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# Reentrant Phase Transitions and Non-Equilibrium Dynamics in Membraneless Organelles

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## Abstract

Compartmentalization of biochemical components, interactions, and reactions is critical for the function of cells. While intracellular partitioning of molecules via membranes has been extensively studied, there has been an expanding focus in recent years on the critical cellular roles and biophysical mechanisms of action of membraneless organelles (MLOs) such as the nucleolus. In this context, a substantial body of recent work has demonstrated that liquid-liquid phase separation (LLPS) plays a key role in MLO formation. However, less is known about MLO dissociation, with phosphorylation being the primary mechanism demonstrated thus far. In this perspective, we focus on another mechanism for MLO dissociation that has been described in recent work, namely a reentrant phase transition (RPT). This concept, which emerges from the polymer physics field, provides a mechanistic basis for both formation and dissolution of MLOs by monotonic tuning of RNA concentration, which is an outcome of cellular processes such as transcription. Furthermore, the RPT model also predicts the formation of dynamic substructures (vacuoles) of the kind that have been observed in cellular MLOs. We end with a discussion of future directions in terms of open questions and methods that can be used to answer them, including further exploration of RPTs *in vitro*, in cells and *in vivo* using ensemble and single-molecule methods as well as theory and computation. We anticipate that continued studies will further illuminate the important roles of reentrant phase transitions and associated non-equilibrium dynamics in the spatial patterning of the biochemistry and biology of the cell.

## Graphical Abstract

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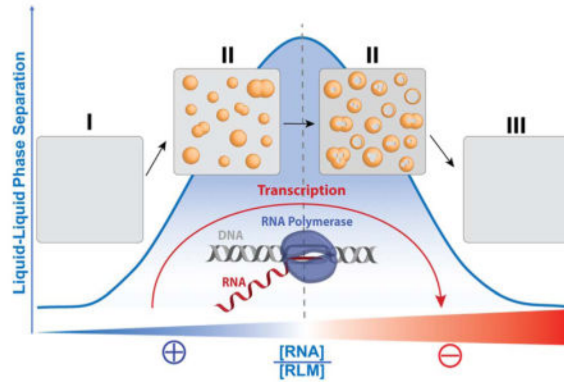


## Introduction

The partitioning of cellular components into distinct compartments plays a crucial role in regulating essential biochemical processes. One way that the cell organizes its intracellular infrastructure is by membrane-bound compartments, such as the nucleus, endoplasmic reticulum (ER), and Golgi apparatus. The lipid-bilayers of these organelles act as semi-permeable barriers that selectively control the transport and concentrations of molecules inside and outside each compartment. In contrast with such cellular structures, membraneless organelles (MLOs) such as the nucleolus, stress granules, P bodies, and Cajal bodies, are fluid/gel-like species that can also selectively partition molecules, thereby exerting substantial effects on cellular biochemistry and function [1–6]. Membraneless bodies were discovered as early as 1835 and 1836 when Rudolph Wagner [7] and Gabriel Valentin [8] respectively observed the nucleolus in neuronal cells. The term nucleolus was coined half a century later, and only recently have MLOs been shown to undergo dynamic cycles of formation and dissolution by a process known as liquid-liquid phase separation (LLPS) [6, 9–11]. These cycles of formation and dissolution of MLOs provide an efficient mechanism for responding to cellular stress or other stimuli. Furthermore, by selectively concentrating and colocalizing biomolecular components, MLOs are believed to facilitate vital cellular processes such as replication, transcription, RNA processing, and other biochemical processes. Rapid progress is being made in understanding these functions of MLOs and their underlying mechanisms.

An important mechanistic underpinning of LLPS is the involvement of weak multivalent interactions. Initial understanding emerged from early polymer chemistry and polymer physics work. In 1920, Staudinger argued that polymeric chains could associate with each other due to their “partial valences” and observed that the polymeric properties were closely related to the size of their primary valence molecules [12]. Later, Flory and Huggins separately developed free energy models of polymer mixing and demixing that laid the groundwork for polymer chemistry [13–15]. From these initial models stemmed numerous condensation polymer chemistry advances [16], which recently have been conceptually applied to a bevy of biological systems [17, 18].

