Coacervates as models of membraneless organelles

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Abstract

Coacervates are condensed liquid-like droplets, usually formed with oppositely charged polymeric molecules. They have been studied extensively in colloid and interface science for their remarkable material properties. The liquid−liquid phase separation underlying coacervate formation also plays an important role in the formation of various membraneless organelles (MLOs) that are found in many living cells. Therefore, there is an increasing interest to use well-characterized coacervates as *in vitro* models that mimic specific aspects of MLOs. Here, we review five aspects – physical and chemical properties, hierarchical organization, uptake selectivity, formation dynamics, and maturation – that are of particular interest and discuss how useful coacervates are to better understand these aspects of MLOs.

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Introduction

Cells contain a vast number of biomolecular reactions and processes that must be tightly regulated and organized for survival. Compartmentalization is a powerful strategy to organize and control these processes. By selectively localizing biomolecules in separated compartments, reactions can be temporarily inhibited or jump-started [1,2], new assemblies can be formed, and noise can be suppressed [3]. In recent years, liquid−liquid phase separation has emerged as a prevalent

mechanism to realize the formation of a particular class of dynamic compartments in cells, called membraneless organelles (MLOs) [2]. MLOs are condensed bodies, usually in a liquid (droplet) state, containing various intrinsically disordered proteins (IDPs) often found together with nucleic acids. However, despite the widespread occurrence of MLOs, many aspects regarding their formation dynamics, aging, and impact on cellular processes remain poorly understood (Figure 1). In order to understand the physicochemical principles underlying MLO functioning, detailed studies are needed in both living cells and model systems, to examine the characteristics and behavior of MLOs *in vivo*, and to elucidate the underlying molecular interactions and assembly processes responsible for those properties. In this review, we evaluate complex coacervates as models for understanding MLOs.

Complex coacervates have shown great potential as models for compartments and materials formed by liquid−liquid phase separation (also termed condensates) [1,4,5]. Coacervation is a term used mostly in colloid science and chemistry to describe the condensation of polymeric molecules into a liquid, hydrated state. The phenomenon was first observed in mixed solutions of *oppositely charged* biopolymers and in solutions of isoelectric proteins with (poly)phenols [6,7]. This process is called *complex coacervation* to highlight the complexation of oppositely charged molecules that drives the condensation, as opposed to *simple coacervation*, which involves a single self-associating molecule (Figure 1). The number of examples of coacervation has grown significantly in the past decade, and now includes peptides, nucleic acids, lipids, small molecules (combined with longer biopolymers), and many synthetic polymers [1,8,9]. The vast majority of examples are unambiguous complex coacervates, but the distinction between simple and complex coacervates is not always as simple. Some ampholytic polymers, for instance, can self-associate to form *auto complex coacervates* (Figure 1), whereas phase-separated droplets of certain aromatic-rich disordered proteins can take up significant amounts of RNA by specific binding, resulting in *mixed condensates* with charged components [2]. In this review, we will use coacervates to describe all these condensed droplets formed by synthetic (bio)molecules, whether driven by charge complexation or other (self-)associating interactions. We have chosen to omit the *in vitro*

Figure 1
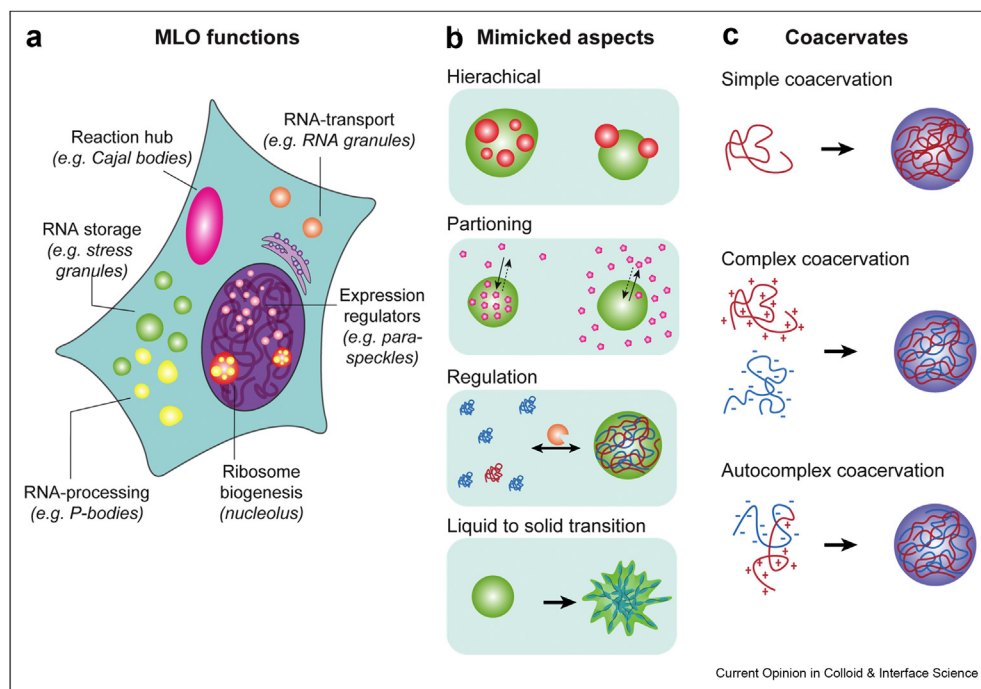
Improving understanding of membraneless organelles by mimicking their characteristic properties and behavior using coacervates. Numerous MLOs have been identified in eukaryotic cells, and they are widely believed to play a role in a variety of functions, as highlighted in (**a**). For most *in vitro* studies, specific aspects of MLOs are generally selected to be mimicked (**b**). To study these aspects in minimal models, simple and complex coacervates consisting of different types of (mostly charged) macromolecules can be used (**c**).

reconstituted MLOs formed by purified proteins that were identified as drivers of MLOs formation *in vivo* from our comparison. These purified proteins can often form droplets *in vitro* in the same way as synthetic coacervates are formed. However, our aim here is to discuss how well 'simple' coacervate models, made of synthetic biomolecules, can mimic the properties, behavior, and functions of MLOs. Nevertheless, the following sections will show that the distinction between coacervates and (*in vitro*) MLOs is not always sharp and gradually fading, as more aspects of MLOs are being mimicked by new generations of coacervates with increasing complexity.

