The synergic effect of water and biomolecules in intracellular phase separation

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Abstract | Phase separation has long been observed within aqueous mixtures of two or more different compounds such as proteins, salts, polysaccharides and synthetic polymers. A growing body of experimental evidence indicates that phase separation also takes place inside living cells, where intrinsically disordered proteins and other molecules such as RNA are thought to assemble into membraneless organelles. These structures represent a new paradigm of intracellular organisation and compartmentalisation in which biochemical processes can be coordinated in space and time. Two thermodynamic driving forces have been proposed for phase separation: the strengths of macromolecule–macromolecule and macromolecule–H_2O interactions, and the perturbation of H_2O structure about different macromolecules. In this Perspective, we propose that both driving forces act in a concerted manner to promote phase separation, which we describe in the context of the well-known structural dynamics of intrinsically disordered proteins in the cellular milieu. We further suggest that this effect can be extended to explain how the partial unfolding of globular proteins can lead to intracellular phase separation.

Two decades have passed since Tolstoguzov first proposed that globular proteins adopt rigid 3D structures to ensure their miscibility (co-solubility) with other biomolecules. In fact it turns out that these compact structures, in contrast to those of 'unfolded proteins' have a lower propensity to interact with other biomolecules and undergo aggregation or phase separation[1,2]. Tolstoguzov recognized the importance of phase separation in cellular processes[1], and we have since developed a detailed (albeit incomplete) understanding of these processes in biological systems[3,4]. It is now widely acknowledged that 'unfolded proteins' — either an **intrinsically disordered protein (IDP)** or one with an **intrinsically disordered protein region (IDPR)** — drive phase separation inside cells to afford a local microenvironment known as a **membraneless organelle (MLO)**[3,5,6]. In recent years, MLOs have attracted increasing attention largely because they can serve as selective microreactors in which specific biochemical processes take place — some components are recruited and concentrated while others are excluded[7,8]. MLOs also exhibit a dynamic liquid nature that allows the rapid and efficient exchange of components[9,10]. Furthermore, most MLOs assemble and disassemble in response to variations in their cellular environment such as post-translational modifications (PTMs)[11–15], pH and temperature[15–18]. This dynamism, made possible by multiple specific and transient interactions known as **quinary interactions**[19–21], constitutes a mechanism by which cells can respond to stress[16–18]. MLOs are also involved in diverse functions including signalling pathways[22], regulation of gene expression[23–25] and RNA processing[25,26] and are found in the nucleus[27,28], cytoplasm[29], mitochondria[30] or chloroplast[31]. In addition, recent work suggests that the deregulation of MLOs is strongly related to some neurodegenerative disorders[32–35].

Already a century before **liquid–liquid phase separation (LLPS)** was observed in cells, it was known that aqueous mixtures of two different polymers undergo a similar phenomenon. Indeed, in 1896 the microbiologist Martinus Beijerink observed that an aqueous solution of gelatine and either agar or soluble potato starch separates into two different phases[36]. This observation went largely unnoticed by the scientific community at the time and it would be another half century before the aqueous two-phase system (ATPS) was rediscovered by Per-Åke Albertsson. In this case, it was found that a mixture of poly(ethylene glycol) (PEG, $M \approx 4000$ Da) and KH_2PO_4/K_2HPO_4 in H_2O (pH 7) separates into two different phases[37]. Mixing aqueous PEG and dextran (a branched glucose polymer) leads to the same result[38] and it turns out that there are several pairs of compounds that undergo phase separation in H_2O, including polymers, salts, ionic liquids and proteins (Fig. 1)[39,40]. These pioneering observations give rise to an extensive body of work aimed at the application of these systems to the separation and isolation of cells, cellular organelles, viruses and biomolecules[41]. Given the knowledge that has since been accumulated, there is now growing interest in applying the ATPS concept to the large-scale

purification of biomolecules[42] and the separation of whole cells — including stem cells and other bioparticles like viruses and nucleic acids[43]. Other possibilities for ATPSs include microscale applications, such as micropatterning for tissue engineering, microarrays and microfluidics for biochemical analysis[44].

Figure 2 | **Hydration and bulk H_2O around a solute.** The solute is surrounded by hydration H_2O (1st and 2nd hydration shell) molecules that are relatively strongly bound and ordered. H_2O molecules beyond the 1st and 2nd hydration shell are generally defined as bulk H_2O. However, there is some evidence of hydration H_2O molecules existing beyond the 2nd or 3rd solvation shell, and their restricted dynamics make them distinguished from bulk H_2O.