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## Role of Complex Coacervation in LLPS and MLO formation

Weak, multivalent interactions between low-complexity sequences (in intrinsically disordered proteins (IDPs) and intrinsically disordered regions (IDRs) [19–23]) have recently been shown to promote phase separation and have been implicated as a mechanism behind MLO assembly [18, 22, 24]. These low-complexity sequences are enriched in positively-charged residues such as arginine and lysine [25], and their phase behavior has been shown to be controlled by RNA, which is also a commons component of MLOs [26]. Repeat sequences in heterogeneous nuclear ribonucleoproteins (hnRNPs) and SR repeat domains in the SRSF family of splicing factors [27, 28] are examples of such species. Studies have shown that such arginine-rich sequences undergo liquid-liquid phase separation (LLPS) via multivalent electrostatic interactions with RNA forming ribonucleoprotein (RNP) droplets/granules. This electrostatically driven process, known as complex coacervation [29], has been hypothesized to provide an important driving force for MLO/RNP granule formation *in vivo* [30, 31].

Complex coacervation is an electrostatically driven mechanism by which the positively-charged arginine/lysine residues of the peptide interact with the negatively-charged phosphate backbone of RNA. Although complex coacervation has been postulated as a mechanism of MLO formation, dissolution of these fluid-like droplets remains less well-understood and the impairment of droplet dissolution has been linked to disease [32]. One potential mechanism of MLO dissolution is through posttranslational protein modifications, such as phosphorylation, which has been shown to regulate RNA granule dynamics in *C. elegans* [33] and alter phase separation of the low-complexity domain of the ALS-linked protein FUS [34]. An *in vitro* model system, composed of a cationic (arginine-rich) peptide and poly-U RNA, further demonstrated that phosphorylation and dephosphorylation can regulate the charge on the peptide, leading to droplet formation and dissolution [29]. Although an attractive mechanism, rapid control of RNP droplets by phosphorylation is complex due to the sequence specificity of kinases and phosphates, as well as the variability of phosphorylation sites in proteins. Due to this, a key question is whether other regulatory mechanisms are operational and necessary at the cellular level.

## Reentrant Phase Transitions - a novel mechanism for MLO dissolution and substructure formation

### Reentrant phase transitions and MLO dissolution

Recently, we proposed an additional regulatory mechanism of RNP droplet dissolution [35]. In this mechanism, low concentrations of RNA aid in the formation of droplets, while higher RNA concentrations drive dissolution of these droplets via a charge inversion mechanism. The mechanism was proposed based on previous observations of DNA condensation upon interaction with positively-charged multivalent ions [36]. This process is driven by short-range electrostatic attraction due to cation binding and leads to charge neutrality. Upon excess counterion binding though, decondensation of this DNA occurs, which is caused by long-range Coulombic repulsion from a charge inversion. This process, known as reentrant condensation, has been observed for DNA and other polyelectrolytes [37, 38]. Although this

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mechanism has been proposed for other systems, it only recently has been theoretically or experimentally examined in RNA or IDP systems. We directly tested this mechanism and observed charge inversion by measuring electrophoretic mobility using dynamic light scattering (DLS), which uses the phase analysis light scattering technique, known as M3-PALS. Figure 1a depicts the molecular level species involved, showing initial charge neutralization followed by charge inversion as poly-U RNA is titrated into the solution of arginine-rich peptide. Figure 1b schematically shows the corresponding phase transitions from Phase I (light, excess peptide) to Phase II (droplets) to Phase III (light, excess RNA). Our results experimentally confirmed that arginine-rich peptide/protein systems in complex with RNA can display reentrant phase transition (RPT) behavior and undergo a charge inversion, and therefore provides a novel mechanism for dissolution of RNP droplets.