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. 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. 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. By highlighting ways in which coacervates mimic MLOs, illustrated by notable recent developments in the field, this review aims to bridge the gap between these two types of condensates. We have organized the discussion in order of decreasing ability of coacervates to mimic the characteristics of MLOs: from (i) the typical (equilibrium) material properties of coacervates and MLOs, which can often be mimicked quantitatively, to (ii) hierarchical organization into subcompartments, (iii) selectivity of biomolecular sequestration and the ensuing potential for catalysis, (iv) dynamics of formation, and ultimately, (v) metastability and aging. In making the comparison, we identify some intrinsic limitations of coacervates and the current gaps between coacervates and MLOs, which will hopefully inspire future research.

Physical and chemical properties
From a physicochemical point of view, one of the most relevant aspects to mimic of MLOs are the material properties (Figure 2), which affect not only the deformability and wetting behavior but also the uptake of client molecules by partitioning and catalytic properties. As coacervates and MLOs are both typically

Figure 2
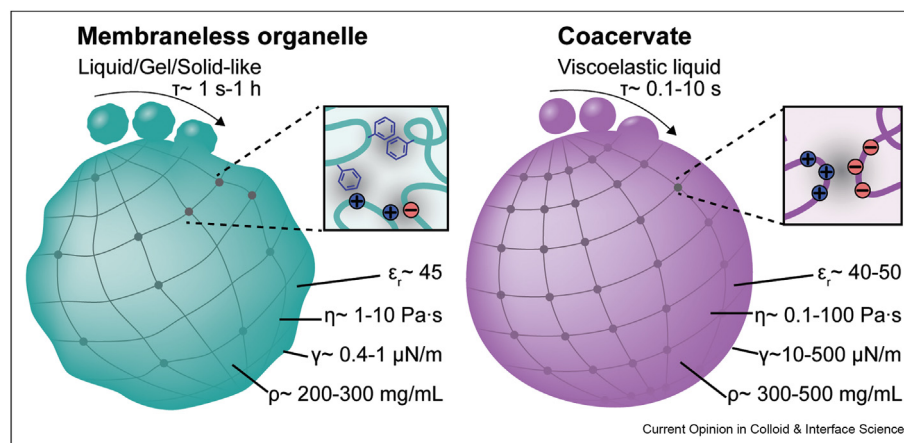
An overview of some relevant physicochemical properties of MLOs versus coacervates, highlighting the parameters: time of coalescence (τ), relative permittivity (ε_r), interfacial tension (γ), viscosity (η), and 'density' (ρ). The similarity in permeability (indicated as mesh) and the higher charge screening of coacervates compared to MLOs (greater number of grey spheres representing ion pairs) is also indicated. This is in contrast to the more variable interaction mechanisms (π-π, cation-π, and charge−charge) that occur within the more chemically complex MLOs (left box).

formed by phase separation of macromolecules in solution, driven by the same types of underlying interactions, it is not surprising that many material properties are very similar, or at least have a similar range. Coacervates are usually dense viscoelastic liquids, extending to gels and solids as their hydration is decreased [10,11]. Depending on composition and salt concentration, complex coacervates can exhibit an elastic response to deformation at short timescales (usually ms-s) [12], but they ultimately flow and fuse as liquids. Their viscosity can vary widely and depends on the chemical structure and length of the components and can be tuned by the salt concentration or temperature. Typically, coacervates have a viscosity of 0.1−100 Pa•s, but much higher viscosities have been reported.

MLOs have similar material properties, although they are not a uniform class. Significant efforts have been made to elucidate the physical state (liquid, gel, glass) specific MLOs are in. For many MLOs, videos showing fusion, flow, dripping, or wetting [13] confirmed that they behave like liquid droplets. The viscosity of liquid MLOs is usually estimated from fluorescence recovery after photobleaching (FRAP) measurements, and typical values are close to 1−50 Pa•s [14,15], well within the range available to coacervates. However, there are various limitations and challenges in using FRAP to quantify viscosity and other properties of MLOs, as highlighted recently [16,17], and recovery should not be taken as proof that liquid−liquid phase separation underlies their formation. Alternative approaches (such as microrheology or fluorescence correlation spectroscopy) should also be considered to derive these parameters,

but also these must be used with caution [15]. Finally, not all MLOs display complete fusion: some keep an irregular shape after merging and show incomplete recovery after photobleaching. These MLOs are better described as soft gels [13], similar to complex coacervates at low salt concentrations. Recent experiments suggest that MLOs can age and undergo a transformation from a liquid state to a more gel-like or even solid aggregate state, which we will discuss below.