At the time of writing, one of the most exciting applications of ATPSs is in the mimicry of intracellular milieu, including the formation of MLOs. This application was enabled through an initial report in 2002 that described how ATPSs in lipid vesicles could mimic living cells[45]. The vesicles,[46] which featured an outer lipid membrane, contained an aqueous PEG-rich region and an aqueous dextran-rich region in which protein preferentially localized. If these two regions are asymmetrically arranged, the vesicles resemble polar living cells such as budding yeast[47]. This experimental work supported earlier independent proposals[48,49] suggesting that phase separation in the cytoplasm could be an important mechanism by which biochemical reactions can be compartmentalised and regulated. Despite these bold proposals and the growing number of chemical ATPSs, liquid–liquid phase separation was first observed in living cells only in 2009. This report describing LLPS in the cytoplasm of *Caenorhabiditis elegans* germ cells during P granule formation[9] was followed two years later by the report of a similar phenomenon in the nucleolus of *Xenopus laevis* oocytes (immature ova)[10]. Since the appearance of these landmark reports, several previously known cellular bodies have been shown to form by phase separation[50]. In most cases they form from a mixture of RNA and proteins, with IDPs and IDPRs apparently having an important role (Box 1). Recently it was proposed that protein unfolded states might also have an important role in the formation of MLOs[21].

Box 1 | **Molecular description of liquid membraneless organelles.**
Many MLOs, such as P-bodies[160], stress granules[161], Cajal bodies and nucleolus[28] or paraspeckles[27,162], comprise diverse IDPs or IDPRs and RNA. Other MLOs, such as insulator or cleavage bodies, are only composed of IDPs or IDPRs[66]. DNA is also found in some MLOs, such as heterochromatin[163]. Two major distinct phase separation mechanisms enable the formation of MLOs: homotypic interactions between like IDPs or IDPRs[15–18,23,60–62,164–167] or heterotypic, multivalent interactions between folded domains and IDPs or IDPRs of two or more unlike proteins[8,26,166,168–170]. IDPs and IDPRs are composed of a set of compositionally distinct regions known as low complexity domains (LCDs) that are crucial for phase separation. Examples of these regions include prion-like domains (PLD)[18,60,171], arginine–glycine–glycine (RGG)[61], phenylalanine–glycine (FG) and arginine–glycine (RG) motifs[15]. RNA-binding domains (RBD) are also components of many IDPs and IDPRs that undergo phase separation[16,17,171]. Binding of RNA to these domains can either inhibit or promote LLPS[16,60,62,117–121]. LCDs interact with each other and with RNA through both electrostatic and hydrophobic contacts, including charge–charge, dipole–dipole, cation–π and π–π stacking[15,62,171,172]. The concept of multivalent associative polymers[173] can be applied to predict critical concentrations at which fused in sarcoma (FUS) family proteins such as TDP-43 and hnRNPA1[171] undergo LLPS. In this model, IDPs are composed by 'stickers' and 'spacers', with the former being responsible for the intermolecular interactions that form the droplets while the latter are responsible for the structural dynamics and material properties of the droplets[174,175]. For FUS family proteins, interactions between Tyr residues of PLDs and Arg residues of RBDs function as stickers, allowing the prediction of their critical concentration for phase separation[171]. The table below describes some examples of proteins that undergo either homotypic or heterotypic LLPS (see Table S1 for a more extensive list).

Type of LLPS	Protein composition	Localization and chemical nature of the intermolecular interactions	Conditions for phase separation (in vitro and in vivo)

Homotypic interactions	FUS[13,33,60,171,182]	Stress granules (cytoplasm). Cation–π interactions mediated by Arg and Tyr residues in LC prion-like domain and RBD.	Low temperature (0–15 °C), depending on [NaCl] (0–250 mM). Phase separated under heat stress (42 °C) in cells. Enhanced by RNA and inhibited by methylation and phosphorylation.
	Ddx4[15]	Germline P granules (cytoplasm). Cation–π interactions mediated by FG and RG LCD from N-terminal domain.	Temperature varies (10–50 °C) depending on [NaCl] (100–300 mM). Attenuated by methylation.
	hnRNPA2[165,183]	Neuronal RNA granules (cytoplasm of neurons). Arg–aromatic interactions mediated by hnRNPA2 LCD.	Phase separation occurred in the presence of 50 mM of NaCl. Decreased by methylation.
Heterotypic interactions	Nephrin, adaptor protein Nck and Neural Wiskott-Aldrich syndrome protein (N-WASP)[169,184]	Membrane cluster. Multivalent oligomerisation/polymerization of Nephrin, Nck and N-WASP. The interdomain linker between two SH3 domains of Nck plays a central role.	Promoted by phosphorylation.
	NPM1 and Surfeit locus protein 6 (SURF6)[170,185]	Nucleolus (nucleus). Electrostatic, multivalent interactions among NPM1 and SURF6 IDPRs. Protein–RNA interactions where also suggested to play a role.	Phosphorylation was proposed to inhibit phase separation.

