Role of Proteins and Divalent Ions in LLPS

Subjects: Biochemistry & Molecular Biology

Contributor: Aneta Tarczewska , Klaudia Bielak , Anna Zoglowek , Katarzyna Sołtys , Piotr Dobryszycki , Andrzej Ożyhar , Mirosława Różycka

Liquid–liquid phase separation (LLPS) is one of the key mechanisms affecting how macromolecular assemblies, including membrane-less organelles (MLOs), are formed and regulated. The molecular and biochemical mechanisms involved in the biomineralization pathway remain puzzling. Additionally, the significance of intrinsically disordered proteins (IDPs), which are an abundant organic component of hard tissues, in the formation of liquid precursors of biominerals remains to be solved. Research on the interactions between proteins and divalent cations is essential for understanding the resulting liquid precursors.

liquid–liquid phase separation | intrinsically disordered proteins | biomineralization

1. Introduction

Liquid–liquid phase separation (LLPS) is one of the key mechanisms affecting how macromolecular assemblies, including membrane-less organelles (MLOs), are formed and regulated [1]. This reversible, thermodynamically-driven process relies on the separation of a homogeneous solution into two distinct liquid phases with different concentrations of solutes. LLPS occurs as a two-phase system based on the concentration of molecules and the physio-chemical parameters of the microenvironment [2]. Phase-separated condensates, especially MLOs, are multicomponent assemblies of proteins and other macromolecules, e.g., nucleic acids [2][3][4]. The interactions between the components are weak, transient, and multivalent [5][6]. The proteins that are found to reside in a condensate may play diverse roles (e.g., scaffold, co-scaffold, clients, and regulators) in maintaining the condensate integrity, composition, and biochemical properties. Scaffolds can self-associate and drive LLPS, so they are primarily responsible for condensate formation. Clients, on the other hand, are low-valency molecules that are recruited to the condensate through their interactions with scaffold biomolecules [7]. Their content may be adjusted to the changing conditions within and outside the condensate [8].

LLPS as a physical process has been known for decades in polymer science, but it has been rediscovered in eukaryotic cells [9]. At present, it appears that it is a universal phenomenon that plays an important role in the interior organization of eukaryotic cells, in the formation of MLOs in prokaryotes [10][11], and during viral life cycles [12]. Notably, some extracellular protein interactions facilitate LLPS [13]. Biomineralization is the process by which organisms produce minerals under biological control. The control of biomineralization is aimed at creating specific minerals composed of inorganic and organic fractions. A very common inorganic component of biominerals is calcium carbonate in the form of various, usually non-calcite polymorphs [14][15]. Although the mechanisms of biomineral nucleation processes have been studied for years, their principles are still enigmatic. Calcite, the most

stable polymorph of calcium carbonate, serves as a model for the primal and accepted-for-years theory of crystal growth, known as a classical theory. Some recent studies, however, showed that the formation of calcium carbonate frequently does not follow the classical model [16][17]. Since 2000, when Gower et al. launched the polymer-induced liquid precursor (PILP) concept of biomineral precursors, it has been widely accepted that the early events of biomineral formation may follow diverse alternative pathways [18]. Moreover, further experimental results concerning calcium carbonate mineralization presented prenucleation clusters as a key precursor phase in mineral formation [19]. Currently, a concept that involves the formation of dense liquid precursors of amorphous calcium carbonate (ACC) via LLPS has become a popular topic of investigation [20][21].

As indicated above, the molecular and biochemical mechanisms involved in the biomineralization pathway remain puzzling. Additionally, the significance of intrinsically disordered proteins (IDPs), which are an abundant organic component of hard tissues [22], in the formation of liquid precursors of biominerals remains to be solved. Research on the interactions between proteins and divalent cations is essential for understanding the resulting liquid precursors. In the available literature, there are only a few examples describing such interactions. However, they may help to understand the functional and pathological phase behaviours in the biomineralization process.

