Published in final edited form as:

J Mol Biol. 2018 November 02; 430(23): 4603–4606. doi:10.1016/j.jmb.2018.09.006.

Phase Separation in Biology and Disease

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It has long been appreciated that cells contain microenvironments not enclosed by membranes. In a classic study [1], Wilson concluded that the cytoplasm of star fish and sea urchin eggs “is a mixture of liquids in the form of a fine emulsion consisting of a continuous substance in which are suspended drops.” More than 100 years later, the concept of the cytoplasm as an emulsion is enjoying a revival, as several nuclear and cytoplasmic bodies have been reported to exhibit liquid-like characteristics: round shape, permeable surface, dynamic internal components, and ability to flow, drip, and fuse [2–5]. These include P granules and other germ granules, stress granules, and P-bodies in the cytosol and nucleoli, paraspeckles, Cajal bodies, and PML bodies in the nucleus [6]. It is now appreciated that these membrane-less, fluid structures form through liquid–liquid phase separation of their components [4,5,7], often including proteins and nucleic acids, especially RNA. However, more recently, the process of phase separation has been associated with many additional, diverse biological structures including signaling centers formed by trans-membrane receptors[8], membrane-bound protein assemblies that initiate endocytosis [9], the semi-permeable barrier within the nuclear pore complex (NPC) [10], extra-cellular matrix protein networks [11], mitotic structures [12], silenced chromatin [13,14], and centers of active gene transcription [15,16]. Thus, while first recognized less than 10 years ago in association with membrane-less organelles (MLOs), the process of phase separation is now known to be linked with widely varied biological process compartmentalized in diverse regions of the cell. However, while these new discoveries have revolutionized our understanding of structural and cell biology, there are many gaps in this knowledge. Importantly, these gaps prevent, for example, explanations of how phase separation mediates the complex biological processes that occur within phase separated bodies, how their specific compositions arise

and are regulated, and how the molecular properties of proteins, RNA, and DNA within them, and interactions between them, give rise to the fluid features of these bodies and how these, in turn, influence function.

The challenges associated with these knowledge gaps are being addressed through the efforts of scientists from across the world, as reflected by the 13 reviews and one original research article assembled in this Special Issue.

MLO Assembly and Composition

Mittag and Parker [17] discuss the molecular interactions that promote the formation of RNA–protein assemblies collectively referred to as RNP granules, with focus on the many different modes of protein–protein interactions that contribute. Proteins are now understood to comprise both folded domains and intrinsically disordered regions, and Mittag and Parker [17] describe how these fundamental building blocks interact in different ways to form the molecular scaffolds that underlie liquid–liquid phase separation within RNP granules. Chong and co-workers [18] discuss the features of RGG/RG motifs found within many disordered protein regions associated with phase separation, containing multiple copies of short regions enriched in arginine and glycine residues. These authors emphasize the versatility of the amino acid arginine in mediating interactions with nucleic acids and of glycine and arginine in self-interaction as well as interactions with other amino acids. This versatility coupled with multivalency and intrinsic flexibility enables multifarious weak and transient interactions between RGG/RG motif-containing proteins and nucleic acids that underlie phase separation. Ruff and co-workers [19] review the phase separation behavior of disordered proteins with repetitive amino acid sequences, which they term archetypal intrinsically disordered protein polymers (IDPPs). A key concept that emerges from studies of IDPPs is that intra-chain interactions that mediate protein compaction also mediate inter-chain interactions that drive phase separation. These authors discuss how intensive solution parameters such as temperature, proton chemical potentials (pH), and hydrostatic pressure can modulate chain compaction and thus phase separation of IDPPs, providing guiding principles to inform our understanding of the broad class of disordered proteins that undergo phase separation. A central question in the field is how the composition of MLOs and other biomolecular condensates is controlled, and Ditlev and co-workers [20] discuss a conceptual framework in which scaffold molecules drive phase separation which, in turn, creates binding valency for recruitment of client molecules. They review the various types of folded domains and disordered regions of proteins, and features of RNA that create different levels of multivalency within scaffolds and clients and, thus, control their respective partitioning within phase separated bodies. The article concludes with a survey of several MLOs and their biomolecular constituents, which illustrate various incarnations of the scaffold/client concept. Finally, Fay and Anderson [21] emphasize the contributions of the RNAs within these condensates to the phase separation processes that mediate their formation. The article reviews the RNA constituents of a wide variety of MLOs in the cytosol and nucleus, and their roles in RNA metabolism, and concludes with arguments that *cis* elements within RNAs are likely key contributors to the formation of these MLOs. Together, this collection of articles addresses cutting-edge molecular concepts that provide a framework for

understanding the interactions between protein and RNA molecules that drive phase separation and the protein sequence features associated with this ability.