It is noteworthy that multiple reports of RNP (or IDP) phase separation have previously included observations that that increasing concentration of RNA (or other interaction partner) leads to a similar rollover behavior in the phase diagram [18, 26, 39–41]. Our mechanistic work now provides a theoretical framework for understanding these types of *in vivo* and *in vitro* results, while keeping in mind that additional effects must further modulate the overall phase behavior in more complex systems in the cell [23, 42, 43]. Additionally, the RPT concept also points to biochemical processes such as transcription as a cellular mechanism for dynamic modulation of droplet organelles.

## Substructure Formation and non-equilibrium dynamics

LLPS provides a mechanism by which cells can organize themselves into distinct sub-compartments [44]. Recent *in vivo* and *in vitro* studies have shown that the nucleolus and RNP granules undergo phase separation and further partition themselves into distinct subcompartments [45]. Over 50 years ago, nucleolar vacuoles displaying distinct subcompartments were discovered in tobacco callus cells using electron microscopy [46]. These observed nucleolar vacuoles formed and contracted in repetitive cycles. Furthermore, the presence of actinomycin D, which inhibits RNA production, impeded vacuole formation. These results led to the postulation that nucleolar vacuoles were related to RNA synthesis in the nucleolus. One potential mechanism for the formation of vacuolar sub-compartments is through a reentrant phase transition. Based on the actinomycin D results hypothesizing that RNA synthesis is tied to vacuolated structures in tobacco callus cells, a reentrant phase transition, which shows that RNA production can mediate the formation and dissolution of ribonucleoproteins, represents a viable mechanism for the formation and modulation of vacuolar substructures. This concept was tested in our recent work [35]. As shown in Figure 2, a jump in RNA from Phase I to II concentrations results in nucleation of droplets of Phase II in a background of Phase I. Similarly, a jump in RNA from Phase II to III concentrations now results in nucleation of light Phase III (vacuoles) in Phase II droplets. These vacuoles showed cycles of formation, growth and ejection, reminiscent of some of the non-equilibrium dynamics described in the earlier tobacco cell work. Thus, the RPT provides a mechanism for both droplet dissociation as well as spatial sub-compartmentalization, both of which likely have important biological ramifications.

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## Emerging and Future Perspectives

As discussed above, much progress has recently been made in the biophysics of liquid-liquid phase separation and its implications for the dynamics and function of MLOs. Nonetheless, many important questions in the field remain to be better addressed. Furthermore, the recent developments and discoveries open a host of additional areas for further investigation. Here, we discuss our views on some of these open questions as well as methods that can be used to address them.

### A few open questions – molecular variables, non-equilibrium dynamics, transcription and cellular functions

This perspective mainly focuses on the theme of reentrant phase transitions (RPTs) and their implications for MLOs. As discussed above, our recent work revealed the role of RPTs in the dissolution dynamics and substructure formation of ribonucleoprotein droplets [35]. While the results are interesting, this early work has only begun to chart out the biophysical characteristics, control elements, and functional contexts of this phenomenon.

It will be important to study a number of these issues further both in a well-controlled *in vitro* context as well as in live cells. While we observed qualitatively similar RPTs in a few simple positively charged peptide systems and RNA/DNA systems, a key next step will be to understand how the observed RPT physics translates into more complex RNA and protein systems. In this context, one simple issue is the role of mulitvalency in RPTs, expanding on the general importance of multivalency in LLPS that we discussed above. A simple theoretical framework presented in our previous work can be built upon theoretically and experimentally, using well-designed model peptide and RNA systems to systematically probe the influence of charged motif mulitvalency on the quantitative characteristics of the RPT. An important expansion on this theme will be to understand the influence of charge patterning and the identity of the charged amino acids [47–49]. IDPs, which are common elements of MLOs, often contain both low-complexity/charged and structured regions [23, 35, 50]. It will be important to understand the influence of these various elements in RPTs. Furthermore, over the past two decades, there has been a substantial growth in discovery and our knowledge of the large diversity of cellular RNAs and their functions. The involvement and characteristics of some of these classes of RNA are being probed in the context of phase separation, and should be expanded in the context of RPTs [51]. Phase separation and RPTs may also be further modulated due to other stoichiometry balancing effects of valencies on the interacting species. For example, the effects of “magic numbers”, an interesting concept with implications in physics and biochemistry [52–54], has been discussed for the case of the pyrenoid [55]. It will be very interesting to test for and understand the functional implications of such additional influences on the phase behavior of biomolecules.