2. Role of Proteins and Divalent Ions in LLPS

First, it was suspected that strong stereospecific interactions between protein components played a major role in the formation of MLOs. However, subsequent studies have shed light on the importance of very weak interactions, e.g., electrostatic, hydrophobic, and π-π interactions [5]. Currently, people know that both strong and weak interactions, which occur simultaneously, contribute to the entire complex interaction network and facilitate condensate formation. Additionally, the interaction of proteins with solvent plays a critical role in the regulation of phase transitions; thus, an important feature affecting protein-induced LLPS is solubility [23]. Most proteins that undergo LLPS have poor solubility in water. Placing such proteins in the structure forming during phase separation is more energetically beneficial than allowing them to come into contact with water [24]. This is particularly important in the case of proteins containing low sequence complexity and a richness in residues that tend to aggregate [25]. Since the physio-chemical properties of the solvent strongly impact protein solubility, LLPS occurs as a function of parameters such as osmolality, ionic strength, pH, or temperature [2][26][27][28].

Another key factor underlying LLPS is multivalence, i.e., the availability of many different binding sites in the molecule. Multivalent proteins can form heterologous electrostatic interactions with different, oppositely-charged proteins or homologous interactions between their repetitive domains [29]. Multivalent proteins have a critical phase separation threshold that is often related to the number of domains it contains and the availability of ligands [24]. Multivalence is especially characteristic for IDPs; therefore, this class of proteins is often involved in promoting phase separation. IDPs do not fold into unique, three-dimensional globular structures under physiological conditions. Changes in the cellular environment and conformational properties allow IDPs to take on numerous conformations induced by the attachment of ligands, binding to the membrane surface, or various types of post-translational modifications [30]. NMR analyses of IDPs after LLPS show that disordered regions of proteins retain conformational flexibility in the condensed phase [26]. Interestingly, IDPs can also form complexes with other

macromolecules or metal ions and consequently undergo, at least in fragments, disordered-to-ordered transitions [31]. IDPs with an inherent propensity for LLPS affect various cellular functions, such as signaling, cell division, intracellular transport, cell cycle control, and regulation of transcription and translation. Unfortunately, in some cases, structural features of IDPs can promote the formation of abnormal conformations prone to aggregation, which in turn causes severe diseases associated with protein misfolding, such as Alzheimer's, Parkinson's, or Huntington's disease [32]. Interestingly, not all fibrous structures cause disease. Amyloid aggregation of a large number of IDPs is associated with the biogenesis of functional amyloids, which positively influence various biological functions, e.g., melanin pigment formation, bacterial biofilm formation, or biominerals [33].

Recently, it was shown that divalent cations also have the ability to tune protein phase behaviour. However, it remains a largely unexplored area. The first report describing LLPS of proteins in the presence of divalent cations comes from 2020. Singh et al. showed that LLPS of tau protein is modulated by zinc ions, which strongly enhance the propensity for tau to undergo LLPS by lowering the critical concentration of protein [34]. Surprisingly, none of the other divalent metal ions tested (manganese(II), iron(II), cobalt(II), nickel(II), and copper(II)) were found to promote the phase separation of tau. However, the mechanism by which zinc ions promote LLPS of tau is not known. Singh et al. suggested that local folding of tau, resulting from zinc binding, could cause an increased density of positive and negative charges within particular regions. This, in turn, would lead to stronger attractive intermolecular interactions, facilitating LLPS [34]. Another proposed theory is that zinc ions promote LLPS of tau by facilitating the formation of transient intermolecular cross-links between protein molecules [34]. However, these suggestions need to be further studied.

Divalent cations can modulate phase transitions both directly and indirectly through interactions with other proteins. EF-hand domain protein 2 (EFhd2) is a conserved calcium-binding protein. It is expressed in various tissues but predominantly in the central nervous system [35][36]. EFhd2 has been found to be associated with tau aggregates in the mouse model, JNPL3, and as a tau-associated protein in Alzheimer's diseased brains [35][37]. Recent studies have shown that EFhd2 modulates the phase transition of tau and directly alters tau liquid phase behaviour to form solid-like structures in vitro, and this phenomenon is controlled by calcium ions [38]. Notably, both EFhd2 and tau, in the absence of calcium ions, lead to the formation of solid-like structures containing both. On the other hand, in the presence of calcium ions, EFhd2 and tau phase separate together into liquid droplets [38].