MLO Regulation and Function

The broad topic of RNA granule regulation and function is addressed from many different perspectives by several author groups. Seydoux [22] discusses the importance of contributions from RNA to phase separation together with proteins with RGG and other types of disordered regions in the formation of P granules. An interplay between RNA-directed phase separation and competitive interactions among numerous proteins, many of which are regulated by reversible phosphorylation, mediates asymmetric formation of P granules in the cytoplasm of newly fertilized embryos. It is speculated that P granules, which exhibit RNA silencing function, may provide an epigenetic mechanism for RNA memory of germline gene expression. A second review article in this area, by Chakravarty and Jarosz [23], also addresses fascinating links between phase separation and epigenetic inheritance in yeast. In this case, phase-separated microenvironments formed by disordered protein regions containing prion elements, which can encode “information” by adopting distinct conformations, are proposed by Chakravarty and Jarosz [23] as settings for conformational switching to create heritable prion states or “strains.” The authors develop their arguments by reviewing mechanisms of prion-based inheritance and discussing the prevalence of prion elements within phase separation-prone intrinsically disordered regions; the key conceptual advance is the proposal that phase-separated micro-environments promote conformational switching to generate biological information in the form of prion strains. Alberti and Carra [24] point out the association of certain MLOs and age-related diseases and note that proteins with disordered and low complexity regions, especially at the high concentrations that exist within MLOs, can experience aggregation and misfolding into fibrillar structures. The protein quality control system (PQS) is tasked with mitigating the deleterious effects of such aggregates in cells, and the authors discuss the interplay between the PQS and MLOs with known associations with age-related diseases. They speculate on the exciting possibility that therapeutic modulation of the PQS may provide a means to treat age-related diseases that arise from protein aggregation within MLOs in the future.

Other articles in the Special Issue illustrate the diverse ways in which phase separation is utilized in biological systems. In one, Zilman [25] discusses the FG-NUP proteins, which are localized in the center of the NPC and mediate the NPC’s diffusional gating function as well as nuclear transport receptor-directed translocation of molecular cargoes through the NPC. In the parlance of polymer physics, the FG-NUP proteins exhibit cohesiveness, which enables them to undergo phase separation and to perform the functions noted above in this condensed form. Another example is provided by elastin, which is discussed by Muiznieks and colleagues [26]. Elastin, an extracellular matrix protein, mediates the extensibility and elastic recoil of large arteries, lungs, and other tissues by forming disordered polymeric assemblies through phase separation. The elastin sequence comprises numerous repeats of modules enriched in non-polar amino acids and glycine, creating low sequence complexity, which are connected by additional modules that form covalent, intra-chain and inter-chain crosslinks. The entropy of phase-separated elastin assemblies is maximized when the ensemble is relatively compact and is reduced when extended under force; elastic recoil is

driven by the entropic force to return to the compact configuration—an example of an entropic spring. The concept of phase separation as a means to perform mechanical work is discussed by Bergeron-Sandoval and Michnick [27], with examples ranging from displacement of cytosolic biopolymer networks, induction of membrane invagination, distortion of other condensates, and bending of filament bundles. In these examples, the energy of multivalent molecular interactions that drive phase separation is utilized collectively to alter the macroscopic structural features of other biomolecular assemblies (e.g., the cytoskeleton, lipid membranes, other MLOs, and actin filaments). Turning to the nucleus, Woodruff [28] discusses ideas on how phase separation may mediate the assembly and function of components of the centrosome, an MLO involved in spindle assembly and chromosome segregation during mitosis. The centrosome is a barrel-shaped structure surrounded by a dynamic protein mass called pericentriolar material (PCM). Components of the PCM and of another mitotic structure, the spindle matrix, have been observed *in vivo* to display liquid-like features and undergo phase separation *in vitro*, leading to an intriguing model wherein PCM and the spindle matrix are viscoelastic compartments that concentrate tubulin and other factors, promoting tubulin polymerization and spindle microtubule assembly. This latter collection of articles illustrates the versatility of phase separation in selectively organizing sets of biomolecules within liquid-like compartments to perform a wide range of essential biological process.

Methods to Study MLOs *In Vitro* and *In Vivo*

Finally, two articles address methodologies used in studies of biomolecules that undergo phase separation. Alberti *et al.* [29] provide a practical “user’s guide” for performing phase separation assays with purified proteins. Phase separation-prone proteins often non-specifically aggregate during recombinant expression and purification and the formation of aggregated species can alter a protein’s behavior in phase separation assays. These authors provide detailed guidelines for recombinantly expressing, purifying, and handling these types of proteins and also discuss common pitfalls in confocal microscopy assays of phase separation. Lastly, Mitrea and co-workers [30] provide an encyclopedia of methodologies for studying phase separation, including methods for studying droplet formation *in vitro* and in cells, for characterizing their material properties in either setting, for studying the conformational properties of proteins before and after phase separation, and for studying the architecture of the transient molecular networks that underlie the process of phase separation. These two articles will be helpful guides to seasoned investigators of phase separation and newcomers to the field alike.

It is now clear that the process of phase separation is a ubiquitous mechanism to reversibly organize biomolecules to perform specialized biological functions in specific locations and at specific times in cells. This Special Issue provides timely accounts of the principles that underlie the process of phase separation by protein and RNA molecules, the diverse biological settings in which phase separation is now known to play roles, and methodologies used to reveal these amazing insights that require the rewriting of structural biology and cell biology text books. We hope that the articles in this collection will serve as guideposts for other investigators to explore the likely myriad other incarnations of phase separation in biology and disease.

Abbreviations used:

MLOs	membrane-less organelles
IDPPs	intrinsically disordered protein polymers
PQS	protein quality control system
NPC	nuclear pore complex
PCM	pericentriolar material

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