Besides the physical state, the chemical composition of MLOs has an important influence on the role of MLOs in the cell. From a coarse-grained point of view, coacervates and MLOs have very similar overall compositions. Estimates of the water content of coacervates vary from 40% for hydrophobic polyelectrolytes [18] to 60−90% for hydrophilic ones [19,20], while the concentration of organic material (e.g. peptides, nucleic acids) is estimated to be around 300−500 mg/mL [19,21]. Similar estimates for MLOs show far greater variation, partly because the quantification of such high concentrations of protein in living cells, for instance, by fluorescence-based methods, is far from trivial [15,22]. In the case of Ddx4, local concentrations of 200−300 mg/mL have been measured by NMR on *in vitro* reconstituted droplets [23]. It is interesting to note that such protein concentrations are far beyond the solubility limit for most proteins [24]. Therefore, the condensed phase could be regarded as a way for cells to store certain proteins in large amounts, and a buffering function of MLOs has been suggested [3].

Derived from this overall composition, the relative permittivity (which is a measure of the local

polarizability of the medium) inside coacervates and MLOs has been estimated to be significantly lower than water. For complex coacervates, this has been expressed as an effective dielectric constant of about 40–50 [25], compared to 45 for Ddx4-based MLOs [26]. This lowered permittivity has significant implications for both partitioning and the intermolecular interactions between molecules inside the dense phase. Most organic client molecules, including peptides and proteins, are less polarizable than water and tend to be concentrated inside coacervates by partitioning [1], and the same is expected for MLOs on this basis. The lower relative permittivity also means that some intermolecular interactions, such as base stacking interactions, are weaker than in pure water, whereas others, such as ion pairing, are stronger. This alteration of intermolecular interaction strengths can have far-reaching consequences. As an example, double-stranded DNA (dsDNA) that is stable in an aqueous buffer can spontaneously dehybridize inside a Ddx4 droplet [27]. The local environment was found to have an equivalent effect as a 4 M GuHCl solution. The same effect was recently also observed in complex coacervates of short peptides, which suggests that it is caused by the effective overall composition and permittivity [28]. Similar effects of altered intermolecular interaction strengths are expected for protein–protein and protein–RNA interactions, which rely on a combination of hydrophobic effects, π-π interactions, cation-π interactions, ion pairing, and hydrogen bonding.

From a more detailed, chemical point of view, the composition of coacervates and MLOs differs significantly. Most complex coacervates are made of highly charged synthetic polymers, peptides, or nucleic acids, whereas the proteins involved in MLO formation have at most 10–20% of charged residues. The remaining 80–90% of amino acids are predominantly polar neutral and aromatic residues, including Gly, Ser, Thr, Asn, Gln, Phe, and Tyr [29,30]. As a consequence, the local charge concentration in complex coacervates is far greater than in most MLOs, which results in a stronger charge screening, although the effect may be negligible in practice, as the screening length is already shorter than 1 nm in physiological conditions. Interestingly, the difference in the amino acid composition does not lead to a difference in permittivity, as discussed above, but it results in easier nonspecific RNA uptake based on charge neutralization in complex coacervates on average [21].

Finally, coacervates have been known for their low interfacial tension, which is not only relevant for their deformability but also for the rate with which they coarsen through Ostwald ripening, and their tendency to wet other interfaces [31] or form multiphase assemblies (see next section). The interfacial tension is related to the strength of the interactions that drive phase separation, which is often set by the salt concentration, and it depends on the length of the components making up the coacervate. Estimates of the interfacial tension of complex coacervates are between 10 and 500 µN/m [32]. For MLOs, the interfacial tension has not been quantified directly *in vivo*, but estimates from *in vitro* reconstituted protein droplets point at much lower interfacial tensions of around 0.4–1.0 µN/m [33,34]. However, we question the relevance of comparison between absolute values of the interfacial tension determined *in vitro*, as the liquid–liquid interface of MLOs *in vivo* may be significantly altered by the presence of large amounts of proteins and other clients in the surrounding outer phase. These proteins can directly alter the interfacial tension by changing the effective solvent properties and indirectly affect the stability and coarsening of MLOs via interfacial adsorption [35] and depletion [36].

Hierarchical organization

Unlike the first examples of complex coacervates, most MLOs are composed of many coexisting biomacromolecules. In concert with their more chemically complex composition, a hierarchical organization into subcompartments has been observed in several MLOs (possible arrangements are shown schematically in Figure 3a). The nucleolus is the most prominent example of a cellular condensate organized into distinct subcompartments (Figure 3a–b), and its hierarchical organization has been linked to its functional role in ribosome biogenesis [37]. The nucleolus in mammalian cells consists of three coexisting phase-separated domains: a fibrillar center, embedded in a dense fibrillar component, which is in turn located inside the granular component. In an *in vitro* reconstituted model system or upon disruption of actin polymerization *in vivo*, these three domains are all liquid-like but with differing properties that underlie their immiscibility [33]. The phase-separated nature of the domains is essential for the shuttling of locally produced rRNAs and other client molecules between the different subcompartments (phases) without a physical barrier, and thus, for ribosome biogenesis. The same is true for other MLOs, such as stress granules, paraspeckles, and Cajal bodies (Figure 3d–e), although a consensus about the physical state of the internal domains has not been reached in all cases [38].

