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Seeing Biomolecular Condensates Through the Lens of Viruses

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Abstract

Phase separation of viral biopolymers is a key factor in the formation of cytoplasmic viral inclusions, known as sites of virus replication and assembly. This review describes the mechanisms and factors that affect phase separation in viral replication and identifies potential areas for future research. Drawing inspiration from studies on cellular RNA-rich condensates, we compare the hierarchical coassembly of ribosomal RNAs and proteins in the nucleolus to the coordinated coassembly of viral RNAs and proteins taking place within viral factories in viruses containing segmented RNA genomes. We highlight the common characteristics of biomolecular condensates in viral replication and how this new understanding is reshaping our views of virus assembly mechanisms. Such studies have the potential to uncover unexplored antiviral strategies targeting these phase-separated states.

INTRODUCTION

Virus assembly is a highly cooperative affair that presents many underexplored drug targets to impede viral infection. A key gap in knowledge is the inadequate comprehension of the hierarchy of molecular interactions and allosteric regulation that orchestrate virus assembly (1–3). Defining virus assembly rules in cells is challenging, as viruses require significant remodeling of the cellular environment, leading to the formation of inclusion bodies, commonly known as viral factories or viroplasms (4–7). These inclusions have been a useful diagnostic marker for human and animal diseases such as rabies (8), influenza (9), and smallpox (10), as well as in plant viral infections (11). Numerous electron microscopy studies indicate that these structures represent virus assembly sites, and breakthroughs in experimentation are beginning to shed light on the mechanisms of their formation and how they interact with cellular processes.

Since the initial discovery that membraneless organelles can arise from the segregation of macromolecules through a mechanism called phase separation (12), numerous comprehensive reviews have been published on the topic of phase separation in biology (13–19). While originally thought to be a purely physical phenomenon, it is now understood that biomolecular condensates serve crucial functions in the biochemistry of cells. Unsurprisingly, phase separation also functions in viral replication, with multiple examples highlighting its involvement in infections by diverse viruses (20–22). The significance of intracellular compartmentalization of viral replication was established long before the phase separation–driven mechanism of formation of viral factories had been proposed (8, 23–29). Many RNA viruses hijack or modulate cellular membraneless organelles, e.g., stress granules (30), to boost their replication. These observations make viral factories an attractive system for studying biomolecular condensates and their functional relevance.

Despite biomolecular phase separation's conspicuous involvement in viral replication, the mechanisms and factors that influence it, and its role in the virus assembly process, are underexplored. In this review, we delve into the current understanding of the role of biomolecular phase separation in viral replication to identify potential areas for future research. We focus on the replication strategies of viruses with segmented RNA genomes, with an emphasis on how the concept of phase separation is reshaping our understanding of viral factories and assembly of RNA viruses. We also describe open questions in this area of research, including challenges and opportunities for antiviral therapies that target biomolecular condensates.

MEMBRANELESS ORGANELLES AND PHASE SEPARATION

The phase separation paradigm proposes that membraneless organelles arise from thermodynamically driven demixing of biopolymers that form microscopically detectable clusters of interacting biopolymers. In a system of interacting molecules, three types of competing interactions arise: solvent-macromolecule, macromolecule-macromolecule, and solvent-solvent. Under good-solvent conditions, macromolecule-solvent interactions are stronger than macromolecule-macromolecule interactions, and the solution remains homogeneously mixed. However, under poor-solvent conditions, macromolecule-macromolecule interactions are stronger than macromolecule-solvent interactions, leading the system to phase separate (14, 19). Therefore, biomolecules will remain miscible in solution until their solubility limit is reached. At this concentration (referred to as the concentration of saturation or c_{sat}), the mixture partitions into a highly concentrated, macromolecule-rich phase and a bulk phase of lower concentration (**Figure 1**). Thus, for a single component fluid, phase separation can be described as a simple density transition. Often, biomolecular condensates initially behave as liquids and undergo maturation, resulting in the loss of fluidity, which is discussed below in the context of viral condensates. Moreover, most

