Liquid-Liquid Phase Separation in Biology

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Abstract

Cells organize many of their biochemical reactions in non-membrane compartments. Recent evidence has shown that many of these compartments are liquids that form by phase separation from the cytoplasm. Here we discuss the basic physical concepts necessary to understand the consequences of liquid-like states for biological functions.

Contents

PHASE TRANSITIONS AND THE FORMATION OF NON-MEMBRANE-BOUND COMPARTMENTS

Cells have a problem: How do they organize complex biochemical reactions in space? They have solved this problem by creating compartments, or organelles, which are distinct chemical environments. A compartment has two important properties. It must have a boundary that separates it from its surroundings, and the components within it must be able to diffuse freely, so that chemical reactions can take place inside. Many compartments are separated by membranes, such as mitochondria, which contain a chemical environment necessary to make ATP (Friedman & Nunnari 2014), or lysosomes (Luzio et al. 2007), which contain components necessary to destroy other proteins. In the case of membrane-bound compartments, it is easy to understand how different compartments can coexist. However, many compartments do not have membranes. Examples are nucleoli, which make ribosomes inside the nucleus (Boisvert et al. 2007); centrosomes (Mahen & Venkitaraman 2012), which nucleate microtubules; Cajal bodies, which make spliceosomes (Gall 2003); and stress granules (Buchan & Parker 2009, Decker & Parker 2012), which take various forms under different stress conditions. In the case of non-membrane-bound compartments, it is harder to understand how the different compartments coexist. Why do the components of these non-membrane-bound compartments not simply mix with their surroundings? Some non-membrane-bound compartments, such as glycogen granules (Stubbe et al. 2005), do not mix because they form cross-linked aggregates. However, these are less suitable for compartments in which the types of chemical reactions common in biology take place, because cross-linked components cannot diffuse freely. What structure or organization could a cell use to organize non-membrane-bound compartments?

Recent observations on several compartments have suggested that the best way to think about them is as liquid drops that coexist with the cytoplasm. The first clear example of a liquid-like compartment was the P granule from *Caenorhabditis elegans* embryos. P granules were identified

by electron microscopy (Wolf et al. 1983) and fluorescence (Strome & Wood 1983) and have long been known to segregate with the germ line of *C. elegans* embryos (Hoege & Hyman 2013, Strome & Wood 1983, Updike & Strome 2010, Voronina et al. 2011). Careful observation showed that they fuse, exchange components rapidly with the cytoplasm, are easily deformed by flows, and have a viscosity similar to runny honey (Brangwynne et al. 2009). All of these properties suggest that they are liquids. Further work showed nucleoli also have liquid-like properties and are approximately 50 times more viscous than P granules. Many non-membrane-bound compartments likely will have the properties of liquid drops (for further discussion, see Brangwynne 2013, Hyman & Brangwynne 2011, Hyman & Simons 2012, Weber & Brangwynne 2012).

In this review, we explain why describing non-membrane-bound compartments as phase-separated, liquid-like droplets can illuminate many of the key properties described above for non-membrane-bound compartments, namely, the formation of small reaction volumes with different chemistry from the outside. Other reviews have focused on the biology and biophysical properties of these liquid-like compartments (Brangwynne 2013, Hyman & Brangwynne 2011, Weber & Brangwynne 2012). This review aims to define the terminology of liquid-like states in cells and how the ideas of soft matter physics can help us to understand the assembly of biological compartments. To this end, we have often used a slightly simplified presentation of the corresponding physics, which does not necessarily provide the complete physical picture. Interested readers are referred to more detailed literature where appropriate.

PHYSICS OF A LIQUID-LIKE STATE

What is a liquid? A liquid is a state of matter in which components can easily rearrange. Roughly speaking, we can distinguish liquids from solids, in which components do not easily rearrange and exhibit a different degree of order (see **Figure 1**). More precisely, in solids, particles are caged; in other words, particles keep their specific neighborhood for a long time. In liquids, particles change their neighborhood quickly. We can illustrate this difference with water, which below the freezing point is a solid crystal and above freezing is a simple liquid. When water is a solid, it cannot be easily deformed, and a piece of ice will maintain its shape. When water is a liquid, it can easily be deformed and even flows (see **Figure 1**). A volume of liquid water will not maintain a given shape in the absence of a container. In both cases, molecules are similarly dense. They are closely packed, and both phases are hard to compress. But in the case of liquid water, molecules move quickly and exchange their neighbor relations with ease, whereas in ice, molecules tend to keep their neighbors; in other words, they are locally caged. Because of the rapid motion in liquids, different components can mix easily. Chemical reactions can occur everywhere within the liquid through the random collision of reactants. This is why chemical reactions in biology tend to take place in liquids.

We are used to thinking of the cytoplasm as a liquid. If you puncture a cell, the liquid cytoplasm will generally flow out. However, we have tended to think of compartments inside cells as more solid-like aggregates, so as to distinguish them from the liquid-like cytoplasm. If many compartments are liquid-like, how can liquid phases stay separate? After all, we are used to liquids being mixtures. If you combine two miscible fluids, such as tea and coffee, the two will mix. They mix because a mixed state has higher entropy than an unmixed state, and thermodynamic systems tend to evolve toward states of higher entropy (for more details, see sidebar, Entropy, Mixing, and Diffusion, and **Figure 2**). However, liquids can also demix. For instance, when you make vinaigrette and leave it, you come back annoyed to find that the oil and vinegar have demixed into two different phases: an oil phase and a vinegar phase. Why is entropy not driving the system to a mixed state? The separation into two phases is driven by the physical interactions between the oil molecules and "vinegar molecules." Specifically, if oil molecules neighbor other oil molecules,