The aim of this Perspective is to compare the relatively well-studied ATPSs with MLOs in order to better understand the molecular mechanisms by which the latter form. The discussion will focus on two well-known models of phase separation: the Flory–Huggins (FH) theory[51–53] and the theory of H_2O structure alteration originally formulated by Zaslavsky[54,55]. In view of the importance of H_2O ordering, we propose here that intracellular LLPS is driven by a balance of enthalpically favourable intermolecular interactions and the increase of solvent entropy on moving to a solute surface to the bulk. This proposal is discussed in the context of H_2O-mediated effects on the association of IDPs and the factors that modulate this process, and we also describe the role of IDPs in phase separation.

[H1] General aspects of liquid–liquid phase separation

The ordering of hydration H_2O molecules has been proposed to be central to phase separation. It has been the subject of several studies[64,85] but there are several unanswered questions concerning the structure of liquid H_2O and how it is affected by the presence of other compounds. The most widely accepted model of H_2O proposes that the liquid state features a three dimensional network of H bonds, in which each molecule binds four other molecules in a tetrahedral structure (Fig. 2). In contrast to ice, in which the H bonds are kinetically stable, in liquid H_2O these bonds only have average life-times in the range 1–8 ps[176–178]. The dynamism of this network means that even in the bulk there will be some defects in terms of dangling H bond donors or acceptors, but these states are very short-lived (<200 fs)[179]. This dynamism is also reflected in the rate at which H_2O responds to the introduction of a solute,

when an almost instantaneous rearrangement of H_2O structure is observed. The nature of this rearrangement depends on the hydrophilic or hydrophobic nature of the solute, but in each case there are two different regions of H_2O: one closer to the compound (hydration H_2O) and the other further away (bulk H_2O). The boundary between the two regions is sometimes difficult to assign but several experimental and theoretical studies confirm that the mobility of H_2O molecules is substantially reduced in the vicinity of other molecules[180,181].

Figure 1 | **Solutions can undergo liquid-liquid phase separation to afford aqueous two-phase systems.** The two components in each system coexist in solution until a critical concentration is reached and the system separates into two different aqueous phases. The different physicochemical characteristics of each phase have been exploited in applications that include the separation of complex mixtures[56]. **a** | An aqueous mixture containing two polymers can separate into two phases that are enriched in the different polymer species. **b** | Likewise, an aqueous salt and polymer mixture can separate into a salt-rich phase and a polymer-rich phase.

Phase separation occurs when the concentration of macromolecular components in a system exceeds a critical concentration such that they accumulate into distinct phases[41]. For LLPS in biological systems, two types of polymer systems are relevant: non-associative or segregative and associative[56]. Segregative polymer systems include phase-separated solutions of two polymers (ATPSs), where the different phases are each enriched in a different polymer (Fig. 3a). For example, the nucleolus is sub-compartmentalised when fibrillarin (FIB1, an enzyme involved in processing pre-ribosomal (r)RNA) and nucleophosmin (NPM1, a protein that binds nucleic acids and is involved in ribosome biogenesis) separate into two distinct coexisting phases[57]. In associative polymer systems, phase separation occurs when a phase rich in both polymers forms inside a polymer-depleted phase (Fig. 3b)[56]. In a chemistry context this is referred to as **coacervation**, which is observed when oppositely charged polymers experience electrostatic attraction and form concentrated coacervate droplets surrounded by a dilute solution[58]. The same process happens in cells when, for example, the negatively charged nephrin intracellular domain (NICD) undergoes complex coacervation with positively charged proteins[59]. Associative LLPS is also possible with aqueous solutions featuring only one polymer species (Fig. 3c). A large number of cellular examples species undergo this type of process, including fused in sarcoma (FUS, a protein that binds DNA and RNA)[60], RNA helicases Ddx4[15] and LAF-1[61], polyadenylate binding proteins Pab1[16] and Pub1[17], translation terminator factor Sup35[18], and heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1)[62].

Figure 3 | **There are three types of aqueous polymer phase separation in biology. a** | Segregative polymer systems comprise two polymers that separate into two distinct aqueous phases, each of which is composed mostly by one of the polymers**. b** | Associative polymer systems comprise two oppositely-charged polymers that interact with each other to form a phase distinct from the surrounding environment or coacervate. **c** | Polymers can also self-associate, forming droplets that separate from the surrounding mixture[56].