These studies will be important not only as an exploratory exercise to chart the fundamental biophysics of LLPS, but will have important implications for biological function and misfunction. The quantitative characteristics of RPTs (e.g., the concentration ranges and shape of the phase diagram) can control the concentration parameters and dynamics of MLO dissolution, likely important in multiple cellular functions and in the cell cycle. It will also be particularly interesting to understand how the spatiotemporal characteristics of the phase-

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separated state of droplets tunes biochemistry of proteins and RNA. A simple (and well discussed) idea is that droplets increase local concentration and therefore can alter the efficiency of interactions and biochemistry in a species-specific manner. In a less trivial mechanism, the altered environment of droplets, for example dielectric constant [56], could also influence the structural and dynamic characteristics of proteins and RNA, and thereby influence their biochemistry [57, 58]. For example, enzyme activity could be higher due to local concentration effects in droplet organelles, but could also be altered due to changes in enzyme structural features. Similarly, a more extended conformation of an IDP in a droplet (such as observed recently [50]) could provide more rapid phosphorylation or other modification. These properties in the context of an RPT could give rise to additional functional effects.

While transcription has previously been associated with the formation of droplet organelles, the RPT findings provide a potential mechanism for transcription to control the cycle of droplet formation and dissolution in a monotonic fashion (Figure 3). Thus, the window-like phase separation behavior observed in the RNA-peptide/protein systems would predict that while increasing RNA concentration by transcription can result in droplet formation starting at RNA concentrations below the I/II phase boundary, it could instead result in droplet dissolution starting at concentrations within phase II (as also depicted in Figure 2). We tested this idea using an *in vitro* transcription reaction in our recent work, with the results indeed following the model-based predictions [35]. These results reveal a potential new mechanism for droplet dissolution in cells. Furthermore, the characteristics of the reentrant phase transition also result in the possibility of further complexity and regulation. In a particularly striking example, a situation of accelerated transcription rates in droplets could couple with the window-like behavior of the RPT to result in a negative feedback loop in the Phase II/III region. This loop in a cellular flux circuit will tend to limit the concentration of the RNA to within the window, since increase in RNA concentration will result in dissolution and reduced transcription.

A key next step is to explore the influence of RPTs in the context of live cells. Chemical biology, electroporation, microinjection or optogenetic methodologies to achieve spatiotemporal control of RNA and transcription reactions in live cells will permit direct and controlled tests of RPTs and their biophysical and biochemical correlates on cellular functions. Finally, direct observation of RPT-related effects and downstream function in native (unmodified) cells will also be an important future direction. One aspect for exploration will be the effects of copy numbers, local concentration and dispersal mechanisms on the occurrence and functionality of RPTs.

