

Review

The solvent side of proteinaceous membrane-less organelles in light of aqueous two-phase systems

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ABSTRACT

Water represents a common denominator for liquid-liquid phase transitions leading to the formation of the polymer-based aqueous two-phase systems (ATPSs) and a set of the proteinaceous membrane-less organelles (PMLOs). ATPSs have a broad range of biotechnological applications, whereas PMLOs play a number of crucial roles in cellular compartmentalization and often represent a cellular response to the stress. Since ATPSs and PMLOs contain high concentrations of polymers (such as polyethylene glycol (PEG), polypropylene glycol (PPG), Ucon, and polyvinylpyrrolidone (PVP), Dextran, or Ficoll) or biopolymers (peptides, proteins and nucleic acids), it is expected that the separated phases of these systems are characterized by the noticeable changes in the solvent properties of water. These changes in solvent properties can drive partitioning of various compounds (proteins, nucleic acids, organic low-molecular weight molecules, metal ions, etc.) between the phases of ATPSs or between the PMLOs and their surroundings. Although there is a sizable literature on the properties of the ATPS phases, much less is currently known about PMLOs. In this perspective article, we first represent liquid-liquid phase transitions in water, discuss different types of biphasic (or multiphasic) systems in water, and introduce various PMLOs and some of their properties. Then, some basic characteristics of polymer-based ATPSs are presented, with the major focus being on the current understanding of various properties of ATPS phases and solvent properties of water inside them. Finally, similarities and differences between the polymer-based ATPSs and biological PMLOs are discussed.

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1. Introduction

The ability, under specific conditions, to undergo liquid-liquid phase transitions (LLPTs) leading to the liquid-liquid phase separation represents a general property of various biological and non-biological liquids. For example, biotechnology widely uses biocompatible aqueous two-phase systems (ATPSs, which are typically polymer-based systems) for multiple purposes [1, 2], such as extraction and purification of biomolecules, bioparticles, cells, and biopharmaceuticals; analysis of the partition behavior of various compounds and their separation; biocatalysis; product removal from the aqueous media during the biotechnological whole-cell biotransformation; extractive fermentation and bioconversions; and characterization and analysis of individual proteins and their interactions with different partners [3–19]. In biology too, the phase separation leading to the formation of various proteinaceous membrane-less organelles (PMLOs) that exist as liquid droplets in cytoplasm, nucleoplasm, mitochondrial matrix, or stroma of the chloroplasts is of utmost importance [20–28]. The common features that clearly link polymer-based ATPSs and biological PMLOs are their liquid-like properties, fluidity, and high water content. In fact, each of the phases in an ATPS typically contains well over 80% water on a molal basis [2]. Liquid droplet phases can be formed in concentrated aqueous protein solutions (typically, in a range of 50–100 mg/ml) *in vitro* [29]. In aqueous mixtures of polyvalent signaling proteins, protein concentrations leading to phase separation *in vitro* can vary from 50 nM to 500 μ M, depending on a particular pair of proteins [30]. It was also pointed out that cytoplasmic concentrations of WASP (Wiskott-Aldrich syndrome protein) and WASP-interacting SH3-domain protein (also known as NCK (non-catalytic region of tyrosine kinase adaptor protein)-interacting protein with SH3 domain), are 3.5–9.0 μ M and 14.5 μ M, respectively, and that the proteins are concentrated ~100-fold in the droplets relative to the bulk [30]. This suggests that although proteinaceous droplets might contain proteins at concentrations of 1–100 mM, they still possess very significant amounts of water. It was also demonstrated that the apparent critical volume fractions for peptides and proteins undergoing phase separation and liquid droplet formation ranges from 3.6% to 16% and to 26% for the aqueous solutions of incretin peptide, lysozyme, and bovine lens γ -crystallins, respectively [31]. In other words, water represents a common denominator of both ATPSs and PMLOs. On the other hand, since polymers inside the ATPS phases and proteins and nucleic acids inside the PMLOs are present at high concentrations, one can expect that these polymeric/biopolymeric constituents might affect solvent properties of water inside the phases, such as electrostatic properties, ability to participate in dipole-dipole interactions, ability of water to serve as donors and acceptors of hydrogen bonds. This article addresses this issue by presenting several pieces of related information. First, liquid-liquid phase transitions in water are discussed. Then, types of biphasic (or multiphasic) systems in water are considered and multifariousness of PMLOs is briefly addressed. Next, we present some basic characteristics of polymer-based ATPSs and focus on the current knowledge pertaining to the properties of phases in ATPSs and consider solvent properties of water in ATPS phases. Finally, we discuss some similarities and differences between the polymer-based ATPSs and PMLOs.

2. Liquid-liquid phase transitions in water

Being the most abundant liquid on Earth, water is characterized by an impressive set of various anomalies including the presence of more

than sixteen crystalline phases [32–34], or the fact that the bulk water density shows a maximum at 4 °C under ambient conditions, leading to lower density in the solid phase [35–38], the volume increase on crystallization to ice I_h, together with the increase in the heat capacity C_p and the isothermal compressibility K_T with decreasing temperature T [32, 33, 39, 40], all of which are very different from the behavior of “normal” liquids [41]. To explain the origin of various water anomalies, several potential scenarios have been proposed [40, 42–44], with the possibility of the existence of the liquid-liquid phase transition (LLPT) being currently the most widely discussed model [41, 44]. For example, by using water-glycerol mixtures to avoid water crystallization, Murata & Tanaka have revealed that this system was able to undergo the transition from liquid I to II in the vicinity of 180 K [45]. The authors also showed that there were detectable differences in density, fragility, glass transition temperature, hydrogen bonding state, refractive index, and structure between these liquids I and II [45]. Importantly, although this study analyzed water-glycerol mixtures, the transition between the two liquids was preferentially driven by the local structuring of water and not of glycerol. Based on these observations the authors suggested that additives, such as glycerol, that introduce frustration against crystallization can be used to access the LLPT that could be hidden by crystallization [45]. It was also pointed out that in hydrated proteins at low temperature, there is a strong coupling between the “dynamical” transition; i.e., a transition of a protein from a glassy state with no conformational flexibility and no biological functions to a biologically functional form of a protein at temperatures ≥ 220 K, and the hydration water, which also shows a similar dynamic transition [46]. In fact, a sudden switch in dynamic behavior of the hydration water on lysozyme from predominantly high-density (more fluid state) to low-density (less fluid state) form was shown to occur at 220 K [46].

Although the LLPT separating two amorphous phases of water, the high-density amorphous (HDA) and the low-density amorphous (LDA) water, is typically observed at very low temperatures (well below the water freezing point at ambient pressure), the presence of noticeable structural differences between the two phases of such supercooled water was observed experimentally [41, 47–57]. It was also pointed out that the noticeable density fluctuations can be experimentally observed in ambient water, and that the detected density differences can be caused by the fluctuations between tetrahedral-like and hydrogen-bond distorted structures related to low and high density water, respectively [54]. Based on these observations a water model was proposed, where there is a temperature-dependent, dynamic equilibrium between the two types of local structures driven by the need of a system for minimizing enthalpy (strong near-tetrahedral hydrogen-bonds) and maximizing entropy (non-directional H-bonds and disorder) [54]. This work also suggested that although the hydrogen-bonding environments in the deeply super-cooled water are characterized by the extreme differences, similar differences can be also found at ambient conditions, and even preserved at the conditions close to the boiling point [54].

In our view, the aforementioned liquid-liquid phase transitions in water that produce the co-existing phases of water with different properties can be related to other phase separation phenomena taking place in aqueous media and leading to the formation of ATPSs and PMLOs. It is also possible that phases of water with different properties are related to the dissimilarity of solvent properties of water in different phases of ATPSs/PMLOs, which defines the partition behavior of proteins, nucleic acids, and small molecules in phase separated systems.

3. Types of biphasic (or multiphasic) systems in water

Phase separation in mixtures of different water soluble compounds in aqueous media represents a rather general phenomenon. In fact, depending on the physico-chemical properties of particular compounds, their aqueous mixture may separate in two or more aqueous phases. For example, the highest number of aqueous 18 phases was reported by Albertsson in mixture of six polymers, such as Dextran sulfate, Dextran, and four hydroxypropyl Dextrans with various degrees of substitution [1]. The list of over 300 phase-separated systems containing from two to six aqueous phases in mixtures of various polymers and surfactants was reported by Mace et al. [58]. Unfortunately, it is currently unknown how the number of phase-forming components is related to the number of phases formed in their mixture. However, all these systems may be considered reversible in regard to concentrations of phase-forming components. In fact, dilution of a system would result in disappearance of the phases and return to the desirable concentrations of the component would result in the formation of the phases. It was also pointed out that such systems may be used to form stable density step-gradients in water with small differences between the densities of the phases (about 0.001 g/cm³) and have been demonstrated to serve as means for separation of nanoparticles [59], different forms of erythrocytes to diagnose sickle cell disease [60] and iron deficiency anemia [60,61], and for isolation of the reticulocyte-enriched fractions from blood [62].

It should be mentioned that all the synthetic aqueous two-phase systems are reversible (meaning that dilution of the mixture results in a homogeneous solution and return to the original composition of the mixture results in formation of original two-phase system) and reproducible (aqueous mixture of the fixed concentrations of all components always separates into two (or more) equilibrium phases of the fixed compositions, no matter if the separation of the phases occurs under centrifugation or by maintaining the mixture at the same temperature for a prolonged time). Despite the aforementioned ability to generate the multi-phase separated systems, phase separation is more often studied, however, in the so-called ATPSs generated in mixtures of two phase-forming compounds (in the presence or absence of certain additives). In such mixtures, two immiscible coexisting aqueous phases are formed once the concentrations of the phase-forming compounds exceed particular thresholds. Under fixed environmental conditions (temperature and composition of the reaction mixture), the process of ATPS formation is spontaneous and completely reversible, and the formed

phase-separated systems are in complete equilibrium, thermodynamically stable, and reversible. However, the rates of phase separation and ATPS formation depend on the composition of a system undergoing phase transition, with PEG-salt systems typically separating relatively fast (minutes) and with two polymer-based systems (e.g., PEG-Ficoll) usually undergoing slow (hours) separation.

There is typically a clear interfacial boundary separating two distinct aqueous-based phases, each preferentially enriched in one of the compound, with the aqueous solvent in both phases. The ATPSs may also be formed in aqueous solutions of a single compound, such as thermoresponsive polymer [63,64], or stimulus responsive polypeptide [65,66], or surfactant [67–69]. Here, once the particular temperature threshold is reached, the two-phase system is formed with one phase enriched in the compound (polymer, polypeptide or surfactant), and the other phase essentially free of the compound. All these systems are unified by the fact the each of the phases typically contains well over 80% water on a molal basis, and yet they are immiscible and differ in their solvent properties (see below).

The components in an ATPS formed by two compounds commonly include two polymers [1, 2, 70–72], single polymer and salt [1, 2, 70–72] or surfactant [73, 74], two different surfactants [75], and ionic liquids [13, 76, 77]. There are also ATPSs formed by proteins and polysaccharides [78–83]. Recently, the interest to such ATPSs increased due to findings that multiple proteinaceous membrane-less organelles (PMLOs), which can be created as a result of highly controlled biological phase separation, are observed in cells in vivo [20–28].

The particular pairs of proteins forming ATPSs are listed in [81] and shown in Table 1. Table 1 illustrate that an important feature of the protein pairs capable of phase separation is that at least one member of the pair contains high levels of intrinsic disorder (i.e., a protein that naturally contain long regions without unique structure, a property that can be identified by application of sequence-based computational tools for the evaluation of intrinsic disorder predisposition of a query protein) or are denatured (i.e., a globular protein that underwent irreversible thermal denaturation). This suggests that, probably, intrinsic disorder and high conformational dynamics play an important role in the overall fluidity of phases in the resulting ATPSs. This is in agreement with recent finding that intrinsically disordered proteins play crucial roles in the biogenesis of the PMLOs [22, 25, 26].

The original list of nonionic and ionic polymers capable of forming ATPS was presented by Albertsson [1], and only a few additions to the list were made recently. The most widely used and studied pairs of

Table 1
Pairs of proteins capable of the phase separation and formation of ATPSs.

System	Conditions of phase separation	Disorder content of protein #1 ^a	Disorder content of protein #2 ^a
Ovalbumin (#1) – temperature-denatured ovalbumin (#2)	pH 6.7; water; 20 °C	18.39% (49)	denatured
Bovine serum albumin (#1) – temperature-denatured ovalbumin (#2)	pH 6.7; water; 20 °C	30.95% (46)	denatured
Bovine serum albumin (#1) – Gelatin/collagen alpha-1 type II (#2)	pH 7.8; 1 ≤ 1 (NaCl); 40 °C	30.95% (46)	83.05% (1213)
Fibrinogen (#1) – SB globulin fraction (#2)	pH 7.9; 1 = 0.4 (NaCl); 25 °C	80.98% (357)	N/A ^b
Ovalbumin (#1) – Glycinin (#2)	pH 6.8; water; 20 °C	18.39% (49)	53.20% (202)
Ovalbumin (#1) – SB globulin fraction (#2)	pH 6.6; water; 20 °C	18.39% (49)	N/A ^b
Bovine serum albumin (#1) – Fibrinogen (#2)	pH 6.6; water; 20 °C	30.95% (46)	80.98% (357)
Bovine serum albumin (#1) – Glycinin (#2)	pH 6.7; water; 20 °C	30.95% (46)	53.20% (202)
Bovine serum albumin (#1) – SB globulin fraction (#2)	pH 4.9; 1 = 0.39 (NaCl); 25 °C	30.95% (46)	N/A ^b
Gelatin (#1) – Legumin (#2)	pH 7.0; water; 40 °C	83.05% (1213)	48.94% (123)
Gelatin (#1) – BB globulin fraction (#2)	pH 7.0; water; 40 °C	83.05% (1213)	N/A ^b
Bovine serum albumin (#1) – Gliadin (#2)	pH 11.0; water; 25 °C	30.95% (46)	80.78% (163)
Ovalbumin (#1) – Casein (#2)	pH 6.6; water; 20 °C	18.39% (49)	50.90% (67)
Bovine serum albumin (#1) – Casein (#2)	pH 6.9; 1 = 0.5 (NaCl); 25 °C	30.95% (46)	50.90% (67)
SB globulin fraction (#1) – Gliadin (#2)	pH 10.5; water; 25 °C	N/A ^b	80.78% (163)
Fibrinogen (#1) – Casein (#2)	pH 6.4; 1 = 0.41; (NH ₄) ₂ SO ₄ ; 25 °C	80.98% (357)	50.90% (67)
Glycinin (#1) – Casein (#2)	pH 6.6; water; 20 °C	53.20% (202)	50.90% (67)
BB globulin fraction (#1) – Casein (#2)	pH 7.0; water; 20 °C	N/A ^b	50.90% (67)
SB globulin fraction (#1) – Casein (#2)	pH 6.9; water; 25 °C	N/A ^b	50.90% (67)
Gliadin (#1) – Casein (#2)	pH 11.0; water; 25 °C	80.78% (163)	50.90% (67)

^a Disorder content was evaluated by PONDR® VSL2 (<http://www.pondr.com/>) as a percent of the residues predicted to be disordered. Values in brackets show length of the longest disordered region in a query protein.

^b Corresponding values are not available, since exact compositions of the BB and SB globulin fractions are unknown.

Table 2
Aqueous two-phase systems used for partitioning of small organic compounds.

Polymer-1	Polymer-2
Dextran	Polyethylene glycol (PEG)
Dextran	Ficoll
Dextran	Polyvinylpyrrolidone
Dextran	Polyvinyl alcohol
Dextran	Ucon (copolymer of ethylene glycol and propylene glycol)
Dextran	Polypropylene glycol
Ficoll	Polyethylene glycol (PEG)
Ficoll	Ucon (copolymer of ethylene glycol and propylene glycol)
Polyethylene glycol (PEG)	Polyacrylamide
Polyethylene glycol (PEG)	Ucon (copolymer of ethylene glycol and propylene glycol)
Hydroxypropyl starch (PES)	Dextran
Hydroxypropyl starch (PES)	Ficoll
Hydroxypropyl starch (PES)	Ucon (copolymer of ethylene glycol and propylene glycol)

nonionic polymers forming ATPS are presented in Table 2. Most of the ATPS formed by the polymers listed in Table 2 may be used for analytical applications. The reasons will be discussed below. In some cases, these ATPS may be used for separation of biomolecules or particles but commonly only on the laboratory scale, since the cost of fractionated polymers (i.e., homogeneous polymers of fixed polymerization degree) is prohibitive for industrial separation purposes.

For industrial separations, the polymer-salt ATPSs are typically discussed. Examples of such systems are listed in Table 3. There is a vast variety of salts capable of forming ATPSs in mixtures with the polymers listed in Table 3. It has been suggested by Ananthapadmanabhan and Goddard [84] that only kosmotropic salts, such as sodium sulfate or phosphate, are able to form ATPS with polyethylene glycol (PEG), but this assumption was found to be incorrect later when PEG-NaClO₄ ATPSs were described [85, 86]. Since phosphates and sulfates present environmental hazard, the search for more environmentally benign salts capable to form ATPSs, being mixed with polymers, intensified lately. As a result, citrates, tartrates, and other organic salts were found to meet the necessary criteria. The advantages of the polymer-salt ATPSs for industrial separation purposes are relatively low cost, high speed of phase separation, and low viscosity of the phases. The disadvantages from the viewpoint of their use for analytical applications are relatively high temperature sensitivity and large differences between the ionic compositions of the coexisting phases (see below). It is also important to emphasize here that the range of polymers and salts concentrations used for the ATPS generation shows large variability, being dependent on the temperature, polymer molecular weight, and additives (such as buffer composition, concentration and nature of buffer salts), as well as on the type, molecular weight, and concentration of the partner (second polymer or salt).

