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EMERGING AREA

Soft active aggregates: mechanics, dynamics and self-assembly of liquid-like intracellular protein bodies

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Intracellular bodies consisting of dynamic aggregates of concentrated proteins and often RNA are a ubiquitous feature of the cytoplasm and nucleus of living cells. Dozens of different types of protein bodies are involved in diverse physiological processes including ribosome biogenesis, RNA splicing, and cell division. Unlike conventional organelles, they are not defined by an enclosing membrane. Instead, these bodies represent dynamic patterns of locally concentrated macromolecules which turn over on timescales of seconds. Here we discuss recent findings suggesting that intracellular protein bodies are active liquid-like drops that self-assemble within an intrinsically structured cytoplasm.

Introduction

The interior of living cells is organized into a variety of subcellular domains that spatially compartmentalize the vast number of simultaneous biomolecular interactions. Such compartmentalization is accomplished in large part by localizing certain molecules within membrane-bound organelles, such as the Golgi apparatus, secretory vesicles, or mitochondria. However, it is increasingly apparent that non-membrane-bound organelles play an important role in localizing specific molecules, often both RNA and protein, to distinct intracellular micro-compartments. Some of these structures are large (>1 μm) and were visualized in fixed samples by early microscopists over one hundred years ago. Examples include the nucleolus, a complex aggregate of RNA and protein that forms in the nucleus around actively transcribing ribosomal gene clusters,1 Cajal bodies (CBs), which are also found in the nucleus and play an important role in RNA splicing,2 and centrosomes, which organize the mitotic spindle of dividing cells. A number of other intracellular protein bodies have recently been identified, primarily as a result of the advent of modern molecular biology techniques, particularly fluorescence imaging using green fluorescent protein (GFP)-tagged proteins. Examples include Processing (GW or P) bodies (discovered 2003), which are found throughout the cytoplasm of cells, and play an important role in translational repression and mRNA degradation;3 and Paraspeckles (discovered 2002), which are small nuclear bodies that play a role in gene expression by retaining RNA in the nucleus4 (Fig. 1). Intracellular protein and RNA appear to have an intrinsic capability for self-assembly into loose aggregates (see Table 1), and it is likely that more such bodies will continue to be discovered.

In addition to physiological protein bodies, there are also a wide variety of pathological protein aggregates that underly diseases such as cataract formation, alzheimers, and the prion diseases.5 These pathological aggregates typically consist of a small number of different proteins, and the study of their self-assembly has been amenable to in vitro biochemical approaches. Disease aggregates typically involve the formation of fibrillar protein amyloids, whose self-assembly has generated considerable interest from researchers from the physical sciences.6 By contrast, the formation of physiological protein bodies has received less attention, likely as a result of their relatively higher degree of biological complexity. Whereas amyloid-like aggregates have been considered within the realm of physical chemistry, physiological protein bodies have been studied largely...
using traditional cell and molecular biology approaches. However, the growth in the field of complex active materials suggests that the recent interest in intracellular protein bodies by physical scientists will only continue to increase. Here I discuss recent findings on the biophysical nature of these protein bodies, and highlight outstanding questions related to their nonequilibrium, “active” behavior in living cells.

Dynamic turnover

A common feature of many of these structures is the rapid turnover of their components. Whereas disease-causing amyloid-like aggregates are relatively stable (for exceptions see ref. 7), physiological protein bodies are highly dynamic. Experiments to probe these dynamics in cells often rely on fluorescence recovery after photobleaching (FRAP), which monitors the exchange of de-activated fluorophores within the structure with fluorescently active fluorophores outside of the structure. These studies have revealed that in a wide variety of protein bodies, the components typically undergo complete exchange with a dilute cytoplasmic pool on timescales of less than 1 minute. These findings are striking considering that these structures may persist for tens of minutes with little change in size or shape.

