Membraneless organelles and how to simulate their molecular structure

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February 11th 2019

Abstract
Membraneless organelles form through phase separation. Such a process can be driven by complex coacervation. Two polymers of opposite charge interact to form a separate phase in solution. This essay describes this in the context of biological phase separation. Interactions of proteins involved in droplet assembly are more complex. To understand the full set of interactions new approaches are required. One such approach might be found in molecular dynamics simulations.

keywords: membraneless organelles, complex coacervation, phase separation, simulation, molecular dynamics

Introduction

Compartmentalization plays a crucial role in life. In biology, lipid membranes have become the characteristic of compartments. They form the boundary between cells and their environment and, in eukaryotes, separate the cytoplasm from organelles (e.g. mitochondria, nuclei and peroxisomes). However, recently the study of a new type of compartment, not bound by lipid membranes, is becoming increasingly popular. These membraneless organelles form in the cytoplasm and nucleoplasm through liquid-liquid phase separation. They add complexity to the cell's intracellular space, fulfilling new roles through the creation of distinct chemical environments.

Before the beginning of the 20th century the use of RNA dyes revealed an intricate network of circular bodies in germ cells (Ritter, 1890). The bodies could be seen due to their increased RNA concentration. In 1899, E.B. Wilson reviewed a discussion on the nature of these bodies (Wilson, 1899). He specifically noted that they came in various sizes, from small spheres to tiny granules. A young ovarian egg would contain an almost homogeneous protoplasm (footnote: protoplasm out-of-use term for the inside of cells, including cytoplasm and nucleoplasm) with only a few spheres and granules. During growth they would increase in size, while new granules spontaneously popped up. Comparing two popular views, Wilson concluded the observed structure was most likely an emulsion and the observed bodies were size gradations of one structure. Wilson was not far off; the study of membraneless organelles considers the cytoplasm and nucleoplasm as emulsions of liquids in different phases.

Phase transitions are common from everyday experience, water can be in solid, liquid, or gas phases. Highly concentrated gaseous water (humid air) can condense into droplets upon decreasing temperature, such as the formation of dewdrops. Even further lowering of temperature will cause the water molecules to order into a crystalline solid, ice. Although biomolecules in cells are much larger than water, they undergo similar phase transitions. This is observed in X-ray crystallography, where proteins often liquidize before ordering into a crystal lattice. Similarly to how liquid oil and water demix through phase separation, proteins in a condensed liquid phase also phase separate from bulk water (Shin & Brangwynne, 2017).

Membraneless organelles are thought to be formed by such phase separation of condensed proteins. Interactions between proteins, and between proteins and RNA are thought to drive this condensation. Bodies formed through the interactions between these polymers are called ribonucleoprotein (RNP) bodies/granules, or RNP droplets. The latter names owes to the exhibited liquid properties, such as fusion of smaller into larger droplets upon contact, and wetting and dripping on nuclei (Brangwynne *et al.*, 2009; Brangwynne *et al.*, 2011). Examples of RNP droplets are nucleoli in the nucleus and germ granules in the cytoplasm (Weber & Hyman, 2012). Additionally, droplets exist that form purely through protein interactions, e.g. purinosomes (Weber & Hyman, 2012). The droplets can be thought of as different liquid phases of the cytoplasm and nucleoplasm, essentially creating an emulsion (Hyman & Brangwynne, 2011). Under the right conditions they condense spontaneously (Nott *et al.*, 2015), which can be dependent on RNA/protein concentration, salt concentration, temperature and more (Nott *et al.*, 2015; Pak *et al.*, 2016).

Although the study of phase separation has only recently gained traction in biology, in polymer chemistry it has long been studied under the theory of (simple/)complex coacervation (Brangwynne *et al.*, 2015; Sing *et al.*, 2017). Complex coacervates are formed through electrostatic interactions between two oppositely charged polymers (Sing *et al.*, 2017). Such a charged polymer is also often called a polyelectrolyte. The interactions between the chains cause them to form a coacervate, creating a separate phase in the solution. In cells however, the assembly of membraneless organelles seems to rely on additional effects (Levine *et al.*, 2017). Besides charged amino acids in protein chains, hydrophobic and aromatic residues are important (Nott *et al.*, 2015; Pak *et al.*, 2016), and specific motifs of residues have also been implicated (Nott *et al.*, 2015). Additionally, (domains of) proteins involved in these complexes are often intrinsically disordered (Levine *et al.*, 2017). Popular theories of complex coacervation only apply to idealized systems, relying purely on electrostatic interactions (Sing *et al.*, 2017). Understanding the combined driving forces that drive liquid-liquid phase separation in cells requires new approaches.