ATPSs may also be formed in aqueous solutions of surfactants at certain surfactant concentrations and temperatures. Homogeneous aqueous micellar solution in this case separates into two aqueous phases with different concentrations and sizes of micelles in the phases. Such ATPS may be formed in a single surfactant solution or in mixtures of different surfactants, and they have been reported to be utilized successfully for separation of proteins and viral particles [68, 87–93]. An interesting group of ATPS are those formed by a single polymer and surfactant, such as PEG-Triton X-100 or Dextran-octylglucoside [73]. These ATPS were used for fractionation of membrane proteins prior to MS-

analysis [94]. There is rather limited number of published studies of these ATPS, and they will not be considered here in any detail.

As the subjects of this review are phase separation in ATPSs, the properties of the coexisting aqueous phases governing distribution of biological compounds between these phases, and similarities and differences between the polymer-based ATPSs and protein-based PMLOs, only polymer-polymer and to lesser degree polymer-salt ATPSs will be discussed below because the studies of the factors affecting these processes were mostly reported for these two types of ATPSs.

4. Biological LLPTs and proteinaceous membrane-less organelles

4.1. Diversity of proteinaceous membrane-less organelles

As it was aforementioned, recently, there is a dramatic increase in the interest to ATPSs among many researchers in the biological community. This is because of an important finding that cells contain multiple proteinaceous membrane-less organelles (PMLOs), which are likely to be created via highly controlled biological LLPTs in vivo [20–28]. In the norm, the intracellular space of a living cell is characterized by inhomogeneity and crowdedness, where biological macromolecules (such as protein, nucleic acids, and polysaccharides) are present at high concentrations, occupying up to 40% of the cellular volume [95–101]. Furthermore, eukaryotic cells contain multiple membrane-encapsulated organelles, such as autophagosomes, chloroplasts (found in plant cells only), rough and smooth endoplasmic reticulum, Golgi apparatus, liposomes, lysosomes, mitochondria, nucleus, peroxisomes, secretory vesicles, and vacuoles, which provide discrete and specialized microenvironments with diverse physicochemical properties and therefore possess a set of specific and well-characterized functions. In addition to these “traditional” membrane-bound organelles found exclusively in eukaryotes, both eukaryotic and prokaryotic cells are known to possess numerous PMLOs. Although the presence of the dark staining granules at one pole of the *Musca domestica* (fly) larvae was mentioned by Ilya Metschnikoff for the first time >150 years ago [102], and although subsequent studies clearly showed that such “polar granules” are present in a variety of insect species [103, 104], as well as in worms [105] and frogs [106], where they play an important role in germ cell specification, the overall importance and versatility of PMLOs were mostly neglected for a long time. In part, this was due to the inability to purify these cellular bodies because of their membrane-less nature and highly dynamic liquid-like structure. The situation is changing now, and it is recognized that the cytoplasmic and nuclear PMLOs represents a large set of diverse cellular compartments, which are implicated in important biological processes. They are cell size-dependent and highly dynamic, often optically observed as spherical micron-sized droplets [107]. Many PMLOs have unique morphologies and specific distribution patterns within a cell, contain specific sets of resident proteins and typically have RNA, and therefore are commonly known as ribonucleoprotein (RNP) granules/bodies or RNP droplets [20].

Below, we are briefly presenting some of the eukaryotic and bacterial PMLOs. Detailed description of several cellular bodies and illustrative examples of proteins typically found in these cellular bodies are given elsewhere [26]. By their localization inside a eukaryotic cell, PMLOs can be classified as cytoplasmic, nuclear, mitochondrial, and chloroplast bodies. There is only one type of PMLO in chloroplasts (chloroplast stress granules (cpSGs)) [108], and mitochondria contain only mitochondrial RNA granules [109]. Cytoplasmic PMLOs include Balbiani body [110], centrosomes [111], cytoplasmic prion protein-induced RNP granules [112], germline P-granules (germ cell granules or polar granules, or nuage) [113, 114], GW/P bodies (glycine- and tryptophan-rich cytoplasmic bodies; also known as mammalian processing (P) or Dcp-containing bodies) [115, 116], mitochondrial cloud [117], neuronal transport RNA granules [118], processing bodies or P-bodies [119], RNP granule (grP body) in arrested or stressed oocytes [120], RNP-rich

Table 3
Aqueous two-phase systems used for partitioning of small organic compounds.

Polymer	Salt
Polyethylene glycol (PEG)	Phosphate, sulfate, citrate, tartrate, etc.
Polypropylene glycol	
Polyvinylpyrrolidone	
Ucon (copolymer of ethylene glycol and propylene glycol)	

cytoplasmic germline granules or chromatoid bodies [116], Sec bodies (that are formed in *Drosophila* cells during the amino-acid starvation by sequestering the components of the ER exit sites, ERES) [121], sponge bodies [122], stress granules (SGs) [123], subcortical aggregates (SCA) [124], temporal asymmetric MRP bodies (TAM) bodies that are present exclusively during mitosis [125], and U bodies containing uridine-rich small nuclear ribonucleoproteins U1, U2, U4/U6 and U5 (U snRNPs) [126].

Nuclear PMLOs include amyloid bodies (A-bodies), [127], Cajal bodies (CBs) [128], chromatin [129], cleavage bodies [130], DNA damage foci [131, 132], histone locus bodies (HLBs), [133], nuclear gems (Gemini of coiled of Cajal bodies) [134, 135], nuclear HSF1 (heat shock factor 1) granules [136], nuclear pores [137], nuclear speckles or interchromatin granule clusters [138], nuclear stress bodies (nSBs) [139, 140], nucleoli [141], Oct1/PTF/transcription (OPT) domains [142], paraspeckles [143], PcG bodies (polycomb bodies, subnuclear organelles containing polycomb group proteins) [144], perinuclear compartment (PNC) [145], promyelocytic leukemia nuclear bodies (PML nuclear bodies) or PML oncogenic domains (PODs) [146], and the Sam68 nuclear bodies (SNBs) [145].

Although prokaryotic cells do not have membrane-encapsulated organelles, bacteria are known to have complex anatomy at the subcellular level, with their cytoplasm being highly organized. One of the most studied examples of bacterial cytoplasmic compartments is the cell poles found in rod-shaped bacteria [147]. In comparison with other areas of the cytoplasm, the cell poles are characterized by the visibly distinct cytoplasm [147] and contain >80 different proteins [148], with polar organizing protein Z (PopZ) playing a central role in assembly of cell poles and controlling polar localization of at least 11 different proteins [147]. It was also pointed out that PopZ forms a large three-dimensional structure at cell poles, and binding partner proteins diffuse into and out of this network [147]. It was also shown that another bacterial protein, FtsZ, which is the main component of the bacterial division machinery, is able to undergo LLPTs related to the formation of the bacterial septal ring [149].

4.2. Major characteristics of PMLOs

As it follows from the list of cytoplasmic, nuclear, mitochondrial, and chloroplast bodies (one should keep in mind though that this list is far from being complete since new subcellular domains are discovered in a regular basis), PMLOs are highly diversified. Besides differences in cellular localization, they have rather different morphologies, possess divergent functions, and are typically characterized by highly dissimilar compositions (in fact, partial overlap was reported for the contents of only a few PMLOs). For example, size of various sub-nuclear bodies ranges from 20 nm to 5 μ m, with Cajal bodies, polymorphic interphase karyosomal associations (PIKAs), promyelocytic leukemia (PML) bodies, paraspeckles, and speckles showing as liquid droplets with the diameter of 0.2–2.0 μ m, 5 μ m, 0.2–1.0 μ m, 0.2–1.0 μ m, and 20–25 nm, respectively. A key spindle pole-associated PMLO, centrosome, possesses characteristic morphology with a core surrounded by electron dense pericentriolar material containing numerous proteins. The core of a centrosome includes a pair of orthogonally arranged, nine-fold symmetrical sub-organelles, centrioles, each representing a 500 nm long cylinder with the diameter of ~200 nm [150]. The amount and composition of the pericentriolar material changes in mitosis, giving rise to the nucleating center for spindle and astral microtubules [150]. Nucleolus that serves as major place of the assembly of ribosomal subunits in eukaryotic cells is a highly dynamic and tightly regulated organelle characterized by noticeable cell cycle-dependent (or pathology-related) changes in size, shape, and morphology [151]. Furthermore, nucleolus undergoes dramatic ultrastructural and morphological changes, as well as molecular modulations and changes in protein composition when a cell is reprogrammed to a new phenotype [152]. Finally, PIKAs (also known as OPT domains) that correspond to sites of sensing or

repair of DNA damage are distinguished by an extreme variability of size and number, being present in nuclei of some cells as a single, large, roughly spherical domain up to 5 μ m in diameter or as dozens of small punctuate foci in other cells [153].

However, despite these functional, compositional, and distributional differences, PMLOs are grouped together based on their membrane-less and highly dynamic nature and also because all of them invariantly contain proteins and often include RNA or DNA. This clearly indicates that proteins, being a common denominator for all the currently known PMLOs, serve as major regulators of the assembly-disassembly cycles, dynamics, morphology, and structure of various subcellular bodies.

Since protein composition dramatically varies between different PMLOs, it is clear that many different proteins located in different cellular regions are able to regulate the biogenesis of different subcellular bodies. A bounding property of PMLO-associated proteins is the presence of significant levels of intrinsic disorder [22, 25, 26, 154–163]. Unfortunately, current literature contains rather limited information on proteins experimentally shown to undergo liquid-liquid phase separation in aqueous solutions alone or in mixtures with other proteins, nucleic acids, or polysaccharides. However, all such proteins shown so far to undergo LLPTs are either entirely intrinsically disordered or are hybrid proteins containing ordered domains and long intrinsically disordered regions [26]. Paragraph below provides a short description of the protein intrinsic disorder phenomenon.

Current literature provides compelling evidence that biological activity of many proteins is not associated with the lock-and-key model, and, instead, many proteins with a wide spectrum of crucial biological do not have specific tertiary structures [164–170]. These proteins/regions are abundantly found in various proteomes [167, 171–177], where they play important roles in recognition, regulation, and control of various signaling pathways [178–180]. In comparison with their ordered counterparts, these intrinsically disordered proteins and regions contain less order-promoting residues, such as Ile, Leu, Val, Trp, Tyr, Phe, Cys, and Asn, and are enriched in the disorder-promoting residues, Ala, Arg, Gly, Gln, Ser, Pro, Glu, and Lys [167, 181–183]. Structurally, intrinsically disordered proteins and regions represent highly dynamic conformational ensembles characterized by amazing structural plasticity and heterogeneity that include combinations of non-foldons (non-foldable protein regions), foldons (independent foldable units of a protein), semi-foldons (regions which are always in semi-folded state), inducible foldons (disordered regions that can fold at least in part due to the interaction with binding partners), and unfoldons (regions that have to undergo an order-to-disorder transition to become functional) [184, 185]. Different parts of intrinsically disordered proteins and regions are ordered (or disordered) to a different degree and this distribution is changing with time [184]. Among important functional features attributed to these structure-less proteins and regions are their ability for high specificity – low affinity binding [186], capability to serve as hub proteins in various protein-protein interaction networks [179, 187–192], to be engaged in promiscuous interactions with unrelated partners [193], to fold at interaction with a partner [194–197], to adopt different structures upon binding to different partners [164, 193, 198–202], to preserve significant levels of disorder even in the bound state [203–209], and to be able to return to their highly dynamic and pliable conformations after the completion of a particular function [184]. Disordered regions contain of various posttranslational modifications and proteolytic attack [210–212]. Finally, disease-associated mutations often lead to the disruption of sites of posttranslational modifications located within disordered regions [213], and, in general, pathogenesis of various human maladies, such as cancer, cardiovascular disease, amyloidosis, diabetes, and neurodegenerative diseases is linked to the misbehavior of intrinsically disordered proteins and regions [178, 214–220].

Since formation disassembly of PMLOs is driven by highly controlled biological LLPTs *in vivo* [20–28], these observations imply that the presence of intrinsic disorder represents an important property that defines

the capability of a protein to undergo LLPT. One of the possible explanations for the critical involvement of intrinsic disorder in biological LLPTs is the ability of disordered proteins and regions to be involved in multiple specific, but weak interactions. One also should keep in mind that phase separation can typically take place only when a specific concentration threshold of the macromolecule undergoing LLPT is reached [83, 221]. Since at any given moment, many different proteins and nucleic acids can be present at high enough concentrations to promote LLPTs, a living cell is capable of possessing of several coexisting liquid phases simultaneously [221–224].

Physically, PMLOs, which lack membrane encapsulation, behave as fluid, liquid-like entities, being able to drip, wet, fuse, and form spherical structures upon fusion [113, 123, 225, 226]. Since the major biophysical properties of PMLOs are rather similar to those of cellular cytoplasm, nucleoplasm, matrix, or stroma (e.g., the density of PMLOs is only slightly higher than that of the surrounding intracellular fluids [227, 228]), these subcellular bodies represent a different liquid state of those intracellular fluids [20]. In fact, PMLOs are characterized by high internal dynamics and fluidity, and are classified as liquid-droplet phases of the corresponding intracellular fluids, such as cytoplasm, matrix, nucleoplasm, and stroma [30, 113, 123, 225, 226, 229].

Being membrane-less, PMLOs are characterized by a highly dynamic nature, with their interior and components being constantly involved in direct contacts with their surrounding cytoplasm, nucleoplasm, mitochondrial matrix, or stroma of the chloroplasts [230, 231]. Despite their absence of enclosing membranes that support the integrity of normal, membrane-bound organelles, PMLOs are characterized by the structural integrity which is supported by dynamic protein–RNA, protein–protein, and protein–DNA interactions [23]. To be able to form liquid-like PMLOs that should rapidly disintegrate in response to changes in their environment, the related components should be engaged in specific but weak interactions. Therefore, among important features of proteins undergoing LLPTs and potentially responsible for the PMLO formation are presence of repetitive units, multivalency, flexibility, enrichment in some specific residues, and accessibility to post-translational modifications; i.e., features commonly found in intrinsically disordered proteins and regions [25, 26]. It is likely that similar interactions are responsible for the biogenesis of PMLOs.

Since currently available information on the physico-chemical properties of solvent inside the PMLOs is rather limited, we are presenting below an overview of the well-studied phase separated systems formed by polymers in aqueous media. These ATPSs can be considered as simple physical models PMLOs, with many regularities related to the formation and properties of ATPs being potentially used for better understanding of the biological LLPTs and resulting PMLOs.

5. Basic characteristics of aqueous two-phase systems

5.1. Phase diagrams

No matter what particular pair of polymers or a single polymer and inorganic or organic salt is used for ATPS formation, the first step in characterization of the system is construction of the phase diagram, where the compositions of the two coexisting phases are presented for various overall compositions of mixtures of two polymers (or a single polymer and salt) in water as illustrated in Fig. 1.

The curved line (the binodal curve) separates two regions of compositions. All compositions below the binodal line correspond to a homogeneous single-phase region, while those above the binodal line correspond to the region of two-phase systems. Any mixture of polymers and/or salts with composition in this region separates into a two-phase system. Points representing the compositions of the top and bottom phases lie on the binodal curve. The line connecting the compositions of the two coexisting phases and the overall composition of the system is called a tie line. The length of the tie line decreases as the concentrations of the two polymers (or polymer and salt) in a

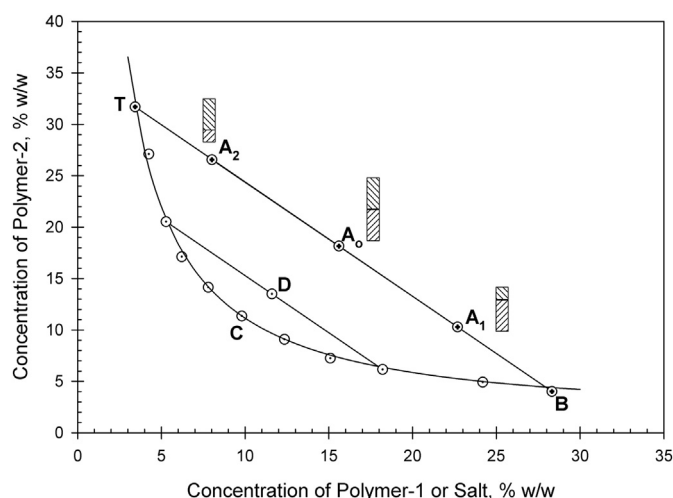


Fig. 1. An example of a typical phase diagram. A_0 , A_1 , and A_2 represent the total compositions of three different systems lying on the same tie-line with different volume ratios and the same compositions of the coexisting phases represented by points B (bottom phase) and T (top phase). Point D represents the total composition of the system lying on the different tie-line. C – critical point.

given ATPS are reduced. At a certain point called the critical point (denoted point C in Fig. 1), the compositions of the two coexisting phases are identical [1, 2, 70–72]. The binodal curve may be determined by turbidimetric titration [232]; i.e., dilution of the polymer mixture until reaching polymers concentrations corresponding to homogeneous solution. Increasing polymers concentrations to the original level results in generation of the ATPS of the same composition. Tie lines may be determined using different analytical assays to measure the polymer and/or salt concentrations in both coexisting phases.