Divalent cations can also modulate the LLPS of transcription factors. It was shown that zinc and copper(II) ions induce LLPS of the F region of the *Aedes aegypti* ecdysteroid receptor [39]. Since this region seems to affect the dimerization of nuclear receptors, the interactions with other proteins, and the stabilization of ligand binding, LLPS of the ecdysteroid receptor might contribute to the regulation of transcriptional activation.

Protein interactions driving LLPS may vary depending on the nature of the amino acid sequence [40]. Proteins are polyelectrolytes that can have both positive and negative charges [41]. Well-described examples of LLPS (often referred to as coacervation) are those based on interactions between polycationic proteins and polyanionic RNA molecules [42]. Less is known concerning the ability of polyanionic proteins to undergo LLPS in a similar charge-dependent manner. Mayfield et al. identified a previously unknown mechanism of calcium-dependent LLPS

occurring within the endoplasmic/sarcoplasmic reticulum (ER/SR) that explains efficient calcium ion buffering and storage [43]. It was shown that calcium ions modulate LLPS of the polyanionic protein and major calcium-binding protein of the SR of skeletal muscle, calsequestrin-1 (CASQ1). The process was reversible and occurred within cells. CASQ1 is an IDP that influences its capacity for LLPS. It was also shown that the LLPS of CASQ1 is regulated via phosphorylation by the secretory pathway kinase Fam20C, which phosphorylates structurally-conserved regions of CASQ1 [43]. Thus, the phosphorylated protein (pCASQ1) more readily entered the LLPS state. Mayfield et al. [43] suggested that this likely arises from the increased disorder and conformational flexibility of pCASQ1. Additionally, they hypothesized that calcium-dependent LLPS of polyanionic IDPs is a widespread and evolutionarily-conserved phenomenon that might represent a major mechanism underlying calcium ion handling and signaling [43].

References

1. Gomes, E.; Shorter, J. The Molecular Language of Membraneless Organelles. J. Biol. Chem. 2019, 294, 7115–7127.
2. Hyman, A.A.; Weber, C.A.; Jülicher, F. Liquid-Liquid Phase Separation in Biology. Annu. Rev. Cell Dev. Biol. 2014, 30, 39–58.
3. Banani, S.F.; Rice, A.M.; Peeples, W.B.; Lin, Y.; Jain, S.; Parker, R.; Rosen, M.K. Compositional Control of Phase-Separated Cellular Bodies. Cell 2016, 166, 651–663.
4. Shin, Y.; Brangwynne, C.P. Liquid Phase Condensation in Cell Physiology and Disease. Science 2017, 357, eaaf4382.
5. Brangwynne, C.P.; Tompa, P.; Pappu, R.V. Polymer Physics of Intracellular Phase Transitions. Nat. Phys. 2015, 11, 899–904.
6. Posey, A.E.; Holehouse, A.S.; Pappu, R.V. Phase Separation of Intrinsically Disordered Proteins. In Methods in Enzymology; Academic Press: Cambridge, MA, USA, 2018; Volume 611, pp. 1–30. ISBN 9780128156490.
7. Banani, S.F.; Lee, H.O.; Hyman, A.A.; Rosen, M.K. Biomolecular Condensates: Organizers of Cellular Biochemistry. Nat. Rev. Mol. Cell Biol. 2017, 18, 285–298.
8. Ditlev, J.A.; Case, L.B.; Rosen, M.K. Who's In and Who's Out—Compositional Control of Biomolecular Condensates. J. Mol. Biol. 2018, 430, 4666–4684.
9. Brangwynne, C.P.; Eckmann, C.R.; Courson, D.S.; Rybarska, A.; Hoege, C.; Gharakhani, J.; Jülicher, F.; Hyman, A.A. Germline P Granules Are Liquid Droplets That Localize by Controlled Dissolution/Condensation. Science 2009, 324, 1729–1732.