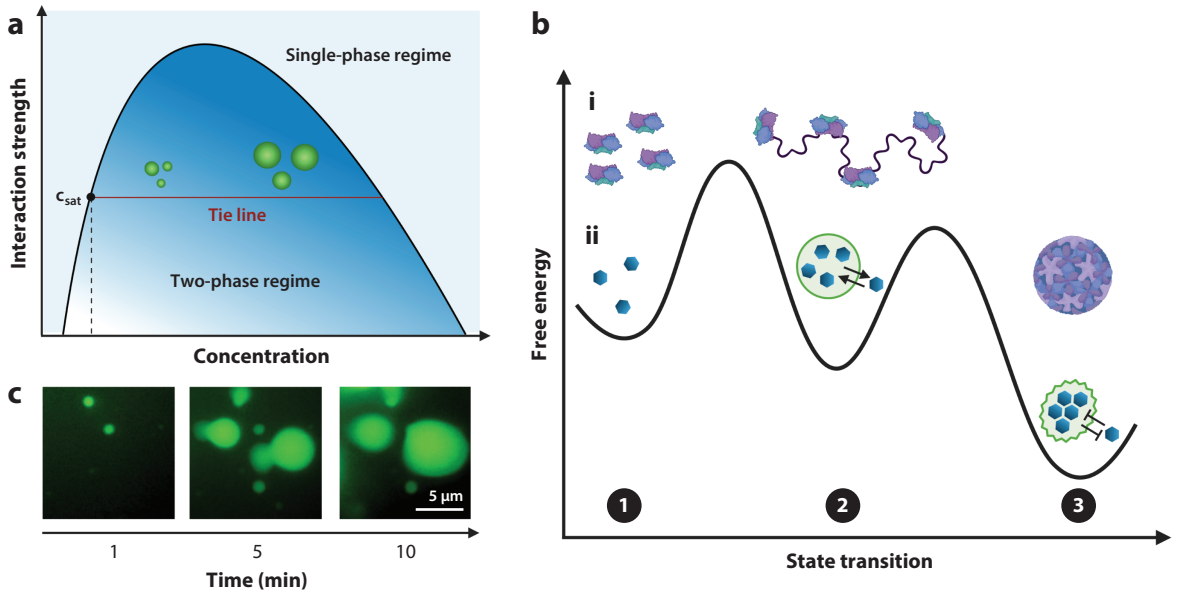

Figure 1

(*a*) A 2D phase diagram of a system containing a single type of biomolecules. The solution remains homogeneous (a single-phase regime) until the saturation concentration, denoted as c_{sat}, is reached. Above this concentration, the system enters a two-phase regime, where it undergoes phase separation into a dense macromolecule-rich phase and a dilute phase depleted of biopolymers. Note that the concentration of macromolecules in both phases remains constant, while the volume of the dense phase increases along the tie line (an example shown as a *red line*). This leads to an increase in droplet size (represented as *green spheres*). (*b*) Parallels between RNA virus assembly pathways and nucleation of biomolecular condensates. The free energy landscape for virus assembly (*i*) and condensate formation (*ii*) is shown, along with the states labeled 1–3. Viral capsid subunits cooperatively assemble around the nucleic acid (*i*), while proteins undergoing phase separation (*ii*) form biomolecular condensates (state 2—dynamic virus assembly intermediates versus dynamic, liquid-like condensates that exchange biopolymers with the dilute phase). These processes lead to the formation of a complete virion (shown in *purple*), with a defined coat protein stoichiometry, or, in the case of condensates, further maturation into less dynamic gel-/glass-like structures with significantly reduced exchange between two phases. (*c*) Two-component phase-separated protein droplets (rotavirus nonstructural proteins NSP5 and NSP2) that dynamically fuse and grow in vitro. The dynamic behaviors, such as fusion, growth, and reversible deformation, are typical features of liquid-like droplets that distinguish these condensates from aggregates. Figure adapted from images created with BioRender.com.

biomolecular condensates represent complex viscoelastic network fluids that result from phase separation coupled to percolation (networking) or gelation (19).

Membraneless organelles vary greatly in their molecular composition, containing variable numbers of interacting partners with poorly defined stoichiometries that may fluctuate. Nucleoli, stress granules, processing bodies, paraspeckles, and Cajal bodies (17, 19, 31) are examples of cellular membraneless organelles. The dynamic nature of biomolecular condensates may provide the cell with a means to rapidly fine-tune reaction kinetics and increase the specificity of biochemical reactions (31–35) in response to temperature and concentration (both biopolymers and small molecules) changes or post-translational modifications (PTMs). Thus, since their initial discovery in cells, biomolecular condensates have been commonly perceived as key mediators of cellular functions, being involved in cell division, gene expression, and stress responses (13).

ARE MEMBRANELESS ORGANELLES ALWAYS LIQUID-LIKE?

Unlike protein aggregates, biomolecular condensates often display liquid-like properties, such as surface tension that gives the droplet a spherical shape, coalescence, and interfacial tension that can

affect the stability and shape of the droplet causing its reversible deformation (13, 14). The growth of condensates may occur as a consequence of fusion events between droplets (**Figure 1**) or due to the phenomenon known as Ostwald ripening. This process is driven by a difference in the chemical potential or concentration of biomolecules and leads to the formation of a dominant condensate that continuously grows by absorbing smaller condensates (36, 37). Such properties of liquid-like condensate allow for rapid exchange of macromolecules with the surrounding environment and enable molecules within condensates to establish transient interactions. Microscopically, not all condensates exhibit a typical spherical droplet-like appearance, and some membraneless organelles engage other membrane-bound organelles, as in the case with TIS11B that forms a network of condensates intertwined with the rough endoplasmic reticulum (ER), known as TIGER compartments (38). Furthermore, phase-separated systems can mature into less fluid structures over time, such as labile fibrils, hydrogels, amyloids, or nonfibrillar aggregates. The observed changes in fluidity often correlate with the molecular composition of membraneless organelles and the PTMs of condensate-forming proteins (15, 18, 39–41). Such material property changes, including loss of fluidity, are commonly seen in condensates. A notable example of the involvement of such regions in phase separation is the nucleation and growth of amyloid fibrils through the formation of ß-strand interactions, which result in the maturation of condensates and loss of their fluidity (13, 42).

CELLULAR TRIGGERS OF BIOMOLECULAR PHASE SEPARATION

Fluctuations in the cellular environment that lead to phase separation include changes in protein concentrations and PTMs (17, 31). Understanding the concept of saturation concentration and determination of c_{sat} (see **Figure 1**) is important for predicting the behavior of a particular experimental system. For instance, attempts to isolate liquid-like viral factories from rotavirus-infected cells have been unsuccessful (43), likely because such condensates can form only at low to high micromolar protein concentrations achieved in intact virus-infected cells (27). Following cell disruption and fractionation, the NSP5 and NSP2 protein concentrations are likely to be below the c_{sat} required for droplets to form.

Furthermore, within the concentrated phase, macromolecules interact in a transient manner, and the strength of these interactions is often modulated by PTMs (44, 45), including serine/threonine phosphorylation (25, 46–49), lysine acetylation (50), arginine methylation (51), and likely others. While PTMs are important inhibitors or enhancers of phase separation, it is less clear whether all known modifications are required for its nucleation or whether they are auxiliary in this process and may occur after condensate formation. Some modifications, particularly those affecting the charge of a condensate-forming protein, are generally more important for phase separation. Therefore, even single charged amino acid substitutions can affect condensate nucleation and morphology (52, 53).

WHAT ARE THE ATTRIBUTES OF CONDENSATE-FORMING BIOMOLECULES?