It should be emphasized that in contrast to typical organic solvent–water biphasic systems, any selected pair of polymers (or a single polymer and salt) in water may form a vast variety of two coexisting phases of different compositions and properties (see below). On the other hand, the overall system composition may be varied along a given tie-line (in Fig. 1 points A_0 , A_1 , A_2), and in this case the two-phase systems will all have the same compositions of the coexisting phases, but varied ratio of volumes of the phases (see in Fig. 1). Any overall system composition located off a given tie line (point D in Fig. 1) forms an ATPS with compositions of the two phases different from those corresponding, e.g. to the system represented by point A_0 .

Any ATPS with extremely high (or low) ratio of the two volumes on a given tie-line would have the overall composition very close to the composition corresponding to point T or point B. Such composition, being in the vicinity of the binodal line, would be extremely sensitive to any changes in such external factors as concentrations of phase forming polymers and additives (salts, co-solutes, temperature, etc.), and this situation appears to be the one describing formation of membrane-less organelles of very small volumes formed in a relatively large volume of cytoplasm or nucleus in the presence of crowding environment and likely fluctuating concentrations of various large and small co-solutes in vivo.

Typically, a given ATPS is prepared by mixing two or more stock solutions of polymers and salts. Each stock solution is diluted by the other stock solutions, and therefore, the dilution factor must be taken into account in order to estimate the desirable concentration of each ingredient in each stock solution. The stock solutions are prepared by weight, and it should be emphasized here that for such hygroscopic polymers as Dextran and Ficoll, it is necessary to prepare the stock solution in water and determine the true polymer concentration by freeze-drying several aliquots or some other method. The experimental protocols recommended for these purposes are given in [233].

Since a phase diagram is a geometrical representation of a two-phase composition, there are two commonly used geometrical descriptors of the position of the ATPS of a particular overall composition on the phase diagram. First, it is important how far away from the critical point the ATPS position is. The reason is that an ATPS with composition close to that of the critical point is highly sensitive on temperature and salt composition. On the other hand, it may be difficult to prepare an ATPS with composition very far from the composition of the critical point. The necessary concentrations of the stock polymer solutions have to be high. Concentrated solutions of many polymers, such as Dextran or Ficoll, are highly viscous. This translates into reduced accuracy of dispensing stock solution in a mixture. In order to describe the distance of the ATPS composition from the critical point, the so-called tie-line length for the ATPS is commonly used [1, 2, 70–72].

The tie-line length, TLL, is described as:

$$\text{TLL} = [(\Delta C_P)^2 + (\Delta C_Q)^2]^{0.5} \quad (1)$$

where (ΔC_P) is the difference between the concentrations of polymer P in the coexisting phases and (ΔC_Q) is the difference between the concentrations of polymer (or salt) Q in the coexisting phases. The larger the TLL value is, the further away the ATPS composition is from the critical point.

Another important characteristic of the phase diagram is the slope of the tie-line, STL, described as:

$$\text{STL} = \Delta C_P / \Delta C_Q \quad (2)$$

where all parameters are as defined above. The STL value characterizes the asymmetry of the phase diagram. It should be mentioned that STL was found to be constant in polymer-polymer ATPSs. In the case of polymer-salt ATPSs, the STL-value changes with the distance from the critical point according to experimental data from some authors [234–237], while it is constant according to the experimental data from the other authors [238–240]. The question remains open and requires further investigation.

The number of phase diagrams reported for polymer-salt ATPSs tremendously exceeds that for polymer/polymer ATPSs. The apparent reason is the limited number of commercially available water-soluble polymers, and, even more importantly, the perspective of practical applications of ATPSs in separation of biotechnological products. As aforementioned, polymer-salt ATPSs separate into two phases much faster, are less viscous, and cheaper than polymer/polymer ATPSs. There is a vast variety of inorganic and organic salts capable to form ATPSs with polyethylene glycol (PEG), polypropylene glycol (PPG), copolymer of ethylene glycol and propylene glycol (Ucon), and polyvinylpyrrolidone (PVP). The data reported commonly include the compositions of the coexisting phases formed by one or two molecular-weight fractions of a given polymer and one or more inorganic or organic salts at three-four different temperatures (typical data can be found in [241–243]).

Tie lines for each ATPS may be assigned by application of the lever arm rule to the relationship between the mass phase composition and the overall system composition. This rule may be expressed by the ratio of segments AB and AT (see Fig. 1) that can be estimated graphically by using the weight ratio:

$$V_{\text{top}} * \rho_{\text{top}} / V_{\text{bottom}} * \rho_{\text{bottom}} = \text{AB/AT} \quad (3)$$

where V and ρ are the volume and density of the top and bottom phases, correspondingly. An example of phase diagrams determined in this manner is described by Huddleston et al. [234]. This approach requires accurate determination of volumes of coexisting phases. Preparation of an ATPS of a rather large volume in a volumetrically calibrated tube is necessary for this purpose as well as measurements of the densities of both phases.

A high throughput approach was suggested by Hubbuch and co-workers [239, 244–246]. An elegant novel solution suggested in [244] is that the tie lines may be determined without measuring volumes of the two phases. It is possible to calculate them just from the densities of the phases and total mass and volume of a given ATPS. The lab-on-a-chip micro fluidic density sensor was integrated with a liquid handling workstation to measure the densities of the phases [244]. A number of phase diagrams for PEG-400-, PEG-600-, and PEG-1000 potassium phosphate ATPSs including those with NaCl additive determined in this manner was reported [244]. The particularly interesting experimental observation was that the slope of the tie-line was constant for each phase diagram determined [244].

The accuracy of the phase composition determination is based on the accuracy of concentrations measurements of the two main ATPS-forming components. It also includes the fact that three points – overall ATPS composition, and compositions of the coexisting phases must be connected by a straight line (tie-line), and it commonly exceeds the accuracy of binodal line determination by turbidimetry analysis. That is probably why the authors reporting binodal lines data only, commonly report how well the data presented are fitted with the so-called Merchuk equation (see, for example, in [234, 247, 248]). It must be emphasized that Merchuk equation [248]:

$$\ln C_P = a + bC_S^{0.5} + dC_S^3 \quad (4)$$

where C_P and C_S are the concentrations of polymer and salt (or polymer-2) on the binodal line is an empirical fitting equation without any physical meaning, and although it fits a lot of experimental binodal lines reported for different polymer-salt and polymer-polymer ATPS, it should not be considered as a verification of the correctness of the experimental data. There is a lot of other even simpler equations that describe the binodal lines even better. The physical model of phase separation in aqueous mixtures of polymers or a single polymer and a salt once generated may provide an equation or a set of equations that would not only fit the binodal curve of an ATPS, but provide some insight in the phenomena that remains an enigma for over 70 years.

It is well known that the polymer molecular weight affects the phase diagram of both polymer/polymer and polymer-salt ATPS. The examples are shown in Fig. 2. The same trend is observed in both types of ATPS, in polymer/polymer (Dextran-PEG in Fig. 2A, data from [2]) and in polymer-salt (PEG- Na_2SO_4 in Fig. 2B, data from [249–251]). It is typical for phase diagrams of both types of ATPSs to shift downward with increasing polymer molecular weight. This shift shows that polymer concentrations required for phase separation decrease with increasing molecular weight of the polymer. This general trend is sometimes considered as supporting the viewpoint that the polymer size and hence the excluded volume effect is driving phase separation [252]. This trend, however, is observed within a set of ATPSs formed with a given polymer only. The effect of the polymer nature on phase separation appears to be not just more important factor than the polymer size, but also clearly contradicts the view of the excluded volume effect as driving phase separation.

The effect of polymer nature on phase diagrams may be observed in Fig. 3. Phase diagrams for Dextran-PEG, -PVP, -PVA, and -Ucon in the same media (0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.4) are plotted in Fig. 3A (data from [2, 253]). It is readily seen that the highest concentrations of polymers required for phase separation are observed for Dextran-PVP ATPS, while the lowest concentrations are required for Dextran-PVA. Molecular weight for PVP is about 12 kDa, and for PVA about 55 kDa. The polymers concentrations needed for phase separation in Dextran-Ucon and Dextran-PEG systems are between those for the aforementioned ATPSs, though the molecular weight of both PEG and Ucon are relatively low – 6 kDa and 4 kDa, correspondingly. Therefore, these data support the notion that the polymer size is less important for phase separation than the polymer chemical nature. Similarly, in polymer-salt ATPSs as can be seen in Fig. 3B (data from

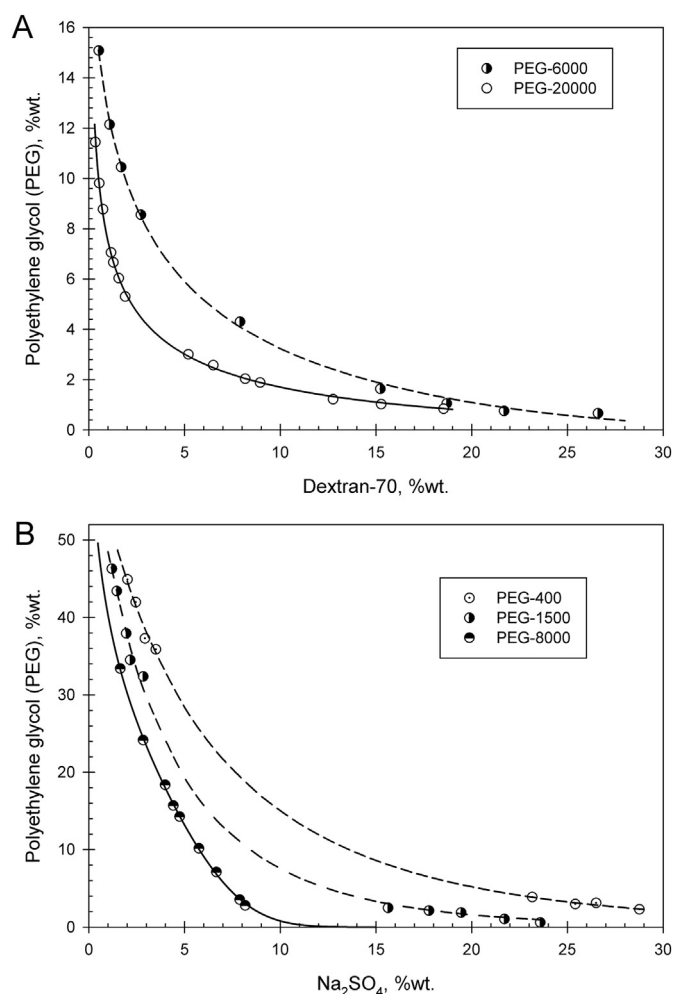


Fig. 2. A. Binodal lines of dextran-polyethylene glycol (PEG) systems formed by dextran-70 (molecular weight of 70,000) and PEG with two different molecular weights: 6000 and 20,000. B. Binodal lines of polyethylene glycol (PEG)-sodium sulfate systems formed by PEG with three different molecular weights: 400, 1500 and 8000. Error bars are the same size or smaller than the symbols.

[254–256]), the concentrations of polypropylene glycol (PPG-725) with molecular weight of 725 Da required for phase separation in aqueous mixtures with sodium citrate are much less than those of polymers with larger size, PEG-4000 and Ucon-4000 (both polymers with molecular weight of about 4 kDa). The binodal lines for these two polymers of the same size in mixtures with sodium citrate are clearly shifted relative to each other. It should be noted that it is currently unknown what chemical or physicochemical features of polymers are important for phase separation in their aqueous mixtures.

The nature of salt in polymer-salt ATPSs has an effect on phase diagram as dramatic as the effect of the polymer nature. Binodal lines plotted in Fig. 4 demonstrate these effects for potassium and sodium carbonates in aqueous mixtures with PPG-400 (Fig. 4A, data from [257, 258]) and for sodium chloride and sodium nitrate in aqueous mixtures with PPG-425 (Fig. 4B, data from [257, 259]). Both differences in the type of the salt cation (Fig. 4A) and type of the salt anion (Fig. 4B) have significant effects on binodal lines.

These effects must be taken into account when pH effect on partitioning of a protein or other solutes in ATPS is studied. In order to change pH, the ratio of salts in a buffer must be altered. An illustration of what happens with PEG-salt ATPSs when pH is varied is shown in Fig. 5. Binodal lines for PEG-10000-KH₂PO₄ (pH < 5.7), PEG-10000-K₂HPO₄ (pH 9–10), and PEG-10000-1:1 mixture of KH₂PO₄ and K₂HPO₄ (pH 6.5–6.7) reported in [260] are presented in Fig. 5. It is

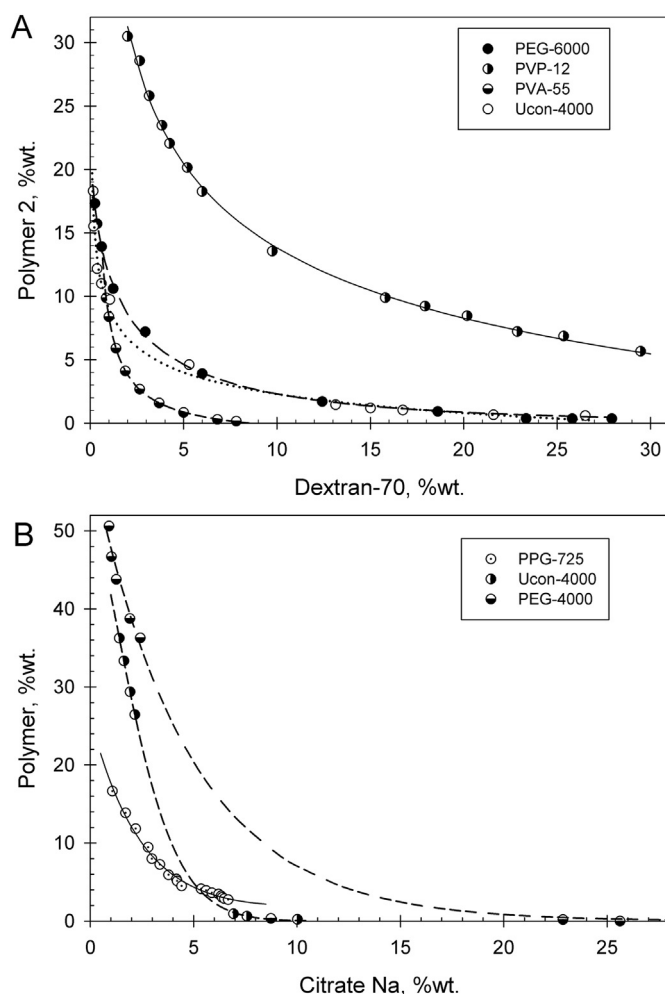


Fig. 3. A. Binodal lines of dextran-70-polymer 2 systems formed by dextran-70 (molecular weight of 70,000) and polymer 2 (polyethylene glycol (PEG) with molecular weight 6000; polyvinylpyrrolidone (PVP) with molecular weight 12,000; polyvinyl alcohol (PVA) with molecular weight 55,000, and Ucon (copolymer of ethylene glycol and propylene glycol) with molecular weight 4000. B. Binodal lines of polymer-sodium citrate systems formed by polypropylene glycol (PPG) with molecular weight 725; Ucon (copolymer of ethylene glycol and propylene glycol) with molecular weight 4000, and polyethylene glycol (PEG) with molecular weight 4000. Error bars are the same size or smaller than the symbols.

obvious that pH change in a PEG potassium phosphate ATPSs with a fixed overall composition leads to very significant changes in the compositions of the coexisting phases. Similar changes are observed in PEG sodium phosphate [261] and in PVP-phosphate buffer [262]. The effect of comparable pH changes in the polymer/polymer ATPSs is much less dramatic but should be taken into account as well.

There are numerous attempts in the literature to consider phase separation in aqueous PEG-salt mixtures in terms of salting-out phenomena [237, 238, 242, 243, 258, 263–272]. These attempts, however, may hardly be viewed as successful in regard to understanding the mechanism of phase separation in polymer-salt ATPSs, since the molecular mechanisms of the salting-out process are unknown. It must be stressed once again that the properties of salts important for their phase separation in aqueous mixtures with polymers are unknown. This can be illustrated by an important observation that despite all knowledge accumulated in this field, it is still unpredictable how the replacement of sodium salt for the same potassium salt or changes in the ratio of phosphate buffer salts, such as Na₂HPO₄ and NaH₂PO₄, would affect the compositions of the coexisting phases in any known ATPSs. Furthermore, although numerous examples exist confirming the importance of salt ions in modifying the water molecules chemical potential resulting

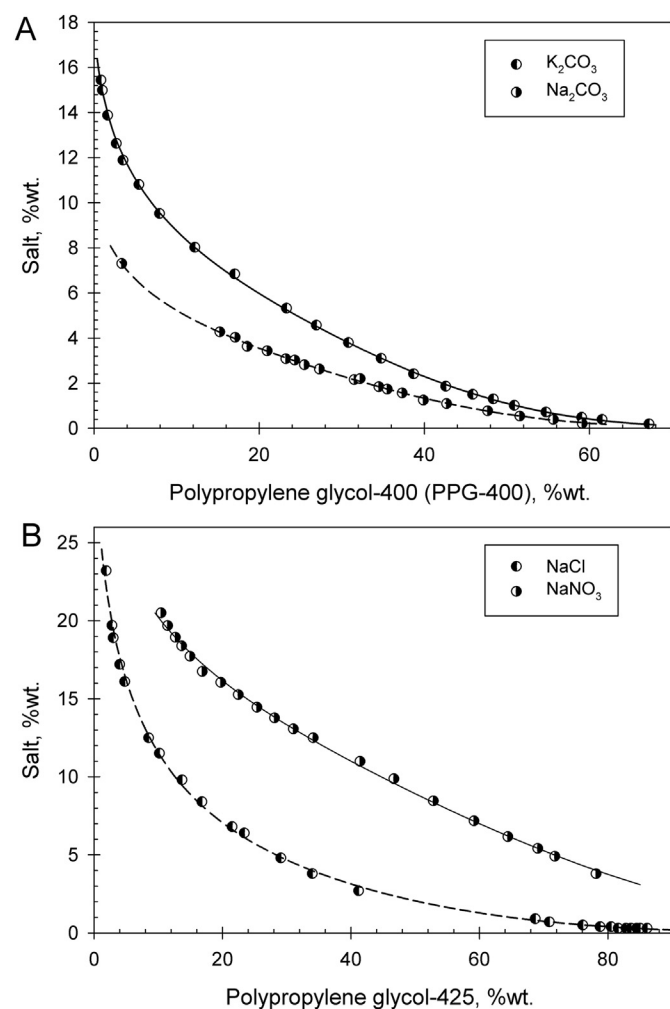


Fig. 4. A. Binodal lines of polypropylene glycol (PPG)-sodium or potassium carbonate systems formed by polypropylene glycol (PPG) with molecular weight 400. B. Binodal lines of polypropylene glycol (PPG)-sodium salts systems formed by polypropylene glycol (PPG) with molecular weight 425 and sodium chloride or sodium nitrate. Error bars are the same size or smaller than the symbols.