One such approach might be found in molecular simulations. Although this approach has not been applied for membraneless organelles, it has been proven useful in understanding complex coacervation in model polyelectrolyte systems. The main question of this essay is therefore: what are relevant factors in biological coacervation and how can simulation be used to study them? To answer this question I will explore the components in cells that are considered critical for assembly of membraneless organelles. Recent studies have examined protein sequences involved in organelles and identified important elements. By comparing these studies I will show common elements that ought to drive phase separation. Then I will discuss the current understanding of complex coacervation in polymer chemistry. Some of the properties of components in biological phase separation, are also found important in this field. Finally, I will explain why chemical theories still appear to lack detail for an understanding of complex coacervation in cells, and assess if molecular simulation approaches could aid.

Intrinsically disordered proteins dictate formation of membraneless organelles

Recent biological studies are starting to uncover the driving forces for phase separation in cells. Evidence indicates that besides electrostatic interactions additional forces are at play. Intrinsically disordered proteins (IDPs) are shown to be important. These proteins are composed of diverse amino acids, indicating a role for driving forces besides electrostatics. Recent studies are beginning to unravel common factors in these sequences.

Membraneless organelles, as found in eukaryotes, often contain both proteins and RNA, although some exist that are pure proteinaceous. Of the former the cytoplasm contains processing bodies (mRNA turnover, decay), neuronal granules and germ granules (Brangwynne *et al.*, 2015). Nuclear are Cajal bodies, nucleoli, and PML bodies. Protein-only droplets are inflammasomes, signalling complexes, purinosomes, centrosomes, postsynaptic density bodies, dynamic inclusion bodies, and transcription factor bodies. Sizes range from 0.1 - 3 micrometers (Crabtree & Nott, 2018). The organelles form through the association of specific molecules. The general function of these droplets is therefore colocalization of molecules in a small space to control the rate of reactions (Brangwynne, 2013). For example, RNA containing droplets have a range of functions in RNA metabolism: storage, splicing, decapping, and degradation (Weber & Brangwynne, 2012). Transcription activation has been indicated through recruitment of protein complexes at the DNA (Boija *et al.*, 2018). Purinosomes are involved in purine biosynthesis, speeding up metabolic reactions. Thus, these organelles can fulfill diverse roles.

Their visualization is relatively easy with microscopy where they appear as spherical droplets (fig. 1). Initial discoveries were made possible with the use of RNA stains (Ritter, 1890). Additionally, large oocytes increase visibility of germ granules (Wilson, 1899). Because the total droplet volume depends on the size of the cell and the concentration of droplet components, a larger cell has bigger/more numerous droplets (Brangwynne, 2013). With the development of advanced microscopy techniques, such as electron microscopy and phase contrast microscopy, visualization is now easily accessible for all types of membraneless organelles (Eddy, 1975; Voronina *et al.*, 2011). Of the latter technique, especially differential interference microscopy (DIC) is popular (Brangwynne *et al.*, 2011; Nott *et al.*, 2015), showing sharp boundaries of droplets (see figure 1; Handwerger *et al.*, 2005).

Obtaining information on physical properties is more difficult however. The small size of nuclei in cultured cells hampers microscopical measurements, because this results in even smaller dimensions of subnuclear structures. Pioneering studies by Handwerger and colleagues have been able to measure density and permeability by isolating oocyte nuclei into mineral oil (Handwerger *et al.*, 2005). These large oocyte nuclei, and subsequently large subnuclear structure, allow density measurements by an interferometer microscope. Cajal bodies, nuclear speckles, and nucleoli turn out to be only slightly more dense than the nucleoplasm. Estimation of protein concentration for the nucleoplasm is ~0.11 g/cm^3, while it ranges from ~0.14-0.22 g/cm^3 for nuclear droplets. Additionally, fluorescently tagged dextrans are only excluded from droplets above dextran sizes of 2000 kDa. Handwerger and colleagues thus concluded a sponge-like structure for these nuclear organelles (Handwerger *et al.*, 2005).