P granule dynamics

The biological importance of these dynamic physical properties was highlighted in a recent study on the localization of intracellular germ granules, using the worm *Caenorhabditis elegans* as a model organism. Germ granules are RNA- and protein-rich bodies that are found in all animals, and play an important role maintaining germ (sex) cells in an undifferentiated state. In the newly fertilized 1-cell embryo, P granules are distributed throughout the cytoplasm. As the embryo polarizes along its anterior–posterior (AP) axis, P granules localize to the posterior half of the embryo. This process is important because upon division the posterior P granule-containing cell becomes the first progenitor germ cell, while the non-P granule-containing anterior cell will differentiate to form somatic tissues such as skin and neurons. It was found that this localization is not due to segregation by cytoplasmic flow or other direct transport mechanisms, but rather relies on a gradient in P granule stability. P granules in the anterior are destabilized and decrease in size, while P granules in the posterior grow in size (Fig. 2). This stability gradient is facilitated by a cascade of nonequilibrium reaction–diffusion processes that establish gradients in polarity proteins along the AP axis. Of particular importance is the non-specific RNA binding protein Mex-5, which appears to destabilize P granules by competitively binding RNA. These proteins thus spatially regulate a transition between dissolved protein and RNA components and their condensed P granule form.

These findings underscore a fundamental biophysical question about such intracellular bodies. Specifically, if P granule localization relies on a kind of phase transition, what is the nature of the condensed phase? P granules were found to exhibit remarkably liquid-like behaviors, including fusion, dripping, and...
wetting (Fig. 3A). Using the results of FRAP molecular mobility measurements of molecules within P granules, and the timescale for droplet fusion, P granule viscosity was estimated to be $\eta \approx 1 \text{ Pa s}$, and P granule/cytoplasm surface tension was estimated to be $\gamma \approx 1 \mu \text{N m}^{-1}$; this small surface tension value is comparable to other macromolecular liquids,10 consistent with the expected scaling relation $\gamma = k_BT/l^2$, where $k_BT$ is the thermal energy scale and $l$ is the characteristic size of the molecules. These findings suggested that upon polarization the AP-axis straddles a phase boundary between a soluble, dissolved phase of protein and RNA and a condensed liquid droplet phase (Fig. 2).

**Origins and generality of liquid-like dynamics**

Of the more than two dozen known P granule proteins, all contain RNA binding domains. These occur predominantly as multiple, modular domains each of which exhibits only weak RNA binding; these would be expected to facilitate the dynamic exchange of components, and could give rise to the bulk P granule fluid properties. But multiple, weak modular domains are typical of RNA-binding proteins,11 which suggests that these liquid-like properties may be found in other protein bodies, particularly those also containing RNA.

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**Fig. 2** Spatially controlled dynamics of P granule assembly. (A) *C. elegans* embryo expressing GFP-tagged P granule protein PGL-1 (green). An overlaid DIC image of the embryo is pseudo-colored in red. In the upper picture the embryo has not yet polarized; P granules are everywhere, but are shrinking. In the lower image the embryo has polarized and P granules have condensed in the posterior. Embryos are 50 $\mu$m long. (B) The growth rates of P granules for the pre-polarization and post-polarization case, as a function of position along the AP axis. (C) Schematic phase diagram showing that the AP-axis crosses a phase boundary upon polarization. Here $\chi$ is an interaction parameter that reflects the relative affinity between P granule components, as compared to the thermal energy scale $k_BT$. The horizontal axis represents the concentration (volume fraction, $\phi$) of P granule components. (A) and (B) adapted from Brangwynne et al.,59 with permission.

**Fig. 3** Examples of liquid-like behavior of intracellular protein bodies. (A) P granules in *C. elegans* can be seen dripping off of a nucleus (N) being sheared by fluid flow. Adapted from Brangwynne et al.,59 with permission. (B) Germ granules (red) being deformed by motor proteins in zebrafish. Note that in the last frame the droplet is released from the larger aggregate and rounds up. Adapted from Strasser et al.,53 with permission. (C) Two nucleoli fusing into one within the nucleus of a newt cell. Adapted from Amenta.13
Consistent with the idea that liquid droplet-like properties are not restricted to P granules, many other protein bodies are also spherical, implying a significant surface tension and internal relaxation. For example, CBs were previously known as “spheres” for their nearly perfect geometric form.\textsuperscript{2} The density and permeability of these structures have been studied in the large nucleus of frog (\textit{Xenopus laevis}) oocytes. An interferometric technique revealed that the macromolecular density of CBs is only \(\sim 30\%\) greater than the surrounding nucleoplasm (\(\sim 100\ \text{mg}\ \text{ml}^{-1}\)). Moreover, CBs were found to be highly permeable to labeled dextran tracer molecules (Fig. 4).\textsuperscript{12} This study concluded that CBs are “semifluid spheres suspended in semifluid nucleoplasm”. Other sub-nuclear bodies are also generally spherical, although the complex chromatin architecture inside the small somatic nucleus likely plays a role in deforming them. Indeed, both speckles (also referred to as B-snurposomes) and nucleoli are highly spherical in the large \textit{X. laevis} oocyte nucleus. Speckles were found to be \(\sim 50\%\) and nucleoli \(\sim 100\%\) more dense than the surrounding nucleoplasm, with a correspondingly lower permeability to inert dextran tracer (Fig. 4).