Although most (if not all) polypeptides can phase separate in vitro, only certain proteins can form condensates under physiological conditions. Some of the attributes of these biopolymers include disordered regions, low complexity regions (LCRs), and multi-domain and multimeric organization. Disordered regions play a key role, as their conformational heterogeneity allows them to sample and engage in diverse, dynamic interactions that sustain the condensate network. LCRs promote the formation of both inter- and intramolecular interactions using repetitive sequences of a limited range of amino acids, while the dynamic nature of these interactions confers

fluidity to the condensate network. LCRs are often solvent exposed and may present residues that can be recognized by cellular kinases and phosphatases. Such regions are rich in polar residues such as asparagine, glutamine, and serine, as well as aromatic residues such as phenylalanine and tyrosine (54, 55). These residues are involved in the formation of short-range dipolar, π-π, and π-cation interactions. Such interactions are perturbed in the presence of aliphatic diols, including 1,6-hexanediol (1.6-HD) (56) or propylene glycol (27), which can both alter the dielectric constant of the solvent and diminish the contribution of the hydrophobic effect (57). Similarly, some LCRs contain charged motifs composed of arginine/lysine or aspartate/glutamate residues that participate in long-range electrostatic interactions, both with themselves (homotypic interactions) and with other charged molecules, including RNAs (heterotypic interactions) (13). Unlike dipole-dipole and hydrophobic interactions, condensates formed primarily by electrostatic interactions are not sensitive to the aliphatic diols (27). Most biomolecular condensates employ various types of interactions, and their relative contribution to the stability of a condensate may change over time. For example, phosphorylation of a scaffold protein can both augment and weaken electrostatic interactions.

Multivalency is another important feature of condensate-forming molecules, as it offsets the entropic cost of phase separation by increasing the interaction energy within the condensate. Moreover, multi-domain organization may contribute to the stereo-specificity of biomolecular interactions within the condensate network. Thus, mutations disrupting oligomerization of condensate-forming proteins often result in phase separation abrogation or significantly affect the c_{sat}.

Not all proteins that are contained within a condensate can phase separate on their own under physiological conditions. Proteins that primarily constitute membraneless organelles and drive their formation are referred to as scaffolds, whereas other macromolecules that are not essential for but may be recruited into a condensate are referred to as clients (17). Such clients can transiently bind to scaffolds and can then be displaced through the modulation of their affinities toward the scaffold.

THE ROLE OF RNA IN THE FORMATION OF MEMBRANELESS ORGANELLES

Prior to RNA encapsidation, viral factories accumulate a vast number of transcripts destined for packaging (58). This aspect of some viral factories, including those formed during rotavirus infection, shares several common features with other ribonucleoprotein (RNP) granules that contain multiple RNAs. The nucleolus is the largest RNP condensate and is responsible for ribosome assembly (59). The central scaffolding component of the nucleolus incorporates multiple copies of the pre-ribosomal RNA molecules. Upon assembly, these structures recruit more than 400 distinct proteins and RNAs, including assembly chaperones and enzymes, creating a unique environment for ribosomal assembly (60).

Other examples of cytoplasmic RNPs include stress granules and P granules that are formed via condensation of nontranslating RNAs and RNA-binding proteins (RBPs). Accurate mimics of stress granules were reconstituted in vitro by adding small amounts of the scaffold protein G3BP to cell lysates, confirming phase separation as the mechanism of their formation (61, 62). Such in vitro reconstitution studies are useful to define the organizing principles of the RNP condensates, including viral condensates.

The formation of RNP granules is influenced by the length, sequence, and structure of RNA and also its concentration and the ratio of RNA to protein (34). At low RNA concentrations, RNA promotes phase separation of multivalent RBPs through electrostatic and cation-π interactions.

Mechanistically, long RNAs can significantly decrease the c_{sat} of multimeric RBPs that bind to them, promoting RNP granule assembly. Like LCRs, long and branched RNAs with large unstructured regions (63) have a higher propensity to form intermolecular interaction networks that can act as condensate scaffolds (16, 64, 65). However, at higher concentrations, RNA may cause dissolution of membraneless organelles, described as a re-entrant phase behavior (66).

THE INNER STRUCTURE OF RIBONUCLEOPROTEIN GRANULES

Originally perceived as lacking internal structure, RNA-rich condensates often contain subcompartments of more viscous core regions surrounded by dynamic protein-rich domains (67, 68). Given the gradient of densities at the interface between two or more phases, it is possible that distinct RNA and protein species interact differently with the scaffold and its clients. Portz and Shorter (69) suggested that the 3D organization of the RNA scaffold aligns RBPs in such a way that they come into close proximity with certain binding partners while preventing interaction with others. Thus, the sub-compartmentalization of RNA-rich condensates may provide an additional mechanism for controlling biochemical reactions by the composition and structure of multiphasic biomolecular condensates (70).

During viral infections, the link between transcription, RNA accumulation, and potentially translation (71) in viral factories suggests that the internal organization of the inclusions produced by overexpression of scaffold proteins alone may differ significantly from bona fide viral condensates, which contain multiple viral RNAs and additional proteins. However, these systems can provide valuable insights into the phase behavior of viral condensate-forming proteins that drive the assembly of viral factories.

EARLY REPLICATION ROTAVIRUS VIRAL FACTORIES ARE RIBONUCLEOPROTEIN GRANULES

Studies of rotavirus replication factories (72), also known as viroplasms, suggest that double-stranded (ds) RNA viruses with segmented RNA genomes provide a highly useful model for investigation of cytoplasmic RNP condensates (27, 43). Each rotavirion produces multiple copies of 11 distinct transcripts that can be either translated or encapsidated prior to their replication (dsRNA synthesis) in viroplasms (**Figure 2**). The amount of RNA released by a transcribing particle is dependent on the multiplicity of infection used, i.e., the ratio of infectious virions per cell, allowing the protein:RNA ratio to be controlled throughout an experiment. As early as 2–4 hpi, the first liquid-like viroplasms begin to emerge, containing all 11 types of viral transcripts, supporting the idea that these early infection stage cytoplasmic inclusions represent RNP granules (27, 58). These spherical granules exhibit liquid-like properties, being able to fuse and relax into a sphere and being capable of rapid (within 30 s) dissociation and reassembly (within 10–15 min) following addition and removal of low concentrations of aliphatic diols, including 1,6-hexanediol or (less toxic to cells) propylene glycol (27). Dissolution of viroplasms results in the release and redistribution of viral transcripts in the cytoplasm of infected cells, demonstrating that early infection stage viroplasms share many similarities with other cytoplasmic RNP granules, such as stress granules.