Inspired by the observations of higher-order organization in certain MLOs, there has been a significant interest in recent years to develop multiphase coacervates with a similar hierarchical organization (Figure 3g–l). The underlying reasoning is that if hierarchical organization is purely the result of physical–chemical interactions leading to phase separation in MLOs, then model coacervates should be able to display the same organization provided that the intermolecular

Figure 3
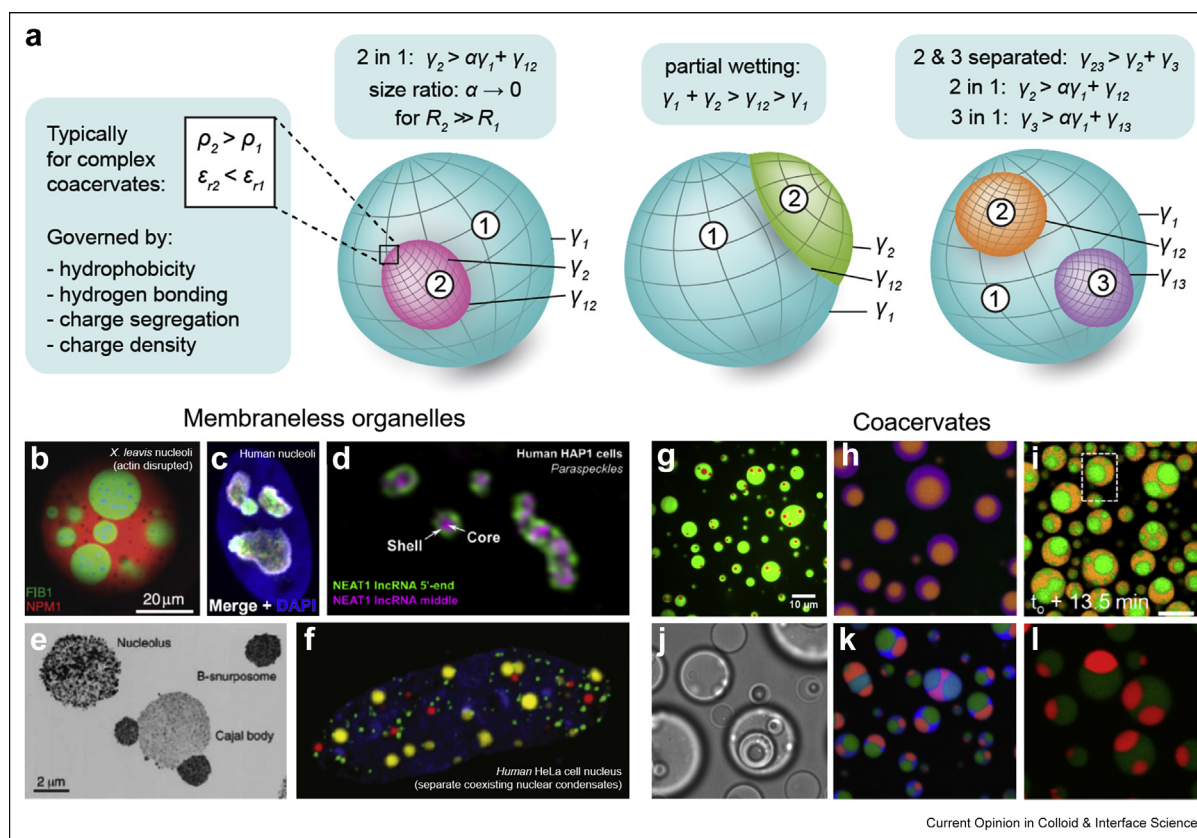
Hierarchical organization in MLOs and coacervates. A schematic illustration of three experimentally observed hierarchical arrangements highlighting the relative magnitude of the interfacial tension (γ), relative permittivity (ε_r), and density (ρ) (**a**). The parameter α is a measure for the relative size of the domains, as defined in Ref. [39]. Various examples of hierarchical organization in MLOs: the nucleolus (**b–c**), paraspeckles (**d**), Cajal bodies (**e**). By contrast, many MLOs also remain separated (**f**). Efforts to mimic these structures in coacervates have used a variety of electrolytes. These examples include ssDNA(red)/PLys(Me)$_3$ core coacervates in a ssDNA/GFP-K$_{72}$ (green) outer coacervate phase (**g**); nested multiphase organization in the 2xRRASL peptide (green)/Prot (red)/pGlu (blue) coacervate system (**h**), and the pLys (green)/Q-dextran (orange)/ss-oligo system (**i**); nested triple coacervates with an ATP/PAH inner core, surrounded by a PSPMA/PDDA shell in a PAA/PDDA outer coacervate phase (**j**); multiphase coacervates with double cores, some appearing partially wetted, observed with PAH, Prot, 2xRRASL, PAA, pGlu, and polyU (**k**); and partial wetting and lens-shaped domains observed in a pLys (red)/pAsp (green)/polyU coacervate system (**l**). Nonstandard abbreviations include protamine sulfate (Prot), poly-uridylic acid (polyU), poly-L-glutamic acid (pGlu). Images were taken with permission from Refs. [13,26,33,38–41].

interactions are well tuned. Once established, coacervate model systems could help to elucidate the principles of multiphase separation and the mutual arrangement of the phases.

Multiple groups have now shown that multicomponent mixtures of charged polymers, nucleic acids, or proteins can undergo multiphase separation and form coacervates with a hierarchical organization [39–43]. Both mixing all components directly, and combining macromolecule pairs into coacervates followed by mixing of the coacervates, yields the same hierarchical organization, and up to three coexisting coacervates have been found [39,40]. Most examples of multiphase coacervates show

complete wetting [39–41,43], which is explained by the low interfacial tension between two coexisting coacervates caused by the chemical similarity between different coacervates (Figure 3a). However, partial wetting has been found in the case of polyallylamine/polyacrylic acid (PAH/PAA) and protamine/polyglutamate double coacervates [40] and in the case of a prion-like domain from FUS and arginine-rich peptide/RNA complex coacervates [42]. The coexisting coacervates are apparently very distinct in these systems, resulting in a higher interfacial tension.

The phenomenon of multiphase coacervate formation is generic and has been realized with many different

combinations of synthetic polymers, peptides, and nucleic acids so far. It has been argued that the critical salt concentration of the coacervate systems can be used as a means to predict the possibility of multiphase coacervate formation [39]. In general, the most hydrophobic coacervate is found at the core of the multiphase assembly, but other interactions can also be found at the core, such as in polyA:polyC condensates [44]. It is interesting to note that the arrangement order of coacervates may depend on their respective size if the interfacial tension is sufficiently low [39].