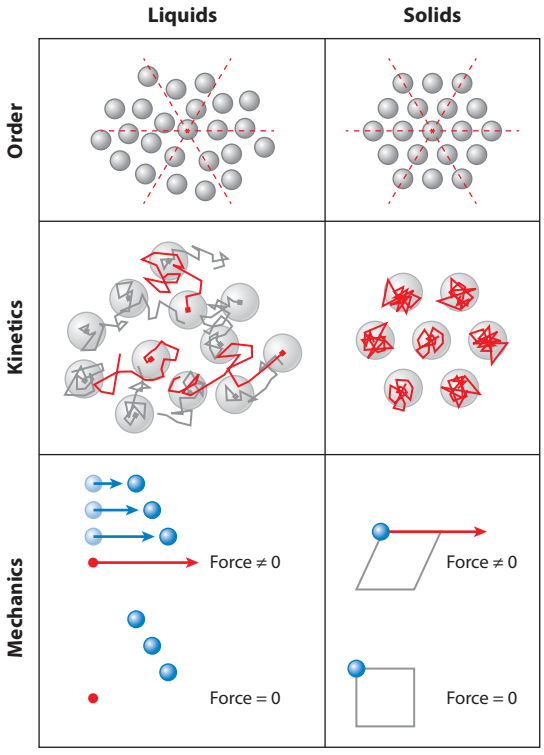

Figure 1

Schematic representation of important characteristics of ideal liquids (*left*) and ideal solids (*right*). Order: For a liquid, there is only short-range positional order. This means that one cannot draw straight lines (*dashed red lines*) along which particles (*indicated by gray spheres*) are separated by approximately equal distances. However, for a crystalline solid, positional order exists over long distances. Therefore, it is possible to draw straight lines along which particles are equally spaced. Kinetics: In liquids, particles rearrange quickly and diffuse. Diffusion allows the particles to move distances far beyond the particle size (particle trajectories are depicted in red and gray). In contrast, particles in a solid are mostly confined to a small cage created by the neighboring particles, and cage rearrangements are extremely rare. Mechanics: Applying forces locally (*indicated by red dot*) that deform a liquid volume leads to particles in different regions moving away from each other (*top*). The corresponding flows (*blue arrows*) can carry small objects for the time the force is applied (light and dark spheres correspond to the time when the force is switched on/off). Locally, the flow velocity is proportional to force and the velocity amplitude is determined by viscosity. In a low Reynolds number flow (Purcell 1977), as is the case in cells, particle motion stops when the force vanishes. There is no memory of the initial configuration (*bottom*). In the case of a solid, application of force leads to the buildup of deformations until forces are balanced by elastic stresses. Particles typically keep their neighborship relations, and the system has a memory of the initial configuration. When the force is removed, the system relaxes back to the initial undeformed state. In other words, probing a certain point in the solid (*sphere*), it returns to the initial position before the force has been applied. Note that real liquids may exhibit a solid-like elastic response during short deformations, a phenomenon called viscoelasticity. Real solids may gradually lose the memory of an initial configuration under strong deformations, a phenomenon called plasticity. Some amorphous solids may also exhibit viscoelastic behaviors, meaning that the force also gives rise to flows.

the system has lower energy than if oil molecules neighbor "vinegar molecules." It is this energy reduction by demixing that opposes entropy-driven mixing (see sidebar, Molecular Interactions Drive Demixing, and **Figure 2**).

Note that in our example, both of the demixed phases (oil and vinegar) consist of many different components. Within each phase, entropy still ensures that the components are well mixed. To

ENTROPY, MIXING, AND DIFFUSION

Multicomponent systems often tend to mix spontaneously and are then found in a homogeneous mixed state. This is a consequence of a system's tendency to increase entropy. Entropy characterizes the amount of disorder in a system. To illustrate the entropy change owing to mixing, let us first consider the entropy before (**Figure 2*a***) and after (**Figure 2*b***) mixing. Consider two volumes that are separated by a partition (indicated in *yellow* in **Figure 2*a***). Each volume is filled by a different type of molecule, represented by red and blue particles, respectively. When the partition is removed, both types of molecules mix and reach a uniform concentration profile (**Figure 2*c***, solid/dashed lines correspond to before/after mixing). The entropy associated with mixing is called mixing entropy, S^{mix}. Therefore, the unmixed state has zero mixing entropy. There are many different ways one can arrange red and blue particles in the mixed state. The mixing entropy measures this number of possibilities. The mixing entropy per unit volume is given by $\frac{S^{mix}}{V} = -k_B \frac{\phi}{v_r} \ln \phi - k_B \frac{1-\phi}{v_b} \ln(1-\phi)$. Here, k_B is the Boltzmann constant and V is the volume of the entire system. The molecular volumes of red and blue molecules are denoted as v_r and v_b. We have introduced the volume fraction ϕ of the red molecule: This volume fraction is defined as the percentage of volume of the box that is occupied by red molecules. Volume fraction is directly related to the concentrations of the molecules. The concentration of red molecules is $c_r = \phi/v_r$, and the concentration of blue molecules is $c_b = (1-\phi)/v_b$. The mixing entropy S^{mix} is shown as a function of volume fraction ϕ in **Figure 2*d***. Note that the entropy vanishes for unmixed states with $\phi = 0$ or $\phi = 1$. In a mixed state with $0 < \phi < 1$, the entropy is positive. The second law of thermodynamics states that entropy increases when processes happen spontaneously. Therefore, the mixing entropy generally drives the mixing of initially unmixed components. To mix, particles must be transported, which typically occurs via diffusion. How is this diffusive transport related to the mixing entropy? In general, particle flux is driven by differences in chemical potential. More precisely, the rate of transport J is proportional to the local gradient of the chemical potential, i.e., $J \propto -\frac{d\mu}{dx}$. The chemical potential can be defined as $\mu := \frac{v_r}{V}\frac{dF}{d\phi}$, where F denotes free energy. The free energy is related to the entropy via $F = E - TS$, where T is temperature and E denotes energy determined by the intermolecular interactions between all components. In the following, we consider the contribution of mixing entropy to the particle flux. To this end, we compute this flux by considering the simple case $E = 0$, in which interaction energies between particles are weak or negligible compared with the thermal energy scale, k_BT. In this case, the free energy simplifies to $F = -TS^{mix}$ and $\mu = -T\frac{v_r}{V}\frac{dS^{mix}}{d\phi}$. The diffusive flux is now proportional to $-\frac{d\mu}{dx} = T\frac{v_r}{V}\frac{d^2S^{mix}}{d\phi^2}\frac{d\phi}{dx}$. Because the entropy function S^{mix} is concave (concave here means that the curvature of the graph is negative in **Figure 2*d***) with $\frac{d^2S^{mix}}{d\phi^2} < 0$, the flux of particles J is proportional to the concentration gradient $J = -D\frac{d\phi}{dx}$. This is the usual description of diffusive transport, called Fick's law, where $D > 0$ denotes the diffusion coefficient. This diffusive transport will give rise to a change in the concentration profiles toward a mixed state of homogeneous concentration, as shown in **Figure 2*c***. Note that this behavior is associated with convex free energy F (see *blue* line with positive curvature in **Figure 4*a***).

distinguish solids from other materials, physicists use the term soft matter (for further reading, see Chaikin & Lubensky 1995 and Doi 2013). Soft matter encompasses many different types of matter that are easily deformable. Examples are liquids, complex fluids, gels, and colloidal systems. Because different definitions of these terms are used depending on the context, we next provide one set of definitions that is useful in the context of biology.