Another aspect of both *in vitro* and cellular studies is the consequence of RPTs for substructure formation and non-equilibrium dynamics in droplet organelles. As we discussed above, we have shown that the RPT mechanism leads to the predicted formation of vacuolar substructure within droplets [35]. An additional prediction is of tunable properties (lifetime) of vacuoles (Figure 2), again observed experimentally [35]. Extension of this understanding for the more complex systems discussed earlier will be useful for understanding the relevance of these substructures in cells [45, 46]. The RPT also resulted in spatiotemporally complex non-equilibrium dynamics, including formation, fusion and expulsion of vacuoles, again a place

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for further exploration. It will also be interesting to understand the diffusion, flows and spatial patterning of molecules that occurs in such situations. Complex flows and patterning are believed to be functionally important in several other cellular and organismal contexts including with active matter [59–67], and these concepts could be extended to this situation. Furthermore, it will be interesting to understand patterning or gradients of molecular (e.g. protein) structure, dynamics, interactions and corresponding function in droplets. For example, the interfacial layers of molecules (at the surface or interface with vacuoles) experience different interactions and therefore most likely have altered conformational and functional properties. While the specific differences are currently unknown, it is quite possible that they contribute to differences in biochemical functionality, based on some the points we have discussed above.

## Methodologies, current and future

We anticipate that studies of the above questions will leverage standard methods in the field, but will also go hand-in-hand with adaptations and developments in other technologies. Thus, use of simple turbidity and imaging studies of phase separation and use of fluorescence recovery after photobleaching (FRAP) to characterize material properties will continue to be expanded upon. NMR studies of protein structural features in droplets have also been emerging [34, 50], and such methods will continue to be improved. These methods could also be used for studies of rapid dynamics of LLPS by combination with techniques such as stopped-flow or microfluidic continuous-flow mixing [68, 69]. Microfluidic methods could also be used for more rapid mapping of phase behavior [70]. More complex dynamics may also be probed by methods such as nanofluidic T-jump [71], such as observed at a molecular level using recently developed technology. Recent studies have also begun to explore the use of single-molecule FRET to study the conformational properties of proteins directly in a droplet environment [50]. While these studies are complex to perform and interpret, continued adaptation and improvement of this and related methods should substantially assist in studies of structural features, stability and interactions in droplet environments. For example, another study used ultrafast scanning fluorescence correlation spectroscopy to show how sequence information in constituent molecules can tune the interior properties of droplet organelles [72]. Single-molecule pulling methods such as optical and magnetic tweezers will also be useful for such studies [73, 74]. Together, such studies using single-molecule, related and complementary methods are expected to provide information about the influence of droplet environments on distributions and stochastic dynamics of structure, interactions and function [75, 76]. Subdiffraction-sized “droplets”, which have been implicated in functions such as transcriptional regulation, can also be studied using these methods, with superresolution and light-sheet microscopy as well as cryo-EM also likely playing an important role [77–79]. Experimental studies are also expected to be accompanied in parallel with theoretical and computational modeling [17, 45, 80–83], which has the potential to substantially improve our mechanistic understanding of MLOs and their functions. Finally, in parallel with shedding light on the mechanisms underlying aspects of cell biology, the above work can also provide new mechanistic and design insight into research on materials (including with the use of unnatural amino acids [84]), origins of life and synthetic cells [85–94].

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## Concluding remarks

In this perspective, we focused on the concept of reentrant phase transitions and associated non-equilibrium dynamics as they pertain to the biochemistry and function of membraneless organelles in cells. This concept, which was recently adapted by us for MLO studies, is particularly interesting in the context of providing mechanistic bases for dissolution of droplet organelles and dynamic substructures within them. These aspects are of particular relevance for the biological functions of MLOs. We concluded with a brief discussion of open questions in the field, including expanded investigations of the RPT model both *in vitro* and *in vivo*, as well as studies of molecular conformational, interaction and patterning properties influenced by RPTs. Novel methods such as single-molecule fluorescence and pulling methods in conjunction with existing and new ensemble techniques will be important in these future studies. Work on RPT and related novel concepts and mechanisms is expected to shed new light on the complex biochemistry and physics of these fascinating mesoscale objects and their functions in cells.

