We measure the interfacial tension in the aqueous two-phase systems with the pendant drop technique.[36] We consider both simple systems and ones with additives of salts and small organic compounds, such as sucrose, sorbitol and TMAO, in order to explore a range of ATPS solvent properties.[18, 25] The same salts and TMAO also affect the formation of stress granules.[37]

Results

We first test relationship (1) for the ATPSs formed by dextran-500,000 and PEG-35,000 with the interfacial tension values reported[30] for different polymer concentrations. We examined the solvent features of water in the phases and found that in these ATPSs the solvent hydrogen bond acceptor (HBA) basicity $\Delta\beta_i$ is indistinguishable from zero, while the solvent hydrogen bond donor (HBD) acidity $\Delta\alpha_i$ changes little as a function of the concentrations of the polymers. We thus expect the interfacial tension to depend primarily on $\Delta\pi^*$, i.e. the difference between the ability of water to participate in dipole-dipole and dipole-induced dipole interactions in the coexisting phases. Figure 1 demonstrates a linear relationship between the logarithm of interfacial tension, γ_i, reported in ref.,[30] and $\Delta\pi^*$ (Supplementary Material, table S2):

$$\log_{10}(1 + \gamma_i/\gamma_0) = 0.72_{\pm 0.02} - 45.9_{\pm 0.7}\,\Delta\pi^*_i. \quad (2)$$

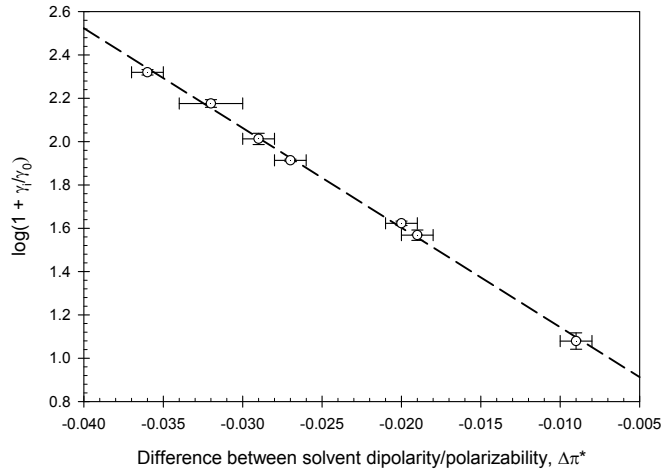

Fig. 1. Interfacial tension in salt-free ATPS depends on solvent dipolarity of the two phases. Logarithm of interfacial tension in dextran-500,000-PEG-35,000 ATPS [30] as a function of the difference between the solvent dipolarity/polarizability, $\Delta\pi^*_i$, of the two phases (Data from table S2 (Supplementary Material), interfacial tension data from [30]).

For these seven experimental points, the correlation coefficient $r^2 = 0.9987$, the standard deviation SD = 0.016, and the ratio of variance F = 3971. Comparing this equation to Eq. 1 implies that for these data with the interfacial tension γ_i values varying from 12 μN/m to ~210 μN/m $\gamma_i/\gamma_0 \gg 1$, or that $\gamma_0 << 12$ μN/m. All of the following surface tension values are larger ($\gamma_i > 40$ μN/m), hence we can approximate $\log_{10}(1+\gamma_i/\gamma_0) \cong \log_{10}(\gamma_i/\gamma_0)$, implying that the parameter γ_0 contributes only a constant offset to $\log_{10}(\gamma_i)$.

However, ATPSs used for protein separation or analysis always include buffer salts, which can affect the interfacial tension.[27] Further, in general the interfacial tension should be sensitive to solvent parameters beyond $\Delta\pi^*_i$. We therefore examined ATPSs formed by dextran-70 and PEG-8000 with 0.01 M potassium/sodium phosphate buffer, pH 7.4 and fixed polymer composition both with and without non-ionic additives affecting the solvent properties of the phases.[25] The interfacial tension values in this system are plotted in Fig. 2 versus the differences between the solvent dipolarity/polarizability, $\Delta\pi^*_i$, and solvent HBD acidity, $\Delta\alpha_i$, of the coexisting phases. The interfacial tension values were measured as described in ref.[36] and the solvent parameters reported previously[25] (Supplementary Material, table S3). We compare these data to the linear relationship in Eq. 1 by multiple linear regression (Supplementary Material, Section S2.5). Two parameters, $\Delta\pi^*_i$ and $\Delta\alpha_i$, are sufficient to describe the behaviour of this system, as:

$$\log_{10}(1 + \gamma_i/\gamma_0) = -15.4_{\pm 4.0}\Delta\pi^*_i - 22.0_{\pm 3.3}\Delta\alpha_i. \quad (3)$$

The lack of a constant term in this fit suggests that in Eq. 1, 0.3 μN/m $\leq\gamma_0\leq$3 μN/m. We will thus approximate $\gamma_0 = 1$ μN/m in the rest of this article. For these four experimental points, r^2 = 0.9958, SD = 0.19, and F = 235.1.