Moreau et al. have shown that an internal liquid domain can also be formed inside a coacervate by segregative phase separation of a small molecule, tetraethylene glycol, inside a complex coacervate of poly(-diallyldimethylammonium chloride) (PDDA) and ATP [45]. Alternatively, internal dilute-phase domains (vacuoles) can be created by perturbation of complex coacervates in an electric field [46]. The inverse arrangement, with complex coacervates droplets forming an inner core phase inside a dextran-rich droplet [47], shows that segregation plays an important role in the hierarchical organization of condensates, a situation that may be similar to that inside the cell. Lu and Spruijt have pointed out that the formation of multiphase complex coacervates from mixtures of oppositely charged macromolecules is, in essence, also caused by segregation between like-charged components of the mixture, causing them to end up in distinct condensed phases [39]. In light of this, the formation of multiple domains in MLOs inside cells likely originates from a similar mechanism of segregation between charged components inside the MLOs. Classical polymer theory predicts that for long macromolecules, a small difference in solvent affinity suffices to induce their phase separation.

These findings in coacervates suggest that basic principles of physical chemistry and phase separation can explain the occurrence of hierarchically organized MLOs in living cells. At the same time, cells also contain many MLOs that remain clearly separated and that do not adopt a hierarchical organization, contrasting the notion that multiphase separation is a generic phenomenon in complex coacervates that occurs in a wide range of systems (Figure 3f). Fully separated but coexisting complex coacervates without stabilizing agents have not been observed yet. This raises the question if the separated nature of some cellular MLOs is the result of their unfavorable interactions, within the framework of interfacial tensions discussed above [37,39], or if it is caused by another factor *in vivo* that is not mimicked by coacervates, such as an active turnover of condensates [48] or a stabilizing layer in the form of adsorbed proteins [35]. In the latter case, synthetic analogs in the form of block copolymers [49] or modified proteins [50] could be used to mimic this situation *in vitro* by stabilizing complex coacervates.

Towards selective uptake: partitioning and enzymatic catalysis

In addition to hierarchical organization, MLO and coacervate physical properties also dictate which client molecules are partitioned into the dense phase. This exclusion or preferential uptake has a direct consequence on downstream biochemical reactions that occur in this unusual microenvironment. The degree of partitioning is set by the molar Gibbs free energy difference of the client molecule between two phases and is influenced by a combination of different driving forces (e.g. polarity, electrostatic, hydrogen bonding capacity, porosity/mesh, the binding capacity of components) outlined in our recent review [1]. In most cases, multiple driving forces control the partitioning of molecules, especially those of higher complexity, into the dense phase.

Due to the often charge-driven nature of coacervation, electrostatic interactions play an important role in determining the partitioning propensity of client molecules into the dense coacervate phase. Here the release of bound counter ions upon complexation is entropically favored and drives the process for a variety of client molecules. Inspired by MLOs that sequester nucleic acids and charged IDPs as client molecules, numerous, usually positively charged coacervate models have successfully harnessed electrostatically driven partitioning for charged client molecules [51,52], as well as RNA [21,28,53,54] and small single-stranded DNA (ssDNA) [55]. Additionally, electrostatic interactions can be enhanced to favor partitioning of client proteins into the dense phase, for example, by modifying their surface charge via succinylation of lysine residues [56,57] or engineering the protein sequence to alter the pI of the protein [58]. In the charge patterning of client proteins, regular distribution of aromatic and charged residues also plays an important role in favorable partitioning into condensates [22,59,60]. These examples of partitioning into coacervates are only minimally selective; that is, clients with the most favorable electrostatic interactions are selected. In contrast, MLOs exhibit a more subtle regulation, in which client proteins can have post-translational modifications, such as phosphorylation, to affect partitioning. Moreover, partitioning into MLOs often involves a combination of driving forces that results in finely tuned selectivity between client molecules of similar chemical composition, such as RNAs with different tertiary structures [61,62].

Multiple driving forces, and therefore selectivity, can be achieved through coacervate compositions that are more chemically and physically intricate. For instance, the mesh size of the dense phase limits the size of