Solids

A solid is a material that can be cast in an arbitrary shape, and the system keeps a memory of this reference shape for very long times (see **Figure 1**). If it is deformed, it will tend to return to the initial shape, unless it breaks. The property with which it resists shape deformation is called shear

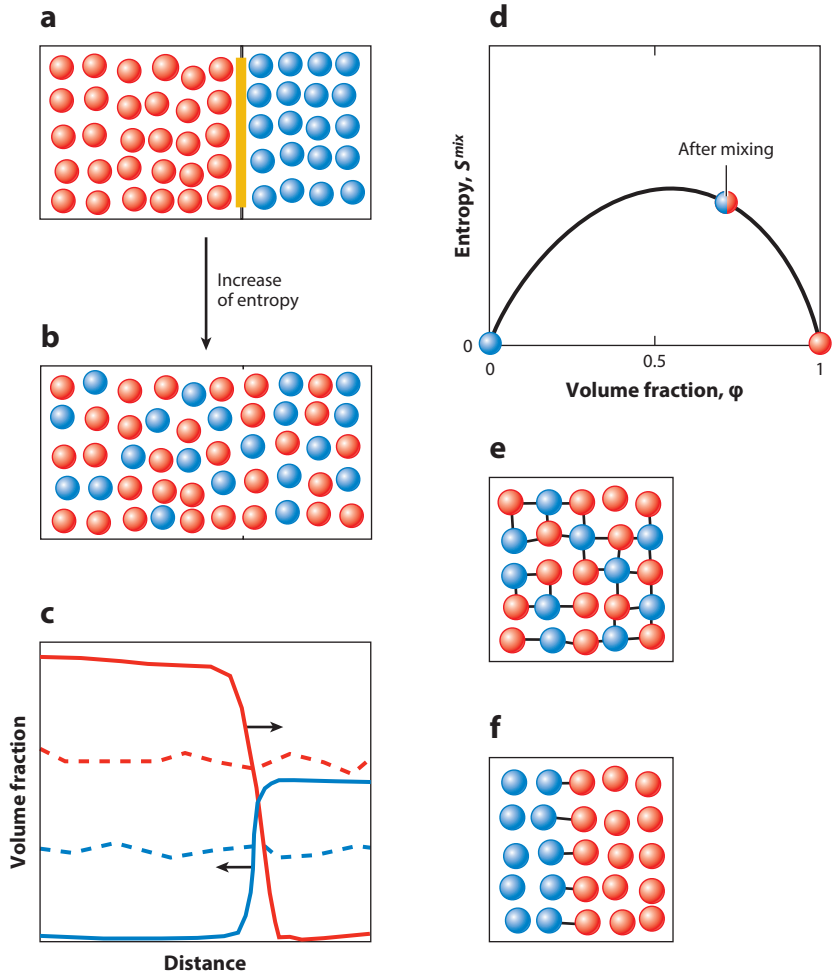

Figure 2

Mixing and demixing. (*a*) Schematic representation of a demixed state where two regions of different compositions are separated by a partition (*yellow*). (*b*) A mixed state, which emerges owing to diffusion after removing the partition. The entropy corresponding to *b* is larger compared to *a*. (*c*) The corresponding spatial profiles of volume fraction for red and blue particles before (*solid line*, *a*) and after (*dashed line*, *b*) the partition is removed. (*d*) Mixing entropy S^{mix} as a function of volume fraction ϕ (for definitions, see sidebar, Entropy, Mixing, and Diffusion). Indicated are the value of S^{mix} corresponding to *b* and $S^{mix} = 0$ for demixed states (*a*). In case of interaction energies that favor like neighbors and disfavor unlike neighbors, a mixed state (*e*) has a larger energy than a demixed state (*f*). This is illustrated by the number of disfavored bonds between red and blue particles.

elasticity. An easily understood example is a piece of rubber, or a steel rod. Both of these can be deformed, but they return to their original shape. This is the crucial difference between a liquid and an ideal solid. Beyond the elastic range, solids can have a range of behaviors, such as plasticity or viscoelasticity; or they break (for more information, see **Figure 1**).

Liquids

A simple liquid rearranges its components at short times; therefore, its shape can be modified easily. The shape is defined by the container or by surface tension (we return to this term later).

MOLECULAR INTERACTIONS DRIVE DEMIXING

In the sidebar Entropy, Mixing, and Diffusion, we discussed that entropy alone drives mixing of several components. Here, we address how microscopic interactions can give rise to demixing of a fluid. As in that sidebar, this question involves the free energy. In the presence of interactions, we must consider the contribution of the interaction energy E to the free energy $F = E - TS^{mix}$. Let us consider interaction energies that favor like neighbors and disfavor unlike neighbors. Such interactions lead to an energy contribution that can be written as $E = \chi V \phi (1 - \phi)$, with $\chi > 0$ denoting a parameter characterizing the strength of interactions between different molecular species, referred to as an interaction parameter. This energy is minimal for either red particles ($\phi = 1$) or blue particles being packed together ($\phi = 0$). It increases in a mixed configuration of particles. This implies that the energy for the configuration depicted in **Figure 2*e*** is larger compared with the one shown in *f*. This is illustrated by the number of disfavored bonds between red and blue particles. The corresponding free energy F is shown in **Figure 4*a*** as a function of volume fraction ϕ (*dark blue line*). In contrast to the free energy in the absence of interactions, which is convex (*light blue* line in **Figure 4*a***), the interactions now imply a region in which the free energy is concave. The existence of this concave region has the following consequence: Consider a homogeneous mixture with a composition ϕ^*, e.g., a mixture similar to the one depicted in **Figure 2*e***. Let us now decompose it into two regions, each of volume fraction ϕ_S and ϕ_D (refer to **Figure 2*f***). The volume fraction of the entire system is then $\phi^* = \lambda_S \phi_S + \lambda_D \phi_D$, with $\lambda_{S/D}$ denoting the relative volume of the two coexisting phases in the system. The corresponding free energy is $F^* = \lambda_S F(\phi_S) + \lambda_D F(\phi_D)$. Now there exists a pair of volume fractions ϕ_S and ϕ_D such that $F^* < F$, as depicted by the dashed line in **Figure 4*a***. Between these volume fractions, F^* is the actual free energy of the system that has demixed in two coexisting phases. In this situation, starting from a mixed state (such as that depicted in **Figure 2*e***), demixing occurs spontaneously.

In other words, liquids do not have shear elasticity. Put another way, when a liquid is put under external force, it has no memory of its previous shape (see **Figure 1**). Therefore, when describing liquids, we use the concept of viscosity rather than elasticity. We can illustrate viscosity with the following example: When liquid flows through a pipe, driven by a pressure difference between the ends, the rate of fluid flow depends on the viscosity of the fluid. Obviously, honey flows slower than water, because it is more viscous. Liquids do have a memory of their volume, so that a liter of water poured from a cylinder will remain a liter. Thereby, liquids are hard to compress. With regard to this property, liquids and solids are similar. This is what makes the difference between liquids and solids so interesting. Although they have very different macroscopic material properties, they can be equally densely packed. In one case the molecules move fast, and in the other they do not.