10. Azaldegui, C.A.; Vecchiarelli, A.G.; Biteen, J.S. The Emergence of Phase Separation as an Organizing Principle in Bacteria. Biophys. J. 2021, 120, 1123–1138.
11. Sołtys, K.; Tarczewska, A.; Bystranowska, D.; Sozańska, N. Getting Closer to Decrypting the Phase Transitions of Bacterial Biomolecules. Biomolecules 2022, 12, 907.
12. Brocca, S.; Grandori, R.; Longhi, S.; Uversky, V. Liquid-Liquid Phase Separation by Intrinsically Disordered Protein Regions of Viruses: Roles in Viral Life Cycle and Control of Virus-Host Interactions. Int. J. Mol. Sci. 2020, 21, 9045.
13. Chiu, Y.P.; Sun, Y.C.; Qiu, D.C.; Lin, Y.H.; Chen, Y.Q.; Kuo, J.C.; Huang, J.R. Liquid-Liquid Phase Separation and Extracellular Multivalent Interactions in the Tale of Galectin-3. Nat. Commun. 2020, 11, 1229.
14. Boskey, A.L. Biomineralization: An Overview. Connect. Tissue Res. 2003, 44 (Suppl. 1), 5–9.
15. Hołubowicz, R.; Porębska, A.; Poznar, M.; Różycka, M.; Dobryszycki, P. Biomineralization--Precision of Shape, Structure and Properties Controlled by Proteins. Postepy Biochem. 2015, 61, 364–380.
16. Demichelis, R.; Raiteri, P.; Gale, J.D.; Quigley, D.; Gebauer, D. Stable Prenucleation Mineral Clusters Are Liquid-like Ionic Polymers. Nat. Commun. 2011, 2, 590.
17. Gebauer, D.; Cölfen, H. Prenucleation Clusters and Non-Classical Nucleation. Nano Today 2011, 6, 564–584.
18. Gower, L.B.; Odom, D.J. Deposition of Calcium Carbonate Films by a Polymer-Induced Liquid-Precursor (PILP) Process. J. Cryst. Growth 2000, 210, 719–734.
19. Gebauer, D.; Volkel, A.; Colfen, H. Stable Prenucleation Calcium Carbonate Clusters. Science 2008, 332, 1819–1822.
20. Qin, D.; He, Z.; Li, P.; Zhang, S. Liquid-Liquid Phase Separation in Nucleation Process of Biomineralization. Front. Chem. 2022, 10, 834503.
21. Avaro, J.T.; Wolf, S.L.P.; Hauser, K.; Gebauer, D. Stable Prenucleation Calcium Carbonate Clusters Define Liquid–Liquid Phase Separation. Angew. Chem. Int. Ed. 2020, 59, 6155–6159.
22. Boskey, A.L.; Villarreal-Ramirez, E. Intrinsically Disordered Proteins and Biomineralization. Matrix Biol. 2016, 52–54, 43–59.
23. Zaslavsky, B.Y.; Uversky, V.N. In Aqua Veritas: The Indispensable yet Mostly Ignored Role of Water in Phase Separation and Membrane-Less Organelles. Biochemistry 2018, 57, 2437–2451.
24. Alberti, S. Phase Separation in Biology. Curr. Biol. 2017, 27, 1097–1102.
25. Martin, E.W.; Mittag, T. Relationship of Sequence and Phase Separation in Protein Low-Complexity Regions. Biochemistry 2018, 57, 2478–2487.