The relationship described by Eq. 3 indicates that in the presence of 0.01 M potassium/sodium phosphate buffer the interfacial tension depends not only on dipole-dipole interactions but also on the solvent HBD acidity.

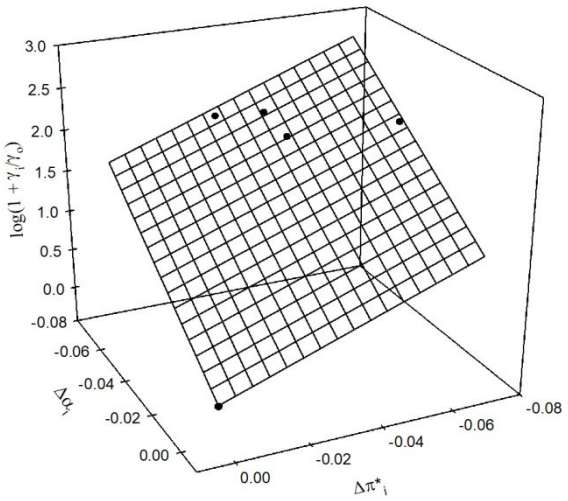

Fig. 2. Interfacial tension in ATPS with 0.01 M buffer depends on two solvent features of the two phases. Logarithm of interfacial tension in dextran-70-PEG-8000-0.01 M K/Na-phosphate buffer, pH 7.4 ATPSs with and without non-ionic additives as a function of the differences between the solvent dipolarity/polarizability, $\Delta\pi^*_i$, and solvent HBD acidity, $\Delta\alpha_i$, of the coexisting phases (Data from table S3, Supplementary Material). The plane corresponds to Eq. 3. Error bars are the same size as/or smaller than the symbols.

Salt additives at concentrations of 0.1 M and above may significantly affect the solvent properties of the phases,[38] as well as the interfacial tension.[27] Therefore, the interfacial tension values were examined in the dextran-70-PEG-8000 ATPS, in the same 0.01 M potassium/sodium phosphate buffer, pH 7.4 but with the addition of 0.215 M NaCl and $NaClO_4$ in two separate series of experiments.

The logarithms of interfacial tension values obtained for the ATPSs containing 0.215 M NaCl are plotted in Fig. 3A versus the differences between $\Delta\pi^*_i$ and the HBA basicity, $\Delta\beta_i$, of the two phases.

The relationship in Fig. 3A may be described by:

$$\log_{10}(1 + \gamma_i/\gamma_0) = -54.4_{\pm 0.4}\Delta\pi^*_i - 1343_{\pm 1.6}\Delta\beta_i, \quad (4a)$$

where γ_i is the interfacial tension in the dextran-70-PEG-8000-0.215 M NaCl-0.01 M pH 7.4 K/NaPB, ATPSs with or without non-ionic additive, and we approximate γ_0=1 μN/m as suggested by the earlier data. For these four experimental points, r^2 = 0.9999, SD = 0.034, and F = 264.

The experimental interfacial tension values are listed in table S4 (Supplementary Material) together with the differences between the solvent features of water in the coexisting phases.[39] The addition of 0.215 M salt clearly increases the interfacial tension in these ATPSs. Multiple linear regression analysis of the relationship between the logarithm of interfacial tension in ATPSs containing 0.215 M NaCl and all differences between various solvent features of aqueous media in the two phases yields the above relationship (Eq. 4a).