Consistent with nucleoli having liquid-like properties, fusion of two or more nucleoli into one larger spheroid has been known for over 100 years (see Amenta,\textsuperscript{13} and references therein). Several other protein bodies appear to exhibit liquid-like behaviors, and such bodies may generally be characterized as liquid droplet phases of concentrated protein and RNA (Fig. 3).

**Insight from phase behavior of proteins and other macromolecules**

The ability of intracellular proteins to self-assemble into liquid phases is not surprising given the fact that, like other macromolecules, proteins exhibit a rich phase behavior.\textsuperscript{14} \textit{In vivo}, protein phase transitions play an important structuring role. A recent study found that as bird feathers mature they concentrate \(\beta\)-keratin proteins, leading to spinodal decomposition which is arrested at a length scale that defines feather color (Fig. 5).\textsuperscript{15,16} In other species the \(\beta\)-keratin proteins appear to phase separate by a nucleation and growth process. The protein phases most well-known to biologists are the protein crystals grown from purified protein solutions \textit{in vitro}, which are essential for mapping protein ultrastructure through X-ray scattering. Nonetheless, solutions of purified protein are also capable of undergoing liquid–liquid phase separation;\textsuperscript{17} important biomedical applications utilize liquid–liquid phase separation of proteins, for example using elastin-like peptides (ELPs).\textsuperscript{18} \textit{In vivo}, ELPs expressed in cells may also exhibit liquid–liquid phase separation.\textsuperscript{19} Moreover, liquid–liquid phase separation of \(\gamma\)-crystalline proteins is thought to play a role in the opacification of the eye lens during cataract formation.\textsuperscript{20} Thus, although there may also be a role for the multiple weak domains found in the RNA binding proteins in many protein bodies, liquid protein phases can also readily form in the absence of them. Indeed, the rich phase behavior of even pure protein solutions allows for liquid protein phases in a variety of contexts, including in living cells.

In considering the biological role such phases may play in cells, it is illustrative to consider the phase behavior of other macromolecules that are thought to play important biological roles. Phospholipid/cholesterol mixtures can undergo 2D liquid–liquid phase separation, which may be the physical basis for membrane organization in cells.\textsuperscript{21} This “raft hypothesis” was inspired by observations \textit{in vitro} showing that upon increasing cholesterol concentration, lipid bilayers separate into a cholesterol-rich liquid-ordered \((L_0)\) phase, and a cholesterol-poor liquid...
disordered phase (Ld). Studies using a model system consisting of giant plasma membrane vesicles (GPMV), which have been shed from living cells, have shown that 2D liquid–liquid phase separation can indeed occur with cell membranes. More recent studies using this system have mapped the critical temperature of this transition using fluctuations in line tension and domain correlation length. Although cell membranes are complex multi-component protein/lipid mixtures, these studies have emphasized the universal nature of this phase separation behavior, independent of the precise nature of the intermolecular attractions.

The lesson from the above lipid-membrane case is that model in vitro or pseudo in vivo systems are likely to prove similarly useful in understanding complex intracellular protein phases. Important insights into the physics of protein phase transitions have come from the study of model colloidal systems using attraction potentials tuned by the size and concentration of crowding agents (depletion attraction). These studies have shown that the phase diagram is sensitive to the relative length scale of the inter-particle attraction, \( \xi = r_g/a \) where \( r_g \) is the size of depletant and \( a \) is the size of the particle; while a highly ordered crystalline phase is stable over a broad range of system parameters, a condensed liquid phase is only stable for \( \xi > 0.25 \). Similar theoretical and experimental studies have shed light on the protein crystallization process, suggesting that crystal nucleation actually begins with the formation of a metastable condensed liquid phase nucleus. Interestingly, formation of ordered amyloid fibrils, for example in prion diseases, may also begin within a disordered "molten globule" of condensed protein.