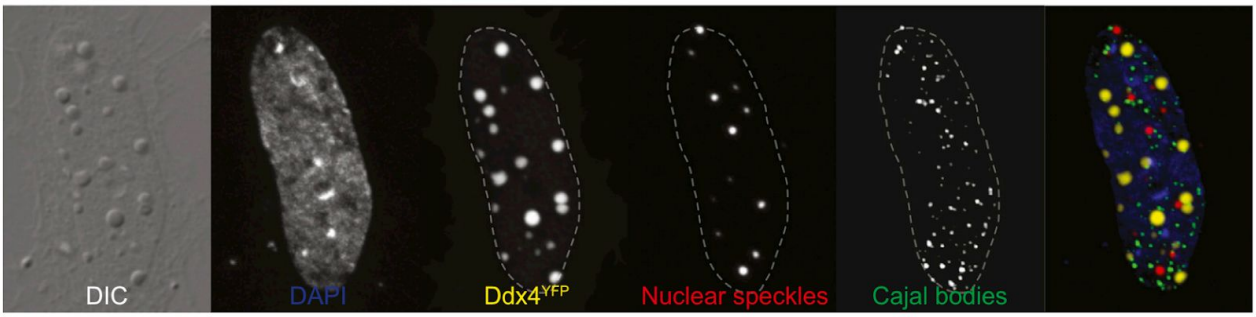

Figure 1. On the left a DIC image of membraneless organelles in the nucleus of a HeLa cell. Middle images contain the fluorescence channels of their respectively named marker: DAP1, Ddx4-YFP, nuclear speckles, and Cajal bodies. Right image shows an overlay of these fluorescent channels with colors as indicated in the middle images. Figure from Nott *et al.*, 2015.

The view of a sponge-like structure has now been abandoned in favor of a liquid droplet view. This view was pioneered by Brangwynne through two subsequent studies of P granules (Brangwynne *et al.*, 2009) and nucleoli (Brangwynne *et al.*, 2011). P granules show typical liquid properties: granules fuse together when they touch, and they flow and drip off nuclei (see figure 2). The same behaviors are observed for nucleoli. Timescales for these behaviors are much larger than for water droplets, they are on the order of ~50 seconds (see figure 2). This indicates a higher viscosity. Indeed, the viscosity of P granules is estimated at roughly 1 Pa·s, or approximately 1000 times as large as the viscosity of water. Such values are comparable to values of colloidal liquids (one substance evenly dispersed in another), or glycerol. A sponge structure is not able to support the observed liquid properties at the larger timescales. Also, the liquid properties dictate that it cannot be a structurally well defined assembly on length scales > 100 nm. Thus, these liquid properties dictate a dynamic nature of the droplet components.

Attempts have been made to demonstrate these dynamics. This can be done using fluorescent recovery after photobleaching (FRAP) experiments where a droplet component is genetically labelled by a fluorescent dye. Local illumination of the droplet can photobleach all the dye within while the environment is left unharmed. The time it takes to recover fluorescence in the body is a property of how easily components in the body can be exchanged with the environment. Brangwynne and colleagues demonstrated the dynamic exchange of a fluorescently labelled PGL-1, a constitutive component of P granules (Brangwynne *et al.*, 2009). The construct is highly concentrated in the bodies. Upon photobleaching, fluorescence is recovered in ~5 seconds. The relatively fast recovery indicates that PGL-1 is dynamically exchanged between the droplet and environment.

This dynamic exchange can be a particularly strong argument for coacervate phases. Such phases are characterized by their liquid nature where only temporary interactions between polymers occur. Brangwynne's study (Brangwynne *et al.*, 2009) however does not provide clear evidence on the dynamic nature of P granules. Although PGL-1 is a constitutive component in the function of P granules, *pgl-1* mutants still have P granules in the cytoplasm (Kawasaki *et al.*, 1998). PGL-1 might therefore only localize to P granules while not being involved in interactions that drive assembly. Thus, no definitive conclusion can be drawn on the dynamics of the polymer components in P granules. Yet, studies by Nott

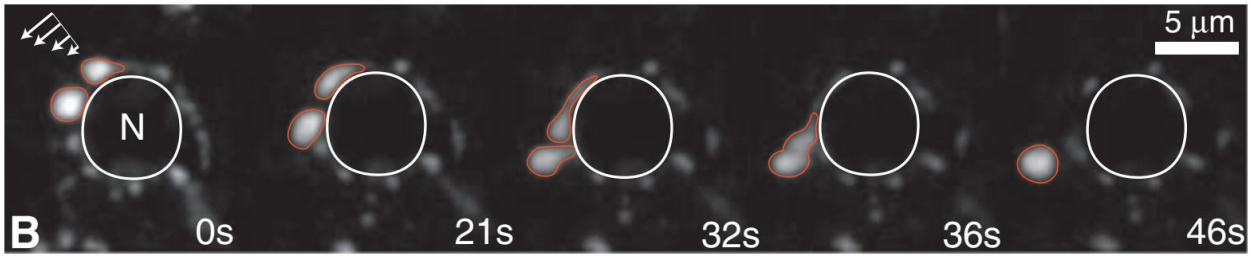

Figure 2. Two droplets first drip off the nucleus along the direction of flow in the cell (indicated in the top left corner). In the later frames the droplets can be seen fusing together. Figure from Brangwynne *et al.*, 2009.