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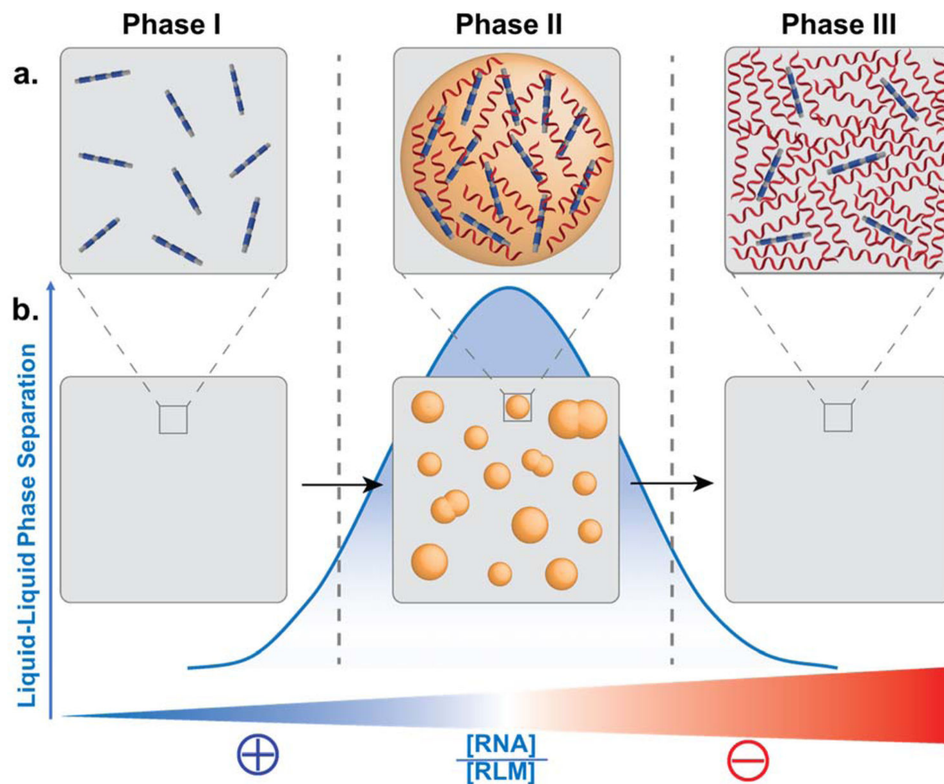


**Figure 1.**

Scheme of reentrant phase transition leads to RNP droplet formation and dissolution. (a) Molecular representation shows the three interaction states in a simple system where RNA initially drives the formation of droplets, but subsequently drives dissolution via a charge inversion mechanism. This results in a change from short range electrostatic interactions to long range interactions. (b) Microscopic view showing that the titration of RNA:RLM ratio results in sequential transitions from a light (Phase I) to a dense (Phase II) to a light (Phase III).

- Arginine Rich Linear Motif - ssRNA

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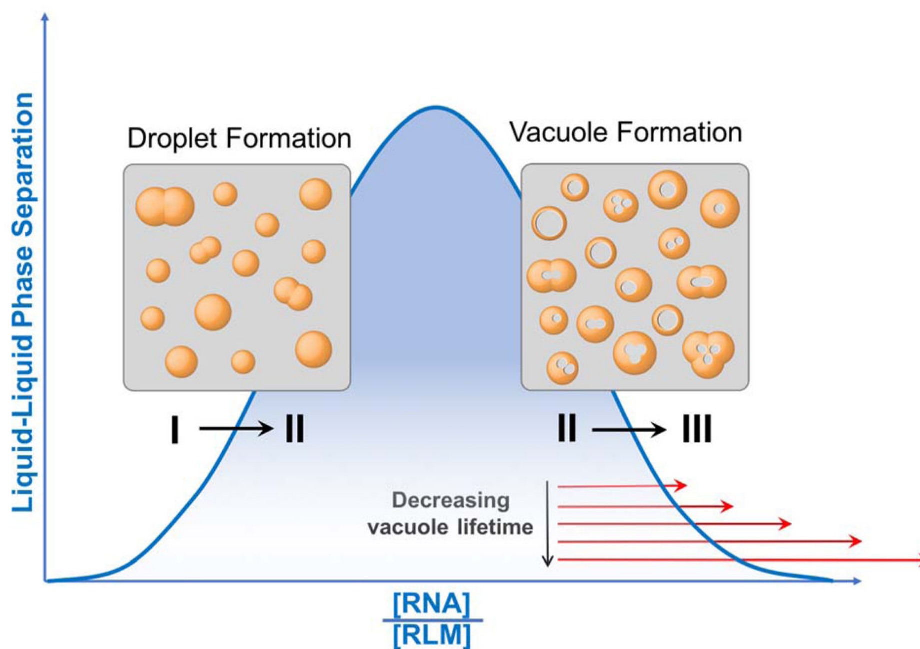