Viroplasm-like inclusions are formed following coexpression of the negatively charged nonstructural phosphoprotein NSP5 (scaffold) and the positively charged RNA chaperone NSP2, the inner capsid viral protein VP2, or both NSP2 and VP2 (73–77). NSP5:NSP2 droplets also form in an in vitro reconstitution system at physiologically relevant low μM concentrations matching those achieved during infection (27, 78). Previous views of viroplasm formation have been dominated by the idea that viral proteins (VPs) are recruited into these structures in a specific order (73, 75–79), yielding a particular multilayered organization (80). While the exact organization of

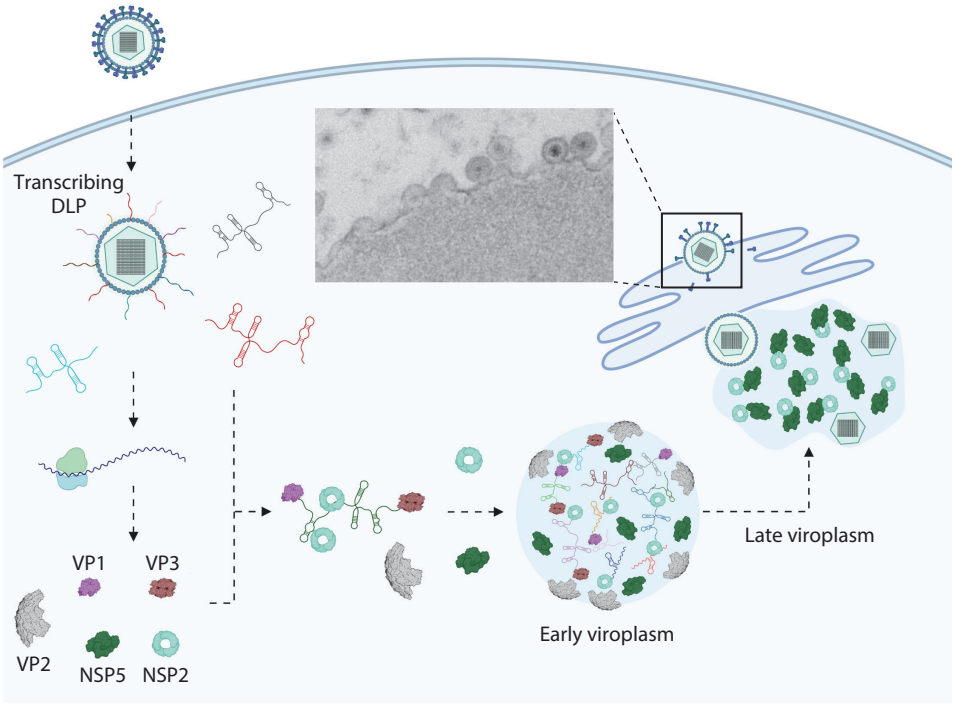

Figure 2

Rotavirus replication cycle. Rotavirus forms triple-layered particles containing 11 distinct double-stranded RNA molecules. Following entry into a cell, the outer protein layer is lost, rendering the remaining double-layered particle (DLP) transcriptionally active. Transcribing DLPs extrude 11 distinct types of transcripts that are initially translated to synthesize nonstructural proteins (NSPs) and structural viral proteins (VPs). The nonstructural protein NSP5 acts as scaffold for the formation of viroplasms and undergoes phase separation upon mixing with the viral RNA chaperone NSP2 (*doughnut-shaped cyan octamers*) at low micromolar concentrations. Such droplets absorb viral transcripts, RNA-dependent RNA polymerase VP1, the inner coat protein VP2, and the capping enzyme VP3. Within the first 6 hpi, such ribonucleoprotein droplets undergo further changes that result in the loss of fluidity, hyperphosphorylation of NSP5 and potentially the acquisition of additional VPs required for the assembly of viral cores containing the RNA, VP1, and VP3 encapsidated by VP2. Upon addition of the second layer of protein termed VP6 (not shown here), a nascent DLP is formed (late viroplasms). Such DLPs acquire a transient membrane envelope, which is lost during the acquisition of the third layer of VP containing VP4 and VP7 within the endoplasmic reticulum (schematically shown). The inset shows an electron micrograph of a negatively stained thin section of a rotavirus-infected cell at 8 hpi. Note the DLPs emerging from the surface of a viroplasm. Figure adapted from images created with BioRender.com.

viroplasms is likely to be more complex than the concentric layers identified by super-resolution microscopy (80), multilayered organization is commonly seen in RNP condensates (18, 81). It is also possible that viroplasmic organization changes over time depending on the stage of infection, NSP5 and NSP2 phosphorylation status (75, 76), and the RNA:protein ratio in the condensate. All of these factors may contribute to the maintenance of the fluidity of viroplasms, as only early infection viroplasms (2–6 hpi) remain spherical and sensitive to 1,6-hexanediol, while larger and less regularly shaped late infection viroplasms are insensitive to such treatments (**Figure 3**). While these results suggest that over time viroplasms undergo maturation, likely stemming from changes in the viroplasm interaction network, it is hard to reveal the exact nature of this phenomenon,