and colleagues (Nott *et al.*, 2015) and Pak and colleagues (Pak *et al.*, 2016) do make a strong case for the coacervate nature of nuage bodies and nephrin nuclear bodies, respectively.

Nott and colleagues studied the assembly of nuage bodies (Nott *et al.*, 2015). The DEAD-box protein Ddx4 is a mammalian RNA helicase located in nuage bodies. The helicase domain of the protein is surrounded by an extended N and C terminus, which are predicted to be intrinsically disordered domains. The domains contain both positive and negative charges. The authors replaced the helicase domain with a yellow fluorescent protein (YFP) (Nott *et al.*, 2015). This mutant showed localization in distinct nuclear droplets (see figure 1). Condensation of droplets could be controlled through temperature and salt concentration. A sudden drop in temperature led to condensation, while a sudden drop in osmotic strength (from 150 mM to 150 µM) dissolved droplets. Returning to initial conditions reversed the effects of both shocks. Such parameters are typical for influencing complex coacervation.

Moreover, their study indicates that Ddx4 drives phase separation of nuage bodies. FRAP experiments on Ddx4-YFP bodies revealed dynamic exchange with the environment, with recovery in less than one second. Moreover, recombinantly expressed and purified Ddx4-YFP could also reconstitute droplets in vitro under near physiological conditions (150 mM ionic strength and 37 °C), indicating these domains of Ddx4 as the driving force for nuage body formation. FRAP on in vitro droplets gave recovery values that were not significantly different from in vivo droplets. These experiments show that a biological droplet forming polymer can be rapidly exchanged. Nuage bodies are thus dynamic, and their condensation can be controlled through temperature and ionic strength. These results strongly argue that nuage bodies phase separate in vivo through interactions of Ddx4 domains. However, a YFP construct with only the N terminal domain was already able to phase separate under the same conditions. This domain contains both positive and negative charges. So, it seems that nuage bodies separate through interactions of a single polymer. This points to phase separation by ‘simple’ coacervation—a process in which a single polymer forms a coacervate.

In contrast, complex coacervation is indicated for the nephrin intracellular domain (NICD). This negatively charged domain of the mammalian adhesion receptor nephrin forms droplets in the nucleus. It associates with non-specific positively charged partners. Pak and colleagues show distinct localization and fluorescence recovery in ~2.5 seconds for their NICD-YFP construct in cells (Pak *et al.*, 2017). Isolated and purified NICD remained soluble under various conditions. Upon addition of a positively charged GFP or poly(arginine) peptide the solution phase separated. In contrast to Ddx4, NICD phase

separates through non-specific interaction with multivalent counterions. Thus, NICD droplets are truly complex coacervates.

Both the studies on Ddx4 and on NICD attempted to reveal which amino acids influenced phase separation. Nott and colleagues investigated a naturally occuring splice variant of Ddx4 (Nott *et al.*, 2015). This splice variant has a block of 34 residues in the N terminus replaced by a single aspartate. This variant was no longer able to phase separate in vitro. In addition, they showed that methylation of only 5-6 arginine groups destabilized droplets significantly. Such methylation of amino acids occurs naturally for Ddx4 and could reduce electrostatic interactions. It is interesting that naturally occuring post-translational modifications of Ddx4 alter its ability to phase separate. This indicates a certain level of control by cells.

They also continued with a bioinformatics approach to unravel important residues (Nott *et al.*, 2015). They studied the amino acid sequence of the Ddx4 terminals in relation to other known IDPs. The ability to phase separate is not a property of all such proteins. Bioinformatics revealed that the number of hydrophobic residues in the disordered tails of Ddx4 is lower than for the average IDP, but the number of charged residues is equal. What dictates phase separation must thus be more complex. A specific feature is the ordering of similarly charged residues into blocks of 8-10 amino acids. They found this feature to be essential. A mutant containing the same overall charge and amino acid composition but without ordering into charged blocks, lost its ability to phase separate in vitro. Such effects are also shown in chemical studies of phase separation (see below). Another feature is the presence of evenly spaced FG and GR motifs. Short-range cation-π interactions between the positively charged arginine and the aromatic phenylalanine could therefore be important for phase separation. Screening in the human proteome revealed the ubiquitous nature of this pattern in IDPs that are known to appear in membraneless organelles. Although these results for Ddx4 are interesting, it should be kept in mind that they might not bear on complex coacervation due to the single polymer involvement.