Figure 4
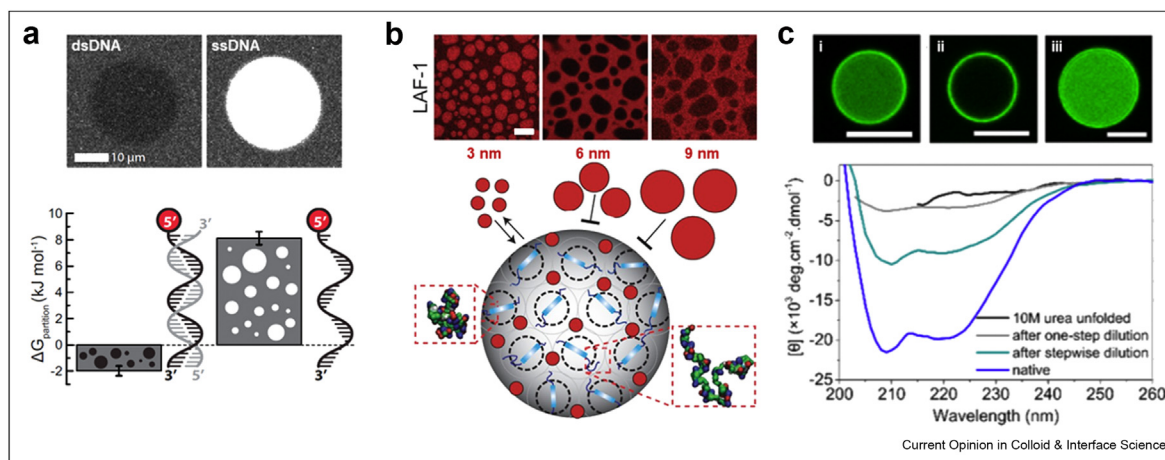
Selective partitioning of DNA and proteins. The organelles formed from the intrinsically disordered N-terminus of Ddx4 proteins excluded double-stranded DNA but allowed single-stranded DNA to permeate the dense phase (**a**, top panel). The DNAs were fluorescently labeled, and the average and SD (error bar) confocal emission intensities from inside and outside the organelles were used to quantify the partition equilibrium coefficient and its corresponding free energy (**a**, bottom panel). The permeability of *in vitro* droplets of LAF1 proteins was probed using fluorescent dextran of different molecular weights (from left to right): 10 kDa dextran permeates the droplets, while 70 and 155 kDa dextran molecules are excluded from the droplets. The schematic below illustrates the void rich nature of LAF-1 protein droplets and their probe-size dependent permeability. The flexible RGG domain in LAF-1 is depicted in blue, and the envelopes defined by the Rg of LAF-1 are shown in black-dash circles (**b**, bottom). In contrast, coacervates made from PDDA/PAA showed the inclusion of native fluorescently labeled BSA protein (i), but the exclusion of denatured protein in 10 M urea (ii). This selectivity was restored when proteins were allowed to refold when urea was diluted 10-fold (iii). The CD spectra (**c**, bottom panel) of native BSA denatured BSA-FITC in 10 M urea and after refolding procedures. Images were taken with permission from Refs. [15,26,35].

molecules that can partition into it [15,27] (Figure 4). In fact, the mesh size of Ddx4 coacervates result in higher partitioning of ssDNA but not dsDNA due to the increased nucleic rigidity and size of dsDNA [26]. Coacervates can display rudimentary selectivity for retaining large clients, such as long templated RNA [54] or DNA-complexed with supramolecular polymers [55], whereas the smaller nucleic acid counterparts are freely diffusible. In addition to mesh size, MLOs also harness the specificity of protein−protein interactions to recruit client components to the coacervate phase, introducing extra functionality to the dense phase [63]. This feature can be engineered into client protein sequences where fused protein tags or interaction domains can drive partitioning into the dense phase [64−66]. Besides engineering clients, the local environment around the coacervate can be tuned to affect selectivity, such as by altering solution pH [67], which can shift the equilibrium in favor of or against the partitioning of certain protein components, where native proteins with patchy surface charge had enhanced uptake. Finally, recent studies on the multiphase organization in complex coacervates have demonstrated that the partitioning of client molecules can be controlled into distinct compartments [39,40]. Multiphase organization could, therefore, reflect another method by which cells achieve selective partitioning. Although the partitioning of client molecules is shaped by a combination of driving forces, the selectivity is underpinned by the chemical complexity of MLOs, which is a feature yet to be matched in coacervate systems.

Selective partitioning of client molecules into the dense phases offers control of downstream enzyme catalysis that can impact MLO function. Synthetic MLOs can be used to modulate cellular activity [68], highlighting the importance of understanding the effects of altered enzymatic activity within and near MLOs. The dense phase of MLOs and coacervates exert two main influences on internalized enzymes (i) the partitioning effect of client molecules altering the local concentrations and availability of substrates and reactants; and (ii) altered enzyme conformation due to the highly charged and crowded microenvironment, thereby altering the energy landscape of the enzymatic reaction. It is difficult to disentangle which of these factors exert the most impact on catalysis, as concentrations of individual components are often not quantified, and the systematic study of these factors is often omitted. Nonetheless, model coacervate systems have successfully encapsulated enzymes [52,69−71] and ribozymes [53,54] that retain catalytic function. The composition and resulting physical properties of the coacervate may impact enzymatic activity: some increase, decrease or have no effect [72]. In addition to affecting internalized protein activity, coacervate formation has been shown to arrest

GFP production due to the sequestration of protein machinery required for translation [73]. So far, it seems possible to realize many outcomes in coacervates that are also possible in MLOs, but the control or predictability of such catalytic processes is still missing.

Formation dynamics and regulation

MLOs are highly dynamic structures that are formed and disassembled within seconds to minutes *in vivo*. The dynamic condensation of MLOs occurs in most cases through nucleation and growth [74] and is believed to result from regulation through a combination of post-translational modifications [75], competitive binding, and chaperone activity of, for example, small heat shock proteins [76]. Equivalent regulation of formation and dissolution remains an elusive feature that is yet to be fully mimicked in coacervates [77]. Coacervates can be used as models to understand the process of condensation upon reversible changes in solubility. Reversible control over coacervate models has largely been studied via two routes: (i) by changing the environment, causing a *passive* change in interactions or (ii) by utilizing enzymatic reactions that *actively* change the phase separating molecules (through covalent modifications) and thereby control coacervation, as summarized in Figure 5.