The shape of a liquid phase is typically dominated by surface tension, which leads to a spherical shape. Surface tension is, as the name suggests, a mechanical tension that exists at the boundary between two phases. It tends to reduce the area of the interface until it reaches a minimum. The minimum area of a drop corresponds to a spherical shape; therefore, surface tension drives liquid drops to be spherical.

So far we have talked about simple liquids. However, most practical liquids are not simple and are better captured by the term complex fluid (Larson 1999). An example of a complex fluid is found in cooking (Harvard Univ. 2012). Here, there are a great variety of different types of liquids with different properties. Dough, butter, cream, vinaigrette, or the foams you get served in fancy restaurants are all examples of different sorts of complex fluids, or soft matter. Each can behave as a liquid. For instance, a round ball of dough, if left overnight in a bowl, will tend to take up the shape of the bowl, with no memory of its previous shape. Several interesting properties emerge from

the discussion of complex fluids. One particularly interesting property is viscoelasticity. In many science shops, you can buy balls of a special polymer (sometimes called silly putty) that bounces when dropped but can flow slowly with high viscosity when you compress it with your hands or leave it on your desk. Therefore, it has the properties of both shear elasticity, like a solid, over short times (the bounce) and viscosity and flow behavior over longer times. It keeps a memory of an initial shape over a finite period of time.

The actomyosin cytoskeleton has often been used as an example of viscoelasticity (Gittes et al. 1997, Humphrey et al. 2002, Janmey et al. 1994, MacKintosh et al. 1995, Shin et al. 2004). When you initially deform an actomyosin gel, for instance, with an atomic force microscope tip, it will initially respond with elastic behavior. If you keep it under force, it will change shape, and it will lose memory of its previous shape. Thus, an actomyosin gel has solid properties at short times and liquid properties at long times; therefore, it is a complex fluid. Another example of a complex fluid is a liquid crystal. A liquid crystal is a liquid in which the components tend to order along a certain direction. In the liquid crystal display of a calculator, you switch the orientation order of polymeric elements with electric fields (Gray & Kelly 1999, Schadt & Helfrich 1971). In biology, a good example of a liquid crystal is a meiotic spindle. A meiotic spindle has liquid-like properties, as it can fuse and deform and its molecular components turn over rapidly. The tubulin subunits in a spindle polymerize into microtubules, which order themselves by aligning along a common axis, and therefore also exhibit order (Gatlin et al. 2010, Inoue 2008, Itabashi et al. 2009, Shimamoto et al. 2011). [For more information on states of matter, the reader is referred to Chaikin & Lubensky (1995) and Doi (2013).]

Gels

In discussing actomyosin, we have introduced the term gel. The term gel is used in different contexts. For instance, it is sometimes used for disordered materials for which the distinction between liquid and solid is ambiguous, such as the low-temperature phase of a lipid membrane (Ranck et al. 1974). However, a gel usually means a cross-linked network of polymeric structures. In a chemical gel, such as rubber, the cross-links are covalent, and thus such gels behave like solids. A physical gel is held together by weaker interactions. Therefore, we usually think of biological gels as typical examples of physical gels, because they are held together by forces that are weaker than covalent bonds. [However, there are also examples of physically cross-linked gels in biology, such as fibrin gels (Münster et al. 2012).] Owing to the weaker interactions, cross-links have a lifetime, and this lifetime distinguishes between solid-like and liquid-like behavior. In the case of actomyosin gels in cells, filaments turn over in approximately 30 s (Fritzsche et al. 2013). Thus, elastic behavior is seen in response to forces at times shorter than approximately 30 s, and viscous behavior is seen at times longer than approximately 30 s.

One important type of gel in biology is a hydrogel (Frey & Gorlich 2007, Peppas et al. 2000). A hydrogel is a gel that has a high water content and cross-linked components that are water soluble. This means that water enters and swells the gel, and squeezing out water requires an external force. Again, hydrogels can be either physical or chemical. For instance, a contact lens is a good example of a chemical hydrogel. Several different biological systems have been described as physical hydrogels. One classic example is the selective filter of the nuclear pore complex (Frey & Gorlich 2007). Another is the formation of structures by RNA-binding proteins (Han et al. 2012, Kato et al. 2012, Kwon et al. 2013, Schwartz et al. 2013). A hydrogel can be a good way to characterize a biological gel, because proteins and other macromolecular constituents of biological structures tend to be water soluble.

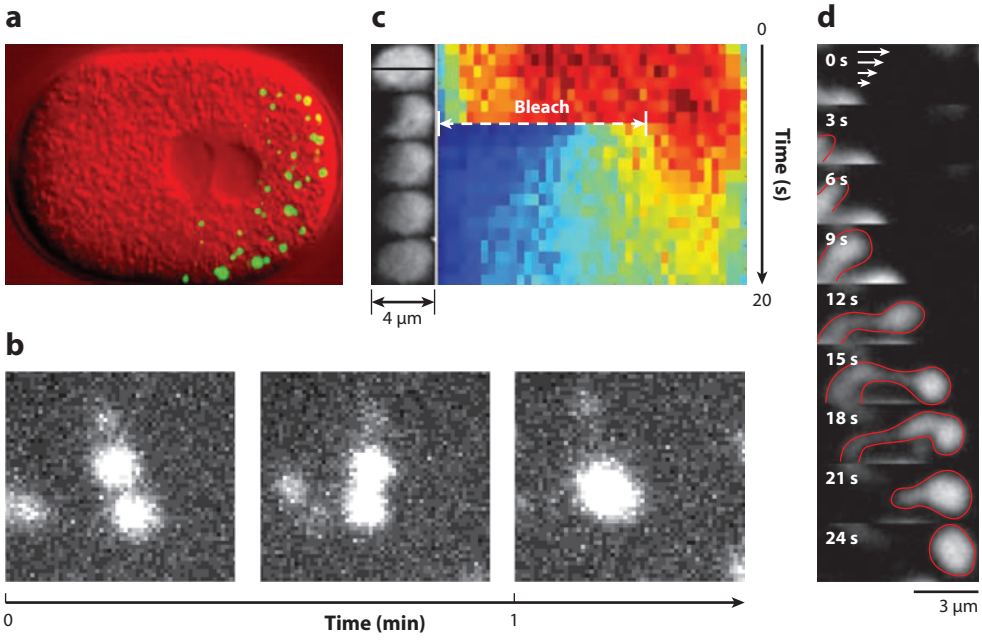

Figure 3

P granules exhibit characteristics of liquid droplets. (*a*) P granules (*green*; GFP tagged) in the cytoplasm of a one cell–stage *Caenorhabditis elegans* embryo. (*b*) Two P granules (*white*) fuse and relax their shape within about one minute. (*c*) Fluorescence distribution before and after photobleaching of a large GFP-tagged P granule (*left*). Kymograph of linear intensity profiles along the anterior-posterior axes (*right*). Red color indicates high intensity and blue corresponds to background intensity. Fluorescence recovery occurs in about 5 s. (*d*) P granule (*red outline*) deformed by sheared flow with a direction indicated by the white arrows. (*a,c,d*) Modified with permission from Brangwynne et al. (2009). We thank Andrés Felipe Diaz Delgadillo for providing the figures shown in *b*.