Phase separations of synthetic polymers and proteins have many features in common. These include a dependence on concentration and external factors such as pH, ionic strength and temperature, as well as physicochemical properties of the components, such as molecular weight, charge composition and hydrophobicity[57,63–65]. In addition to these, the structural disorder that is characteristic of many polymers and proteins (IDPs or IDPRs) appears to be a? key property common to the majority of the phase-separating systems[64,66]. There are several globular proteins that have been observed to undergo phase separation in vitro[67–72] but have yet to be observed to experience the same process in cells. One possible reason for this is the concentration dependence of LLPS. Indeed, phase separation occurs above certain concentrations, when the intermolecular interactions among polymers or among IDPs/IDPRs are energetically favourable[51,52,73]. The disorder and flexibility of synthetic polymer and IDP chains means that these moieties present a large surface area for interactions, and result in IDPs having a lower threshold for phase separation than is the case for more ordered and compact systems. This concept has been elucidated theoretically[74] and has experimental precedent in the behaviours of aqueous systems featuring two polymers (ATPS) or two proteins in which at least one is intrinsically disordered. These

systems undergo phase separation at concentration thresholds lower than systems featuring two compact macromolecules, such as globular proteins[2,48]. Moreover, the larger exposed surface area of disordered solutes not only enables a greater number of solute–solute interactions but also a greater number of solute–solvent interactions. In this regard, it is important to understand the comprehensive molecular interplay that leads to phase separation, including interactions between polymers and solvent.

LLPS and **protein aggregation** leading to **amyloid formation** are frequently referred to as protein self-assembly processes, although their function and mechanisms are distinct[75]. LLPS involves a reversible, dynamic equilibrium between two thermodynamic states of a solute: the dissolved (DS) and droplet forms (Fig. 4a). Protein aggregation and consequent amyloid formation involves kinetically-trapped intermediate states (amorphous aggregates and oligomers) and a final more thermodynamically stable fibril state that renders the process irreversible[75,76]. Dysregulation of MLOs can cause their components to aggregate into amyloids (Fig. 4b)[33,34]. In such a **liquid-to-solid phase transition** (LSPT) process, the droplets become persistent over time, and their inability to disassemble contributes to their increasing toxicity[32–35].

Figure 4 | **Free energy diagram for LLPS and protein aggregation. a** | Inside the cell, IDPs can exist in a dissolved, free state or in a droplet phase. The free energy difference between these two states is small such that the balance can be easily tipped by several factors including concentration, pH, temperature or post-translational modifications (PTMs). Dysregulation of the LLPS process can lead to toxic liquid-to-solid phase transitions (LSPT)[157]. **b** | Protein aggregation pathways can also involve kinetically trapped states such as partially folded states, oligomers and amorphous aggregates that grow into thermodynamically more stable amyloid fibrils. These latter states are detrimental to cells[158]. The nucleophosmin DNA-binding domain (shown in orange/blue, IDP) (Protein Data Bank identifier: 2LLH), native myoglobin (red) (structure predicted using the SWISS-MODEL server, based on PDB ID: 2IN4) and partially and total unfolded myoglobin (representative conformation obtained by molecular dynamics simulation at 500 K, during 100 ns, using Gromacs software package[159]) structures were selected for visualisation purposes and may not represent biologically-relevant interactions.

[H1] Models of polymer and protein phase separation

The thermodynamic driving force for both segregative and associative polymer systems is most frequently explained by invoking the Flory–Huggins (FH) formalism. This mean-field theory predicts that the phase transition of a polymer solution depends on the nature of the chain–solvent, chain–chain and solvent–solvent interactions[51–53]. Flory introduced a parameter χ to account for the energetic cost of having the polymer chain sites being occupied by solvent molecules instead of interacting with another chain. When $\chi < 0$, the chain–solvent interactions are energetically favoured and the polymer is dissolved, the system being in a **good solvent** regime. When $\chi > 0$, the chain–chain interactions are energetically favoured over the chain–solvent interactions, such that the polymer adopts a compact conformation as part of a **poor solvent** regime. Ultimately, χ reaches critical values ($\chi >> 0$), whence enthalpic changes (ΔH) outweigh the entropy of mixing (ΔS_{mix} of ideal solutions), resulting in a positive contribution to the free energy of mixing (ΔG_{mix}). As a consequence, the system separates into a polymer-rich phase surrounded by a polymer-depleted phase[51–53]. This theory has been adapted to systems containing two polymers[77], and in particular has been used to rationalize the phase separation of two neutral polymers observed in aqueous solution[78]. Remarkably, recent studies have applied this model to predict phase diagrams of intracellular proteins[15,62,79–81]. Besides considering ΔH, the χ parameter also includes an entropic component (ΔS) that accounts for both the entropy of the protein and solvent[53]. However, the individual role of each of these entropies to phase separation cannot be discriminated. In addition, FH theory has inherent limitations that stem from its simplicity — it treats polymers as charge-neutral homogeneous chains and can therefore not account for electrostatic contacts and sequence variations[82]. Other theories have been developed for complex coacervation (Overbeek and Voorn theory (OV))[83] and for the dependence of phase separation on the distribution of charged residues within IDPs[84] (random phase approximation theory), and these have been compared to the classical FH theory[82,84].