26. Mitrea, D.M.; Kriwacki, R.W. Phase Separation in Biology; Functional Organization of a Higher Order. Cell Commun. Signal. 2016, 14, 1.
27. Wang, B.; Zhang, L.; Dai, T.; Qin, Z.; Lu, H.; Zhang, L.; Zhou, F. Liquid–Liquid Phase Separation in Human Health and Diseases. Signal Transduct. Target. Ther. 2021, 6, 290.
28. Franzmann, T.M.; Alberti, S.; Morimoto, R.I.; Hartl, F.U.; Kelly, J.W. Protein Phase Separation as a Stress Survival Strategy. Cold Spring Harb. Perspect. Biol. 2019, 11, a034058.
29. Uversky, V.N. Intrinsically Disordered Proteins in Overcrowded Milieu: Membrane-Less Organelles, Phase Separation, and Intrinsic Disorder. Curr. Opin. Struct. Biol. 2017, 44, 18–30.
30. Chen, J.; Kriwacki, R.W. Intrinsically Disordered Proteins: Structure, Function and Therapeutics. J. Mol. Biol. 2018, 430, 2275–2277.
31. Bhattarai, A.; Emerson, I.A. Dynamic Conformational Flexibility and Molecular Interactions of Intrinsically Disordered Proteins. J. Biosci. 2020, 45, 29.
32. Alberti, S.; Dormann, D. Liquid–Liquid Phase Separation in Disease. Annu. Rev. Genet. 2019, 53, 171–194.
33. Mukhopadhyay, S. The Dynamism of Intrinsically Disordered Proteins: Binding-Induced Folding, Amyloid Formation, and Phase Separation. J. Phys. Chem. B 2020, 124, 11541–11560.
34. Singh, V.; Xu, L.; Boyko, S.; Surewicz, K.; Surewicz, W.K. Zinc Promotes Liquid-Liquid Phase Separation of Tau Protein. J. Biol. Chem. 2020, 295, 5850–5856.
35. Vega, I.E.; Traverso, E.E.; Ferrer-Acosta, Y.; Matos, E.; Colon, M.; Gonzalez, J.; Dickson, D.; Hutton, M.; Lewis, J.; Yen, S.H. A Novel Calcium-Binding Protein Is Associated with Tau Proteins in Tauopathy. J. Neurochem. 2008, 106, 96–106.
36. Ferrer-Acosta, Y.; Rodríguez Cruz, E.N.; Vaquer, A.d.C.; Vega, I.E. Functional and Structural Analysis of the Conserved EFhd2 Protein. Protein Pept. Lett. 2013, 20, 573–583.
37. Ferrer-Acosta, Y.; Rodríguez-Cruz, E.N.; Orange, F.; De Jesús-Cortés, H.; Madera, B.; Vaquer-Alicea, J.; Ballester, J.; Guinel, M.J.-F.; Bloom, G.S.; Vega, I.E. EFhd2 Is a Novel Amyloid Protein Associated with Pathological Tau in Alzheimer's Disease. J. Neurochem. 2013, 125, 921–931.
38. Vega, I.E.; Umstead, A.; Kanaan, N.M. EFhd2 Affects Tau Liquid-Liquid Phase Separation. Front. Neurosci. 2019, 13, 845.
39. Więch, A.; Tarczewska, A.; Ożyhar, A.; Orłowski, M. Metal Ions Induce Liquid Condensate Formation by the F Domain of Aedes Aegypti Ecdysteroid Receptor. New Perspectives of Nuclear Receptor Studies. Cells 2021, 10, 571.
40. Wang, J.; Choi, J.-M.; Holehouse, A.S.; Lee, H.O.; Zhang, X.; Jahnel, M.; Maharana, S.; Lemaitre, R.; Pozniakovsky, A.; Drechsel, D.; et al. A Molecular Grammar Governing the Driving Forces for

Phase Separation of Prion-like RNA Binding Proteins. Cell 2018, 174, 688–699.

41. Alberti, S.; Gladfelter, A.; Mittag, T. Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates. Cell 2019, 176, 419–434.
42. Dutagaci, B.; Nawrocki, G.; Goodluck, J.; Ashkarran, A.A.; Hoogstraten, C.G.; Lapidus, L.J.; Feig, M. Charge-Driven Condensation of RNA and Proteins Suggests Broad Role of Phase Separation in Cytoplasmic Environments. Elife 2021, 10, e64004.
43. Mayfield, J.E.; Pollak, A.J.; Worby, C.A.; Xu, J.C.; Tandon, V.; Newton, A.C.; Dixon, J.E. Ca2+-Dependent Liquid-Liquid Phase Separation Underlies Intracellular Ca2+ Stores. bioRxiv 2021.