Some similar features are however also observed for NICD (Pak *et al.*, 2016). NICD contains clusters of negative charge along its sequence. Mutants of NICD with either increased or decreased charged clusters showed that stronger charge density promoted phase separation with charged GFPs, similar to the observation in Ddx4 (Nott *et al.*, 2015). Additionally, they showed that mutants with shuffled NICD regions but same overall amino acid content formed droplets nearly as well as the WT. The overall content of the sequence is therefore more important than the specific sequence. This is also supported by double random deletions in NICD, which almost exclusively reduced droplet formation. A statistical analysis of these deletion mutants revealed that deletion of Tyr lead to the strongest loss of NICD bodies, followed by Arg, Leu, Met, and Trp. This shows aromatic and hydrophobic residues also play important roles in phase separation of NICD bodies.

In summary, liquid phenotypes seem to be a general property of membraneless organelles. Such properties argue for these organelles to be in a phase separated state. For some of the organelles, the specific involvement of IDPs has been demonstrated. By showing that Ddx4 domains and NICD can also condense into droplets in vitro, these peptides appear to be the driving forces for assembly of nuage bodies and NICD droplets, respectively. Although NICD clearly forms droplets through complex

coacervation, Ddx4 does so by simple coacervation. The amino acids of these proteins contain charged residues that are specifically grouped into blocks. Such properties can improve coacervation, as has also been demonstrated in polymer chemistry (see below). However, these studies also indicate roles for hydrophobic and aromatic residues. The importance of these amino acids in coacervation is not well understood yet.

Diverse parameters contribute to complex coacervation

The chemical study of complex coacervates describes the conditions under which two oppositely charged polymers can interact to form a separate phase in solution. This process is influenced by multiple parameters. One interesting parameter for this essay is the influence of charge blocks. Such charge dense clusters on polymers promote coacervation, similar to the charge blocks in IDPs. Theories of complex coacervation are only focused on the charges in polymers and only function for idealized complexes.

Complex coacervates are assemblies of oppositely charged polymer species. Mixtures that form assemblies are described to exist of four non-water molecules: a polycation, a polyanion, a cation, and an anion. The electrostatic interactions between the two polymers drive the liquid-liquid phase separation (Sing et al., 2017). One of the resulting phases is polymer-dilute, while the phase of the coacervate is polymer-rich. The polymer-rich phase is liquid-like but more viscous, i.e. water is still retained. Importantly, the electrostatic crosslinks between the polymers are of temporary nature (Sing et al., 2017). This owes to the dynamic nature of the complexes: there is no one-to-one exclusivity and chains can be replaced and exchanged between the polymer-rich and polymer-dilute phases.

Formation of these complexes has been shown to be influenced by multiple parameters. Their influence can be measured through their effects on the thermodynamics of the process. Isothermal titration calorimetry (ITC) can be used to measure enthalpy changes during reactions (Priftis *et al.*, 2012). Additionally, the Gibbs free energy and entropy increase can be derived from the measured data. Such experiments on solutions of oppositely charged polypeptides, poly(l-ornithine) and poly(glutamate), show thermodynamics are influenced by salt concentration, pH, temperature, polymer length, and polymer concentration (Priftis *et al.*, 2012). Charged polymers in solution will be initially surrounded by monovalent salt ions. The step of complexation releases these ions which causes a gain in the translational entropy of the ions. Higher salt concentrations for example will increase the electrostatic screening of salt and reduce the relative gain in translational entropy (Chollakup *et al.*, 2013; Priftis *et al.*, 2012). In contrast, at high temperatures the translational energy of free polymers dominates, resulting in solvation of coacervates (Nott *et al.*, 2015). pH influences the ionization state of the interacting groups (Priftis *et al.*, 2012). Finally, increasing polymer length and concentration promote coacervation (Chollakup *et al.*, 2013; Priftis *et al.*, 2012). The effects of these parameters can be displayed in a phase diagram (see figure 3).