Figure 5

Current Opinion in Colloid & Interface Science
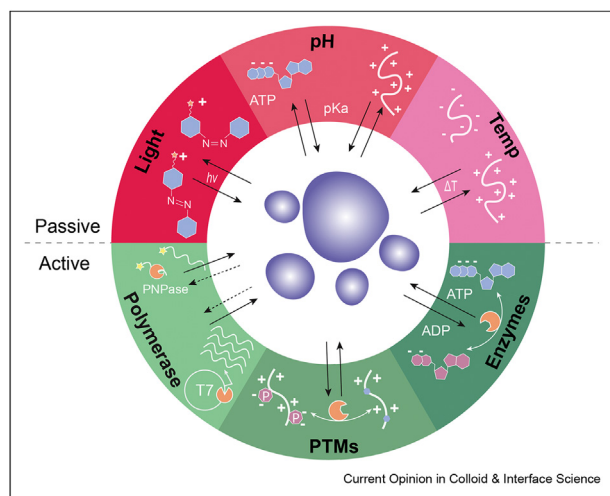
Passive and active control over coacervation. Top, passive control from left to right: light can be used to control coacervation through the conversion of a positively charged azobenzene with DNA. Increasing or decreasing the pH can be used to neutralize either the polyanion (ATP pH ~ 4) or polycation (pLys pH ~ 11) involved in complex coacervation. Several examples showed that temperature could inhibit or induce coacervation. Bottom, active control from left to right: polymerization reactions such as extending RNA through PNPase or T7-polymerase of a plasmid can induce coacervation with a cationic species. Serine phosphorylation through post-translational modifications (PTMs) has been used to control the coacervation of a cationic peptide. Through metabolic enzymatic reactions, concentrations of ADP and ATP can be varied, which controls their coacervation with pLys.

Passive control through environmental changes

Altering environmental conditions can be considered a passive way to control coacervation as it does not require internal regulation. Various environmental parameters — such as temperature, pH, salt and light — have been used to control coacervation. For the most part, these parameters can also affect living systems, and MLO formation could provide a mechanism to survive these fluctuations by temporarily storing sensitive biomolecules in protective compartments [78].

Heat stress is one of the triggers known to induce stress granule formation. Phosphorylation of translation initiation factors by stress-activated kinases is believed to be a key mechanism that leads to inhibition of translation and stress granule assembly [79,80]. However, other mechanisms, including a direct effect of temperature on protein solubility, cannot be ruled out. For understanding whether changes in temperature could have a direct effect on phase separation, coacervate model systems could be used. Several examples of temperature-sensitive coacervates have been reported, including poly(styrene sulfonate) (PSS) and poly(diallyl dimethylammonium bromide) (PDADMAB) [81], lysine-rich elastin-like polypeptide (ELP) and hyaluronic acid [82], and spermine and poly-U [83]. In all these cases, coacervation started at temperatures above $25-30$ °C, and lower critical solution temperature (LCST) behavior was observed. In general, these systems contain higher levels of hydrophobic moieties (e.g. PDADMAB and ELP) [81,82] or involve RNA with a more compact secondary structure at low temperatures, such as polyU [83]. On the other hand, dissolution of coacervates at $40-60$ °C following upper critical solution temperature (UCST) behavior has been reported by Kim et al. using protamine, an arginine-rich polypeptide, with either citrate or triphosphate [84].

Most *in vitro* MLO studies consider a constant pH in a narrow regime between pH $7-8$ (physiological), while pH changes could affect side chain charges [85]. The pH-sensitive phase boundaries for pLys/ATP coacervates were shown to occur between pH 2 and pH 11 [51]. Recently, two research groups exploited these phase transitions to control coacervation by pH within liposomes [31,86]. Although the coacervate system is identical, the approaches to disassembling the coacervates highlighted the two different phase boundaries: Tang and coworkers neutralized the positive charge of pLys above pH 11 [86], while Dekker and coworkers neutralized the negative charge of the ATP nucleotide below pH 5 [31]. Similar pH changes are unlikely to take place in the cytoplasm of living cells, but the development of coacervate systems with more diverse component chemistries such that their phase behavior can be controlled in a narrower pH range near-physiological values could further narrow the gap in understanding the role of pH on MLO dynamics.

Finally, light is a powerful tool to obtain spatiotemporal control over coacervation. Using an azobenzene trimethyl-ammonium bromide (azo-TAB) molecule as the positively charged component, Mann's group showed reversible light-controlled coacervation with double-stranded DNA [87]. Light-activated PhoCl protein cleavage has been used to induce the coacervation of RGG-mScarlet proteins [88]. Where light might go in the direction of active control over coacervation, it is not the most logical approach when mimicking MLOs. However, light can be used as a powerful tool to induce the formation of artificial light-responsive MLO (OptoDroplets) in living cells, as shown by Shin and coworkers (see Section Metastability for more details) [89]. Enzymes, on the other hand, offer a more physiological approach to control coacervation.

Active control by enzymes

While MLO formation induced by variations in external conditions (passive) could serve as a mechanism to protect biomolecules from damage, most MLOs are actively controlled by enzymatic processes, conformational changes, or chaperones that can hold or release phase separating proteins. Efforts to mimic such enzymatic control over phase separation have recently resulted in the first enzymatically controlled coacervates, in which metabolic enzymes were used to produce or hydrolyze ATP. Nakashima et al. used the pLys/ATP coacervate system, combined with two kinase enzymes to controlled both coacervation and dissolution, depending on whether ATP was produced or consumed [90]. Deshpande et al. used this same enzymatic system to control coacervation in liposomes and used different enzymatic reactions to control the dynamic formation of coacervates [70].

The products of enzyme-catalyzed elongation or oligomerization reactions can also promote coacervation (Figure 5). For instance, a re-entrant phase transition was observed in a system where RNA is produced by T7 polymerases [91]. RNA-peptide coacervates were formed until a certain threshold concentration was reached. Beyond this threshold, coacervates started to dissolve due to an excess of RNA. This model system has interesting implications for MLOs as many condensates in the nucleus are involved in RNA transport and processing. Spoelstra et al. were able to control coacervate formation through the use of the polynucleotide phosphorylase (PNPase) enzyme that extends the 5′-end of RNA [92]. Although the initial RNA primer was too short to induce phase separation, extending the RNA eventually increased charge interactions that resulted in coacervation. Dissolution through nuclease activity could be a route to actively control dissolution, as shown for the protease trypsin on poly-(L-lysine-L-serine) and poly-L-glutamic acid

coacervates [93]. These examples demonstrate that enzymes can provide routes to actively control the formation and dissolution of coacervates in a rudimentary way. In addition, recent work demonstrating that chemical reduction and oxidation can be used to control the assembly of micelles with complex coacervate cores by changing the effective valency of the constituents could open the way for similar control of coacervation through redox enzymes [94].