EVIDENCE FOR LIQUID-LIKE STATES IN CELLS

Having defined the differences and similarities between liquids, solids, and gels, we now discuss recent observations and theory that suggest why a liquid-like state is an appropriate concept to describe certain intracellular compartments. This can be illustrated by considering P granules in *C. elegans* embryos (see **Figure 3*a***). Initially they were called granules because of their particulate appearance, but closer inspection of their dynamics (Brangwynne et al. 2009) reveals that they are better described as liquids for the following reasons:

1. Two P granules can fuse after touching, and the two P granules together revert back to a spherical shape (see **Figure 3*b***).
2. P granules can also be seen to drip off nuclei. In other words, P granules deform in shear flows in a manner similar to that of liquid droplets (see **Figure 3*d***).
3. Although they exchange material with the cytoplasm, as measured by fluorescence recovery after photobleaching, they are spherical. As mentioned above, the spherical shape is driven by surface tension.
4. If you photobleach half a P granule, it will recover through internal rearrangement (see **Figure 3*c***).

Therefore, over timescales of seconds, P granules have all the key signatures of a liquid state. They fuse, they drip, they are spheres, and they rearrange their contents within seconds (see **Figure 3*b–d***). For any non-membrane-bound compartment in a cell, the turnover properties are sufficient to specify that it is a liquid. The caveat, however, is that fluorescence recovery after photobleaching measurements usually follows only a subset of the components in a given compartment. But some components may not turn over, because the compartment itself contains a solid gel-like scaffold, within which other components can move freely. Here, the ability of two compartments to fuse helps distinguish solid gels from liquids.

A further example of a liquid-like compartment is the nucleolus of *Xenopus* germinal vesicles (Brangwynne et al. 2011). A nucleolus is a site of ribosome production inside the nucleus and consists of hundreds of proteins and RNAs (Boisvert et al. 2007). It is a classic example of a non-membrane-bound compartment and must execute the extremely complex process of making a ribosome. Material must be transported into the nucleolus, diffusion-limited reactions must take place inside the nucleolus, and assembled ribosomal particles must leave. Examination of the dynamics of *Xenopus* germinal vesicle nucleoli shows that they fuse and turn over rapidly (Brangwynne et al. 2011). Therefore, although nucleoli are considerably more viscous than P granules, they both have liquid-like properties (Brangwynne et al. 2009, 2011).

Many other non-membrane-bound compartments likely have the properties of liquids. Candidates are the many different nuclear speckles such as Cajal bodies, sites of DNA repair, and telomeres. Potential liquid-like cytoplasmic compartments are stress granules and P bodies (Wippich et al. 2013). Many compartments in a cell form rapidly and are disassembled when not required. Also, a surprising number of proteins involved in metabolism and stress responses form cytoplasmic puncta in yeast (Narayanaswamy et al. 2009, Petrovska et al. 2014). It will be fascinating to examine each one of these compartments to ask whether their formation also represents examples of liquid-phase separation, and then to work out the rules that lead to liquid-liquid demixing.

CONSEQUENCES OF LIQUID-LIKE PHASES

We began this review by describing the required properties of a non-membrane-bound compartment. Compartments must remain separated and do not dissolve in the cytoplasm. They must allow transport in and out of the compartment and must ensure sufficiently fast diffusion within the compartment so chemical reactions can take place. We now describe how a liquid-like state naturally provides all these requirements.

Diffusion

The fast dynamics of molecular rearrangement in a liquid implies that all components diffuse and are well mixed (for a discussion, see, e.g., Doi 2013). For instance, if you add some blue dye to a beaker of water, the molecules will mix by diffusion until the dye concentration is equally distributed and entropy is maximized. When the dye is first added to the water, the local concentration of dye is high. All the molecules undergo random movement. This leads to a net flux of molecules from high to low concentration, which emerges from the statistics of many randomly moving molecules. This transport driven by a concentration gradient is called diffusive flux. Diffusion and mixing are of particular importance for chemical reactions in cells, which require that reactants be transported to and from the sites of reaction and also that all reactants stay well mixed. Chemical reactions in biological systems require that all molecules of all types should stochastically meet at all locations. Diffusion provides both for stochastic interactions and for transport when local concentration imbalances build up. This transport brings in the reactants and transports out the products. Diffusion and mixing tend to equalize concentrations (see sidebar, Entropy, Mixing, and

Diffusion, and **Figure 2**). Therefore, cells usually must expend energy to maintain concentration differences within the cytoplasm or within a compartment, for instance, by using other means of transport, or source sink systems by local synthesis and degradation.

Both diffusion and chemical reactions are driven by differences in chemical potentials of the molecular species (for the relationship between entropy and chemical potential, see sidebar, Entropy, Mixing, and Diffusion). The basic definition of chemical potential is an energy per molecule, characterizing the work that must be performed to add one molecule of a certain type to a system. Chemical potential describes the tendency to change the number of a system's component molecules. Therefore, if the chemical potential is higher, there is more of a tendency to reduce the number of molecules of a certain type. Each individual (molecular) species has its own chemical potential, so a complex mixture is characterized by a set of chemical potentials, each of which describes the tendency of one type of molecule to move in or out of a local region. Therefore, gradients of chemical potential, which within a given phase stem from differences in concentration, drive diffusive fluxes (see sidebar, Entropy, Mixing, and Diffusion, for more details).

Phase Separation

To make a non-membrane-bound compartment, it must be separated from the liquid cytoplasm, and this can be achieved through liquid-liquid demixing. The idea of liquid-like states either separating from the cytosol or in cell membranes is a powerful way of thinking about cellular subcompartmentalization. For instance, phase separation allows the components to become rapidly concentrated in one place in the cell. Entry of proteins or other regulators into droplet phases could lead to rapid disassembly of liquid compartments. A small increase in concentration of components could allow reactions to start without any other regulatory event. Depletion of components from the cytoplasm as they segregate into the condensed phase could stop reactions in the cytoplasm itself. An interesting example is the sequestration of mTORC1, which is sequestered in P granule–like structures, referred to as stress granules. Upon activation of the DYRK3 kinase, stress granules dissolve, releasing mTORC1 for signaling (Wippich et al. 2013).