Besides the above mentioned parameters, charge patterning on polymers also influences coacervation. Unlike the homopolymeric polyelectrolytes (consisting of monomers with identical charges) often used in chemical experiments, proteins are build up of diverse amino acids. Advances in synthetic chemistry have recently allowed sequence defined polymerization. Chang and colleagues have used this to demonstrate the effects of charge patterning in synthetic polypeptides (Chang *et al.*, 2017). An anionic homopolymer, poly(glutamate), was lead to interact with multiple species of a cationic poly(glycine-co-lysine). The cationic polymers consisted of repeats τ of the positive (lysine) and neutral (glycine) monomer, where τ denoted the size of the repeats (with $\tau = 2, 4, 8, 16, 24$). $\tau = 2$ was a repeat

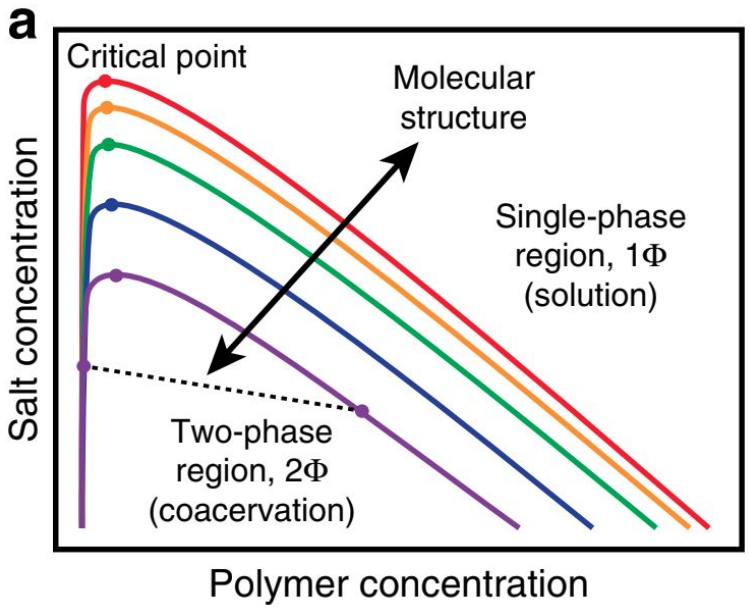

Figure 3. A phase diagram shows the region of coexistence of two phases under different salt and polymer concentration. The region of two phase coexistence 2Φ is indicated under the curve, where the dotted line connects salt and polymer concentration in the polymer-dilute and polymer-rich phase. The line connecting the points is angled because the salt concentration is not equal for the coacervate and supernatant phase. A phase diagram can potentially be multidimensional, or show multiple curves for different polymers, degrees of polymerization, etc. The critical point indicates the salt concentration at which phase coexistence is no longer possible. Figure from Chang *et al.*, 2017.

of one positive and one neutral monomer, $\tau = 4$ a repeat of two positive followed by two neutral monomers, etc. Overall charge was the same for all species. An increase in the blockiness of charge (increase in τ) monotonically increases the critical salt concentration (CSC) at which complex coacervates dissolve. In a phase diagram the CSC determines the height of the curve (fig.). Above this salt concentration polymers can no longer phase separate.

An increase in the CSC can be explained by the thermodynamics of salt ions. The translational entropy of these ions effects coacervation of charge patterned polymers. ITC showed that the driving force for coacervation of the different τ species is entropic (Chang *et al.*, 2017). Blocky polymers will have condensed counterions densely packed at the charged patches. Release of counterions will therefore give a greater increase of translational entropy, making the coacervate phase more favorable. This increases the CSC. Thus, charge-dense regions in polyelectrolytes promote complex coacervation.

Theoretical description of complex coacervates

An ideal analytical theory on complex coacervation should be able to capture the effects of all these parameters. One of the most popular is the Voorn-Overbeek model. This theory attempts to predict phase diagrams through a mean-field approach (Sing *et al.*, 2017). A mean field replaces all the interactions on one specie by a single averaged effect. Field theoretic approaches, including RPA, improve on Voorn-Overbeek by taking into account fluctuations in the field, e.g. configurations and conformations (Sing et al., 2017). Field theory also brings problems. These approaches do not converge well for

highly-charge dense polymers (Sing et al., 2017). This is a problem for the interchanging neutral and charge dense regions in block copolymers. Also it has not been possible so far to apply such methods to large-scale heterogeneous systems.