Nevertheless, a significant gap between MLOs and coacervates remains, as biological systems often control coacervate formation through post-translational modifications (PTMs) of the IDP condensate components. For instance, the methylation of Arg residues in FUS proteins was shown to disassemble condensates [95]. To the best of our knowledge, there has been only one coacervate model of this phenomenon: Aumiller Jr. and Keating showed that serine phosphorylation of [RRASL]$_n$ peptides can reversibly control coacervation [96]. Expanding this approach to other (synthetic) coacervate models may be challenging, as many enzymes have specific recognition sequences based on protein chemistry.

Finally, one of the most challenging mechanisms of control over the formation dynamics to mimic is the conformational changes triggered by host-guest bindings to proteins. There are several biological examples where binding of ATP triggers conformational changes that play a role in phase separation, as suggested for stress granules [97], or RNA binding to form a P-granule gradient [98]. To our knowledge, there are no coacervate systems that can mimic such a switch on host-guest binding to control coacervation.

Figure 6

Current Opinion in Colloid & Interface Science
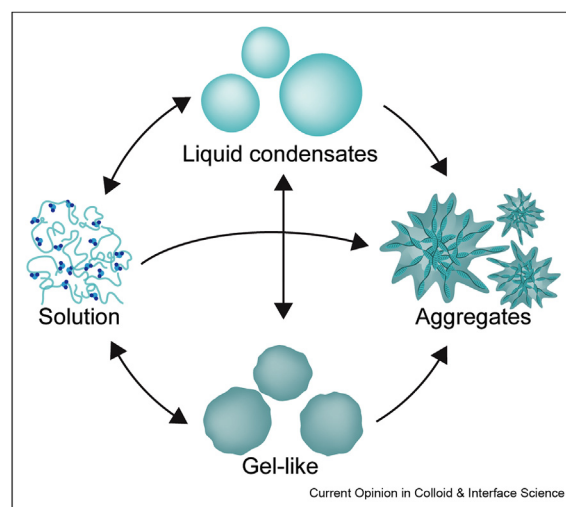
Various phases that are accessible to metastable coacervates.

Metastability: liquid-to-solid phase transitions

Once formed, certain MLOs can undergo phase transitions from the liquid state to gel-like or solid forms (Figure 6). Liquid-to-solid phase transitions are of interest as they are linked to the emergence of various pathologies and the regulation of cellular processes [99,100]. Phase transitions of the metastable liquid (coacervate) state occur when long-range associative forces (such as electrostatics) facilitates local rearrangements that enable solid-like assemblies (such as cross-β structures stabilized by close-range, directional interactions) to form over time [4]. This results in a lower free energy state where the system becomes gel-like or forms solid aggregates that can no longer revert back to the dense liquid phase. These liquid-to-solid transitions in metastable liquid condensates can be considered a case of nonclassical nucleation, where proteins can self-assemble from a condensed liquid state instead of directly from a solution [101]. Formation of the close-range interactions involved in the formation of solid aggregates can be facilitated by increasing local concentrations of proteins or by introducing crowding agents [36]. The effect of increasing associative interactions between phase separating proteins on the aging and solidification of droplets was nicely illustrated in MLO models containing light-triggered Cry2 protein associations, called OptoDroplets [89]. OptoDroplets showed a decreasing fluidity as Cry2 associations increased with higher light exposure. The gels formed were initially reversible but can age into irreversible aggregates. The crowded coacervate provides an environment where close-range interactions can be amplified, which can then lead to phase transitions.

MLOs that undergo liquid-to-solid phase transition are, in fact, moving away from the very nature of coacervates, as previously described. Accurately modeling this feature, therefore, poses a fundamental challenge to coacervate systems. Several recent examples suggest that a form of liquid-to-solid transitions might be possible in coacervates when the external conditions are changed *after* coacervation. Strengthening the internal ion-pair interactions inside coacervate by salt-induced dehydration leads to dynamic arrest and yields irreversible gel-like states [11]. DNA hybridization can also induce liquid-to-solid transitions in pLys-DNA coacervates, which result in the reversible formation of solid aggregates [102]. Last, pH-sensitive peptides that facilitate intermolecular charge transfer can also age to form irreversible solid aggregates [103]. All of these solid states do not quite resemble the morphologies or characteristics of liquid-to-solid transitions in MLOs, leaving a discernible gap to be filled by coacervate models.

Conclusion

Understanding how biomolecular processes are organized and regulated in living cells is of relevance for a wide range of topics. Appropriate *in vitro* models of MLOs can help to elucidate the roles and characteristics of these dynamic, droplet-like structures by enabling *in vitro* experiments in controlled conditions that would not be possible *in vivo*. Coacervates — a term stemming from colloid science — share important characteristics with MLOs, and as highlighted in this review, can successfully mimic certain properties. Since coacervates are formed by liquid—liquid phase separation, driven by the same attractive interactions as is often the case in MLOs, they have comparable material properties, and they exhibit the same potential for hierarchical organization, selective sequestration, and in principle, dynamic formation. However, 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 [104–106]. 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.

However, 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 [48], and they can literally shape MLOs [33,38] 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 [92,107].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could

have appeared to influence the work reported in this paper.

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