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COMPLEX COACERVATION AT THE ORIGIN OF LIFE: COMPLEX COACERVATE AS PROTOCELLS & MEMBRANELESS ORGANELLES

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ABSTRACT

Complex coacervation [formation of a dense macroion-rich phase (the coacervate) in equilibrium with a dilute macroion-poor phase (continuous phase or supernatant)] is a particular case of associative phase separation that occurs when oppositely charged macroions (or polyelectrolytes) are mixed. Since the pioneering work of Bungenberg de Jong and co-workers on gelatin–acacia gum complex coacervation in the 1920–40s, coacervates have received increasing research interest because a variety of mature and emerging technologies depend critically on the association of oppositely charged polymers or particles. Such association could involve charge complexation in solution. Analogous to membraneless organelles, complex coacervates are water droplets dispersed in water and formed by spontaneous liquid–liquid phase separation (LLPS) of an aqueous solution of two oppositely charged polyelectrolytes to form a dense polyelectrolyte-rich phase (coacervate) and a more dilute solution. The literature on complex coacervation is daunting because of its size, breadth of discipline (spanning physical chemistry, bio-chemistry, colloid science, chemical, biological, biomedical, and materials engineering, among others), and engagement of both natural and synthetic polymers in a myriad of combinations with other charged and uncharged components. While not aiming at an exhaustive coverage of the field, in this review, we restrict our discussion in describing and contextualizing the recent advances in the science and engineering of polymer-polymer complex coacervates, outlining complex coacervate as protocells & membraneless organelles.

Complex coacervation has a long history in the field of cell biology. The first protocells may have been complex coacervates of abiotic macromolecules that served as liquid containers for early anabolic processes.[1] Historically, one particularly contentious topic surrounding complex coacervation was the potential for these phase-separated compartments to serve as a type of protocell that could form the basis for the evolution of life.[2-9] Despite this hypothesis, originally put forth by Oparin,4,[9-11] has had on many studies on the origin of life, the importance of coacervates declined rapidly, mainly because it seemed in stark contrast with the presence of well-defined membranes that separate cells from the outside world as well as those that separate the cellular interior into organelles. It is only over the past few decades that evidence has accumulated observing the existence of organelles not enclosed in membranes, so much so that membraneless organelles (MLOs) are now considered essential components of eukaryotic cells.[12-14] They have been shown to constitute a more dynamic way to sequester (sometimes temporarily and reversibly) cellular components from the rest of the cell. Membrane and MLOs can be respectively assimilated by analogy to a grape (membrane organelle) that encloses its seeds and to oil droplets in an aqueous solution (MLOs). These findings have thus renewed broad interest in Oparin's proposal[15] and have led to new experimental efforts to address the origin of life. For instance, in 2019 Jia et al. started with prebiotically available α-hydroxy acids and prepared polyester droplets that would segregate proteins and RNA in a fashion compatible with origin-of-life conditions.[16] More recently, it was shown that phase separation may help in transforming abiotic ornithine residues into arginines, thus allowing the formation of a dsDNA-binding protein.[17] In modern cell biology, regulated intracellular liquid–liquid phase separation of bio- macromolecules is known to play fundamental roles in organizing the cytoplasm, assembling transient signaling complexes, and sequestering metabolic pathways.[18]

In the past few years, coacervate research has seen a tremendous development, in part inspired by the rapid advances in the field of MLOs and the need for model systems that are simple enough to allow systematic and quantitative investigation of MLO characteristics. MLOs represent a rich and still poorly understood variety of phase-separated subcellular structures such as the

nucleolus and germ granules.[19-21] These indispensable organelles are formed as a result of liquid−liquid phase separation (LLPS), primarily by the process of complex coacervation, i.e., interactions between charged polyelectrolytes such as proteins and nucleic acids. MLOs exhibit liquid-like material properties[22] and tend to be highly dynamic, as there is a continuous internal diffusive rearrangement of the coacervate material as well as an exchange of components with the surroundings.[21,23] A number of cytoplasmic and nucleoplasmic MLOs comprised of RNA and protein, such as nucleoli[24] and P granules,[21] have been reported to have liquid phase characteristics.[19, 25-32] An increasing number of other MLOs are candidates for liquid phase separation, including Cajal bodies, nuclear speckles, para-speckles, and PML bodies in the nucleoplasm and stress granules and germ granules in the cytoplasm.[33,34] Since coacervates and most MLOs are both formed through liquid-liquid phase separation, driven by the same attractive interactions, coacervates have a clear potential to mimic material properties, hierarchical organization, and sequestration of MLOs. This has inspired scientists to utilize the self-assembling and crowded nature of coacervates to engineer synthetic cells[35] and artificial organelles,[18,36,37] which are capable of mimicking specific biological features including compartmentalization and communication.[38,39]