**Depletion in cells**

The cytoplasm is a highly crowded environment, and the depletion attraction is also likely to play a significant role in cells; for example, crowding may promote the assembly of nuclear bodies such as nucleoli. In general, the length scale of the intracellular depletion attraction may be comparable to the size of the molecules themselves, i.e., \( \xi = 1 \), suggesting that liquid phases may be intrinsically stable within the cytoplasm. Indeed, the depletion attraction (also known as "macromolecular crowding") has been suggested to lead to phase separation in cytoplasm. Nonetheless, the specificity of protein/protein and protein/RNA interactions must play a central role in giving rise to bodies of defined composition. The extended conformational flexibility of RNA, together with the modular RNA binding domains, could also play a role in providing an extended domain of attraction within the intrinsically crowded cytoplasm. However, the fact that under some conditions proteins alone can form stable liquid phases underscores the challenge of elucidating the precise molecular/structural requirements for liquid phase separation. **In vitro** protein studies which move towards incorporating the full molecular complexity of intracellular protein bodies, analogous to the bottom-up approach towards a quantitative understanding of the mechanics and dynamics of the cytoskeleton, are likely to play a key role; a promising example is the recent mapping of the phase diagram of more complex mixtures of lens proteins.

**Control over size and number of intracellular bodies**

As with microstructure control in industrial materials processing, tight control over the number and size of intracellular protein bodies is in many cases essential to their function. For example, cells with more than two centrosomes have disorganized mitotic spindles and are at risk for chromosome mis-segregation and cancer. Centrosome growth is thus tightly constrained by the requirement of a paired centriole seed, of which only two are mature around tandem rDNA gene repeats; the number of these sites sets a maximum of 10 nucleoli in humans, although typically less are observed due to fusion events. CBs can also undergo both fusion and fission events, and exogenous immobilized CB components are capable of nucleating CBs. However, mechanisms underlying regulation of CB number remain unclear.

The size of intracellular bodies can also play a critical biological role. For example, the size of nucleoli is strongly correlated with cell growth rates; nucleolar size is often used as an indication of the aggressiveness of tumor growth. Moreover, the size of centrosomes was recently shown to determine the size of the mitotic spindle, which must be developmentally regulated to accommodate different cell sizes. Small centrosomes appear to form in small cells simply due to the smaller mass of available centrosomal proteins. The total mass of P granule material is similarly determined by cell size: upon polarization of the 1-cell embryo, P granules begin growing in the posterior but quickly deplete soluble protein pools and the growth rate returns to zero. The finite volume of cells thus plays an important role in constraining the final size of intracellular protein bodies. Ostwald ripening, in which surface tension causes larger bodies grow at
the expense of smaller ones, has recently been reported to play a role in vitro in the growth of amorphous protein crystal precursors, and could also play a role in setting the growth dynamics and number of intracellular bodies. To my knowledge such behavior has not yet been documented in cells and may be limited by the relatively small surface tensions involved; for example, at a given position in the embryo small P granules are not observed to shrink at the expense of larger ones.

**Complexity and active dynamics of intracellular bodies**

This emphasis on equilibrium thermodynamic concepts and the classic liquid-like features of intracellular protein bodies should not distract attention from the fact that these are highly complex materials that can operate far from equilibrium. For example, although in some cases the dynamic turnover of components has been considered within an equilibrium framework, it is clear that for many intracellular bodies these dynamics reflect a distinctly nonequilibrium behavior. The protein nucleostemin is a GTP hydrolyzing enzyme (GTPase) whose localization to the nucleolus is sensitive to cellular GTP levels; other nucleolar proteins also exhibit GTP-dependence. Many protein bodies also contain ATPases, including a number of RNA helicases involved in unwinding RNA. Fluctuating motion within living protein bodies thus will not be strictly thermal, but will exhibit features of the collective activity of these nonequilibrium processes; associated transport processes could in turn be affected, as seen in other nonequilibrium biological contexts.