A change in the type of salt changes the relative importance of the different solvent properties. The logarithms of interfacial tension in the dextran-70-PEG-8000-0.215 M $NaClO_4$-0.01 M K/NaPB, pH 7.4 ATPSs with and without non-ionic additives are

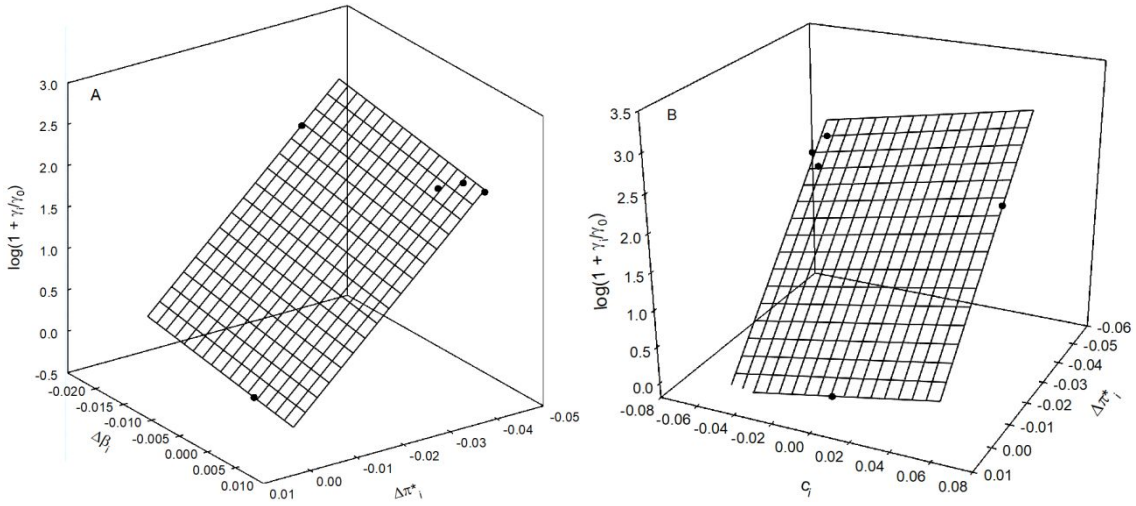

Fig. 3. Interfacial tension in ATPS with 0.215 M salt depends on two solvent features of the phases. Logarithm of interfacial tension in ATPSs formed by dextran-70-PEG-8000 in 0.01 M K/Na-phosphate buffer, pH 7.4 with and without non-ionic additives, with two different salt additives: (A) 0.215 M NaCl as a function of differences between the solvent dipolarity/polarizability, $\Delta\pi^*_i$, and solvent hydrogen bond acceptor basicity, $\Delta\beta_i$; the plane corresponds to Eq. 4a, and (B) 0.215 M $NaClO_4$, as a function of differences between the solvent dipolarity/polarizability, $\Delta\pi^*_i$, and electrostatic properties, c_i, of the coexisting phases, the plane corresponds to Eq. 4b (Data from table S4, Supplementary Material). Error bars are the same size as/or smaller than the symbols

plotted as a function of the differences between the solvent dipolarity/polarizability, $\Delta\pi^*_i$, and electrostatic properties of the two phases, c_i, in Fig. 3B.

The relationship shown in Fig. 3B may be described by:

$$\log_{10}(1 + \gamma_i/\gamma_0) = -58.8_{\pm 1.6}\Delta\pi^*_i + 4.4_{\pm 1.1}c_i, \quad (4b)$$

where γ_i is the interfacial tension in the dextran-70-PEG-8000-0.215 M $NaClO_4$-0.01 M K/NaPB, pH 7.4 ATPSs with and without non-ionic additives; $\Delta\pi^*_i$ and c_i are as defined above, and we approximate $\gamma_0 = 1$ μN/m as suggested by the earlier data. For these four experimental points, $r^2 = 0.9989$, SD = 0.099, and F = 902. Equation 4b was obtained by multiple linear regression analysis as before.

The difference between the electrostatic properties (ion-ion and ion-dipole interactions) of the phases does not appear to play a role in the interfacial tension in ATPSs with 0.215 M NaCl but does contribute to the interfacial tension in ATPSs with 0.215 M $NaClO_4$. This disparity may arise because the distribution of $NaClO_4$ in dextran-PEG ATPS is known to be more asymmetric than distribution of NaCl.[17]

Because the most extreme distribution of salt in ATPSs is observed in the systems formed by a single polymer and salt,[17] we examined interfacial tension in the different type of ATPS formed by PEG and Na_2SO_4; specifically, PEG-8000-Na_2SO_4 ATPSs in the 0.01 M sodium phosphate buffer (NaPB), pH 6.8 with and without additives of sucrose and sorbitol. The difference between electrostatic interactions (parameter c_i) of this system[40] is about ten times larger than for any of the other systems, so this parameter may be expected to dominate their behaviour. As demonstrated in Fig. 4, the logarithm of interfacial tension is indeed found to be linearly related to parameter c_i.