Still other theories of complexation are based on Manning condensation. This approach invokes counterion release theory, which is very adept at describing the gain of translational entropy upon complexation of single polyelectrolytes (Sing *et al.*, 2017). Its downside is however applicability to the larger complex coacervates that do not have one-to-one chain correspondence. Although field-theoretic approaches are most promising, especially in polymer chemistry, it seems there is not yet an encompassing theory for complex coacervation. For additional information on complex coacervation theories the reader is referred to Sing et al., 2017.

The theories described here have some general limitations as well. The assumptions that are made in them only allow representation of highly idealized complexes. For example, all species are assumed to be composed of charged spheres in a dielectric medium. The type of coarse-graining used by these theories is called a restricted primitive model (RPM) (Sing *et al.*, 2017). In such models, atomistic interactions are neglected, and complexation is based only on electrostatic interactions. Thus, the theories are fit for highly idealized systems. For polymer chemistry such theories might suffice (although boundary cases also encounter problems here; Sing *et al.*, 2017), for biological complexes these theories lack detail.

Can simulations further an understanding of biological phase separation?

Models often used for simulating complex coacervates are RPMs. Additionally, theories in this field implement this type of coarse-graining almost exclusively. Such models simplify the multivalent polymers to a string of charged beads, surrounded by single charged beads from salts. This type of coarse-graining allows for much larger simulations, which are often needed for complex coacervation.

Indeed, RPMs have proven useful in the simulation of charge density. The experimental study of block copolymers of Chang and colleagues was also supported by RPM simulation (Chang *et al.*, 2017). The charge patterned polymers were computationally modelled as interchanging neutral and charged beads. Simulation results were used to calculate phase diagrams. The results matched to experiments showing extension of the coexistence region for larger charge blocks. In a similar fashion RPM can predict that polymers with more charge spacing (i.e. less charge density) have a smaller coexistence region (Radhakrishna *et al.*, 2017). Additionally, such simulation also show counterion release as a driving force for complexation (Chang *et al.*, 2017; Radhakrishna *et al.*, 2017). At higher salt concentrations it can also be shown that the structure becomes more diffuse (Lytle *et al.*, 2018). RPM can thus be used to simulate general properties of complex coacervates.

Moreover, RPMs indicate that the dense intracellular environment might promote droplet assembly (Hyman & Brangwynne, 2012). The high concentration of protein in the cytoplasm causes molecular crowding. RPM simulations of solutions containing neutral polymers, on top of polycations and polyanions, are used to simulate molecular crowding (Lytle *et al.*, 2018). Neutral polymers separate to the supernatant phase in such simulations. The coacervate is more stable with higher concentration of neutral polymers. It increases the CSC at which structures dissolve. Stabilization could be enforced through counterpressure in the supernatant phase by the neutral polymers. The dense protein environment inside cells might in a similar way promote phase separation.

Clearly such models can be extremely useful. Still, they are limited due to some coarse assumptions. RPM is limited to considering electrostatic interactions as the only driving force for complex coacervation. Simulating the additional interactions that are potentially important in intracellular phase separation requires other approaches. Hydrogen bonding in protein-protein interactions requires atomistic models (Marrink & Tieleman, 2013). Atomistic models make use of molecular dynamics simulations, which predict particle movement, or Monte Carlo simulations, which predict states based on Boltzmann probabilities.

To consider some examples Pak and colleagues extended their study of NICD with MC atomistic simulations (Pak *et al.*, 2016). They used ABSINTH (Vitalis *et al.*, 2008) to model interactions of NICD. ABSINTH is an implicit solvent force field (dielectric constant 78.2) specifically designed for modelling conformations of IDPs (Vitalis *et al.*, 2008). Atomistic simulations of NICD confirmed their experimental in vitro assembly conditions. NICD molecules without multivalent counterions repulsed each other due to their negative charge. This was quantified through radial distance between NICD peptides. These NICD-only simulations showed the polypeptides to be at maximum distance from each other. On the

other hand, simulations together with polylysine or polyarginine showed association of NICD with the polycation and a shorter distance between NICD molecules. The simulations support that this IDP can form a complex coacervate only in the presence of positively charged partners. However, their simulation analysis is lacking consideration of the interactions of the protein's hydrophobic and aromatic residues.