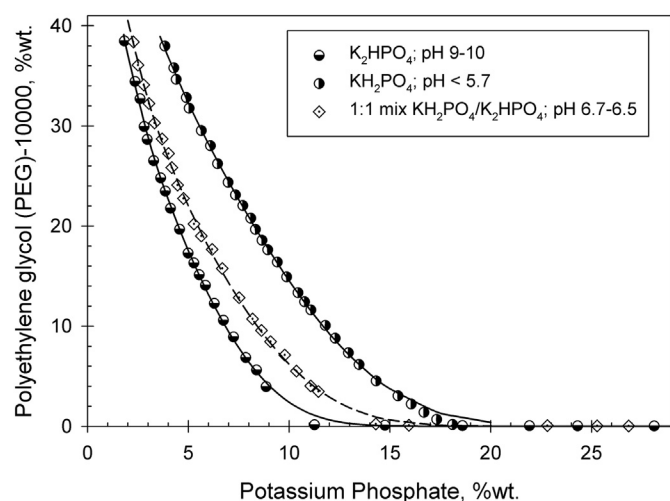


Fig. 5. Binodal lines of polyethylene glycol (PEG)-potassium phosphate systems formed by polyethylene glycol (PEG) with molecular weight 10,000 and different potassium phosphates or their mixture with pH indicated. Error bars are the same size or smaller than the symbols.

in phase separation of the polymers in the solution, the exact mechanisms of salting-out and salting-in effects remain elusive. We cannot, for example, predict what compounds would be salted out and salted in by such well-studied salts as NaCl or $NaClO_4$ [273].

Effects of salt additives on phase separation in ATPSs were studied in polymer/polymer systems [2] much more than in PEG-salt systems, though a few examples were reported [249, 274, 275]. In polymer/polymer ATPSs, the salt additives effects depend on the particular polymers pair, as well as on the type and concentration of the additive(s). The salt effects on phase diagrams of Dextran-PVP and Dextran-PVA ATPSs are much more significant than those in Dextran-PEG or Dextran-Ficoll ATPSs (see in [2], pp. 103–116). In order to compare the effects under discussion in polymer/polymer and polymer-salt ATPSs, the binodal lines for Dextran-70-PEG-6000 (see in [2], pp. 552–558) and PEG-8000- Na_2SO_4 [275] in the presence of the same salt additives at the same concentration of 0.5 M are presented in Fig. 6. It is readily seen that binodal lines in both cases shift down with addition of a given salt and the shift is much more drastic in PEG-sulfate than in Dextran-PEG ATPSs. At smaller additive concentrations the trend is the same but the binodal line shifts are less significant (see in [2], pp. 553–557). It should be mentioned that the similar salt additives affect binodal lines in Dextran-PVP and Dextran-Ficoll ATPSs in completely different

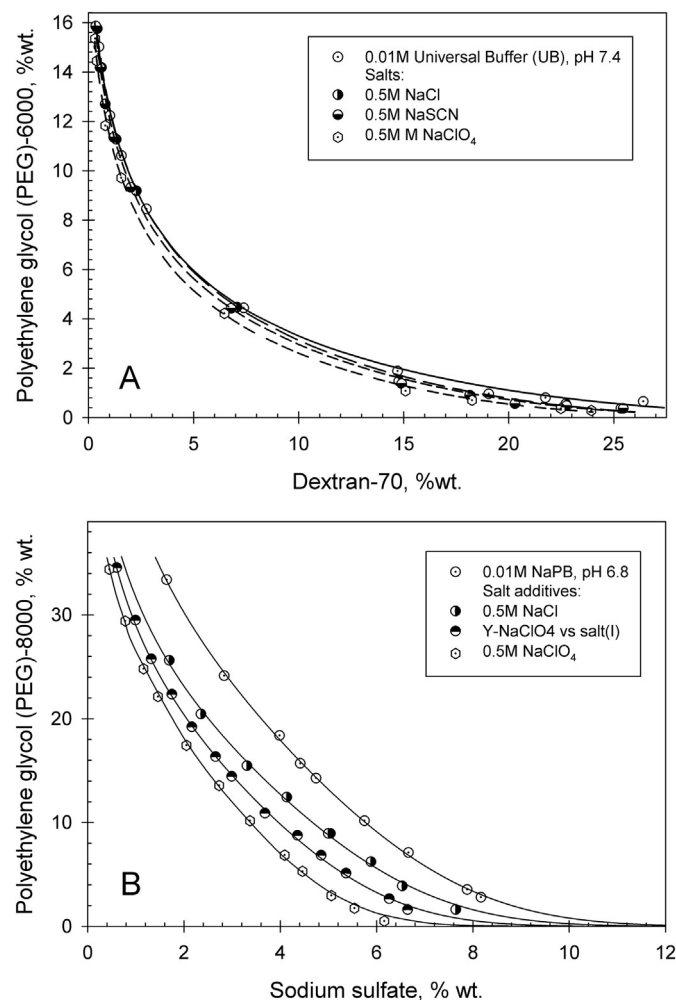


Fig. 6. A. Effects of addition of various sodium salts on binodal lines of polyethylene glycol (PEG)-dextran-70 systems formed by polyethylene glycol (PEG) with molecular weight 6000 and dextran-70 with molecular weight 70,000 in 0.01 M universal buffer, pH 7.4. B. Effects of addition of various sodium salts on binodal lines of polyethylene glycol (PEG)-sodium sulfate systems formed by polyethylene glycol (PEG) with molecular weight 8000 in 0.01 M sodium phosphate buffer, pH 6.8. Error bars are the same size or smaller than the symbols.

manner: KSCN, KClO_4 and KCl induce upward shift of the binodal line (see in [2], pp. 103–122). Again, the mechanism of salt additives effects on phase separation in ATPSs remains essentially unknown.

It is hard to explain why the attention of researchers in the field was lately attracted to studying the temperature effect on phase separation in polymer-salt ATPSs. The experimental data accumulated so far on the temperature effects on phase separation in ATPSs demonstrate essentially opposite effects in polymer-salt and polymer-polymer ATPSs. Increasing temperature in polymer-polymer ATPSs always results in the upward shift of binodal line as illustrated in Fig. 7A for Dextran-70-PEG-6000 ATPS and increased amounts of both or at least one of the two polymers. This observation was explained (see [2], pp. 99–103) based on the assumption that phase separation in these ATPSs is caused by polymer-induced effects on the structure of hydrogen bond network in water. Since temperature increase reduces the stability of the water structure, higher amounts of polymers are needed to form the immiscible water structures [276]. In polymer-salt ATPSs, the temperature effect is opposite as shown in Fig. 7B for PEG-4000- $(\text{NH}_4)_2\text{HPO}_4$ ATPS [238]. An increase in the temperature results in the downward shift of binodal line, which is in agreement with the suggestion [277] that phase separation in polymer-salt ATPSs is similar to that observed in aqueous solutions of nonionic thermoresponsive polymers [278]. This observation does not help in explaining how the phase separation occurs. It may be recommended, however, to consider the

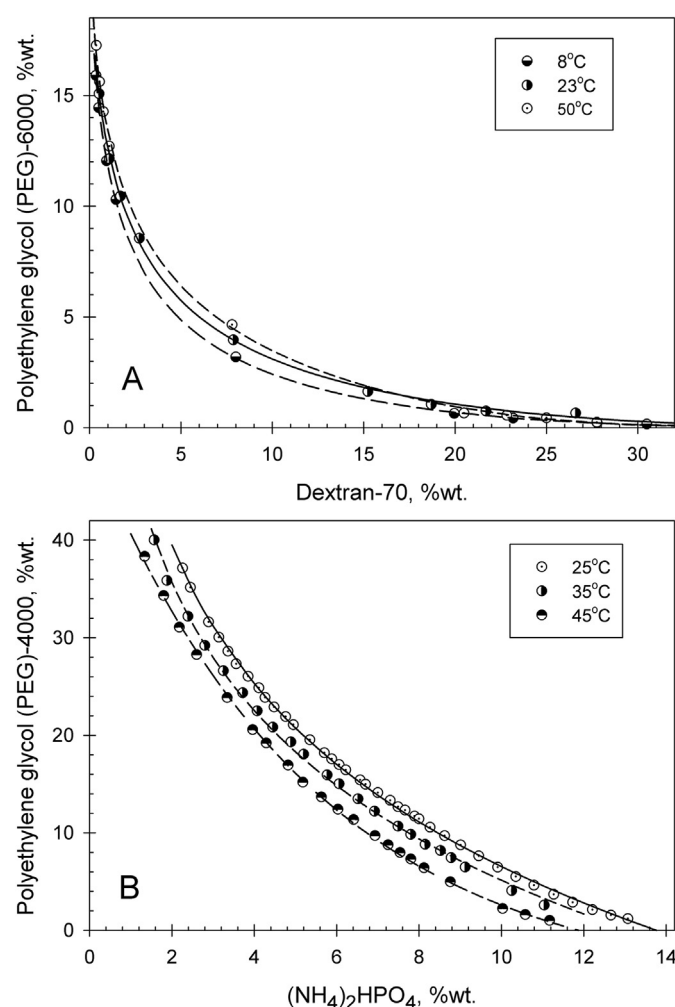


Fig. 7. A. Effect of temperature on binodal line of polyethylene glycol (PEG)-6000-dextran-70 system formed by polyethylene glycol (PEG) with molecular weight 6000 and dextran-70 with molecular weight 70,000. B. Effect of temperature on binodal line of polyethylene glycol (PEG)-4000- $(\text{NH}_4)_2\text{HPO}_4$ system formed by polyethylene glycol (PEG) with molecular weight 4000. Error bars are the same size or smaller than the symbols.

experimental data accumulated for ATPSs in the framework of a larger amount of information and theoretical models already developed in the field of aqueous solutions of thermoresponsive polymers [278].

It should also be noted that all the aforementioned factors (polymer molecular weight, salt additive type and concentration, temperature, etc.) generally affect phase compositions of an ATPS even if the binodal line appears to be unaffected. As an illustration, the slopes of tie lines (STL, see Eq. (2) above) in Dextran-PEG-6000 (see [2], p.102) and in PEG-1500-KOH, PEG-1500- $(\text{NH}_4)_2\text{SO}_4$, and in PEG-1500- ZnSO_4 (see [240]) ATPSs are plotted in Fig. 8 as functions of temperature. One should note here that the behavior of analyzed systems was fully reversible, since cooling them down resulted in a complete restoration of the properties of these systems observed before the heating. The STL changes are clearly different in different ATPSs, but similar changes occur as functions of salt additive concentration, ionic composition, and other factors. The most important general experimental observation in this regard is that any change in overall polymer and/or ionic composition of an ATPS or in external temperature results in changes in the compositions of the coexisting phases. These changes cannot be predicted at present due to the lack of understanding of the mechanisms of phase separation in aqueous polymers and salts mixtures, but these changes must be taken into account when the partitioning data are considered.

A variety of attempts were reported in the literature to use different thermodynamic models for the description of phase diagrams. Current local-composition models, such as Non-Random Two-Liquid (NRTL) model [85, 235, 237, 251, 263, 279–294], Wilson model [85, 282, 284, 295], the group contribution model (UNIFAC) [279, 290], activity coefficient lattice model UNIQUAC [85, 279, 296, 297], as well as those based on Flory-Huggins theory [294, 295, 298], were not able to describe phase diagrams with good accuracy and cannot predict what pairs of polymers or polymer and salt would form two-phase systems in water. It seems that all the attempts to use different thermodynamic models for description of phase diagrams in ATPSs were only partially successful mostly because we currently do not understand the physical forces driving phase separation. These forces clearly involve all types of interactions of polymers and salts with water and each other. Until some theoretical model explaining the interplay between these interactions is developed, phase diagrams will remain just an empirical characteristic of quantitative relationship between the overall composition of a given ATPS and the compositions of the coexisting phases.

It was shown previously for the 1:1 mixture of Dextran and polyvinyl alcohol that even in the case when two polymers are completely compatible (i.e. miscible) in dry state, their mixture in water may form an ATPS (see in [2], pp. 141–152). This fact confirms a suggestion

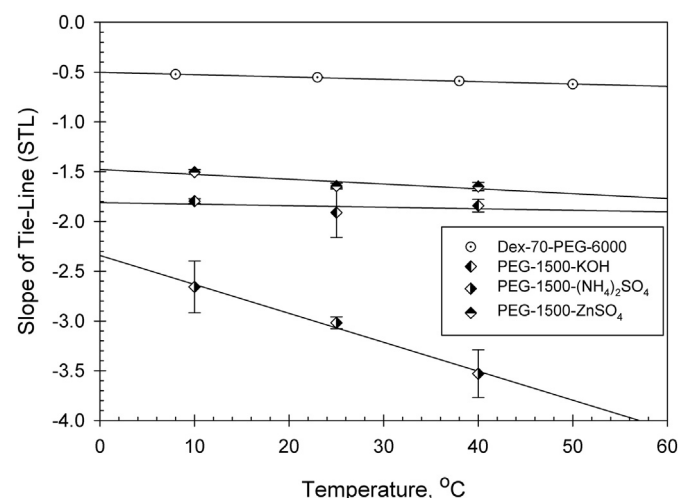


Fig. 8. Temperature dependence of the slope of tie-line (STL) for several ATPSs.

that phase separation in aqueous mixtures of two polymers originates from different effects of polymers on the water structure, resulting in the formation of immiscible hydrogen bonds networks of the same aqueous nature [276].

Phase diagrams for a lot of different ATPSs, in particular, for polymer-salt ATPSs, were reported in the literature over the last two decades (over 400 papers published over 1994–2016 according to the Scopus search). There are several general conclusions that can be drawn so far:

- The slope of a tie line in any polymer/polymer ATPS is constant and specific for the polymer nature and molecular weight, temperature, and salt composition used, in contrast to polymer/salt ATPS, where the constancy of the tie line slope remains questionable;
- Temperature dependence of phase diagrams is less pronounced in the polymer/polymer than in the polymer-salt ATPSs. The accumulated data so far did not provide any deep insight into the mechanism of phase separation in ATPSs.

Two main questions remain unanswered:

- What physicochemical properties of polymers (including proteins, nucleic acids, and polysaccharides) and salts govern their abilities to form two-phase systems in water, and
- Why the composition of an aqueous mixture of two polymers (or single polymer and salt) may be varied along the same tie-line with formation of the coexisting phases with identical compositions of the phases, while any deviation of the overall composition of the mixture from the tie line results in changes in the compositions of the two phases.

Any approach to answering these two questions would be extremely helpful in gaining better insight into mechanism of phase separation.

Analysis of phase diagrams for different ATPSs is important for two reasons. First, it is important fundamentally for understanding mechanisms of phase separation in different ATPSs. Second, it is desirable to manipulate conditions for distribution of proteins and other soluble compounds in ATPSs in a rational manner. In regard to the first issue, the phase diagrams information accumulated so far failed to provide better insight into mechanism of phase separation. The second issue – manipulating distribution of solutes in ATPSs is obviously related to the properties of coexisting phases. These properties may be explored in any ATPS with or without detailed phase diagram information. This information is clearly valuable but not crucial for understanding and manipulating solute partitioning in ATPSs. This controversial and likely to be debatable conclusion will be substantiated below.

5.2. Properties of phases in ATPSs

There are a lot of experimental results reported in the literature on the properties of the coexisting phases in various ATPSs. All these results, no matter what particular technique has been used, show unambiguously that the properties of the phases are different and, for a given ATPS, the differences increase with increase in the distance from the critical point on the corresponding phase diagram. This behavior is expected, because the differences between the compositions of the phases increase. Therefore, any characteristics of the difference between each property of the two phases include description of the particular composition of the overall composition of a given ATPS. It has been established also that essentially all the different properties of the phases in a given ATPS are interrelated, and that complicates the description. Several of the most important properties of the phases are discussed below sequentially, and the interrelationships between these properties

and their role in distribution or partition behavior of proteins, nucleic acids, and other biological compounds are considered after the description of all these properties.

5.2.1. Hydrophobic properties of coexisting phases

Partition behavior of soluble compounds in any two-phase system is characterized by a partition coefficient or distribution coefficient defined as the ratio of concentrations of a compound in two immiscible phases at equilibrium. In the chemical and pharmaceutical sciences, the two phases are often restricted to mean two immiscible solvents. Organic solvent-water systems, such as 1-octanol-water or hexane-water, are often used, and distribution or partition coefficient (D or P) is defined as the ratio of a compound concentration in the organic solvent phase to that in aqueous phase.