Beyond the historical debate, phase-separated and coacervate-like materials have been increasingly discussed in the context of cellular compartmentalization. Improvements in microscopy and labelling strategies has led to the discovery of a tremendous range of membraneless cellular compartments that harness liquid-liquid phase separation to drive functionality. Such compartmentalization has been typically associated with interactions between intrinsically disordered proteins (IDPs) and oligonucleotides. The formation of stress granules has been observed as a mechanism for cells to arrest certain metabolic pathways while retaining the enzymatic machinery for later use.[40] Granule formation has also been associated with loci of transcription[19,41,44] and ribosome biogenesis, such as nucleoli.[19] Compartmentalization also enables passive noise filtration, which can further help to increase the predictability of transcriptional outputs.[44]

In addition to the potential benefits of compartmentalization, aberrant phase transitions have also been correlated with disease states. FUS is a prion like IDP associated with the neurodegenerative disease ALS that has been shown to form liquid compartments as a result of stress and/or DNA damage. However, aging experiments demonstrated that mutations in FUS associated ALS resulted in an accelerated liquid-to-solid transition.[45] While it should be noted that complex coacervation is not the driving force behind the formation of all membraneless organelles, there is tremendous potential for parallel scientific exploration in the space between pure biology and pure materials science.

The compartmentalization afforded by coacervation can also be harnessed to define micro- or nanoscale reaction chambers. Coacervate droplets and coacervate-core micelles have been used to entrap enzymes to create nanoreactors and potentially increase the reaction efficiency and/or operational stability of the encapsulated proteins.[46-48] In one example, a higher thermal tolerance was achieved for encapsulated trypsin, along with an increased reaction rate when compared to native trypsin.[46] In another example, the encapsulation of such constructs have the potential to be used to enable enzyme replacement therapies. Alternatively, these reactors can selectively uptake nanoparticles or other small molecules to enable *in situ* chemical synthesis.[48,49] For instance, nanoreactors containing poly(ethylene glycol)-*b*-poly(*α,β*- aspartic acid) (PEG-*b*-PAsp) and homo-cationer poly([5-aminopentyl]-*α,β*-aspartamide) (Homo-PAsp-AP) were capable of activating prodrugs on location at tumor tissue sites.[48]

MLOs play versatile roles in regulating the cellular biochemistry, and their malfunctioning is associated with protein-aggregation diseases including Alzheimer's disease.[50-53] With new examples being discovered at a rapid pace, it is increasingly becoming clear that liquid−liquid phase separation (LLPS), primarily by the process of complex coacervation, plays a crucial role in an especially wide variety of cellular processes such as DNA compaction and chromatin organization,[54-57] selectively filtering specific biomolecules,[58] stress regulation,[19,59] transcription regulation,[60-63] polarity establishment,[21] photosynthesis,[64] endocytosis,[65] cell signaling,[66] and cell adhesion.[67] While some functionalities such as sequestering and concentrating specific molecules to assist biochemical reactions are recurring and established themes, many other questions are just starting to get investigated. For example, it is as of yet quite unclear whether, and if so how, MLOs physically manipulate their local environment, e.g., mechanically remodel membranes. The interaction between MLOs and membranes is gathering interest but has not yet been widely studied. Recent work has indicated the role of coacervates in endocytosis and cell adhesion,[67,68] pointing out the potential of MLOs in exerting forces on lipid membranes.

In *Caenorhabditis elegans* (*C. elegans*), the liquid-like P granules that act as mRNA exporters have been reported to directly wet the nuclear membrane,[69] possibly enhancing transport. Membrane-bound phase-separated protein cluster shave also been shown to be involved in a variety of signaling pathways, modulating signal transduction as well as recruiting cytoskeletal elements.[70-73] These recent studies indicate previously unknown roles served by MLOs, including that of mechanical work.[74] Membrane-bound coacervates could serve as localized sites for the production of lipids or