None of these newer theories account for the influence of H_2O-mediated polymer–polymer or protein–protein interactions. These effects were considered by Zaslavsky and co-workers, who

proposed that phase separation occurs because polymers alter the structure of H_2O to the point that it forms two different and mutually incompatible H_2O structures[54,55,85]. This proposal was developed in order to explain previous experimental studies suggesting that H_2O-mediated structural changes induced either by the ATPS components (polymers, salts, ionic liquids)[86–90] or factors such as temperature and urea concentration[54] modulated phase separation. Similarly, bulk H_2O was shown to have different properties (acidity and basicity, dipolarity and polarizability) in the presence of amino acids, globular proteins and IDPs[91–93]. In accordance with these observations, it was proposed that the alteration of H_2O properties by the presence of IDPs or IDPRs plays a key role in intracellular phase separation[85]. These experiments on H_2O in the presence of proteins were conducted using solute concentrations of 1.5 %w/v[91,92], values much smaller than the cellular concentration of macromolecules, which is estimated to fall in the range 30–40 %w/v[94]. Thus, in cells we might expect approximately 20-fold larger alterations in the structure of bulk H_2O. Consensus exists regarding the capacity of proteins to alter the dynamics of surrounding solvent[95], although the extent of such perturbation is still under debate[96–99]. Nuclear magnetic resonance (NMR) spectroscopy, time-resolved vibrational spectroscopy and dielectric relaxation spectroscopy have been applied to H_2O and show that its dynamics inside different microbial cells are diverse, with 15–45% of H_2O molecules having slower dynamics in comparison with the bulk[100,101]. Such slower motion happens mostly on the surface of proteins[101], which is consistent with the idea that H_2O can be well structured when at macromolecular surfaces[102,103]. Thus, in both IDPs and globular proteins, hydration H_2O was found to have greater order and lower mobility relative to bulk H_2O[104,105,106,108,112]. This higher ordering of H_2O when at a protein surface has been proposed to have a key role in protein aggregation and amyloid formation[109]. Amyloid fibrils form by a primary nucleation polymerization step, in which protein monomers assemble and form oligomers that continue to grow into protofilament and further fibrils either by adding monomers one at a time or associating with another oligomer[110,111]. Time-resolved fluorescence spectroscopy and computational studies indicate that both oligomer formation and consequent protofilament and fibril grown can be driven by a positive change in entropy associated with liberating confined H_2O molecules from the protein surface to the bulk[106,108,112–114,153].However the role of H_2O, was found to be different for hydrophilic and hydrophobic sequences[113,114]. MD simulations showed that protofilament formation is 1000 times slower for hydrophilic N-terminal peptide of Sup35 in comparison to hydrophobic Aβ-peptides due to the formation of metastable long-live structures in the former. These structures are stabilized by interactions between H_2O and the surface of the β-sheets[114]. Additionally, a different role of H_2O was suggested in the aggregation and consequent amyloid formation of tau (a protein that stabilized microtubules)[153]. Tau monomers are composed by a repeat domain that includes four repeat smaller domains (R1 to R4) and a projection domain which contains two N-terminal repeats and two proline-rich domains[186]. R1 to R4 compose the core domain that forms β-sheet structures in the fibers whereas the rest of the domains remain disordered during fibrillation. Tau fibrillation was driven by an increased mobility of H_2O (and therefore increased entropy) around the unstructured region of the fibres when compared to the monomeric form[153]. Other study has shown that tau aggregation is driven by H_2O expulsion of the R3 of core domain upon association with adjacent tau[187]. Collectively these results suggest that the role of H_2O entropy in protein aggregation may involve different mechanisms acting simultaneously, depending on the sequence and disorder degree of the protein.

Given the increased evidence for an intrinsic relation between dysregulation of LLPS as a possible promoter of protein aggregation and amyloid formation[186,157], the potential role of hydration H_2O on phase separation cannot be ignored. Indeed, Sup35 and tau, for which H_2O-mediated amyloid formation was investigated[114,153,187], were also found to phase-separate inside cells[18,167,186]. Remarkably, N-terminal domain of Sup35[18] and both N-terminal domain and repeat domain of tau[167,186,188] were found to be crucial for their LLPS. Another IDP recently observed to phase separate inside the cells is α-synuclein[189].Both LLPS and LSPT of this protein were driven by intermolecular hydrophobic interactions between the residues of amyloidogenic nonamyloid β-component (NAC) domain[189]. Accordingly, NAC domain was previously found to drive α-synuclein aggregation and amyloid formation, possibly through the release of confined H_2O to the bulk[108]. One could think that, in this particular case, water expulsion may play a crucial role in the α-synuclein LLPS→LSPT process. Nevertheless, when we speak of liquid protein droplets, it is important to keep in mind that there is still