Partition coefficients of organic compounds with different chemical structure in organic solvent-water systems vary over several orders of magnitude [299–303]. Therefore, the partition behavior of solutes in these systems is commonly characterized by the $\log D$ or $\log P$ values. The $\log P$ term represents the partition coefficient of the solute in its un-ionized form, and the distribution coefficient ($\log D$) for the same solute refers to the ratio of the sum of the concentrations of all forms of the compound (ionized plus un-ionized) in each of the two phases. For ionizable compounds, the $\log D$ parameter is pH- and ionic strength-dependent [304].

It is well established in studies of partitioning of organic compounds in organic solvent-water systems that different structural fragments of molecules are characterized by fragment-specific constant contributions in $\log P$ or $\log D$ values [299–305]. In order to compare the relative hydrophobicity of different organic solvents the contribution of a methylene group, CH_2 , into $\log P$ is convenient to use. The methylene group is hydrophobic and the free energy of transfer of this group from water to an organic solvent is a good measure of the relative hydrophobicity of the solvent assuming the properties of aqueous phase saturated with the organic solvent are constant.

Partitioning of a homologous series of compounds with aliphatic alkyl chain is typically studied for this purpose. An example of typical experimental data is illustrated in Fig. 9 [300, 306]. Partition coefficients ($\log P$) for fatty acids in octanol-water system and for aliphatic alcohols in octanol-water and cyclohexane-water systems are plotted as functions of the aliphatic chain length. The linear curves observed in Fig. 9 may be described as:

$$\log P_i^j = A_i^j + E^j \cdot N_i \quad (5)$$

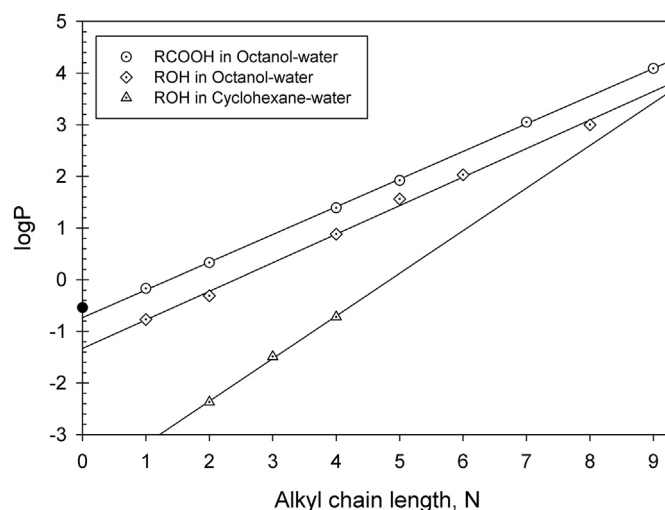


Fig. 9. Logarithms of partition coefficients for aliphatic alcohols and fatty acids in organic solvent-water biphasic systems at pH 7.4 as functions of the alkyl chain length.

where P_i^j is the partition coefficient of i^{th} compound in j^{th} solvent-water system; E^j is the contribution of a CH_2 group into $\log P_i^j$ value, and A_i^j is the contribution of a polar group into $\log P_i^j$ value. It should be noted that the slopes of the curves for both series of fatty acids and aliphatic alcohols are the same within the experimental errors limits. For the curves in Fig. 9, the slopes (parameter E) values are: 0.536 ± 0.003 for fatty acids and 0.55 ± 0.02 for alcohols in octanol-water system, and 0.83 ± 0.02 for alcohols in cyclohexane-water system. Parameter E values for different solvent-water systems are different [307]. It should also be noted that in two cases, for fatty acids in octanol-water system and for aliphatic alcohols in cyclohexane-water system, first member of the series (formic acid and methanol, correspondingly) do not fit the relationships observed. The likely reason is that the methyl group in both these compounds is in close proximity to the polar group and it affects its contribution into $\log P$ value.

The partition coefficient of a soluble compound is related to the free energy of transfer of the compound between the equilibrated phases (for details see [2], pp. 162–167):

$$\Delta G_{\text{water} \rightarrow \text{solvent}}^{\circ} = -RT * \ln P_{\text{solvent/water}} \quad (6)$$

where $\Delta G_{\text{water} \rightarrow \text{solvent}}^{\circ}$ is the free energy of transfer of a solute from aqueous phase to the organic solvent phase; $\ln P_{\text{solvent/water}}$ is the natural logarithm of the solute partition coefficient defined as the ratio between the concentrations of the solute in organic and aqueous phases; R – universal gas constant, and T – absolute temperature. Free energies of transfer of a CH_2 group from water to organic solvent calculated with Eq. (6) vary from -433 ± 6 cal/mol CH_2 for methyl ethyl ketone-water system to -1127 ± 31 cal/mol CH_2 for cyclohexane-water system [2].

The choice of a homologous series of compounds to be used for analysis of the relative hydrophobicities of the coexisting phases in ATPS is restricted by low solubility of compounds with long aliphatic alkyl chains. Willauer et al. [308] used a series of the carbon-14 labeled aliphatic alcohols (methanol, ethanol, n-propanol, n-butanol, and n-pentanol) to estimate the hydrophobic properties of the phases in PEG-salt and Dextran-PEG ATPSs. This series of compounds provides reliable estimates of the relative hydrophobicity of the phases (see below) but presents a challenge of performing experiments with radiolabeled solutes, and does not provide important information in regard to electrostatic properties of the phases (see in next section). Homologous series of ionized compounds, such as sodium alkyl sulfates, alkyltrimethylammonium bromides, and alkylbenzyltrimethylammonium chlorides were used successfully for the same purpose by Zaslavsky et al. [309]. In order to assay the concentrations of these compounds in the phases of polymer/polymer ATPS, it was necessary to use extraction protocols – inconvenient and laborious procedures [309]. It has been noted, in particular, that it is typical for charged compounds with relatively short alkyl chain to deviate from linearity described by Eq. (5) [309]. This deviation varies with ionic composition of an ATPS and illustrates the influence of a charged polar group on hydrophobic properties of methylene groups in proximity to the polar group. In order to simplify the analytical procedures, it has been suggested to use a series of sodium salts of dinitrophenylated (DNP-) amino acids with aliphatic alkyl side-chains (DNP-glycine, DNP-alanine, DNP-norvaline, DNP-norleucine, and DNP-amino-n-octanoic acid) [310, 311]. These compounds are water soluble and colored which makes it convenient to assay their concentrations in the phases using direct absorbance measurements in UV-VIS spectrophotometer at ~ 360 nm. It has been shown [310, 311] that the equivalent number of CH_2 groups, N_c (see in [2], pp. 162–167) instead of the numbers of carbon atoms in the alkyl side-chains, N_i , should be used in Eq. (5) because the N_c parameter takes into account the influence of the charged polar moiety in DNP-amino acid Na structure on the hydrophobic character of methylene groups in its proximity.

In contrast to organic solvent-water systems both phases in ATPS are of the same aqueous nature, and usually the ratio of the compound concentration in the upper phase to that in the lower phase is used as the compound partition coefficient, K , though this choice is not based on any physical consideration. Typical relationships observed in ATPS for the aforementioned series of Na salts of DNP-amino acids are presented in Fig. 10. The data presented in Fig. 10 show that the difference between the relative hydrophobicity of the phases (the slopes of the linear curves) varies in ATPS with the type of ATPS, molecular weight of polymer, salt composition, etc. The data reported in the literature for polymer-salt ATPS show that the free energies of transfer of a CH_2 group in PEG-salt and Ucon-salt ATPS without additional salt additives vary from -57 ± 1.4 cal/mol CH_2 in PEG-2000- Na_2SO_4 ATPS [249] to ca. -700 cal/mol CH_2 in PEG-2000- K_2CO_3 ATPS [308]. These data, unfortunately, are quite limited. Therefore, there are only two general conclusions to be made. First, the difference between the relative hydrophobicities of the coexisting phases may vary depending on the ATPS composition and exceed similar differences observed in organic solvent-water systems. Second, the difference between the relative hydrophobicities of the phases in polymer-salt ATPS increases with the

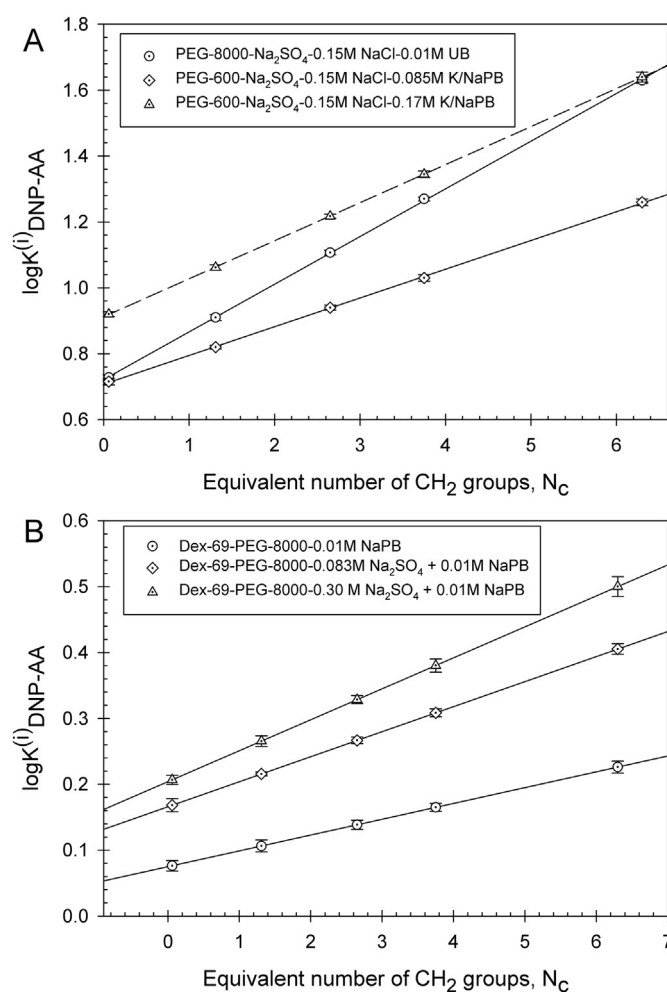


Fig. 10. A. Logarithms of partition coefficients of sodium salts of dinitrophenylated (DNP-) amino acids with aliphatic alkyl side-chain in aqueous polyethylene glycol (PEG)-8000-sodium sulfate two-phase systems of various ionic compositions as functions of the equivalent number of methylene groups in the alkyl side-chain. (UB – universal buffer; K/NaPB – sodium/potassium phosphate buffer, all buffers with pH 7.4). B. Logarithms of partition coefficients of sodium salts of dinitrophenylated (DNP-) amino acids with aliphatic alkyl side-chain in aqueous dextran-polyethylene glycol two-phase systems of various ionic compositions as functions of the equivalent number of methylene groups in the alkyl side-chain. (NaPB – sodium phosphate buffer, pH 7.4). All systems are formed by dextran-69 with molecular weight 69,000 and polyethylene glycol (PEG) with molecular weight 8000.

distance from the critical point on phase diagram, i.e. with increasing difference between the polymer and salt concentrations in the coexisting phases. Due to the limited amount of experimental data it is impossible currently to discuss the effect of polymer nature and molecular weight, though the salt nature clearly influences the $\Delta G^\circ(\text{CH}_2)$ value quite significantly.

It has been also shown that salt additives may affect the difference between the hydrophobicity of the two phases in PEG- Na_2SO_4 ATPS [249, 312, 313]. The data again is rather limited. The only definite conclusion to be made is that the type and concentration of salt additive affects the difference between the relative hydrophobicities between the coexisting phases in PEG- Na_2SO_4 ATPS. The likely possible reason for the salt additive effect on the $\Delta G^\circ(\text{CH}_2)$ -value may be the salt-induced change in the polymer concentrations in the two phases. The question of the origin of this effect remains open, however.

Some indications in favor of the above assumption may be obtained from examination of the data reported in the literature on the differences between the relative hydrophobicities of the coexisting phases in polymer-polymer ATPS. It should be mentioned that the $\Delta G^\circ(\text{CH}_2)$ -values in multiple polymer/polymer ATPS, such as Dextran-PEG, Dextran-Ficoll-400, Dextran-PVP, and Dextran-PVA in the presence of different salt additives, were reported and discussed previously (see [2], pp. 162–196). Additional data obtained in polymer/polymer ATPSs formed in mixtures of different pairs of polymers confirmed that the $\Delta G^\circ(\text{CH}_2)$ -values vary from ca. 13 ± 1.2 cal/mol CH_2 in PEG-8000-Ficoll-70 ATPS to 140 ± 11 cal/mol CH_2 in PEG-8000-Ucon-3900 ATPS are generally lower than in polymer-salt ATPSs [314]. The ATPS formed with Ucon are the only ATPS of this family with the $\Delta G^\circ(\text{CH}_2)$ -values of the order of magnitude close to those observed in polymer-salt ATPS. The polymer nature in this case seems to be more important than the polymer molecular weight or concentration. As an example, Ucon total concentration in Dextran-Ucon ATPS is lower than that of Ficoll in Dextran-Ficoll-70 ATPS with the essentially the same overall concentration of Dextran-75 in both ATPS, and the difference between the relative hydrophobicities of the two phases in the Dextran-Ucon ATPS exceeds that in Dextran-Ficoll-70 > 4-fold. It has been established previously that the $\Delta G^\circ(\text{CH}_2)$ -value increases in a given ATPS with increasing difference between the polymer concentrations in the coexisting phases similar to polymer-salt ATPS [2].

Effects of salt additives on the difference between the relative hydrophobicities of the coexisting phases in polymer/polymer ATPS was studied in detail in [249, 315–317]. Some of the data reported in [315] are presented graphically in Fig. 11. The data presented in Fig. 11 show that the nature of polymers forming ATPS is most important for the $\Delta G^\circ(\text{CH}_2)$ -value, and the salt additive type and concentration is of secondary importance. The ranges of the $\Delta G^\circ(\text{CH}_2)$ -values for ATPS formed by different pairs of polymers overlap depending on the particular salt additive and concentration used and vary from ca. 10 up to over 200 cal/mol CH_2 .

The $\Delta G^\circ(\text{CH}_2)$ -value is clearly a measure of the relative affinity of the coexisting phases for a hydrophobic CH_2 group, and hence it may be used as a measure of the difference between the relative hydrophobicities of the two phases. The free energy of transfer of a solute or a moiety, such as a CH_2 group, is generally considered to represent the difference of the relative free energies of cavity formation between the phases [2, 308, 318]. This consideration is based on the model of transfer of a solute between two immiscible phases which includes formation of a cavity in the target phase to accommodate the solute, and the solute-solvent interactions occurring once the solute is located in the cavity. Therefore, the $\Delta G^\circ(\text{CH}_2)$ -value may represent the difference between the energies of cavity formation in the two phases and/or the difference between the CH_2 group interactions with the solvent in the two phases. For the hydrophobic CH_2 group interactions with the solvent in both aqueous phases of different composition are clearly hydrophobic hydration interactions. It was suggested recently that the hydrophobic hydration is determined by the mean energetics of aqueous medium [319].

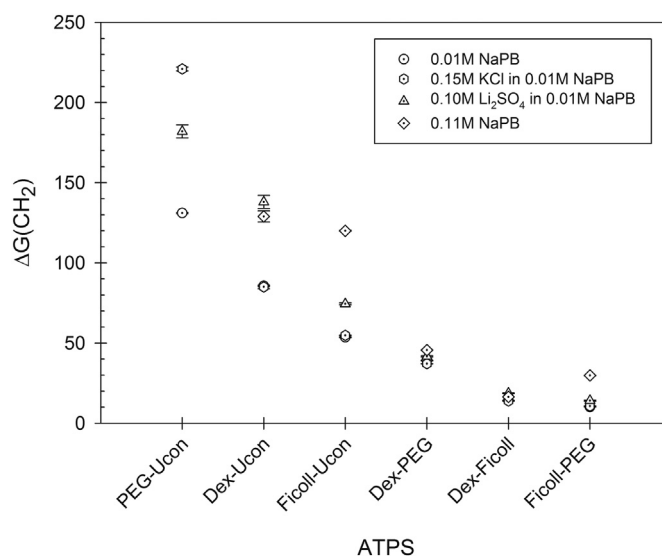


Fig. 11. Differences between the relative hydrophobicities of the coexisting phases (expressed in terms of the free energy of transfer of a CH_2 group) in various aqueous two-phase systems formed by two polymers indicated in the presence of different salt additives (NaPB – sodium phosphate buffer, pH 7.4).

Analysis of the $\Delta G^\circ(\text{CH}_2)$ -values in different ATPS by itself does not allow one to answer the above question – what particular properties of the media in the two phases does the $\Delta G^\circ(\text{CH}_2)$ -value represents. This question will be discussed below.

Summing up, it may be concluded that the difference between the relative hydrophobicity of the phases in polymer-polymer ATPSs is much lower than those observed in organic solvent-water systems and lower than in polymer-salt ATPSs. It depends on the nature of polymers used for ATPS formation and their concentration as well as on the ionic composition. Presentation of actual ranges of the differences between hydrophobic properties of the phases in polymer-polymer and polymer-salt ATPSs is rather meaningless at the moment, since these differences have been reported for a limited number of systems, and even in the cases when these differences were reported, it was shown that they vary significantly depending on the polymers and salts concentrations. Finally, the observed dependence of hydrophobic properties of the aqueous media in the phases of ATPS on the polymer and ionic composition of the media implies that the relative hydrophobicity of any compound (including proteins and nucleic acids) is an intrinsic property of the compound only partially, and it varies with the composition of its aqueous environment. This conclusion actually is implied by the definition of the hydrophobicity/hydrophilicity as a measure of the interactions of the compound with its aqueous environment. This conclusion, however, is commonly ignored in the literature.