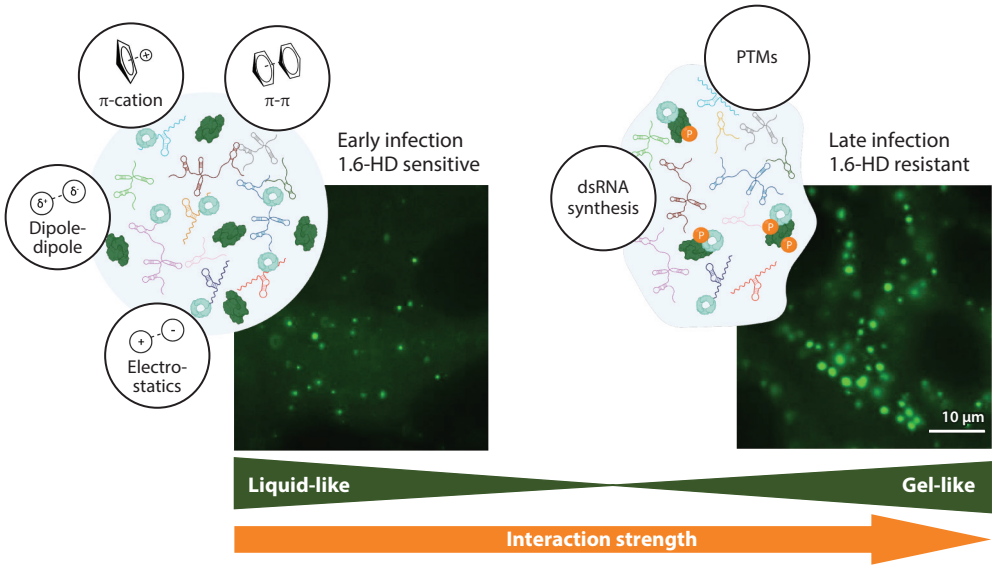

Figure 3

Maturation of viroplasms. Weak multivalent interactions (shown on the *left*) drive the phase separation of nonstructural proteins NSP5 and NSP2 as well as partitioning of RNAs and viroplasmic proteins into the early infection stage (2–6 hpi) liquid-like viroplasms that are sensitive to aliphatic diols [1,6-hexanediol (1.6-HD) and propylene glycol]. These inclusions increase in size and become less round over time, concomitant with NSP5 hyperphosphorylation, double-stranded (ds) RNA synthesis, and the production of nascent double-layered particles. Such mature viroplasms are resistant to aliphatic diol treatments, suggesting that the interaction network of these late infection stage condensates has changed. The insets show widefield images of early (*left*) (see **Supplemental Video 1**) and late (*right*) (see **Supplemental Video 2**) infection stage viroplasms tagged with NSP5-EGFP. Figure adapted from images created with BioRender.com.

Supplemental Material >

and how it is linked to the internal reorganization of the viroplasmic condensate. Such changes could be due to the strengthening of bonds in the condensate network resulting from the increased affinity between the negatively charged hyperphosphorylated NSP5 and positively charged NSP2, and/or decreased affinity between NSP5/NSP2 and RNA. The conversion of the single-stranded RNA into the dsRNA form in viroplasms during RNA encapsidation also could be responsible for the decreased fluidity, as dsRNA leads to a larger fraction of dynamically arrested RNP condensates (82). Single-molecule fluorescence in situ hybridization (smFISH) imaging studies of viroplasms suggest that the earliest detectable viroplasms contain very little, if any, viral RNA, but RNA content in viroplasms rapidly increases by 4–6 hpi (58). Similarly, NSP5 phosphorylation impairment results in the lack of detectable viral RNA accumulation in viroplasms (53). It is likely that multiple factors contribute to the changes in material properties, and it will be interesting to understand how these material property changes are related to fluctuations in protein and RNA composition as well as PTMs.

WHAT DETERMINES THE SIZE OF VIRAL FACTORIES IN CELLS?

Intuitively, liquid condensates should eventually fuse into a single large condensate, as is often observed in vitro. Indeed, in vitro reconstituted NSP5:poly-arginine droplets will fuse over time (27), as expected from basic thermodynamics principles. Given that viroplasms are NSP5:NSP2 condensates, what determines their finite (up to several microns) size in cells? Simulations suggest that the form of phase separation that produces multiple droplets, known as emulsification, can occur when the timescale of molecular diffusion is orders of magnitude faster than the timescale

for condensate bond formation (37). Moreover, freely diffusing molecules may be incapable of joining the condensate because a fraction of binding sites is unavailable due to dynamic PTMs or newly acquired protein or RNA clients, explaining why smaller viroplasms do not fuse indefinitely. Interestingly, factories formed by reovirus, another nonenveloped segmented dsRNA virus, appear to form larger structures that can fuse and form intricate networks in the cytoplasm (71, 83). It is possible that smaller viral RNP condensates than can be observed by light microscopy are formed in rotavirus-infected cells prior to the assembly of microscopically identifiable condensates. Despite their nanoscopic sizes, such RNP clusters could still meet the characteristic features of condensates (84) or form the functional nanometer-sized precursors for the assembly of viral factories, as observed by super-resolution DNA-PAINT microscopy of rotavirus-infected cells (58). Advances in imaging of viral factories should provide further answers to many questions about the RNA stoichiometry and internal organization of these early infection stage RNPs.

SEGMENTED RNA GENOME ASSEMBLY IN VIRAL CONDENSATES

Rotaviruses are not the only segmented dsRNA viruses that employ phase separation for constructing viral factories. Similar phenomena have recently been reported for bluetongue virus (85) and infectious bursal disease virus (86), and it is possible to extend to reoviruses, considering their liquid-like behavior (71, 83, 87, 88). Given the segmented nature of the genomes of *Reoviridae* family viruses (89), and the importance of multivalent RBPs (85, 90–93) in the formation of viral factories, is it possible that these granules also function in genomic RNA assortment? Tauber, Tauber, and Parker (94) proposed that RNP granules provide an environment that is highly conducive to the formation of intermolecular contacts between the RNAs. Molecular crowding, depletion attraction, and a highly polar environment of the interior of membraneless organelles (33) can contribute to the stabilization of intermolecular RNA contacts (33, 95, 96) or act as an ATP-independent helicase (33). Indeed, many condensate-forming RBPs possess RNA chaperone (27, 94, 97) or helicase (98–100) activities. RNA-RNA interaction networks increase the valency for recruiting multivalent RBPs (16, 34, 96, 101), and such condensates are often stabilized by the arginine-rich LCRs that engage in electrostatic and cation-π interactions with RNAs. The stronger interaction of arginines with double-ring purines relative to single-ring pyrimidines may explain some sequence specificity in the composition of RNP granules (102).