In the case of P granules, the complex set of proteins and RNAs that make up the P granule segregates from many other components that remain in the cytoplasm. In this process, two complex mixtures are formed that do not mix with each other but coexist as P granule and cytoplasm. This means that the components in the P granule have a higher affinity with each other than they do with respect to cytoplasmic molecules. This difference in affinities drives the phase separation (for more information, please refer to the sidebar, Molecular Interactions Drive Demixing; to **Figure 2**; and to Bray 1994, de Gennes 1979, Doi 2013, Safran 1994). This is counterbalanced by the entropy-driven tendency of all components to mix (see sidebar, Entropy, Mixing, and Diffusion). Both phases are mixtures of all components, but one phase is strongly enriched in a subset of molecules.

In the section on diffusion, we discussed how concentration differences are equalized by diffusive flux that is in turn driven by gradients in chemical potential. If this is true, then why are two different phases stable? Should not the difference in concentration of any molecular species between the two phases be equalized by diffusive flux? Normally, if you bring two mixtures with different compositions together, diffusion will mix the two mixtures (see sidebar, Entropy, Mixing, and Diffusion, and **Figure 2**). To understand this, we must think about the interfaces between phases, which are known as phase boundaries. Interestingly, in these phase boundaries, diffusive fluxes are not generated by concentration differences across the phase boundary. This is because there is no chemical potential difference across the interface. It is possible to have two phases with different composition in which the chemical potentials are equal because the chemical potential changes

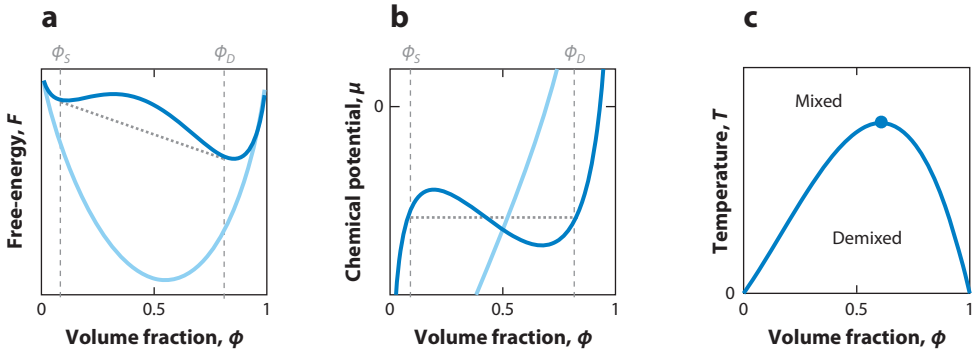

Figure 4

Thermodynamics of mixing and demixing. (*a*) The free energy F as a function of volume fraction of the red molecules ϕ (the volume fraction of blue molecules is $1 - \phi$). Both molecular species mix in the absence of interactions, $F = -TS^{mix}$ (*light blue convex curve*). In the presence of interactions disfavoring the close proximity of different species (see sidebar, Molecular Interactions Drive Demixing, and **Figure 2**), demixing can occur (*dark blue curve*). The range of volume fraction where demixing occurs is the interval $[\phi_S, \phi_D]$ with the free energy F^* of the demixed state indicated by the dotted line. (*b*) The chemical potential μ as a function of volume fraction corresponds to the free-energy functions shown in *a*. In case of demixing, the chemical potential can be equal for two different compositions (*dashed lines*), and thereby a demixed state with these compositions is thermodynamically stable. In the case of mixing (*light blue line*), each value of the chemical potential corresponds to a different composition. (*c*) Phase diagrams for a binary mixture depicting mixed and demixed states. The diagram depicts temperature T versus composition ϕ. The critical point, i.e., the composition corresponding to the largest temperature where a demixed state can exist, is indicated by a blue dot.

nonmonotonically with concentrations (see **Figure 4*b***). This means that there can exist two concentrations with the same chemical potential. More strongly, we can say that phase separation occurs if there are two different compositions of all molecular species with the same chemical potentials in both phases.

The fact that there are no diffusive fluxes that tend to equalize concentration across the boundary does not mean that there is no diffusion across the boundary (see sidebar, Concentration Gradients Across Phase Boundaries Do Not Imply Diffusive Fluxes). Molecules move stochastically in and out of the two different phases, but with equal numbers of molecules going one way or the other. Therefore, if the chemical potential in one phase is raised by, for instance, adding components (this could happen by synthesis or by chemical reactions), then molecules will diffuse into the other phase until the chemical potentials are equalized again. The study of the interface between different phases is extensive and can have important consequences for transport across the phase boundary (Anderson 1989, Lyklema 2005, and references therein). Transport across phase boundaries in biological systems has not been explored and will be an important topic for future studies in both biology and physics.

In our typical example of liquid-liquid demixing—a vinaigrette—oil and vinegar demix in two phases. Both vinegar and oil consist of many components. This separation can be characterized by a phase diagram (see **Figure 4*c***). For a given composition and temperature, the diagram shows whether the solution is a one-phase mixture or whether it separates into two phases. The line defines where demixing happens.

In the case of a vinaigrette, phase separation of oil and water is driven by a hydrophobic effect. What interactions between proteins and other biomolecules could drive phase separation

CONCENTRATION GRADIENTS ACROSS PHASE BOUNDARIES DO NOT IMPLY DIFFUSIVE FLUXES

Here we devote attention to the question: Why can two phases of different compositions coexist? Should the difference in concentration of molecular species not be equalized by diffusive fluxes? Diffusive fluxes are driven by gradients of chemical potential, μ (see sidebar, Entropy, Mixing, and Diffusion). In the case of mixing, concentration gradients are equalized by diffusion. This corresponds to the free energy and chemical potential functions shown in **Figure 4*a*** and **4*b*** as *light blue* lines. However, in the case of phase separation, a region of negative curvature appears in the free energy function, leading to a nonmonotonic chemical potential (**Figure 4*a*** and **4*b***, *dark blue lines*). The chemical potential at volume fractions ϕ_S and ϕ_D are equal. In a phase-separated state, the two coexisting phases, solute (S) and droplet (D) phase, adopt these volume fractions ϕ_S and ϕ_D, respectively. Most importantly, in equilibrium, the chemical potential is constant everywhere in space, i.e., within the phases and across the interface. As a consequence, the particle flux vanishes everywhere, in particular across the interface. Despite the existence of a concentration gradient $d\phi/dx$, there is no diffusive flux across the phase boundary because $\frac{d\mu}{dx} = 0$ (see **Figure 5*c***). There are two cases in which the chemical potential is constant in space: a single droplet embedded in a homogeneous phase (**Figure 5*a***) or two homogeneous phases separated by a flat interface (**Figure 5*b***). The only difference between these cases is that for a flat interface the pressure is also homogeneous, whereas for spherical objects, such as bubbles and droplets, there is a pressure jump across the interface. Specifically, the inner part of the droplet acquires a higher pressure compared with the outside, $p^{in} - p^{out} = \gamma \frac{2}{R}$, called the Laplace pressure (**Figure 5*d***). This equation is the Laplace law, where γ denotes surface tension and R the droplet radius. The Laplace law follows from the balance of normal forces at the interface. It is the Laplace pressure that governs the ripening of a system consisting of multiple droplets to eventually reach one of the cases shown in **Figures 5*a*** and **5*b***. This can be seen by considering two droplets of different size, such as those depicted in **Figure 5*e***. The chemical potential depends not only on composition but also on pressure, because it characterizes a tendency of a molecular species to enter or leave a given volume. Therefore, the chemical potentials in two droplets of different sizes differ because they have different Laplace pressures. Specifically, the chemical potential in the smaller droplet is bigger than the one in the larger droplet. This implies a diffusive particle transport from the smaller to the larger droplet since particles are driven along gradients of the chemical potential (for this, refer to sidebar, Entropy, Mixing, and Diffusion). In other words, the larger droplet grows at the expense of the smaller shrinking one and thereby drives the system into the final one-droplet state (**Figure 5*a***). This phenomenon is often referred to as Ostwald ripening.