The linear relationship presented in Fig. 4 is described by:

$$\log_{10}(1 + \gamma_i/\gamma_0) = 0.23_{0.04} + 3.18_{\pm 0.06}\, c_i. \quad (5)$$

where we approximate γ_0=1 μN/m as suggested by the earlier data. For these three experimental points, $r^2 = 0.9996$, SD = 0.012, and F = 2544.

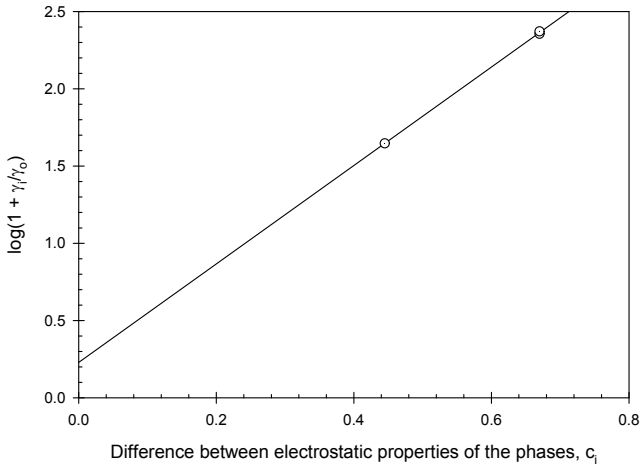

Fig. 4. Logarithm of interfacial tension in PEG-8000-Na_2SO_4-0.01 M Na-phosphate buffer, pH 6.8 ATPSs with and without non-ionic additives as a function of the difference

between the electrostatic properties, c_i, of the coexisting phases (Data from table S5, Supplementary Material).

Discussion

Our data show that the interfacial tension in ATPSs depends on the solvent properties of the coexisting phases. Measurements on salt-free ATPSs (Eq. 2) also support the effects of dipole-dipole interactions on the interfacial tension.[41] The data show that in ATPSs with the relatively high concentration of salt distributing unequally between the two phases the interfacial tension is affected by the difference between the electrostatic properties of the phases (Eqs. 4b and 5). This finding supports the conclusion of Vis *et al*.[42] about the charge effect on the interfacial tension in aqueous two-phase system formed by non-ionic dextran and charged protein gelatin.

Phase separation in aqueous mixtures of two polymers is commonly observed as turbidity appearing from the formation of micro-droplets of one phase in the other. For liquid-liquid phase separation, whether in water, cytoplasm or nucleoplasm, at least some properties of the phases must differ, forcing the emergence of interfacial tension.[43] Aqueous solutions of different polymers may differ quite significantly with respect to their solvent properties.[44] The examples of HBD acidity and dipolarity/polarizability in aqueous solutions of individual polymers[44] are given in table S6 (Supplementary Material).

Solvent properties of aqueous media, such as dipolarity/polarizability, HBD acidity, and HBA basicity, in solutions of individual proteins, such as human small heat shock protein HspB6[45] and different dehydrins,[46] differ even more than those observed in individual solutions of phase-forming non-ionic polymers.[44]

Effects of phase-forming polymers PEG-4500 and Ucon-3930 on water in solutions of individual polymers at concentrations between 0 and 40 %wt. were examined[47] by Fourier-transform infrared spectroscopy and polarized Raman spectroscopy. The IR-spectra of water in polymer solutions were analyzed based on the model[48] describing the OH band profile as six superimposed Gaussians, each with a different height, width, and peak position (Supplementary Material, Section S4). Each Gaussian was assigned to a particular subpopulation of water with different number of hydrogen bonds (from zero to four). This classification is complicated by some Gaussian sub-bands representing mixtures of subpopulations of water, e.g., with 2 and 3 or 3 and 4 hydrogen bonds. The relative intensity of the Gaussian sub-bands was found[47] to change with polymer concentration, and for particular sub-bands the relative intensity was established to correlate strongly with the particular solvent features of water (π^*, α, and β) in polymer solutions (Supplementary Material, Section S4, table S7). For simplicity we may consider each subpopulation of water existing at equilibrium with other subpopulations but as an independent part of overall H-bond network with specific properties with respect to water-water interactions. The coefficients of Eq. S1 listed in table S8 (Section S4, Supplementary Material) characterize the contribution of these independent parts of H-bond network into a given solvent property. Data in table S7 indicate that the changes in the solvent properties observed in polymer solution result from changes in relative amounts of various independent parts of the H-bond network induced by the polymer. Hence the solvent features of water change in polymer solution due to the polymer effect on the relative concentrations of water subpopulations with different numbers of hydrogen bonds.