By selectively recruiting viral transcripts destined for assortment and packaging, such condensates would minimize spurious RNA-RNA interactions with noncognate RNAs (96). RNA imaging studies suggest that rotavirus viroplasms contain viral RNAs and exclude polyadenylated messenger RNAs (**Figure 4**). The selective partitioning of RNA to viroplasms is likely determined by conserved *cis*-RNA elements recognized by the viral RNA-dependent RNA polymerase VP1 (58), as a temperature-sensitive VP1 mutant (tsC) that fails to accumulate VP1 in viroplasms under nonpermissive temperatures (103) also shows impaired viral transcript partitioning into viroplasms (58).

RNA-PROTEIN COACERVATION AS A PARADIGM FOR VIRUS ASSEMBLY

The current paradigm of RNA virus assembly extrapolates from the observations that viral coat proteins containing arginine-rich motifs can self-assemble around RNAs (2, 104). Biophysical studies revealed that the formation of viral capsids proceeds via compaction of encapsidated RNAs, driven by either the conformational rearrangement of the RNA (3) or the charge-driven condensation of coat proteins' arginine-rich terminal regions (104–107). For simple RNA viruses, thermodynamic studies led to the understanding of phase diagrams for assembly in vitro. For

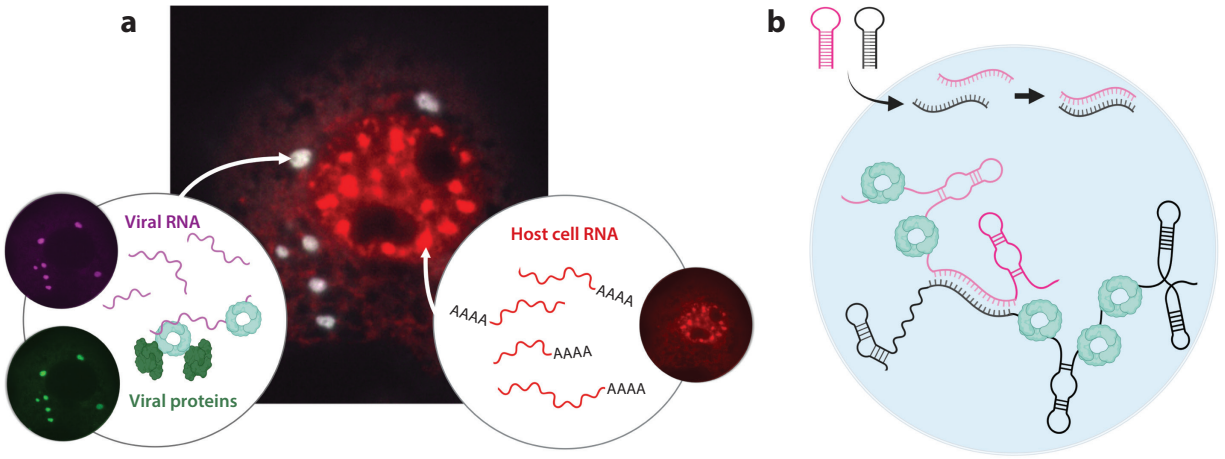

Figure 4

RNA selectivity of viroplasms. (*a*) Viral RNA (*magenta*, *left*) and nonstructural protein NSP5-tagged viroplasms (*green*, *left*) partition to viroplasms (*overlay*, *white*). Poly-adenylated cellular messenger RNAs (*red*, *right*) are excluded from viroplasms but can be detected as separate RNA-containing foci in the nucleus. Images represent single-molecule RNA fluorescence in situ hybridization of the viral RNA and poly-adenylated RNAs. Panel *a* adapted from Reference 58. (*b*) Membraneless organelles can absorb and melt structured RNAs, promoting intermolecular RNA-RNA interactions. Small RNA stem-loops are absorbed by a condensate (*green*), where they can re-anneal, forming a new RNA duplex. Larger RNAs containing complementary sequences (*black* and *magenta*) can stably base pair, in the process assisted by RNA-binding proteins (ring-like RNA chaperone NSP2 shown as *green doughnuts*). Promiscuous RNA-RNA interactions may be prevalent in granules containing a diverse set of RNAs in a high local concentration (96). Such interactions can be limited via the selective recruitment of cognate RNAs and through the action of viral RNA chaperones that promote sequence-specific interactions between the individual RNAs comprising a multisegmented RNA genome. Viral transcription may also promote the assembly of such granules due to the high local concentration of RNAs that can recruit more RNA-binding proteins. Figure adapted from images created with BioRender.com.

example, changes in the ionic strength or pH weaken the electrostatic repulsion between the amphiphilic coat proteins, allowing attractive hydrophobic interactions to overcome the energy barrier and drive assembly of cowpea chlorotic mottle virus (108) or tobacco mosaic virus (106). Based on these observations, McPherson (109) hypothesized that restructuring of the coat subunits on the fluid, micellar surface of the RNA-protein condensate leads to symmetrical icosahedral capsids, akin to processes that occur on the surfaces of growing crystals. The proposed model argues that the electrostatically modulated affinity of coat proteins for the RNA produces a nucleoprotein condensate, in which an ordering transition transforms the disordered condensate into an ordered virion (109, 110). The condensate model does not exclude the presence of high-affinity packaging signals found in genomes of many RNA viruses (2, 104), as such RNA elements would control the nucleation of condensation on the viral genome during packaging.

A plausible model is that capsid assembly is initiated on viral RNAs at protein concentrations that are initially low. The presence of a cognate RNA would lower the c_{sat} required for condensation. Consequently, additional protein subunits would be partitioned into the growing condensate, leaving little protein that could otherwise participate in the formation of noninfectious RNA-free particles. This idea is supported by multiple observations that overexpression of viral coat proteins generally yields empty particles, and no partially completed particles or their intermediates expected for sequential assembly pathways are observed by electron microscopy in cells or in vitro (109). Thus, there are many similar aspects shared by the liquid phase of a condensate and the coat subunits in the micellar intermediate formed during virus assembly, which also is driven by transient hydrophobic interactions and electrostatics, high local concentration, and fluidity (109).