in a biological context? A common type of phase separation that is studied for proteins is the coexistence of a protein crystal with a solution, often used in the context of protein structure determination. The interactions between proteins in a crystal are driven by strong stereospecific interactions (Durbin & Feher 1996).

At high protein densities, when molecules are densely packed, it is quite common to get a protein crystal or a jammed state, in which molecules cannot rearrange. In computer simulations and experiments, crystalline and jammed states have been found (Fusco & Charbonneau 2013, George & Wilson 1994, Haas & Drenth 1999). Furthermore, liquid states were also possible. To get a liquid state in simulations, one must have a significant concentration range, where the system does not go into this densely packed state and the components are loosely associated by attractive interactions (Asherie et al. 1996). These attractive interactions are characterized by valency, interaction strength, and interaction range. All these parameters are important in creating or not creating a liquid state. Favorable for a liquid state are long-range interaction, moderate

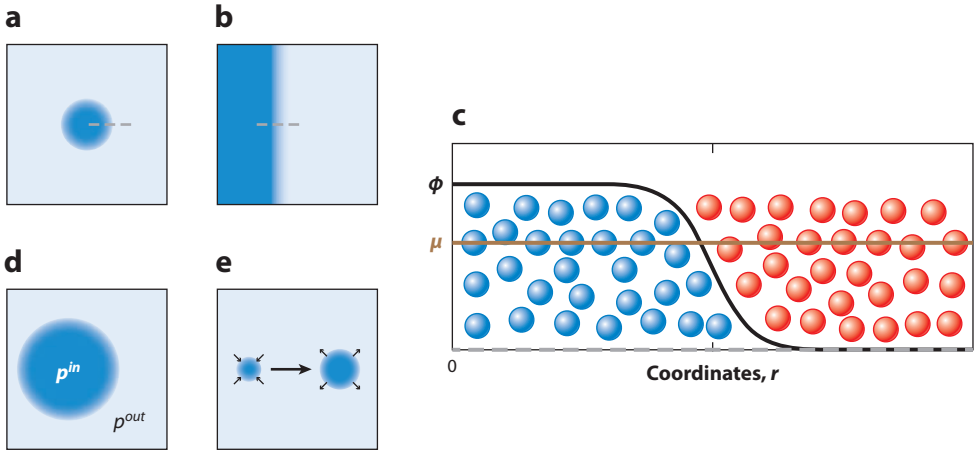

Figure 5

Coexistence of two phases of different composition. In equilibrium, there are two cases in which the chemical potential is constant in space: (*a*) a single droplet embedded in a homogeneous phase or (*b*) two homogeneous phases separated by a flat interface. Here we depict only the field of the blue particles' volume fraction ϕ. Note that the red particles' volume fraction is given as $1 - \phi$. (*c*) The volume fraction of the blue particles ϕ (*black line*) as well as the chemical potential (*brown line*) across the interface (coordinates along the interface, r, are indicated by dashed gray lines in *a* and *b*). (*d*) A droplet exhibits a larger pressure inside, p^{in}, compared to outside, p^{out}, owing to the curvature of the interface. (*e*) Two droplets of different sizes undergo Ostwald ripening; i.e., the larger droplet grows at the expense of the smaller shrinking one (the flux of droplet material is shown by the black arrow).

valency, and moderate binding energy (Asherie et al. 1996). Relating this to proteins, the valency would come from multiple binding sites in the same protein. Indeed, in a recent groundbreaking study, Li et al. (2012) showed in vitro that multivalent weak interactions between signaling proteins can drive the formation of liquid drops. By generating engineered Nck and N-WASP proteins in vitro, Li et al. were able to show that these proteins form liquid droplets, in which the concentration of the proteins in the drops was approximately one hundredfold higher than in the surrounding aqueous medium. The range of interaction could depend on several aspects of the molecular organization of the proteins. For instance, the degree of disorder and structural flexibility of the protein could be important (Jonas & Izaurralde 2013, Malinovska et al. 2013).

The physical chemistry of polymers can give us clues about how protein disorder and structural flexibility could contribute to liquid-like states. The range of interactions depends on more than the range of the bare physical interaction, such as those mediated by electrostatic forces. One example is colloidal beads coated with polymers that form a brush-like structure on the surface (Kodger & Sprakel 2013 and references therein). Swelling and collapse of the brush and its resulting changes in thickness set the range of interactions such that the thicker the brush, the longer the range. This is because when two beads approach each other, the brushes will interact over a range defined by the thickness of the deformable brush. These systems are used in polymer chemistry to stabilize colloidal liquids. We could imagine that polymer brushes on colloidal particles are models for proteins that have both globular and disordered domains. More generally, the interplay of van der Waals forces, electrostatics interactions, and depletion forces together with the effects of polymer brushes will contribute to the liquid-like nature of colloidal systems (Lin et al. 2000, Russell et al. 2012). Understanding which aspects of protein chemistry lead to liquid-like states is one of the most important problems in the physical chemistry of the cytoplasm.

DYNAMICS OF PHASE SEPARATION: COARSENING AND THE IMPORTANCE OF NUCLEATION

So far we have discussed how phase separation can be a powerful mechanism to organize cellular compartments. However, a cell faces several challenges when harnessing phase separation. The first challenge is how to initiate the growth of a droplet, also known as nucleation. The second challenge is that the size of the emerging droplets is hard to control.