These data imply that the 'simple' aqueous mixture of two phase-forming polymers at the concentrations below the binodal line (Fig. S3, Supplementary Material, Section S3) may be more complex than a homogeneous solution. We assume that the two polymers form different polymer-specific water hydrogen bond network domains, which have dissimilar solvent properties. The dissimilarity between the domains increases with increasing polymer concentrations in the mixture. Two types of domain exist in the polymer mixtures until the polymer concentrations exceed a threshold beyond which the domains become immiscible, at which point the emerging interfacial tension leads to the formation of micro-droplets, eventually coalescing into separate layers controlled by the density of the phases. Data presented here show that the most important interactions are dipole-dipole, dipole-induced dipole, hydrogen bonding, ion-dipole, and ion-ion interactions which are affected differently by the polymers and additives. It should be mentioned also that the size of the domains is likely to depend on the molecular weight or size of each polymer as follows from the known dependence of binodal line position on phase diagram upon the molecular weights of phase-forming polymers.[12, 17]

Formerly described mechanisms of liquid-liquid phase separation in biological systems, which are always in aqueous media, do not include any active role of the aqueous medium[1-9] with the only exception of ref.[49] The current work suggests that neglecting the properties of the aqueous medium would oversimplify the characterization of liquid-liquid phase separation processes within the even more complex cytoplasm or nucleoplasm.

Experimental

Materials

List of the materials and provenance given in table S1 (Supplementary Material).

Methods

Preparation of aqueous two-phase systems

Aqueous two-phase systems were prepared as described in refs.[25, 39, 40, 50] The details are provided in Supplementary Material, Section S2.1.

Solvatochromic studies

The solvatochromic probes 4-nitroanisole, 4-nitrophenol, and Reichardt's carboxylated betaine dye were used to measure the dipolarity/polarizability, π^*, hydrogen bond acceptor (HBA)

basicity, β, and hydrogen bond donor (HBD) acidity, α, of the media in the separated phases of ATPS. The measurements were performed as described previously.[15, 25, 39, 40]
The detailed protocols are described in Supplementary Material, Section S2.2.

Electrostatic properties of the phases

The difference between the electrostatic properties of the coexisting phases is determined in each ATPS by partitioning a homologous series of sodium salts of dinitrophenylated (DNP-) amino acids with the aliphatic alkyl side-chains of the increasing length, alanine, norvaline, norleucine, and α-amino-n-octanoic acid as described previously.[15, 18, 25, 38-40] The detailed description is provided in Supplementary Material, Section S2.3.

Interfacial tension measurements

Interfacial tension of each ATPS was determined using pendant drop tensiometry as described in ref.[36] Detailed protocols of the measurements and analysis are provided in Supplementary Material, Section S2.4.

Multiple linear regression analysis

The linear relationship between the logarithm of interfacial tension of ATPS with a given ionic composition with or without non-ionic additives was confirmed using Eq. 1. Detailed protocol used see in Supplementary Material, Section S2.5.

Conclusions

From measurements in different aqueous two-phase systems we found that the interfacial tension values are strongly correlated with the solvent properties of coexisting phases, such as the dipolarity/polarizability, hydrogen bond donor acidity, hydrogen bond acceptor basicity, and electrostatic properties (ion-ion and ion-dipole interactions).
From this we infer that, in a mixture of two phase-forming polymers, two different types of water domains coexist until the polymer concentrations exceed a threshold beyond which the domains become immiscible, and the emergent interfacial tension leads to phase separation.
The physical intermolecular forces that underlie aqueous/aqueous phase separation in our model experiments also exist in the cell. Although in vivo processes are more complex, the physical phenomena of phase separation explored in this work models those in biological systems. The role of such intermolecular forces in real biological systems remains to be experimentally explored.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

ART, EEK and EKM acknowledge support by the National Science Foundation under grant no. CHE-1808281.

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