5.2.2. Electrostatic properties of coexisting phases

As mentioned above in phase diagrams section, the ionic compositions of the two phases in ATPS are interrelated with polymer compositions of the phases and are generally different in any particular ATPS ([2], pp. 116–122). An interfacial electrostatic potential difference arises in ATPS as the result, and ion-dipole and ion-ion interactions in the two phases are different due to different ionic compositions in the phases (see details in [2], pp. 196–216).

It has been suggested to use the intercept A-value in Eq. (5) for sodium salts of DNP-amino acids in ATPS as a measure of the difference between the electrostatic properties of the coexisting phases [2]. The DNP-NH-CH-COO[−]Na⁺ group is obviously imperfect as a probe of electrostatic properties of the phases as in addition to electrostatic ion-dipole and ion-ion interactions it may participate in hydrogen bonding and other polar interactions with the media [2]. Any other charged moiety would have similar drawbacks, however, and the group under

discussion is not only convenient, but there is also plenty of experimental data accumulated in the literature for this group (see below).

In order to estimate the parameter A_i^j in Eq. (5) on the scale similar to the $\Delta G^\circ(\text{CH}_2)$ -values used to estimate the differences between the relative hydrophobicities of the ATPS phases we may replace Eq. (5) with:

$$\ln K_i^j = A^j + E^j \cdot N_{\text{IC}} \quad (7)$$

where K_i^j is the partition coefficient of a sodium salt of i^{th} DNP-amino acid in j^{th} ATPS, N_{IC} is the equivalent number of CH_2 groups in the alkyl side-chain of i^{th} DNP-amino acid; A^j and E^j are contributions of polar charged group and of a CH_2 group into $\ln K$ of sodium salt of DNP-amino acid, correspondingly. The free energy of transfer of the polar charged group under consideration is:

$$\Delta G^\circ(\text{ionic group}) = -RT \cdot A^j \quad (8)$$

where all the parameters are defined above.

The free energy of transfer of a polar ionic group $\text{DNNP-NH-CH-COO}^-\text{Na}^+$ from lower to upper phase of polymer-salt ATPS varies from 347 ± 0.6 cal/mol ionic group in PEG-8000- Na_2SO_4 ATPS with ~ 0.5 M NaSCN additive [313] to 1550 ± 35 cal/mol ionic group in Ucon-3900- NaH_2PO_4 ATPS [320].

It should be noted that the affinity of the polar ionic group for the polymer-rich upper phase exceeds that for the salt-rich lower phase by 600–1550 cal/mol ionic group, much more than for a nonpolar CH_2 group (see above). On the other hand, the trends observed for $\Delta G^\circ(\text{CH}_2)$, such as an increase in value with increasing distance from the critical point of the phase diagram and effects of salt and polymer types used, appear to hold for $\Delta G^\circ(\text{ionic group})$ in polymer-salt ATPS.

The data reported on the effects of different salt additives on differences between the electrostatic properties of the two phases in PEG- Na_2SO_4 ATPS showed that the salt additives affect the differences between the electrostatic and hydrophobic properties of the phases quite differently [249, 312, 313]. Increasing salt additive concentration in the ATPS under discussion always increased the absolute $\Delta G^\circ(\text{CH}_2)$ -value, while similar trend for the $\Delta G^\circ(\text{ionic group})$ -value is observed only in the presence of NaH_2PO_4 additive, the opposite trend is displayed in the presence of NaSCN and NaClO_4 additives, and initial decrease of the difference between the electrostatic properties of the phases is observed in the presence of NaCl up to concentration of ~ 0.54 M after which the difference increases with increasing NaCl concentration. In PEG-600- Na_2SO_4 ATPS increasing buffer concentration results in increasing difference between the electrostatic properties of the phases and NaCl and NaSCN salt additives in this system decrease the difference in question.

The data reported so far for the electrostatic properties of the phases in polymer-salt ATPS are quite limited and much more data must be obtained to draw general conclusions. The only conclusion that can be made unambiguously is that the electrostatic and hydrophobic properties of the coexisting phases are not interrelated.

The difference between the electrostatic properties of the coexisting phases in polymer/polymer ATPS are generally much smaller than in polymer-salt ATPS. It is obviously due to much less significant difference between the ionic compositions of the two phases in the latter ones. The free energy of transfer of a polar ionic group $\text{DNP-NH-CH-COO}^-\text{Na}^+$ from lower to upper phase in polymer-polymer ATPS of the same ionic composition (0.15 M NaCl in 0.01 M NaPB, pH 7.4) [314] vary from 2 ± 10 cal/mol ionic group in Dextran-75-Ucon-3999 ATPS to 134 ± 8 cal/mol in Dextran-70-Ficoll-400 ATPS [2] to -176.6 ± 0.4 cal/mol ionic group in PES-100 (hydroxypropyl starch)-PEG-8000 ATPS.

Similarly, effects of salt additives in polymer/polymer ATPS on the difference between the electrostatic properties of the coexisting phases are relatively small [315]. It is important to note that the different salt additives affect the electrostatic properties of the phases in polymer/polymer ATPS differently depending on what particular polymers are

employed for forming an ATPS. As an example, the effect of 0.1 M Li_2SO_4 additive on the difference between the electrostatic properties of the phases in Dextran-Ucon and Ficoll-Ucon ATPSs slightly exceeds that of 0.1 M Na_2SO_4 additive, but just the opposite is true in Dextran-Ficoll ATPS. It is possible that the effects of phase forming polymers on the dielectric properties of aqueous media in the phases of ATPS may influence the electrostatic properties of the phases but very limited studies of these properties even in individual polymer solutions were reported so far [321, 322].

The only general conclusion following from the data currently at our disposal is that the electrostatic and hydrophobic properties of the aqueous media in the coexisting phases of a given ATPS are not interrelated and may be affected by type and concentrations of phase forming polymers and ionic composition of an ATPS.

5.2.3. Solvent properties of phases in ATPS

It has been shown previously (see [2], pp. 268–276) and confirmed later [253, 313, 317, 318, 323–326] that partitioning of solutes from small organic compounds to proteins and nucleic acids in ATPS generally does not involve their interactions with phase forming polymers. Therefore, it has been suggested [2] that unequal distribution of soluble compounds in ATPS is driven by differences between solute-solvent interactions in the coexisting phases. As it has been shown above, the hydrophobic and electrostatic properties of the coexisting phases in ATPS are different. These properties, however, do not include all possible types of solute-solvent interactions.

The solute-solvent interactions for any given solute are governed by the properties of solvent, though the contributions of different solvent properties would depend on the solute structure and physicochemical features. According to Cabot and Hunter [327], most quantitative approaches to the study of solvation phenomena have focused on the use of specially designed spectroscopic probes sensitive to changes in their environment [328–330]. The most widely used term for solvent classification is polarity. This is a very poorly defined term which according to the current definition is the sum of all possible specific and non-specific interactions between the solvent and any potential solute, excluding interactions leading to chemical transformations of the solute [330, 331]. The solute-solvent interactions include multiple types of interactions, such as electrostatic, dipole-dipole, dipole-induced dipole, hydrogen bonding and electron pair donor-acceptor interactions. It is especially important that polarity describes the potential behavior of the solvent in a relationship with the solute which is not an absolute property of the pure solvent [331]. There is a large number of different polarity scales based on different probes and spectroscopic techniques (NMR, IR, UV/Visible absorption and emission spectroscopy, etc.) [327]. According to Ab Rani et al. [331], there is no single measure of polarity; all the polarity scales are estimates and different scales provide different estimates for the same solvent. There is no useful concept of 'right' or 'wrong' when comparing these scales. The test of an empirical polarity scale is its usefulness in explaining and/or predicting other solvent dependent phenomena [331].

Solvent polarity of aqueous media in solutions of several ATPS forming polymers, such as Dextran, Ficoll, and PEG of different molecular weights was measured using the water soluble carboxylate-substituted anionic betaine Reichardt's solvatochromic dye and thymol blue [332], a number of different sulphonaphthalein dyes, fluorescein and eosin [333]. Solvent polarity of the phases of Dextran-Ficoll and Dextran-PEG ATPS was estimated with the same solvatochromic dyes and found to be different in the coexisting phases [321]. The differences between solvent polarity of aqueous media in the two phases of polymer/polymer ATPS together with the differences in the hydrophobic and electrostatic properties of the phases discussed above served as a basis for the suggestion [2, 334] that polymer/polymer ATPS may be viewed as similar to organic solvent-water biphasic systems with the distinction of both phases being of the same aqueous nature.

Any single parameter polarity scale cannot represent the multitude of possible solute-solvent interactions. Therefore Kamlet and Taft developed multi-parameter polarity scales based on Linear Solvation Energy Relationship (LSER) including three scales of hydrogen bond donor acidity (α) [335], hydrogen bond acceptor basicity (β) [336], and dipolarity/polarizability (π^*) [337]. Combination of these three scales describes ability of a given solvent to participate in solute-solvent interactions, i.e. solvent polarity, much better than any single parameter polarity scale. The LSER model used by Kamlet, Taft, and their coworkers may be described as:

$$(XYZ) = (XYZ)_0 + s\pi^* + a\alpha + b\beta \quad (9)$$

where (XYZ) is the solute property (solubility, reaction rate, equilibrium constant, the logarithm of a gas/solvent or solvent/solvent partition coefficient, etc.) in a given solvent; $(XYZ)_0$ is the same solute property in a reference state, s , a , and b are the solute-dependent coefficients characterizing the respective influence of the π^* , α , and β terms on the (XYZ) property under study. The examples of successful use of Kamlet-Taft approach are numerous and beyond the scope of this review.

The solvent dipolarity/polarizability (π^*) scale introduced by Kamlet and Taft was generated using seven solvatochromic dyes with strong and symmetric solvatochromic absorption spectra [337]. The π^* -values obtained for these seven dyes were averaged. In total, 45 dyes were used to generate π^* -values for over 200 solvents [331]. The purpose of using multiple dyes was to avoid getting dye-specific π^* -value. In order to examine π^* -values for aqueous media in polymer solutions and in phases of ATPS only a very limited number of water soluble solvatochromic dyes may be employed. Selection of the particular solvatochromic dye for studying dipolarity/polarizability (π^*) of ionic liquids was based on the arguments valid for studying solvent properties of aqueous media in ATPS [331].

Clearly, the dye used to measure the π^* -value affects both the absolute value and, for ionic liquids with very similar values, the relative ordering. It should be noted again that π^* is not a fundamental physical property of a solvent, but a guide to the effect of the solvent upon solute species that are sensitive to interactions with the solvent dipoles and, in case of ionic liquids its ions. The precise π^* -value has no fundamental physical meaning.

For our studies, we selected 4-nitroanisole; it is commercially available, chemically stable, and water soluble. The experimental details of solvatochromic measurements and how to calculate π^* -value from the wavelength of maximum absorbance of 4-nitroanisole in solution are described in [315, 324, 338, 339]. It should be stressed here that multiple absorption spectra determination over the spectral range of 240 to 600 nm with two-three separately prepared solutions or phases of ATPS are important, and using software packages for determination of the wavelength of maximum absorbance for the dye in each solution is strongly recommended.

The solvent dipolarity/polarizability π^* -values in aqueous solutions of polymers commonly used for ATPS formation reported in [324] are graphically illustrated in Fig. 12A as functions of polymers concentrations. Some of the data from [324] presented in Fig. 12A indicate that the solvent dipolarity/polarizability (π^*) of aqueous media increases with the polymer concentration for all polymers examined except Ucon. The polymer effect on the solvent dipolarity/polarizability (π^*) characterizing the interactions of aqueous media with solute dipoles and induced dipoles decreases in the following sequence:



at both concentrations of 30 and 40%. In order to analyze the possible role of the established influence of polymers on the solvent properties of aqueous media it is important to consider how significant these effects are. The dipolarity/polarizability parameter, π^* , of aqueous media

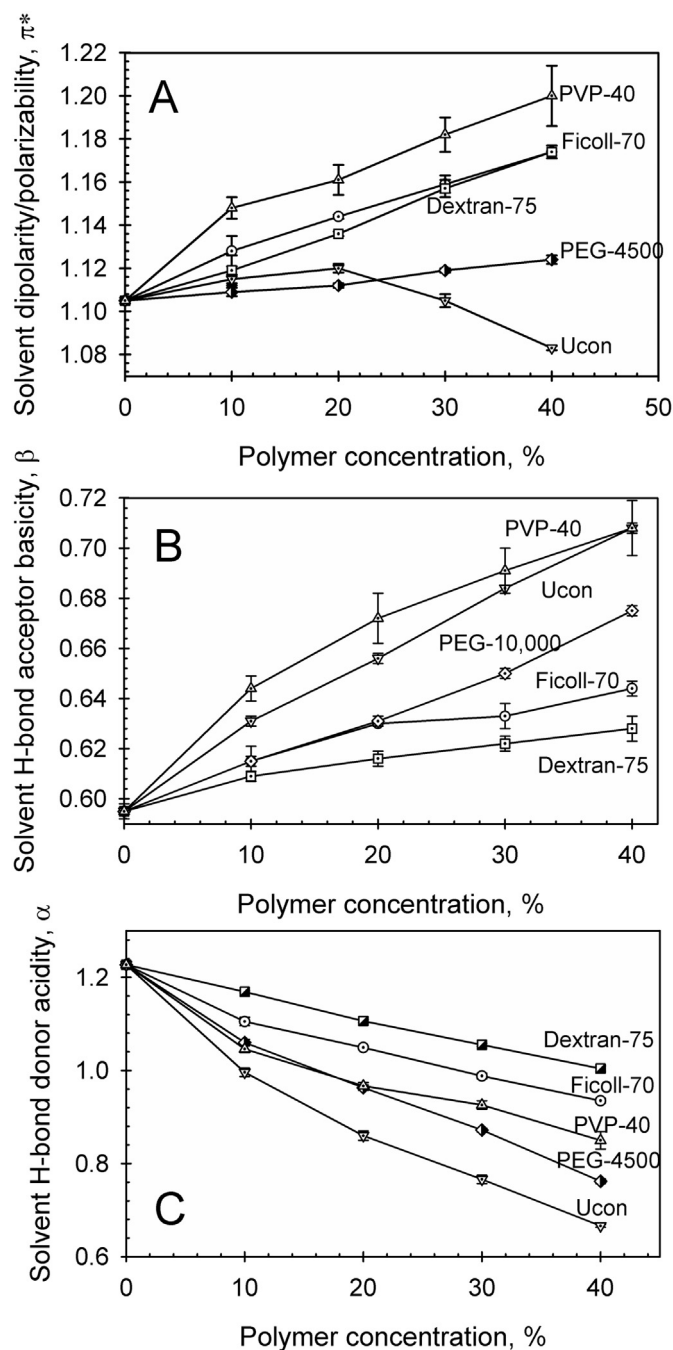


Fig. 12. Effects of polymers on solvent properties of aqueous media in solutions of several polymers forming ATPSs. **A.** Dependence of the solvent dipolarity/polarizability π^* -values in aqueous solutions of polymers commonly used for ATPS formation on polymer concentrations. **B.** Dependence of the solvent hydrogen bond acceptor basicity (β) in aqueous solutions of polymers commonly used for ATPS formation on polymer concentrations. **C.** Dependence of the solvent hydrogen bond donor (HBD) acidity (α) in aqueous solutions of polymers commonly used for ATPS formation on polymer concentrations.

in 40% Ucon solution differs from that of the polymer-free media by 0.022 [324]. The difference between the π^* values for such organic solvents as methanol and ethanol is 0.06 [340]; i.e., three times larger. In the ATPS formed by Dextran-75 and Ficoll-70 [323, 341], however, the difference between the π^* values for the coexisting phases amounts only to 0.003, and this difference affects the distribution of small compounds and proteins between the two phases [342–344]. It should be mentioned that as demonstrate the data in [345] osmolytes affect the solvent dipolarity/polarizability of water in their solutions in the

manner similar to observed in polymers solutions, though at higher concentrations.

The aforementioned arguments in favor of using a single solvatochromic dye for π^* -value measurements may be used for hydrogen bond acceptor basicity (β) measurements. 4-Nitrophenol was chosen as the probe for the β measurements. The β -value is calculated from the wavelength of maximum absorbance for the dye and the π^* value in each solution as described in detail in [324]. The solvent hydrogen bond acceptor (HBA) basicity β -values in aqueous solutions of polymers commonly used for ATPS formation reported in [324] are graphically illustrated in Fig. 12B as functions of polymers concentrations. Some of the data are presented in Fig. 12B and indicate that the solvent HBA basicity (β) of aqueous media increases with the polymer concentration for all polymers examined. The polymer effect decreases in the sequence:

$$\begin{aligned} \text{PVP-40} &= \text{Ucon} > \text{PEG-10K} \\ &= \text{PEG-4.5K} > \text{PEG-600} > \text{Ficoll-70} > \text{Dextran-40} > \text{Dextran-75}, \end{aligned}$$

at both concentrations of 30 and 40%. It should be noted that this sequence is different from the one found for the polymer effect on the solvent dipolarity/polarizability. The polymers effects on the HBA basicity β of aqueous media are rather small. The difference between the β values for aqueous media in 40% Dextran-75 solution and for polymer-free media is 0.033. Similar difference for methanol and ethanol is 0.15 [340]. On the other hand, the difference between the HBA basicity β for aqueous media in the coexisting phases of Dextran-75-PEG-600 is just 0.005 [323, 341].