Nucleation

Nucleation can occur spontaneously via a random fluctuation (known as homogeneous nucleation). If molecules stochastically come together in the right configuration, it may be enough to start a new droplet. Homogeneous nucleation is a rare event; therefore, its timing is hard to control (Binder & Stauffer 1976, Huang et al. 1974, Sarkies & Frankel 1971). Nucleation may also happen at a preexisting site (Malinovska et al. 2013), also referred to as heterogeneous nucleation. Examples are a preassembly of some of the molecules, or the use of a special structure: a ribosomal RNA in the case of a nucleolus (Grob et al. 2014), a centriole in the case of centrosome (Gönczy 2012, Zwicker et al. 2014) and chromatin in the case of a spindle (Heald et al. 1996). With the help of such structures, nucleation can be efficiently controlled. In addition, nucleation control also allows for the control of the number of droplets. For instance, there must be exactly two centrosomes in a cell, and this is controlled by two centriole pairs, each of which nucleates the formation of one centrosome (Gönczy 2012, Zwicker et al. 2014).

Size Control

The size of droplets can be controlled in several ways. One way is to stop the coalescence (fusion) process. For instance, in the case of nucleoli, the actomyosin network can stop coalescence because the mesh size of the network is much smaller than the size of the nucleolus (Feric & Brangwynne 2013). If the actomyosin meshwork is removed, the nucleoli fuse into a super nucleolus that sinks owing to gravity (Feric & Brangwynne 2013). Surface effects can also be used, for instance, in milk, where surfactants stabilize the oil-water emulsion (Pelan et al. 1997). The effects of surfactants in biology are not yet explored. Finally, extra components, which dissolve only in the droplets (Webster & Cates 1998), as well as chemical reactions, can be used to stabilize small droplets against Ostwald ripening (Zwicker et al. 2014).

Ostwald ripening (Doi 2013, Exner & Lukas 1971, Lifshitz & Slyozov 1961, Ostwald 1900) is driven by gradients in chemical potential created by different Laplace pressures between droplets of different sizes (see sidebar, Concentration Gradients Across Phase Boundaries Do Not Imply Diffusive Fluxes, and **Figure 5** for more details). Because smaller droplets exhibit a larger Laplace pressure, and thereby a higher chemical potential, there is diffusive transport from small to large droplets (see sidebar, Entropy, Mixing, and Diffusion, and **Figure 2**). This implies that small droplets shrink at the expense of growing large droplets. If you cannot control the actual coarsening process, the final size can be controlled by the number of molecules used to build the phase (limiting component) (Decker et al. 2011). The relationship between molecule number and droplet size has been discussed in recent reviews (Brangwynne 2013, Goehring & Hyman 2012).

ACTIVE LIQUIDS

So far, we have highlighted the consequences of the thermodynamics of liquid mixtures. However, because the liquid phases provide environments in which chemical reactions happen constantly,

the liquid is inherently active. In other words, rather than relaxing to equilibrium, the phase stays in an active state of persistent reaction rates and molecule fluxes. The fact that there are inherent reactions has several consequences beyond the simple picture that we have described. One of the consequences is that even if we have strong interactions, say, of the order of 20 $k_B T$, ATP hydrolysis can be used to constantly form and break bonds between molecules, thus keeping the system in fluid phases. Another advantage is that in the presence of chemical reactions, Ostwald ripening can be suppressed (Zwicker et al. 2014). ATP hydrolysis can drive active transport processes that can aid, for instance, in concentrating molecules to facilitate phase separation in certain regions or to generate gradients of supersaturation that can be used for droplet segregation (Lee et al. 2013).

In the context of actomyosin gels, ATP hydrolysis also powers the force generation of myosin motors, which introduces active mechanical stresses in the liquid-like gel. Such mechanically active liquids can exhibit spontaneous flows and active mechanical properties (Humphrey et al. 2002, Mizuno et al. 2007). (For more information on active liquids, we refer the reader to Kruse et al. 2004, 2005; Marchetti et al. 2013; Ramaswamy 2010, and references therein.)

CONSEQUENCES OF LIQUID-LIQUID PHASE SEPARATION FOR DISEASE

The fact that liquid-liquid phase separation tends to concentrate proteins comes with inherent dangers. Foremost among these is that the high protein concentration will tend to trigger aggregation processes or jamming, leading to solid gels or even crystals. These would no longer provide the necessary environment for chemical reactions. The cell copes with such aggregation processes using deaggregases (Doyle et al. 2013, Pickett 2006, Tyedmers et al. 2010) and will also regulate the dynamics of the compartments by, for instance, phosphorylation and dephosphorylation (Wippich et al. 2013). However, under certain conditions, such as metabolic syndrome, or in the presence of mutant proteins that aggregate more easily, a cell may not be able to dissolve the aggregates or limit their growth. Such variation in the liquid properties can be seen during *C. elegans* development (Hubstenberger et al. 2013). Indeed, many diseases of the brain are characterized by toxic aggregates, such as amyloid formations in Alzheimer's disease (Brundin et al. 2010), synuclein plaques in Parkinson's disease (Shulman et al. 2011), or plaques seen in amyotrophic lateral sclerosis (Robberecht & Philipps 2013). These proteins likely are normally meant to form liquid-like phases, but in the case of disease they end up taking more solid-like properties. In other words, the original compartments form by liquid-liquid demixing, and the disease state could form by a liquid-solid phase transition (see Hyman & Brangwynne 2011, Li et al. 2013, Malinovska et al. 2013, Shulman et al. 2011, Weber & Brangwynne 2012 for further discussions).

EVOLUTION OF LIFE

One of the most interesting questions in science is how life first appeared. The original experiment of Miller and Urey demonstrated that complex macromolecules could form in environments that are thought to mimic early earth and that contain only simple building blocks (Hyman & Brangwynne 2012, Oparin & Morgulis 1938). In many cases, these macromolecules are similar to those that are important for modern biochemistry. The question still remains of how this early chemistry evolved into self-replicating structures. In the 1930s, Alexander Oparin proposed the idea that the first step in the origin of life would be the phase separation of these macromolecules into liquid coarcevates (Lazcano 2010, Oparin & Morgulis 1938). Indeed, the question of how biological macromolecules form organized assemblies was posed at the dawn of biochemistry (Wilson 1899). This led to a physicochemical description of the cell, using ideas of colloid chemistry to

describe large-scale organization of macromolecules. Biologists considered the cytoplasm to be densely packed with liquid colloid particles that constituted a separate phase, distinct from the surrounding aqueous environment. The recent discovery of liquid-like states in cells suggests that this is a feasible proposition and that the non-membrane-bound compartments may be remnants of ancient structures that served to spatially confine and organize chemical reactions.

One could imagine the following scenario: Macromolecules would have formed constantly in the primordial soup. Once a certain subset tended to phase separate, they would form a small droplet, which would attract more of their kind, and the droplet would grow. In this droplet, reactions may have happened that were not possible outside because there the concentrations were too low. There are two possibilities: The reaction products would stay inside, and the drop would grow, or the reaction products would prefer to leave the drop. In this second case, the system would become a reaction center that would take in material and release some products. If different types of drops grew from the waste products of the other drops, this would stimulate an ecosystem.

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