membrane proteins and could, due to their strong interaction with the membrane, perhaps even be engineered for transmembrane transport that would otherwise require complicated machinery. Future research on coacervate−membrane interactions could produce more refined manifestations of the interplay between cell-sized compartments and condensates. However, most contemporary coacervate models lack the chemical and compositional richness of MLOs, which explains why there remains a gap between coacervates in MLOs in terms of selectivity, regulation, and metastability. We need to identify some intrinsic limitations of coacervates and the current gaps between coacervates and MLOs, which will hopefully inspire future research. Most coacervate models are based on compositionally simple components whose phase separation is driven by a single type of interaction (e.g., charge complexation). They lack the chemical and compositional richness of many MLOs, which often contain many co-assembled proteins, each with a unique arrangement of amino acids and potential for interactions. This complexity explains, to a large extent, the superior selectivity, actively regulated formation and dissolution, and even the metastability seen in some MLOs. In order to mimic these features of MLOs better, reconstituted and designer proteins are being used to successfully create *in vitro* droplets with the same molecular composition as MLOs.[75,76] Using principles from polymer coacervation, a wide range of de novo synthetic peptides were designed with tunable coacervation properties. By systematically analyzing the coacervate phase behavior, Dzuricky[77] provide fundamental insights into the link between sequence and coacervate properties, bridging the gap between condensates *in vitro* and *in vivo*. With increasing complexity, the boundaries between coacervates and (artificial) MLOs slowly fade away, and the more protein-based coacervate droplets will be capable of mimicking the characteristics of MLOs. By using such a bottom-up approach, we will be able to establish which level of complexity is required to mimic each property of an MLO.

Additionally, one crucial aspect that has so far been lacking in almost all *in vitro* models is the out-of-equilibrium nature of the cellular environment. Cells are fundamentally active, and a constant turnover of chemical energy governs the formation, stability, and arrangement of cytoskeletal structures, protein complexes, and also MLOs. Active processes can keep droplets stable or proteins soluble,[78] and they can literally shape MLOs[79,80] and alter their physical state by fluidization. Recapitulating these processes in coacervate models to understand the underlying physical effects is a major challenge for the coming years, of which the first steps are being made. Spoelstra[81] described an alternative strategy to use a UDP-polymerizing enzyme to create enzyme-controlled active coacervates, and the first study in which transient, nonspherical coacervate shapes are reported, depending on enzyme activity. Additionally, Donau[82] reported active peptide-RNA coacervates that are formed transiently upon the addition of a carbodiimide chemical fuel. This is the first report of enzyme-free active coacervate droplets, which display emerging self-dividing behavior shortly before they dissolve.

CONCLUSION AND FUTURE PERSPECTIVES

The importance of their exotic character attracted scientists even beyond the field of colloidal chemistry like A. I. Oparin, a Russian biologist, who cited de Jong's work, mentioned the similarity to proto-cells and coacervates, proposed that life on Earth first formed in coacervate droplets,[83] and some recent studies are heading towards the same direction.[84-88] He summarized his ideas in a famous book entitled *The Origin of Life*. The central argument of this book was that life might have originated inside coacervates containing myriad different organic molecules. Oparin observed that coacervates, intended as small droplets of high concentrations of organic molecules, often form autonomously even in dilute solutions. He therefore suggested that coacervation could have been the mechanism through which a fluid phase would separate in the 'primordial soup'. Over the next few decades, Oparin and co-workers demonstrated chemical enrichment within the droplets, in-situ enzymatic reactions, and droplet growth and fission reminiscent of cellular life.[89] This 'metabolism-first' approach, however, provided no clear connection to genetic evolution and information propagation via nucleic acids that would have been a key step at the onset of life. Their experiments also presumed the existence of large macromolecules and polymers that are unlikely to have existed in a prebiotic environment.

In order for coacervates to be viable protocells, with the ability to sustain both chemical and genetic evolution, they must be able to form from small molecular weight molecules, particularly nucleotides and their activated derivatives. This was first demonstrated in 2011 by Koga *et al*.,[84] who showed that coacervate microdroplets could be formed from nucleoside triphosphates (ATP), diphosphates (ADP, FAD, NAD), and monophosphates (AMP) when mixed with short (2–10 amino acid (aa)) lysine polypeptides (OLys) that might plausibly be produced by prebiotic processes.[90,91] This study showed that phase separation of small molecular weight ions has many similarities to complexation of larger polyelectrolytes. The dependence on electrostatic interactions, for example, is shown by the increase of the critical concentration required for coacervation (CCC) with decreasing negative charge from ATP>ADP>AMP. In addition, they found that increasing the molecular weight of Poly(diallyldimethylammonium) (Poly (DADMAC)) from 150 to 275 kDa increased charge neutralization(with ATP) from 70 to 90%, These results suggest that increased hydrophobicity, decreased solubility and increased orientational freedom from

longer polymer chains all contribute to increasing charge neutralization at the CCC.

NOTES

The authors declare no competing financial interest.

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