a lot of H_2O present, which is thought to enable IDPs to remain disordered and dynamic even when concentrated[79,115,116]. For example, an elastin-like polypeptide (ELP) droplet is approximately 62.5w/w% H_2O[115] and Ddx4[79] droplets are 73v/v% H_2O. In view of this, H_2O may have a crucial role in the modulation of DS ⇌ LLPS→LSPT processes. Because H_2O is, in general, more ordered around protein´s surface, their regulated release from the surface to the bulk ($\Delta S^{H_2O}_{surface\text{-}to\text{-}bulk} \lessdot 0$) upon protein association may be an unfavourable thermodynamic force for ΔG_{mix} ($\gtrdot 0$) and promote LLPS. This process in conjugation with favourable intermolecular protein-protein interactions ($\Delta H \gtrdot 0$) would be sufficient to induce phase separation (Fig. 5a). Previous studies using FH model were able to quantitatively determine changes in the ΔH and ΔS upon phase separation of N-terminus Ddx4[15]. At 50 mM of salt, a positive ΔH (~1 kJ mol^{-1}) and negative ΔS (~ -7.5 J mol^{-1} K^{-1}) were observed, attributed to favourable electrostatic interactions and increased mobility of protein or its associate H_2O in the droplet state[15]. Another work observed that tau-RNA droplet formation occurs with a negative ΔS (~ -10.8 J mol^{-1} K^{-1} (FH-based OV)), suggested to be mainly linked to $\Delta S^{H_2O}_{surface\text{-}to\text{-}bulk}$[143].

In the same line, dysregulation of LLPS could lead to $\Delta S^{H_2O}_{surface\text{-}to\text{-}bulk} \lessdot\lessdot 0$, and promote LSPT. Such idea may be appreciated if one considers the sequence dependence exhibited by LLPS→LSPT. For example, one could expect different H_2O entropy roles for both polar and hydrophobic domains in LSPT process, as observed for Sup35 and Aβ-peptides[114]. In situations where aggregation is thermodynamically favoured, water expulsion from the hydrophobic regions would promote LSPT whereas water bound to polar IDPRs would act as a shield, slowing down aggregation process. In addition, H_2O entropic contribution in the surface of different regions (disorder or structured) of the protein would also be critical for LLPS→LSPT, as it was observed for tau aggregation and consequent fibrillation[153,187]. Indeed, factors such as chaperone recruitment[33] or addition of **hydrotropes** (e.g. ATP)[140] must balance the H_2O entropy gain by both release of H_2O from the structure regions and increased H_2O mobility in the surface of disordered regions, in order to prevent LSPT.

Figure 5 | **Phase separation inside cells is driven by intermolecular interactions and H_2O entropy.** **a** | When the concentration of a protein (brown and blue) reaches a critical value, the favourable protein–protein interactions and the concomitant release of H_2O molecules (shown in red) from the protein surface to the bulk become sufficient to overcome the entropy of mixing. In this case liquid–liquid phase separation (LLPS) is thermodynamically favoured. **b** | Similar intermolecular forces may also drive mixtures of proteins and RNA (green) to give a phase rich in the two biomacromolecules. Here, the nucleophosmin DNA-binding domain (Protein Data Bank identifier: 2LLH) and 16S rRNA (PDB ID: 1PBR) were selected for the purposes of visualisation and the number of H_2O molecules was kept constant during phase separation processes.

. Recent experimental studies of RNA self-assembling and forming droplets in vitro and in vivo[122–124] formed the basis of a hypothesis that RNA could have a role in the formation of many membraneless organelles through a balance of protein–protein, protein–RNA and RNA–RNA interactions[125]. Molecular dynamics (MD) simulations indicate that H_2O molecules at an RNA surface, as is the case with a protein surface, are more ordered and move more slowly relative to the bulk[126,127]. Therefore, either alone or in combination with IDPs, RNA can drive phase separation with a net decrease in enthalpy due to favourable intermolecular interactions, as well as an additional entropic gain from the solvent becoming more disordered **(**Fig. 5b**)**. Overall, the release of every ordered H_2O molecule from the surface of a macromolecule into the bulk will be entropically favoured. Nevertheless, not every protein-bound H_2O molecule is more ordered than bulk H_2O, as is exemplified by H_2O molecules weakly H-bonded inside a protein cleft[128]. In such a case, desolvation of the protein is entropically unfavourable. Moreover, there are some H_2O molecules at the macromolecule surface that mediate its interactions with surrounding macromolecules and so are not released on binding[129].

[H1] Phase separation, protein structure and H_2O dynamics

Several factors can affect the interplay between H_2O structure, dynamics and protein–biomolecule interactions. Thus, the nature and concentrations of salt(s) present, pressure, osmotic stress, crowding,