Such condensates can provide the necessary kinetic control for the assembly reaction by concentrating reactants within the dense phase and lowering their chemical potential and mobility (13, 111, 112) to minimize nonspecific binding and aggregation of VPs.

VIRAL CONDENSATES AND INTERACTIONS WITH CELLULAR COMPONENTS

Another new area of research is how viral condensates interact with organelles and other cellular components to mediate viral replication and assembly functions. Some common themes emerge in the ways in which viral condensates use lipid membranes, in the form of either lipid droplets (113–116) or ER membranes (117), to maximize virus production.

Serendipitously, Donald Caspar suggested that "a plausible place to look for virus disruption in the cell is in lipid-containing structures, since such an environment could affect stabilizing interactions in a way similar to nonpolar solvents and detergents. Membranes would seem to be appealing candidates for such structures" (109, referencing 118). Membrane surfaces may facilitate the ordered addition of hydrophobic protein subunits, preventing their premature association and aggregation.

Indeed, studies of nonspecific interactions between lipids and disordered low-complexity proteins reinforce the idea that in the presence of anionic lipids, such proteins form lipid-protein clusters at a 30-fold lower protein c_{sat} relative to the protein alone. Such lipid-induced condensates are formed due to interfacial ordering of the protein and show less dynamic protein organization than canonical, lipid-free protein condensates (119). Interestingly, the rotavirus scaffold protein NSP5 contains a largely disordered LCR and a highly conserved amphipathic helix that may participate in such lipid-induced ordering. It also is possible that lipid droplets and ER membranes participate in distinct stages of viral assembly and maturation, the former by nucleating the assembly of viroplasms (115) and the latter by providing the environment for the final assembly of outer-layer proteins VP7 and VP4, which are acquired by the particle once the transient membrane envelope is lost (120).

Considering viral factories as condensates can clarify apparently contradictory evidence about the role of various cellular components in the formation of these structures. Numerous questions have been raised about mechanisms of rotavirus viroplasm formation and the role of lipids, microtubules (121), and stress granules (122, 123) in this process. The partitioning of membranes and organelles in phase systems strongly depends on their surface properties (e.g., charge and hydrophobicity). Given the dynamic nature of viral factories, with surface charges likely to change throughout their maturation and alterations in protein:RNA composition, the observed association of certain types of organelles (e.g., ribosomes) and cytoskeletal elements may be due to their differential partitioning among viral condensates. The specific and nonspecific adherence of viral condensate-forming proteins to microtubules (124) and tubulin (125) can result in orientation within the fluid phase of cytoplasm of even those phases not in contact with solid supports (126). Future studies of viral factories that employ higher temporal and spatial resolution, combined with biophysical modeling and in vitro reconstitution assays, will provide a unifying model of when, why, and how viral factories hijack cellular components.

ANTIVIRAL DRUG DISCOVERY THROUGH MODULATION OF BIOMOLECULAR CONDENSATES

There has been growing interest in targeting biomolecular condensates as a potential therapeutic strategy for neurodegenerative and viral diseases. This approach has the potential to offer several benefits over traditional antiviral therapies, including broad-range specificity, partially due to

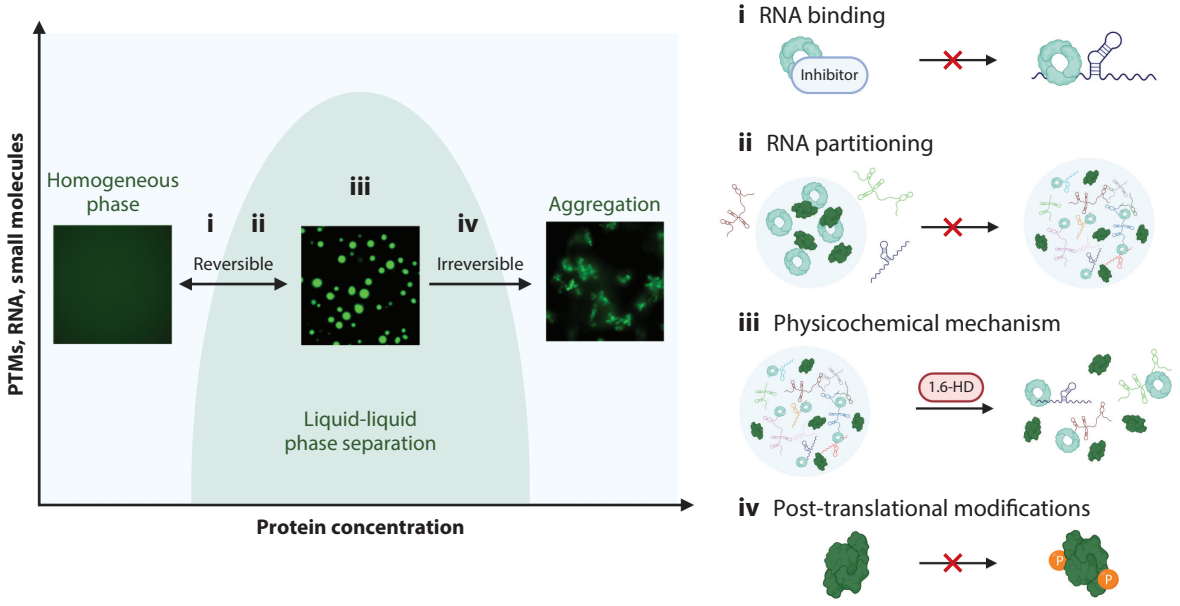

Figure 5

Targeting viral biomolecular condensates. Phase separation of viral proteins and RNAs can be modulated by altering the interaction strength [by post-translational modifications (PTMs) or additional RNAs or small molecules that can increase or decrease the affinities] and protein concentrations. A homogeneous mixture of a single phase (*left inset*) can demix reversibly into two separate phases of different concentrations but at matching chemical potentials [liquid-liquid phase separation (*green droplets*, *middle inset*)]. At higher concentrations of interacting molecules or at increasing interaction strength, a second phase transition can lead to irreversible formation of aggregates (*right inset*). Based on an understanding of phase behavior of viral condensates, several approaches can be exploited to target the formation of viral factories. These include (*i*) rationally designed inhibitors of protein-protein and protein-RNA interactions; (*ii*) targeting RNA partitioning into viral condensates; (*iii*) modulation of physicochemical environment that makes the formation of condensates less energetically favorable, e.g., in the presence of aliphatic diols; and (*iv*) targeting PTMs including, e.g., scaffold phosphorylation or dephosphorylation. Circled Ps denote phosphate groups, and 1.6-HD denotes 1,6-hexanediol. Figure adapted from images created with BioRender.com.