**Figure 2.**
Schematic of vacuole formation due to the reentrant phase transition. The left side shows nucleation of Phase II droplets during a transition from Phase I to Phase II. On the right side, vacuole formation results from nucleation of the light Phase III droplets inside the dense Phase II droplets during the transition from Phase II to Phase III. Vacuole lifetimes can further be controlled depending on jumps in RNA concentration with respect to the arginine-rich linear motif (RLM) concentration.

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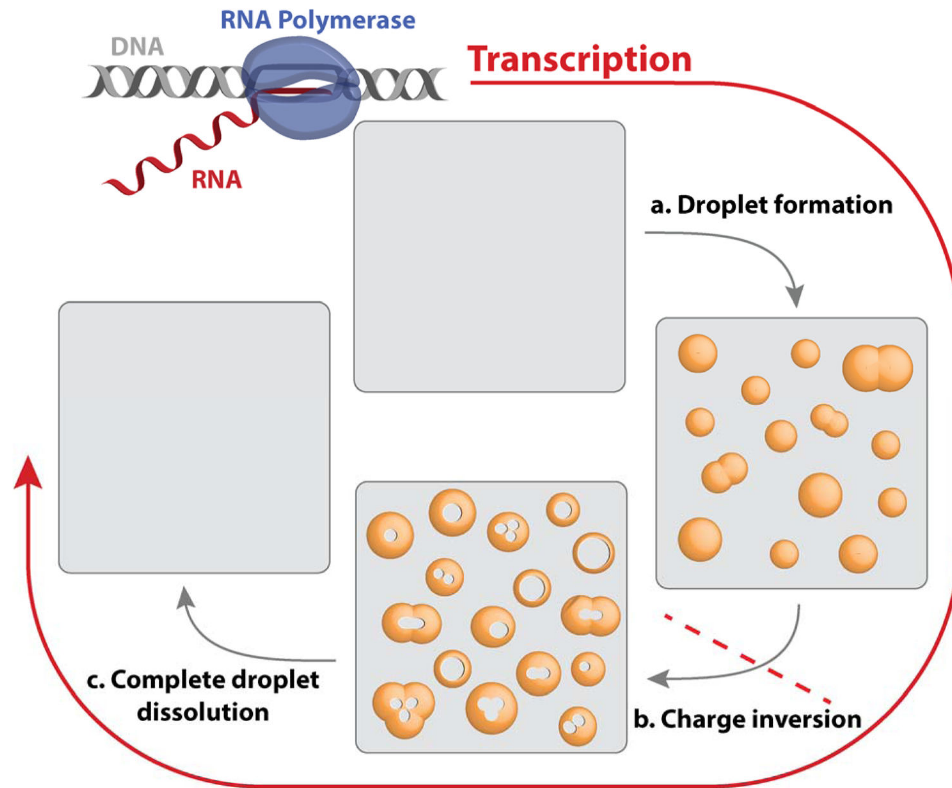


**Figure 3.**
Schematic depicting the potential role of transcription. Transcription, which drives RNA synthesis, can drive droplet formation, charge inversion and subsequently vacuole formation, and complete droplet dissolution.