pH, ATP concentration and temperature can directly lead to changes in the DS ⇌ LLPS →aggregation processes. For example, the presence of salts affects both LLPS and LSPT of aqueous protein solutions by changing the hydration energies[131]. Hydrostatic pressure can also affect H_2O structure and thereby modulate LLPS of lysozyme solutions[132]. Along the same lines, the competition between protein–H_2O and protein–**osmolyte** interactions controls protein stability and phase behaviour in solution[133,134]. Molecular crowding was also shown to promote LLPS by enhancing protein–protein or protein–RNA interactions[134–136]. Nevertheless, it has not been determined if this process is H_2O-mediated or not, although there is evidence that the presence of crowders can induce the release of H_2O from a protein surface[137,138]. Similarly, pH affects the hydration of proteins and hence protein–protein interactions[139], though the function of H_2O in this process associated to LLPS remains largely unknown. ATP has also been shown to help solubilize protein molecules and prevent their LLPS and aggregation[140]. Again, the exact way in which this hydrotropic action affect hydration of both DS and LLPS remains unexplored. A more well-studied aspect is the role of H_2O in temperature-induced phase separation[141]. A classic example is the coacervation of ELPs[115,116,142], a process driven by the entropic cost of solvating hydrophobic ELP domains at high temperatures, in combination with favourable intermolecular interactions[115]. Similarly, it has been suggested that the temperature-dependent phase separation in mixtures of RNA and tau is favoured by an entropic gain associated with the release of H_2O bound to tau, which presents a hydrophobic surface that is not favourable for H_2O binding[143]. Remarkably, solutions of IDPs with a high content of hydrophilic residues, at high temperature, collapse due to an unfavourable solvation free energy[144]. Temperature-driven phase separation has been observed for some IDPs, including Ddx4[15], FUS[33,60], Pab1[16] and Pub1[17]. Overall, these results suggest that H_2O can have an active role in temperature-dependent phase separation.

The thermodynamics of interactions involving H_2O are also relevant when we consider phase separation driven by intermolecular interactions between globular partially unfolded states and IDPs[21]. As is the case with Pab1[16] and Pub1[17], the thermally driven partial unfolding of some globular proteins leads to the exposure of hydrophobic regions. When two proteins present such regions then they will associate because of attractive intermolecular interactions, leading to the exclusion of H_2O molecules from these regions, for which there is no strong thermodynamic driving force to solvate. Thus, above a certain critical protein concentration, the intermolecular forces can lead to phase separation (Fig. 6). Temperature-induced protein partial unfolding was previously considered to be responsible for driving the formation of reversible and specific protein aggregates in response to heat stress[145]. Other reversible metabolic enzyme assemblies were found to form in response to low pH conditions, such as those present during nutrient starvation[146,147]. Indeed, when the cytoplasm becomes acidified a cell can enter **dormancy**, a state in which proteins are organised into multiple assemblies that lead to a liquid-to-solid-like phase transition[148]. Strikingly, this phase transition is accompanied by a reduction of the cell volume, which has been suggested to be related to the expulsion of H_2O molecules when high-order assemblies form[148]. Thus, these observations indicate that the role of H_2O in higher order macromolecular assemblies — including MLOs, filaments or reversible protein aggregates — cannot be neglected and warrants future attention.

Figure 6 | **Thermally-driven phase separation of partially unfolded globular proteins.** When present at high temperatures (for example, in a cell experiencing heat stress), globular proteins (red and orange) may partially unfold and expose their hydrophobic residues. Favourable intermolecular interactions between these domains and other IDPRs (blue), as well as the entropic cost of solvating the newly exposed hydrophobic residues, promote liquid–liquid phase separation (LLPS). Fused in sarcoma RNA-binding domain (blue, Protein Data Bank identifier: 2LA6) and the tRNA aminoacylation cofactor Acr1p (orange)-glutamine tRNA synthetase GluRS (red) complex[145] (PDB ID: 2HRK) were selected for the purposes of visualisation.

Our present discussion has so far only accounted for H_2O molecules that are at the surface of the proteins and belong to the first or second hydration shells (≈4–8 Å). However, the five-helix bundle protein $\lambda^*_{6\text{-}85}$ and ubiquitin have been shown to be accompanied by extended dynamic hydration shells that extend 10 and 18 Å beyond the protein surface, respectively[96,97]. The crowded cellular environment substantially reduces the free volume, such that the distinction between hydration H_2O and bulk H_2O

becomes somewhat blurred[97]. The extended hydration structure and dynamics have become topical in view of recent observations indicating key roles of distant hydration H_2O molecules, in particular how they mediate protein folding and unfolding[96] or protein–protein interactions[149]. Advanced terahertz and far-infrared spectroscopic studies have shown that the extended H_2O network structure and dynamics surrounding human serum albumin are substantially changed when even a short chain PEG interacts with a protein[150]. Another ultrafast 2D infrared spectroscopy study described a subtle transition from independent-to-collective hydration (picosecond) dynamics over a range of ~30 Å around a lysozyme in the presence of both polymer and protein **crowders**[151]. Interestingly, MD simulations revealed that changes in temperature or the electric field exerted by dissolved biomolecules perturb the microstructure of a H_2O network and eventually the inner dynamics of two protein molecules as they approach each other[149]. Together, these observations strengthen the notion that not only the vicinal but also remote H_2O cooperatively modulate protein–protein interactions, although how this affects LLPS remains to be explored. Therefore, the nature of H_2O structure and dynamics, and its influence on IDP interactions and LLPS and/or aggregation inside cellular environments, are stimulating topics for future consideration.