For measurements of the solvent hydrogen bond donor (HBD) acidity (α) the water soluble solvatochromic Reichardt's carboxylated betaine dye sodium [2,6-diphenyl-4-[4-(4-carboxylato-phenyl)-2,6-diphenylpyridinium-1-yl]]phenolate] was used as a single probe. The α -value is calculated from the wavelength of maximum absorbance for the dye and the π^* value in each solution as described in detail in [324]. The solvent hydrogen bond donor acidity α -values in aqueous solutions of polymers reported in [324] are graphically illustrated for some of the data in Fig. 12C as functions of polymers concentrations. These data indicate that the solvent HBD acidity (α) of aqueous media decreases with the polymer concentration for all polymers examined. The polymers effects on the solvent HBD acidity α of aqueous media are quite significant. The difference between the α values for aqueous media in 40% Dextran-40 solution and for polymer-free media is 0.223, much larger than the difference between the α values for methanol and ethanol of just 0.10 [340]. In polymer/polymer ATPS the differences between the HBD acidity α of the aqueous media in the coexisting phases varies from 0.0 in Ficoll-70-PEG-6000 to 0.181 in Dextran-75-Ucon ATPS, depending on the polymer and salt composition (see below) [323, 341]. It should be mentioned that the data reported in [324] agree with those reported previously by Kim et al. [346] in regard of the PEG effect on the hydrogen-bond donor (HBD) acidity of water. It should be added that the data in [345] show that osmolytes affect the solvent HBD acidity of water in their solutions similarly to polymers, although at higher concentrations.

The polymers induced changes in the solvent properties of water in aqueous solutions may be explained by the reported recently data [347] that under crowding conditions (polymer concentration ~ 30%wt.) hydration shells for the polymer overlap implying that water in the solution is affected by the polymer. It means that water in the coexisting phases of polymer/polymer ATPS is represented to a large degree by hydration water, and the phases may be viewed as crowded solutions in such ATPSs, as Dextran-Ficoll, Dextran-PVP, and Dextran-PEG to a smaller extent (see below).

It has been shown [315, 348] that the differences between the solvent dipolarity/polarizability (π^*), hydrogen bond donor acidity (α), and hydrogen bond acceptor basicity (β) values of the phases of polymer/polymer ATPS vary depending on the type and concentrations of

the polymers, although the ionic composition of ATPS is also of (secondary) importance.

It has also been found that the difference between the hydrophobic character of the two phases may be described in terms of the solvent properties of the phases as [348]:

$$E^j = k_1^j \Delta \pi^* + k_2^j \Delta \alpha + k_3^j \Delta \beta + k_4^j A \quad (10)$$

where superscript j denotes the ionic composition of ATPS; and k_1^j , k_2^j , k_3^j , and k_4^j are constants.

The relationships described by Eq. (10) indicate that the difference between the relative hydrophobicity between the phases of the polymer/polymer ATPS depends to the most part on the solvent dipolarity/polarizability, π^* , solvent hydrogen bond acidity, α , and solvent hydrogen bond basicity, β , of the aqueous media in the coexisting phases. The relationship determined in the presence of Na_2SO_4 includes additionally parameter A representing the difference between the electrostatic properties of the phases likely due to pronounced polarization effect of Na_2SO_4 on water in its solutions [349]. It seems reasonable that the Gibbs energy of hydrophobic interaction in an aqueous medium resulting in rearrangement of the highly cooperative hydrogen-bonds network would involve all types of solvent-solvent interactions. The observed differences in the contributions of different types of interactions depending on the ionic composition of ATPS may be explained by that the homologous series of sodium salts of DNP-amino acids used includes compounds with short alkyl side-chains for which the proximity of the charged carboxyl group may influence the average contribution of a CH_2 group into $\log K$ value [348].

It should be mentioned that studies of the solvent properties in several polymer-salt ATPS reported by Huddleston et al. [338, 339] showed noticeable differences between the solvent hydrogen bond donor acidity (α) in the coexisting phases and very small if any differences between solvent dipolarity/polarizability (π^*) and hydrogen bond acceptor basicity (β) in PEG-2000- $(\text{NH}_4)_2\text{SO}_4$ ATPS. Ferreira et al. reported [312], however, quite noticeable differences between all the solvent properties (π^* , α , and β) of the phases in PEG-8000- Na_2SO_4 with NaCl additive at different concentrations. The data in [312] show that the differences between the solvent properties of the coexisting phases in PEG-8000- Na_2SO_4 ATPS are not much larger than in polymer/polymer ATPS. It has been shown [315–317] that the difference between the hydrophobic properties of the phases in this case also may be described in terms of the solvent properties of the phases according to Eq. (10). The data reported [312, 338, 339] on the solvent properties of polymer-salt ATPS are limited even more than those for polymer/polymer ATPS [315, 321, 332, 333, 348], hence it is impossible to draw any general conclusion as yet.

Aforementioned suggestion that the phases of polymer/polymer ATPS and polymer-rich phases in polymer-salt ATPS may be viewed as crowded solutions should be addressed here in more detail. This issue is important from two viewpoints. First, it explains the common observations of high stability of proteins and nucleic acids in polymer/polymer ATPS, as it is well established that crowding conditions generally enhance stability of biomacromolecules. Second, it explains why the solvent properties may be different in the coexisting phases of ATPS. It is generally accepted that protein folding, protein/protein interactions, and other biochemically important processes in vivo may differ from those in dilute solutions commonly used in laboratory experiments [95–98]. One of the reasons is believed to be the high overall concentrations of biological macromolecules that may occupy up to 40% of the cellular volume [95–101]. The term “macromolecular crowding” is used to stress that the influence of high macromolecule concentrations results from the steric interactions of crowding agents with the biomolecules of interest. The crowding molecules are supposed to be inert toward the protein or nucleic acid under study. They physically occupy a significant fraction of the solution volume, leaving only restricted space

available to biomolecules, hence the term “excluded volume effect” is often used [95–101].

The experimental data accumulated and reviewed in the literature show that the excluded volume effect is not the only factor affecting the behavior of biomolecules in a crowded environment [96–98, 219, 350]. In order to explain some experimental observations inconsistent with the excluded volume effect, it was suggested that there are “soft” interactions, such as electrostatic, hydrophobic, and van der Waals interactions between the crowding agent and the protein, in addition to hard non-specific steric interactions [97, 98]. This hypothesis allows one to explain the experimental data by a balance of attractive as well as repulsive crowding agent/protein interactions [97, 98, 351–353]. It is generally ignored that there is a third component in all crowded solutions – water which is known to be important for all the biochemical processes (protein folding, aggregation, protein/protein interactions, etc.) [354]. The commonly used macromolecular crowding agents include Dextran, Ficoll, PEG, and PVP, though proteins, such as albumin or lysozyme are sometimes used as well. It was mentioned above that under crowding conditions there is an overlapping of hydration shells for the crowding agent implying that water in the solution is affected by the agent [347]. It has been suggested that small compounds may display excluded volume effects exceeding those of macromolecules [355]. This hypothesis apparently agrees with the numerous experimental data showing that the small organic osmolytes, such as glucose, sucrose, trimethylamine N-oxide (TMAO), etc. may display crowding effects and therefore are often called molecular crowders [352, 356–358]. It should be noted that the applicability of the excluded volume model to the description of the effects of osmolytes on protein behavior was considered in [359], where the effects of three osmolytes (glucose, sucrose, and raffinose) at high concentrations on the reversible homodimerization of α -chymotrypsin were investigated. The authors used two models to explain the osmolyte-mediated stabilization of α -chymotrypsin homodimer and considered both binding interactions (transfer-free energy analysis) and steric interactions (excluded volume model). This analysis revealed that the observed stabilization of the protein homodimer can be described by the thermodynamic non-ideality arising from molecular crowding by osmolytes [359]. Osmolytes in particular are well known not to engage in direct interactions with proteins.

It is unavoidable to admit that the role of the excluded volume effect as a cornerstone of the theoretical views on crowding phenomena is overestimated. The representation of macromolecular crowders (including proteins) as hard spheres is obviously a very crude approximation. The view that all proteins interact with typical synthetic macromolecular agents, such as PEG, Dextran, Ficoll, PVP, etc., contradicts all the experimental data reported so far in the studies of protein partitioning in ATPS formed by various pairs of these polymers [343, 360]. It should be also emphasized here that the results showing the presence of direct interactions between proteins and macromolecular crowding agents are typically based on the models used for the interpretation of the experimental data (this issue was discussed in detail in [324]). The data on polymer-induced changes in the solvent properties of aqueous media in their solutions (or phases of ATPSs) discussed above should be taken into account when the results reported in the studies of protein-polymer interactions are considered [97, 361–364].

It has been reported that various osmolytes induce changes in the solvent dipolarity, polarizability, HBD acidity, and HBA basicity of water similar to polymers though at higher concentrations [345]. Analysis of the multiple results reported in the literature regarding crowding effects of osmolytes and macromolecular crowding agents on proteins and nucleic acids showed that these results may be described in terms of the solvent properties of water (solvent dipolarity/polarizability and HBD acidity, in particular) changed in the presence of these agents and osmolytes [365].

As indicated by Ferreira et al. [365], the obtained relationships do not provide unambiguous experimental evidence, but they clearly support

the assumption that polymer (or osmolyte) induced changes in the solvent properties of aqueous media may play an important role in macromolecular crowding effects. It was suggested that the solvent properties represent one aspect of the structure of water in the polymers and osmolytes solutions [365]. The data accumulated so far do not allow one to answer the most important question – if the macromolecular crowding effect is the effect of crowding agent-induced changes on the properties of aqueous media or a combination of size-exclusion effect together with the solvent restructuring effects [365, 366]. It should be mentioned that the proteins examined (very limited number) so far indicate that they also are capable of inducing changes in the solvent properties of water and these changes may exceed those observed in the presence of nonionic polymers [367, 368]. The issue is complicated not only by our current limited views of the water structure but also by the essentially complete lack of knowledge of relationship between specific properties of biological macromolecules and the solvent properties of aqueous media. At this time, we may suggest the combination of the two effects results in the experimentally observed changes in biomolecule behavior in crowded solutions. The relative importance of the two types of the effects may be specific for the protein or nucleic acid under analysis [369, 370]. Therefore, this issue is of the same importance for behavior of proteins and nucleic acids in ATPS and it will be discussed in the next section.

5.3. Partition behavior of proteins and nucleic acids in ATPSs

As aforementioned, partition behavior of compounds (including proteins) in a given ATPS is characterized by partition coefficient, K , defined as the ratio of the compound concentration in the upper phase to that in the lower phase. Partition coefficient of any compound is commonly increases or decreases with increasing concentrations of polymers forming an ATPS with increasing distance from the critical point on the phase diagram. Logarithm of the compound partition coefficient is linearly related to the difference between the concentrations of a given phase-forming polymer in the two phases [2].

Partition coefficients of nonionic compounds in ATPS with the same ionic composition, but formed by various pairs of nonionic polymers reported in [341] typically vary. As an example, partition coefficient, K , values for 4-nitrophenol amounts to 1.335 ± 0.005 in Ficoll-70-PEG-6000 ATPS and 7.53 ± 0.04 in PEG-8000-Ucon (both ATPS with the same ionic composition of 0.15 M NaCl in 0.01 M NaPB (sodium phosphate buffer), pH 7.4. For 4-hydroxyacetanilide the partition coefficients vary from 1.237 ± 0.007 in Dextran-75-Ficoll-70 ATPS to 4.20 ± 0.02 in Ficoll-70-Ucon ATPS (both ATPS of the aforementioned ionic composition) [341]. Partition coefficients of ionic compounds may experience even greater variations under similar conditions. As an example, partition coefficient of sodium salt of dinitrophenyl-amino- n -octanoic acid varies from 0.977 ± 0.005 in Ficoll-70-PEG-10,000 ATPS to 14.7 ± 0.5 in PEG-8000-Ucon ATPS [348]. For proteins, the partition coefficients vary even more [360]. Partition coefficients for α -chymotrypsinogen vary from 0.0098 ± 0.0005 in PEG-8000-Ucon ATPS to 2.71 ± 0.02 in Dextran-75-PEG-8000 ATPS, while for human transferrin it varies from 0.0084 ± 0.0003 in Dextran-75-PEG-8000 ATPS to 7.145 ± 0.003 in PES (hydroxypropyl starch)-100-Dextran-75 ATPS (all ATPS of the aforementioned ionic composition) [360].

In spite of the wide ranges of the partition coefficients measured in ATPS formed by various pairs of nonionic polymers, analysis of partition coefficients of small organic compounds and proteins reported in [341, 348, 360, 371–373] showed that partition coefficients of all different compounds from individual amino acids to proteins determined in multiple ATPS of the same ionic composition may be described as:

$$\log K_i^j = S_s^i \Delta \pi^* + B_s^i \Delta \alpha_j + A_s^i \Delta \beta_j + C_s^i c_j \quad (11)$$

where K_i^j is the i -th 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, $\Delta\beta$ 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^i , A_s^i , B_s^i , and C_s^i are constants (i^{th} 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 j denotes the ATPS used; the difference for each solvent property is determined as the one between the upper and lower phases.

It should be noted that the above Eq. (11) is applicable to compounds being partitioned in an ATPS of a fixed polymer and ionic composition containing additives of various osmolytes, such as TMAO, sorbitol, sucrose, and trehalose [342, 343]. The established constancy of the introduced above solute specific coefficients for a given compound across numerous (up to 20) ATPS formed by various pairs of different nonionic polymers shows unambiguously that there are no direct interactions between compounds (including proteins) and polymers used to form ATPS.

Additional evidence in favor of the above conclusion was reported in [344], where it was shown that increasing concentration of trimethylamine N-oxide (TMAO) in a given ATPS from 0 to ca. 2.0 M results in quite dramatic changes in partition behavior of some compounds including proteins. As an example, the partition coefficients of human albumin and bovine α -chymotrypsinogen A increase from 0.518 ± 0.005 and 2.491 ± 0.013 in the TMAO-free ATPS to 3.909 ± 0.049 and 7.575 ± 0.052 in ATPS containing 1.95 M TMAO respectively. For the ribonuclease A, the partition coefficient under same conditions changes from 0.885 ± 0.003 to 1.506 ± 0.005 , while for concanavalin A it changes just from 0.235 ± 0.001 to 0.252 ± 0.001 [344]. TMAO is well known not to be engaged in direct interactions with proteins, and its concentration effect on the protein partition behavior is clearly originates in TMAO effect on the solvent properties of the coexisting phases [344]. The effect of TMAO concentration is compound specific [344], and it agrees with Eq. (11) (see above). The aforementioned data show additionally that the protein molecular weight is not of primary importance for the protein partition behavior governed by the nature and spatial arrangement of the solvent exposed groups and their interactions with the aqueous media in the two phases [344].

An attempt to predict partition coefficients of proteins in ATPSs of various polymer and ionic composition based on structural features of proteins derived from the proteins crystal structures essentially failed [374]. In this study, partition behavior of 10 different proteins in 29 various ATPSs were analyzed in terms of 57 different structural features [374]. It has been found that three structural features describe partition coefficients of all 10 proteins in a given ATPS quite satisfactory, however, these features vary in different ATPSs [374]. It has been concluded that there is no unique set of protein structural features derived from the proteins crystal structures that may describe partition behavior of proteins in various ATPSs [374]. It was suggested that the information gained from the crystal structure of a protein cannot provide insight in regard to the protein responsiveness to different microenvironment [374]. It was also hypothesized that the analysis of protein partition behavior in different ATPSs may provide different complimentary information [374]. This suggestion seems to be in agreement with the different sensitivity of various ATPS to various structural changes in proteins.

It has been demonstrated in numerous examples (see, e.g. in [375, 376]) that in order to detect or analyze and quantify structural differences between closely related proteins it is necessary either design particular specific conditions displayed by a given ATPS or use several ATPS with different ionic compositions. This approach enables one to detect and monitor changes in the posttranslational modifications in a biomarker, such as prostate specific antigen (PSA), for improving clinical detection of prostate cancer [376, 377], detect differences between various batches of a protein [375], etc. Single point mutations may be

readily detected with this approach [378]. It should be mentioned also that partition of nucleic acids mostly in polymer-salt ATPSs with the purpose of isolation of various forms of nucleic acids has been reported by multiple authors [64, 245, 379–391]. Sorting of single-wall DNA-wrapped carbon nanotubes by partition in polymer-polymer ATPSs should also be noted here [392–394].

It should be added that in the case of ATPSs formed by charged polymers, as likely is the case in the proteinaceous membrane-less organelles, the electrostatic interactions of proteins and nucleic acids with the phase-forming components may play an important role. At the same time, the data reported for Hofmeister series of ions show that the effects of ions on the solvent properties of water still play an important role [316]. Therefore, it seems likely that all types of interactions between biological macromolecules and these molecules and aqueous media are important for their distribution between cytoplasm or nucleus and membrane-less organelles.

6. Similarities and differences between the polymer-based ATPSs and PMLOs

Fig. 13 shows that there is a noticeable morphological similarity between PEG-Dextran, PEG-Ficoll, Dextran-Ficoll, and PEG- Na_2SO_4 ATPSs, all of which are also relatively similar to PMLOs. All these entities appear as liquid-embedded liquid droplets. Differences in size between the polymer-based ATPSs and biological PMLOs (stress granules in this case) can be explained based on the aforementioned “finite container size” model, where finite size of a container places restrictions on the droplet size that can grow until the surrounding molecular concentration is depleted, thereby establishing an equilibrium with the dilute phase at a given droplet volume fraction [20].