Another study with less focus on biological proteins, did look at hydrogen bonding interactions. Hoffmann and colleagues used atomistic MD to reveal β sheet formation of polypeptides (Hoffmann *et al.*, 2015). Poly(lysine) and poly(glutamate) will form these structures through interchanging layers of the positively and negatively charged chains. The sheets are stabilized by hydrogen bonds of the backbones. This tight formations will likely result in precipitation, because chains cannot be exchanged with the environment. Replacement of the poly(glutamate) with a racemic poly(glutamate) (monomers of both D and L chirality) destabilizes β sheet formation and could result in a coacervate phase. Racemic poly(glutamate) is more globular and coil rich allowing better access of ions and water. Although they do not contain monomers of different chirality, such globular structures are also likely for IDPs. I hypothesize that the low complexity domains of IDPs could form such structures as well—contributing to the dynamic properties of membraneless organelles.

These studies show that atomistic simulations can reveal important details of complex coacervation. The downside of atomistic models is their size and time scale. Atomistic simulations of NICD only contained two peptides of ~200 residues (Pak *et al.*, 2016). Biologically relevant proteins are often of such sizes and atomistic simulations might therefore be too small. To broaden the understanding of biological coacervation a simulation method is required that can deal with size scales and simultaneously include interactions of multiple amino acids.

Coarse-grained MD simulations could in these respects be a good option. Typical MD simulations early 2018 could include up to 150000 atoms (approximately 10x10x10 nm^3) and could be simulated on the order of microseconds (Marrink *et al.*, 2019). The use of coarse-grained MD could be 2-3 orders of magnitudes more for timescale and size (for example ~ 50x50x50 nm^3; Marrink et al., 2019). Such dimensions still do not come close to the actual sizes of membraneless organelles, but could suffice for simulating physical interactions. Even for dynamic exchange, which is on the order of seconds, coarse-grained simulation can suffice if it can show exchange of electrostatic crosslinks. Moreover, the use of multiple bead types in coarse-grained MD force fields, such as Martini (Marrink & Tieleman, 2013) extends the range of interactions compared to RPMs. With these bead types the properties of most amino acids can be approximated. This can be used to understand the contribution of aromatic and hydrophobic residues in biological phase separation. Additionally, water beads in these force field can importantly show inclusion of water in the coacervate phase—an important property for its liquid nature.

Though application of this method can be promising, investigation of IDP simulation is required first. The flexible nature of IDPs makes it very difficult to experimentally obtain a molecular structure. Therefore, the usual approach of coarse-grained MD where an atomistic structure is converted to a coarse-grained structure, cannot be applied here. Accurately sampling of conformations of these proteins might require adjustment in the parameterization of force fields. Conformations of IDPs generated by an atomistic force field like ABSINTH (Vitalis *et al.*, 2008) could be used to supply the required information.

In summary, simulation of membraneless organelles is a trade-off between a detailed description of interactions and sufficient size scales. Ideally atomistic simulations would be used to gain an exact understanding of interactions, but their size and time scales are insufficient. RPM-based simulations can handle larger systems, but lack a description of the diverse interactions required for proteins. Coarse-grained MD might therefore fill the gap that is left between these two approaches. However, application of this approach is still an open field and will require some initial efforts to review its usefulness.

Conclusion

The presence of membraneless organelles is ubiquitous in eukaryotes. All these organelles behave as liquids, though they are more viscous than water. Nuage bodies exhibit all the liquid properties of coacervates but phase separate only through interaction of Ddx4, thus showing simple coacervation. NICD bodies on the other hand seem a true example of complex coacervates in cells: here NICD requires positively charged partners to phase separate. Both studies on Ddx4 and NICD emphasize a role for IDPs. These specific IDPs contain charged blocks, which are known from chemistry to promote coacervation. However, it is not only the charged residues in these proteins that are important for phase separation: aromatic and hydrophobic residues are also implicated. The physical effects of these residues on coacervation are not yet understood.

In chemistry interactions between such oppositely charged polymers are described by complex coacervation theory. Multiple parameters dictate the phase diagrams that describe the conditions for phase separation. Chemical theories model these phase diagrams but no generally applicable theory exists yet. Recent chemical study found that blocks of charges on polymers promote complex coacervation. This seems especially interesting in biology, where charge patterned IDPs seem involved in assembly of membraneless organelles. These PRM-based theories cannot incorporate other interactions besides electrostatics. Extension of chemical theories is required to accommodate the diverse forces that amino acids carry.

Thus, molecular simulation approaches are currently better suited to incorporate these interactions. Atomistic models have demonstrated their significance, but the time and size scales are too small to understand dynamic exchange. In this respect coarse-grained MD might be better fit. It can potentially simulate IDPs, while maintaining sufficient size and time scales. It will be exciting to see if this method can elucidate these forces. If so, it will lead to an understanding of a whole new type of compartmentalization in living systems.

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