considerable structural similarity in the architecture of the capsid proteins between most icosahedral viruses (127). In principle, targeting weak protein-protein interactions involved in virus assembly is not a new concept. Molecules that target the self-assembly of virus capsids are collectively termed core protein allosteric modulators, with the heteroaryldihydropyrimidine compounds being a classical example of this class of compounds (128). Modulation of the strengths of interactions within condensate networks provides an effective means of arresting dynamic condensates (82), disrupting their formation by increasing the c_{sat} or disrupting the driving interactions altogether, or causing condensate aggregation or hardening (129). Molecules that produce these changes in condensates will thus be referred to as condensate modulators (**Figure 5**).

Because condensates are permeable to many small molecules, an interesting strategy is to use inhibitors of viral transcription or replication that have higher partitioning in a specific condensate. This approach was demonstrated to be viable for increasing the concentration of the antineoplastic drug cisplatin preferentially in condensates and thus influencing drug activity (130). Undoubtedly, optimizing condensate partitioning of an antiviral molecule would be valuable for the development of improved therapeutics.

Because viral inclusions may function in innate immune evasion mechanisms (131), disrupting these structures offers further advantages over traditional antiviral therapies. Importantly,

inhibition of phase separation of SARS-CoV-2 nucleoprotein was shown to boost the antiviral response and limit viral pathology (131).

Targeting biomolecular condensates also poses several challenges. A common criticism of this approach is that due to the lack of specificity in the interactions that drive the formation of membraneless organelles (132), it will be difficult to identify compounds with a reasonably high therapeutic index. However, unlike single component droplets, biomolecular condensates are far more complex systems that display sequence-, composition-, and topology-specific internal networks (19) that give rise to dynamic interactions between the individual components that will self-assemble into a nascent virion. This complexity is exemplified by several reports revealing that single point mutations of scaffold proteins can significantly affect the formation of viral condensates, impeding viral replication (53) or conferring resistance to drugs that modulate hardening of such condensates (129). Thus, even relatively weak protein and RNA binders that modulate hydrophobic and nonionic interactions may have critical implications for viral replication due to high cooperativity of viral condensate formation and capsid assembly.

Another challenge for targeting biomolecular condensates in antiviral therapeutics is the potential for off-target effects. Biomolecular condensates are involved in multiple cellular processes, and as such, therapies that target these structures may have unintended consequences. However, modulation of host membraneless organelles may reveal new therapeutic targets to interrupt viral replication because many viruses appear to require their formation for efficient replication (30). We hope that targeting cellular condensates may become a powerful approach for exposing the Achilles' heel of viral replication.

CONCLUSIONS AND FUTURE PERSPECTIVES

Viruses are not only "pieces of bad news wrapped up in protein" (Sir Peter Medawar) but also wonderful model systems for studying complex biological and biophysical phenomena. Intriguingly, many aspects of virus assembly resemble the assembly of ribosomes in the nucleolus, the most conspicuous cellular condensate. The assembly of ribosomes requires hierarchical remodeling of RNAs and coassembly of multiple proteins starting in the nucleolus and ending after nuclear export into the cytoplasm. More than 400 proteins and RNAs contribute to the formation of the nucleolar condensate. While viral factories are less complex in their composition and protein diversity, they may be more tractable systems amenable to in vitro reconstitution and biophysical characterization. Such studies will shed light on virus assembly mechanisms and also provide a scope to better understand how nascent ribosomes progress along a dynamic biogenesis pathway before they reach maturity.

Arguably, biomolecular condensates have become a topical area of study attracting many investigators from various disciplines. Phase separation is an inevitable consequence of macromolecular synthesis in cells. It is possible that biomolecular condensate formation during viral infections is an evolutionary spandrel, a feature that arises as a by-product of the evolution (133), rather than as an adaptation to a specific function. Phase separation may arise due to the physicochemical constraints on viral gene replication that attain high transcriptional and translational levels within a relatively short interval, particularly at high multiplicity of infection. Given their inherently high propensity for phase separation, overexpression of intrinsically disordered viral proteins would inevitably result in their phase separation.

Considering that biomolecular phase separation is a physical phenomenon, is it under any evolutionary selection? Could it be that during viral infection, the cytoplasmic concentrations of free VPs and nucleic acids must remain below the c_{sat} to maintain effective viral replication and assembly? Are there evolutionary constraints on the sequence and composition of viral

condensate-forming proteins, and how diverse are they between strains infecting distinct hosts? Future studies involving deep learning (55), high-throughput microfluidics (134), super-resolution and live-cell image correlation imaging (135), cryogenic–electron tomography, and advanced biophysical modeling of condensates (136) will help answer these questions.

D'Arcy Thompson in his work *On Growth and Form* wrote, "whenever phenomena and structure in living forms can be explained in terms of the balance of purely physical forces, such explanations must be considered first above all." The phase separation framework is now able to explain many phenomena previously observed in viral replication. It is high time that we delve deeper into understanding how nonliving viruses exploit such physical principles to improve our fundamental understanding of life.

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AUTHOR CONTRIBUTIONS

A.B. wrote the manuscript. J.A. prepared the figures, contributed ideas, and provided feedback on the manuscript.

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