[H1] Concluding remarks and future perspectives

Phase separation inside cells is emerging as one of the most relevant and enlightening discoveries of the 21st century because it may provide an answer to the question of how cells achieve the efficient organisation of biochemical reactions. However, there is much to be learned about the implications of this phenomenon on cellular functions. One way to accomplish this is by using the knowledge gained from more than 50 years of research on model systems such as aqueous polymers that undergo in vitro phase separation. In this Perspective, we have described both the model (ATPS) and native systems (intracellular LLPS) in terms of the thermodynamics that drive phase separation. Inspired by the central role that H_2O plays in protein association events, we propose here that intracellular LLPS may result from a synergetic effect between both intermolecular interactions and H_2O entropy. In this regard, future in vivo[101], in vitro[108] and in silico[107] studies will be necessary to understand the hydration structure and dynamics of IDPs inside and outside of MLOs. The major aim of these studies will be to determine which factors (for example, pH, ionic strength, osmolytes and crowders) modulate protein hydration and structural flexibility. It will also be important to explore these notions in the light of the partitioning behaviour of the components inside or outside MLOs[85,152].

We advocate further studies on the role of H_2O entropy in aberrant liquid-to-solid phase transitions induced by aging or different stresses[32–35], given the already crucial role of H_2O on the pathological aggregation and fibrillation of some IDPs or IDPRs[106,108,112]. Indeed, H_2O expulsion favours protein aggregation and amyloid formation[109], such that retaining H_2O inside droplets would be an advantageous strategy to prevent detrimental aggregation[116] because the high protein concentration within MLOs inherently favours aggregation[32]. We envisage that in the near future, neutron scattering[153], time-resolved fluorescence[108,112], NMR[154] and terahertz spectroscopy[155,156] may be widely applied to probe the hydration states of components during LLPS and after transition to solid toxic protein aggregated form in cell mimics or in cells themselves.

[H1] Glossary

Amyloid fibrils

Highly ordered structures that result from protein aggregation and oligomer formation or association. These structures are bound together by interactions between β sheets. They are associated with several neurodegenerative diseases such as Parkinson's, amyotrophic lateral sclerosis and Alzheimer's.

Coacervation

A liquid–liquid phase separation process that leads to the formation of a colloidal phase of concentrated solutions of charged or neutral molecules including synthetic polymers, polyelectrolytes, polysaccharides and proteins.

Crowders

Polymers and proteins that can be used in vitro to mimic the highly concentrated and heterogeneous environment within cells.

Dormancy
When facing conditions that are not ideal for growing, cells arrest their division cycle, entering a dormant state that involves biomolecular reorganisation and diminished metabolic activity.

Hydrotrope
Solute compose by both a hydrophobic and hydrophilic sequences that solubilize hydrophobic compounds in water.

Intrinsically disordered protein/intrinsically disordered protein region (IDP/IDPR)
A protein or region within a protein that does not have a well-defined 3D structure and exhibits high structural flexibility.

Liquid–liquid phase separation (LLPS)
A process that involves two solutes demixing and forming two new phases of different composition. This is thought to be the basis for the formation of membraneless organelles.

Liquid-to-solid phase transition
Under aging or stress conditions, the liquid compartments of MLOs can change to a different, solid phase because their components (proteins) aggregate and eventually form amyloids.

Membraneless organelle (MLO)
An intracellular compartment without a membrane, formed through phase separation due to the heterogeneous distribution of biomolecules. It exhibits a liquid nature and provides a microenvironment that can serve a defined function, such as RNA metabolism.

Osmolyte
A small molecule, such as a polyol, amino acid or methylamine, that alters protein folding and stability under osmotic stress conditions.

Poor and good solvent regime
These two concepts of polymer solutions reflect the favourable or unfavourable interactions of polymer chains with the solvent. In the poor solvent regime, intra and intermolecular interactions of the polymer are favoured in comparison to chain–solvent interactions and so the solvent is defined as poor. The inverse situation applies to good solvent regime.

Protein aggregation
A phenomenon involving intermolecular interactions between misfolded proteins. This is usually the origin of amyloid formation and consequent diseases.

Quinary interactions
Weak, specific and transient interactions between proteins and other biomolecules that appear to have a crucial function in cellular organisation.

[H1] Author contributions
All authors contributed equally to the preparation of the manuscript.

[H1] Competing interests statement
The authors declare no competing interests.

[H1] Publisher’s note

[H1] ToC blurb

Intracellular phase separation is a major regulatory mechanism in several biochemical processes. This Perspective describes the contribution of H_2O and biomolecules to this phenomenon in the framework of two well-known models.

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