Being the outputs of liquid-liquid phase transitions, both PMLOs and phases in the polymer-based ATPSs are classified as liquids. PMLOs and ATPSs have high water content and are spontaneously formed at appropriate conditions. They can exist as droplets of one phase inside of another phase that can fuse over time. Both are sensitive to the environment and have properties that can be modulated by rather subtle changes in their surroundings. ATPSs and PMLOs affect solvent properties of aqueous media, thereby creating conditions for the partitioning of various compounds. As a result, similar to the polymer phases of ATPSs possessing different “affinities” to different solutes that leads to their non-equal distribution between phases, PMLOs can efficiently “exclude” or “attract” specific cellular compounds. In fact, it was pointed out that since different PMLOs are formed by different intrinsically disordered proteins and their different partners, it is likely that the interiors of these organelles are characterized by rather different solvent properties, leading to the preferential inclusion and exclusion of specific solutes [26]. Here, the enrichment or depletion of a given PMLO in specific components might be (at least in part) driven by the preferential diffusion (partitioning) of these components to a phase formed by specific intrinsically disordered proteins and their partners. Such enrichment/depletion in specific molecules may define functionality of that membrane-less organelle [26].

Being formed, polymer-based ATPSs represent macroscopic entities with easily detectable boundary separating phases. In other words, ATPS formation represents a global LLPT affecting the entire volume containing polymer mixture. It seems that PMLOs are the results of more localized LLPTs and cannot grow infinitely. In fact, all currently characterized membrane-less bodies or organelles range in size from tens of nm to tens of μm [28]. It was also pointed out that the size of PMLOs scales with the size of a cell in which they are present [20]. This phenomenon was explained by the “finite container size” model, where in a container of finite size, a droplet can grow until the surrounding molecular concentration is depleted, thereby establishing an equilibrium with the dilute phase at a given droplet volume fraction [20]. In other words, the total droplet volume depends on “the size of

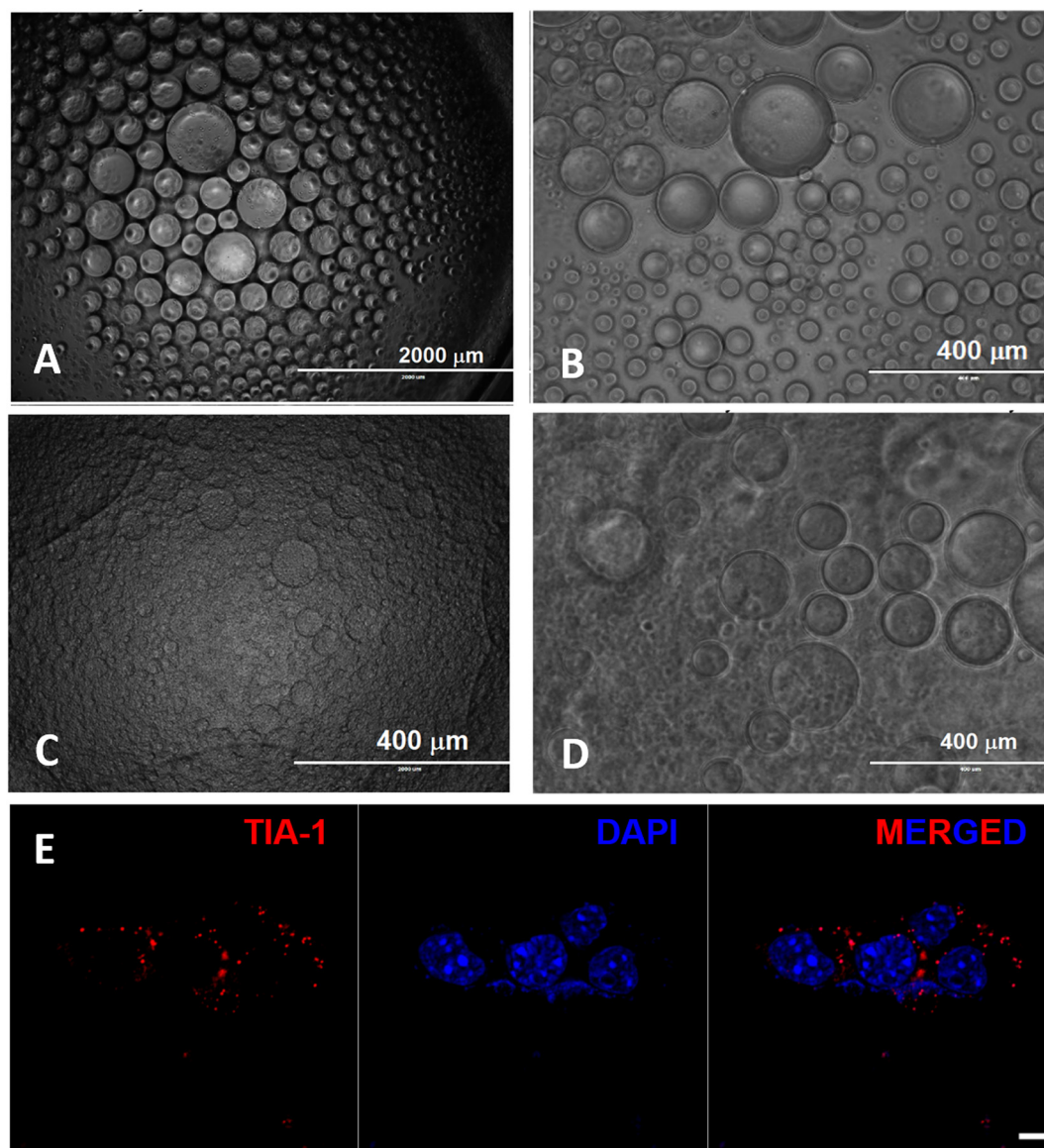


Fig. 13. Comparison of the morphological peculiarities of several ATPSs and a PMLO (stress granules). **A.** PEG-12000 – Na₂SO₄ ATPS. **B.** PEG-12,000 – Ficoll-400 ATPS. **C.** PEG-12,000 – Dextran-75 ATPS. **D.** Dextran-75 – Ficoll-400 ATPS. **E.** Stress granules. HT22 cells were treated with 0.5 mM of NaAsO₄ for 1 h at 37 °C to induce stress granule formation. They were then washed with 1 × PBS, fixed in 4% paraformaldehyde (w/v), and permeabilized with the 1 × PBS containing 0.1% of Triton X-100. Blocking was then performed with 1 × PBS containing 10% donkey serum. The cells were then incubated in the solution containing primary antibody TIA-1 (1:100, Santa Cruz, Dallas, Texas) overnight at 4 °C. TIA-1 functions as an RNA binding protein that is involved in the promotion of stress granules [406]. Secondary antibodies used were Alexa-Fluor 594 fluorescently labeled (1:500, Fisher Scientific, Waltham, MA) and were incubated for 1 h at room temperature. DAPI was added at a concentration of 1:5000 in 1 × PBS for 5 min at room temperature. Coverslips were subsequently mounted onto glass slides using Prolong Gold mounting reagent (Life Technologies, Carlsbad, CA). An Olympus FluoView confocal microscope was used for imaging. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the container (and the precise location within the phase diagram), which is a generic feature of such phase transitions” [20].

Each separated phase of a typical polymer-based ATPS, which is formed by mixing of two polymers or a polymer and a salt at concentrations exceeding a specific threshold, preferentially contains one polymer. However, macromolecular compositions of PMLOs are more complex. Although some individual proteins can undergo LLPTs and can form specific droplets at appropriate conditions in vitro (e.g., human heterochromatin protein 1α (HP1α) [395], *Drosophila* HP1α protein [396], a member of the DDX4/VASA subfamily of the DEAD-box helicase family, Ddx4 protein [107], LAF-1 (P-granule-related DDX3 RNA helicase from *Caenorhabditis elegans*) [156], RNA-binding protein TIA-1 [157], heterogeneous nuclear ribonucleoprotein (hnRNPA1) [397], intrinsically disordered regions of proteins found in RNP granules (Pub1, eIF4GII, Lsm4, FUS, and hnRNPA1) [157], microtubule-binding repeats of the Alzheimer disease-related protein tau [398],

to name a few), formation of many PMLOs requires more than one macromolecule. In fact, because of their macromolecular compositions, where one can find proteins and RNA, many PMLOs are often addressed to as RNP granules/bodies or RNP droplets [20]. Furthermore, proteomes of all PMLOs (eukaryotic [163] and bacterial [147, 148]) analyzed so far are characterized by rather large size. It is likely that not all proteins found in PMLOs are directly related to the assembly of these subcellular structures, and at least some of them are present in PMLOs because of their preferential partitioning into these subcellular bodies. In other words, based on their relation to the LLPTs, PMLO-related proteins can be grouped into two major categories, “drivers” that are directly involved in the PMLO formation and “passengers” attracted to PMLOs by their specific solvent properties of aqueous media. To some extent situation with biological PMLOs resembles aqueous multiphase separated systems formed in mixtures of more than two polymers. In fact, as it was reported by Albertsson, a mixture of six polymers

(Dextran sulfate, Dextran, and four hydroxypropyl Dextrans with various degrees of substitution) can form 18 phases [1]. This indicates that each individual phase in such a multi-phase separated system contains more than one polymer and therefore can be considered as an oversimplified model of a biological liquid droplet with complex composition.

Phases of polymer-based ATPSs are thermodynamically stable. Being formed, they will not change, if environmental conditions are not changed. However, some PMLOs are metastable systems that can undergo “maturation” and “age” into gels or glass [399]. In fact, biogenesis of PMLOs is a highly dynamic process, and, being formed, many PMLOs exist for limited time. Within this physiological time-frame of their normal existence, PMLOs are reversible and can undergo disassembly process when not needed anymore. However, under pathological conditions, the biogenesis of PMLOs can be altered, and they could exist for prolonged time, leading to some irreversible changes in the system. In fact, although “freshly” generated droplets formed by the proteins undergoing LLPTs can easily disassemble by changes in their environment, over time, these droplets can mature to more stable states [157, 397], likely due to the formation of amyloid-like fibrils characterized by very high conformational stability. This is because many proteins found in RNP granules contain not only RNA-binding domains but also prion-related low complexity sequences capable of pathogenic aggregation [157]. In other words, since aggregation and fibrillation are strongly concentration-dependent, dysregulated PMLOs (i.e., PMLOs existing for the time-period exceeding the aforementioned physiologically safe time-frame), with their locally increased concentrations of specific proteins, might inadvertently serve as a kind of amyloid “incubators”.

To the most part, polymers forming ATPSs are inert molecules that do not have specific biological activities and are not (typically) engaged in physical interaction with each other and with other constituents of the system (e.g., molecules subjected for partitioning) [323]. On the other hand, by default, proteins undergoing LLPTs leading to the PMLO formation are biologically active molecules that have various functions. This clearly is related to the fact that different PMLOs are created to have different biological functions [26]. Furthermore, it was hypothesized that the functionality of a given PMLO is pre-determined by the nature of the intrinsically disordered proteins responsible for its formation [26]. On the other hand, the liquid-like properties of PMLOs could play a role in regulation and facilitation of functions of their constituents, which are accumulated within droplets at high concentrations but remain dynamic [26]. For example, a low-density structure of PMLOs in the nucleus of *Xenopus* oocyte was suggested to facilitate the access to macromolecules within these PMLOs from the nucleoplasm [227]. Furthermore, some PMLOs are known to act as liquid-phase micro-reactors accelerating the cytoplasmic reactions due to the increased concentrations of related active RNA and protein components [20, 385, 400].

6.1. Solvent properties of PMLOs

Unfortunately, amount of currently available information on what solvent properties one might find inside the PMLOs is very limited. In a systematic study of the effect of various additives on solvent properties of water in aqueous solutions of elastin-like peptide (ELP, which in this case was a 206 residue-long polypeptide with the MGH (GVGVP)₄₀-GWP amino acid sequence) it was shown that in the solutions containing 0.90 mM ELP in 0.01 M NaPB, the dipolarity/polarizability values π^* of solvent that characterize the ability of solvent to be involved in dipole and dipole/induced dipole interactions with a solute as well as the HBD acidity values α of solvent which are the measure of the ability of solvent to participate as donor in hydrogen bonding with a solute were within the corresponding ranges observed for non-ionic polymers – between those observed for Ficoll-70 and Dextran-75 at the same concentration [368]. On the other hand, the values of the solvent HBA basicity β of aqueous media that describe the ability of solvent

to participate as acceptor in hydrogen bonding with a solute, were very small – close to that observed for PEG-10,000 [324, 368].

One of the peculiar features of the ELP-based systems is that the corresponding GVGXaaP pentapeptide repeat-containing polypeptides are able to undergo an inverse temperature transition, where the increase in temperature leads to the increase in ordered structure of a polypentapeptide (which is mostly random coil-like at room temperature). This heat-induced structurization of the polypentapeptide is accompanied by an increasing disorder of water molecules [401–404]. This is different from the thermal behavior of globular proteins that usually undergo temperature-induced denaturation, which is a transition to a state with the decreased ordered structure. Since ELP is able to undergo temperature-induced reversible LLPT characterized by the phase transition temperature, T_i (which is known to be inversely related to ELP concentration, the number of the GVGXaaP pentapeptide repeats, the hydrophobicity of the variable Xaa residue of the pentapeptide repeat [401–404], and changes in the solvent composition [405]) the correlation between the effect of various additives on solvent properties of water in aqueous ELP solutions and on T_i values was analyzed [368]. This analysis revealed that the temperature of phase transitions in the ELP solutions in the presence of different additives can be described in terms of the solvent properties of the ELP solutions, with the solvent's dipolarity/polarizability serving as the primary solvent property affecting the phase-transition temperature of ELP solutions in the presence of various additives [368].

In another recent study, it was shown that the effects of another protein (human small heat shock protein HSPB6) on the solvent features of water were either similar to those of nonionic polymer, such as Ficoll, Dextran, PEG, etc., and small organic osmolytes (in regard to water's ability for dipole-dipole interactions), or exceed them (in regard to hydrogen bonding donor ability and hydrogen bond acceptor ability) [367].

Since indicated effects of proteins on solvent properties of aqueous media are comparable or exceed effects of polymers forming ATPSs, one can hypothesize that the changed solvent properties inside PMLOs might play a defining role in partitioning of various cellular components (proteins, nucleic acids, polysaccharides, and small molecules) to these cellular bodies. For example, it was shown that the microenvironments created by LLPT might control the dynamic distribution of single-stranded and double-stranded DNA in a phase separated system formed by the intrinsically disordered N-terminal tail of the Ddx4 protein (Ddx4^{N1}, an N-terminal region of human Ddx4 containing residues 1–236), which is a member of the DDX4/VASA subfamily of the DEAD-box helicase family, and is serve as a primary constituent of nuages or germ granules. In fact, Ddx4^{N1} was shown to form phase-separated organelles/droplets both in live cells and in vitro, and the double-stranded DNA was preferentially excluded from these droplets, whereas the single-stranded DNA was significantly concentrated inside the Ddx4 droplets [107].

7. Concluding remarks

In this perspective article, advances in our knowledge of ATPSs and PMLOs are discussed together with remaining open questions. It is established that in ATPSs, solvent properties of aqueous media in the coexisting phases differ in regard to their hydrophobic and electrostatic properties, as well as the properties of water to participate in dipole-dipole and hydrogen bond interactions, where water serves as a donor and acceptor of hydrogen bonds. It is determined that partition behavior of solutes in such systems is governed by the aforementioned differences between the solvent properties of water in the coexisting phases. Partitioning of solutes (including proteins) in such systems is not affected by direct interactions of the analyzed solutes with phase-forming polymers. It is also found that certain nonionic additives capable to influence the solvent properties of water in the phases may drastically change partition behavior of proteins and other solutes.

It follows from the aforementioned advances that studies of partitioning of solutes in aqueous two-phase systems enable one to obtain information about relative contributions of different types of solute-water interactions into partition behavior of the solutes. This information may advance our understanding of solute-water interactions and the solute behavior in biological systems. Such information, in particular, shows that the crowding effects in cells may be caused by the influence of the crowding agents on the solvent properties of water and consequently on the properties of proteins and nucleic acids more strongly than the so-called volume exclusion effect.

Phenomenon of biological liquid-liquid phase separation leading to the formation of PMLOs is also introduced, and variability of PMLOs is discussed. It is indicated that ATPSs and PMLOs have many common features. Both of these phase-separated entities are formed as a result of the liquid-liquid phase transitions and are characterized by liquid-like properties, fluidity, and high water content. It is indicated that although there is a sizable literature on the properties of the ATPS phases and solvent properties of water inside these phases, much less is known about PMLOs. Currently available information suggests that similar to ATPSs, the interior of PMLOs is characterized by changed solvent properties of water. It is hypothesized that these changed properties of water inside the PMLOs can drive partitioning of various compounds (proteins, nucleic acids, organic low-molecular weight molecules, metal ions, etc.) between the PMLOs and their surroundings.

Altogether, for both polymer-based ATPSs and biological PMLOs, there is an interesting interplay between the solvent properties of aqueous media and LLPTs, where changes in solvent promote LLPTs leading to the formation of ATPSs or PMLOs, which, in their turn, change solvent properties of aqueous media inside the ATPS phases and inside PMLOs.

Despite their remarkable similarity, ATPSs and PMLOs have noticeable differences. For example, formation of the polymer-based ATPSs is a completely reversible process, and their phases are thermodynamically stable and do not change under fixed environmental conditions. However, PMLO formation can be irreversible, since some PMLOs are metastable systems, which, being present for a prolonged time, can undergo irreversible transitions into gels or glasses. In fact, many PMLOs are present in the cell for limited time only and easily disassemble when not needed anymore. Alterations in the normal PMLO biogenesis can cause them to be present for prolonged time, resulting in the irreversible changes ultimately associated with pathological transformations.

Conflicts of interest

There are no conflicts of interest to declare.

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