The complexity of intracellular protein bodies is also reflected in the fact that many exhibit a high degree of internal structure. Nucleoli contain three distinct structural domains: fibrillar centers (FC) that contain the ribosomal genes (rDNA), a dense fibrillar component (DFC) that contains the newly transcribed rRNA, and a granular component (GC) consisting of small particles of protein and rRNA undergoing further modification. How this core–shell structural organization is maintained, and how nucleoli might restructure during fusion events (such as that in Fig. 3C), remain open questions. Interestingly, changes in temperature can significantly perturb the organization of these nucleolar domains, which may be related to changes in transcriptional activity. Upon direct pharmacological inhibition of rRNA transcription, the different structural domains of nucleoli strongly segregate away from one another (Fig. 6). At least three different types of separate cap-like structures segregate off of the remaining GC: so-called dark nucleolar caps that are described as “concave”, “convex” light nucleolar caps, and fibrillar caps. This process is thus reminiscent of a liquid–liquid demixing process, in which the different resulting phases appear to have different wetting properties. However, this is an active, ATP-dependent segregation process; moreover, it involves incorporation of normally nucleoplasmic proteins, in addition to a redistribution of nucleolar-associated proteins. Dissecting the biophysical driving forces behind the complex, active structural organization of the nucleolus clearly represents a challenging task for the future.

**Implications for active control**

If intracellular protein bodies behave as active liquid-like droplets, what are the implications for their biological function? Condensed liquid droplets bring together specific molecules in high concentrations, but without restricting their mobility or conformation within the droplet. Macroscopic liquidity implies microscopic mobility, and these concentrated molecules interact at high rates within the small droplet volume. Protein bodies thus play a role as intracellular micro-reactors, concentrating reactive components and thereby increasing the rate of molecular interactions. This is consistent with recent findings that suggest that while CBs are not necessary for proper biological functioning in many contexts, formation of CBs is essential under conditions in which the rates of RNA modification reactions must be optimal, such as during rapid embryonic development.

The function of protein bodies as liquid micro-reactors may be sensitive to nonequilibrium features within the cell. On timescales of tens of seconds, the observed deformation behaviors (e.g. Fig. 3) are consistent with purely viscous relaxations. However, as with other entangled biopolymer systems, we expect that intracellular protein bodies will actually exhibit a frequency-dependent viscoelastic response. In particular, the dynamics on shorter timescales (i.e. seconds and faster) will likely show elastic signatures; this will especially be the case for bodies containing a high degree of entangled RNA. Their function as small, fluid...
reaction volumes thus raises the intriguing possibility that the time-dependent viscoelasticity of protein bodies could be tuned biologically to regulate the rates of these reactions. Unlike non-living aggregates, intracellular bodies have the unique ability to tap into the free energy of nucleotide hydrolysis. This energy could be utilized to control the rate at which protein and RNA interact within the drop. Thus, intracellular protein bodies may share features with other active biopolymer systems, such as the actomyosin cytoskeleton. Here, for example, force generation by ATP-hydrolyzing myosin motors has been reported to either liquefy or stiffen actin gels, depending on the nature of motors and crosslinks. Similar behaviors may also be possible in these active fluid-like intracellular bodies.

Conclusions

Dozens of different types of protein- and RNA-rich bodies are found throughout the cytoplasm and nucleus of living cells. Moreover, the large (~1 μm) dynamic assemblies that are readily seen using conventional microscopy represent only a subset of many smaller dynamic assemblies throughout the cytoplasm. It is increasingly clear that many of these structures represent a liquid-like droplet phase of concentrated protein and RNA. There may thus be strong similarities between this kind of cytoplasmic phase structuring and the 2D liquid–liquid phase separation observed in lipid bilayers. Intracellular protein bodies may represent an analogous liquid–liquid phase separation, in this case forming 3D cytoplasmic “protein rafts”. The nonequilibrium aspects of cytoplasmic bodies, specifically the ATP- and GTP-dependence of the activities of many of the constituent molecules, allow for an additional layer of control over their dynamics and self-assembly. Understanding the full set of control parameters underlying their self-assembly may suggest biomedical interventions to treat diseases associated with protein bodies, in addition to pointing the way towards novel biomimetic compartments for drug delivery and other biomedical interventions.

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